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REVIEW ARTICLE

New perspectives of the role of skeletal muscle derived extracellular vesicles in the pathogenesis of amyotrophic lateral sclerosis: the 'dying back' hypothesis

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Abstract

Amyotrophic lateral sclerosis (ALS), is a progressive neurodegenerative disease that affects nerve cells in the brain and the spinal cord, and is characterized by muscle weakness, paralysis and ultimately, respiratory failure. The exact causes of ALS are not understood, though it is believed to combine genetic and environmental factors. Until now, it was admitted that motor neurons (MN) in the brain and spinal cord degenerate, leading to muscle weakness and paralysis. However, as ALS symptoms typically begin with muscle weakness or stiffness, a new hypothesis has recently emerged to explain the development of the pathology, that is, the 'dying back hypothesis', suggesting that this degeneration starts at the connections between MN and muscles, resulting in the loss of muscle function. Over time, this damage extends along the length of the MN, ultimately affecting their cell bodies in the spinal cord and brain. While the dying back hypothesis provides a potential framework for understanding the progression of ALS, the exact mechanisms underlying the disease remain complex and not fully understood. In this review, we are positioning the role of extracellular vesicles as new actors in ALS development.

KEYWORDS

amyotrophic lateral sclerosis, extracellular vesicles, neuromuscular junctions, skeletal muscle, biomarkers

1 | INTRODUCTION

Motor neurons (MN) are specialized nerve cells located in the central nervous system (CNS) and the peripheral nervous system (PNS). They play a crucial role in controlling voluntary muscle movements and are responsible for transmitting signals from the brain and spinal cord to muscles throughout the body. In Amyotrophic Lateral Sclerosis (ALS), both upper and lower MN degenerate, resulting in progressive muscle weakness, paralysis and eventually, respiratory failure. ALS is a relatively rare disease, affecting 6–9 people per 100 000 individuals annually. These prevalence rates can vary depending on factors such as age, gender, genetic predisposition and environmental influences. Genetic mutations, such as those in genes like C9orf72, SOD1 and others, have been linked to familial forms of ALS, which constitute about 5%–10% of all ALS cases. Environmental factors such as exposure to toxins, heavy metals and certain pesticides have also been implicated in increasing the risk of ALS. Overall, ALS remains a challenging disease to study and understand due to its complex aetiology and relatively low prevalence. Several cellular factors have been described as being involved in the onset of pathology and amongst them, oxidative stress has a central role leading to mitochondrial dysfunction, protein misfolding, DNA damage and ultimately, neuronal death. In addition, reactive astrocytes and microglia, the support cells of the nervous system, become activated in response to oxidative stress and promote

neuroinflammation in ALS, leading to the release of pro-inflammatory cytokines which further exacerbate neuronal damage. Also, alterations in proteasome and autophagy degradation pathways can lead to the accumulation of toxic protein aggregates, contributing to neuronal dysfunction and death. Glutamate excitotoxicity is another feature characterizing ALS pathology, caused by excessive glutamate signalling that increases extracellular levels of glutamate and neuronal damage.

Since its discovery, ALS has been considered a neurodegenerative disease, in which the main damage was mainly ascribed to MN (Eisen, 2021). However, recent findings support the involvement of non-neuronal cells in the disease progression, such as microglia, astrocytes and peripheral monocytes (Calafatti et al., 2023). More importantly, as ALS symptoms typically begin with muscle weakness or stiffness, a new hypothesis has recently emerged to explain the development of the pathology, that is, the '*dying back* hypothesis' which suggests that this degenerative disease starts at the connections between MN and muscles, resulting in the loss of muscle function (Dadon-Nachum et al., 2011). Over time, this damage extends along the length of MN, ultimately affecting their cell bodies in the spinal cord and brain. While the *dying back* hypothesis provides a potential framework for understanding the progression of ALS, the exact mechanisms underlying the disease remain complex and not fully understood. Amongst the possible actors involved at the beginning and in the spread of the disease at the systemic level might be the extracellular vesicles (EVs). These membrane-enveloped nanoparticles are released by all cell types and can mediate intercellular communication (Kalluri & LeBleu, 2020). By carrying and transferring lipids, proteins and RNA from one cell to various cell types, EVs affect the homeostasis of the recipient cells and modulate the cell microenvironment. In ALS, EVs have been implicated in several aspects of the disease, which are described in this review (i.e., propagation of pathological proteins, neuroinflammation and neuronal dysfunction). In addition, we will also describe how muscle EVs could be interesting candidates to consider in the '*dying back* hypothesis'.

2 | EXTRACELLULAR VESICLES (EVs): CLASSIFICATION, BIOGENESIS AND FUNCTIONS

EVs are a set of heterogeneous, bilayered particles found in different biological fluids and released by almost all cell types. EVs carry several components, such as proteins, nucleic acids and lipids, to mediate short- and long-distance intercellular communication (Buzas, 2023). Classifying EVs into distinct categories is currently difficult due to their great heterogeneity and overlapping size. Classically, EVs have been classified according to their biogenesis pathways as exosomes or small EVs, originating from the endosomal pathway (between 30 and 100 nm in size); microvesicles or large EVs, originating from the budding of the plasma membrane (between 100 and 300 nm in size); apoptotic bodies, larger than 1000 nm, which are generated during apoptosis (Kalluri & LeBleu, 2020).

Exosomes originate as intraluminal vesicles (ILVs) during the maturation of endosomes inside the multivesicular bodies (MVBs) (Figure 1a). Exosome biogenesis is a finely regulated multi-step process involving multiple factors. Specific sorting complexes selectively mediate the formation of protein and lipid cluster microdomains via ESCRT (Endosomal Sorting Complex Required for Transport)-dependent or ESCRT-independent mechanisms. In ESCRT-dependent biogenesis, ESCRT-0 and ESCRT-I contribute to the formation of ubiquitinated transmembrane cargo microdomains and, via ESCRT-II, to the recruitment of ESCRT-III for their budding and fission (Colombo et al., 2013). Among ESCRT-independent mechanisms, ceramide formation from sphingomyelin results in spontaneous membrane curvature and generation of microdomains (Figure 1a) (Kajimoto et al., 2013). Tetraspanin family proteins, such as CD63, CD81, and CD9, are also involved in ESCRT-independent cargo sorting (Charrin et al., 2014) (Figure 1a). The dynamics of exosome release rely on a balance between autophagy to maintain the cell's nutritional homeostasis and the release of proteins and toxic lipids from the cell (Xing et al., 2021). When autophagy is triggered, MVBs are fused with lysosomes or are otherwise directed to the plasma membrane via microtubule transport to export their content. Microtubule transport is modulated by the activity of RAB GTPases (Borchers et al., 2021). RAB27A and RAB27B, RAB11 and RAB35 have been identified as important regulators of exosome secretion, as they are involved in the docking of MVBs to the plasma membrane and in the rearranging the sub-membrane cytoskeleton (Borchers et al., 2021). The final stage of fusion of MVB with the plasma membrane, which enables the release of exosomes, is further regulated by soluble NSF attachment protein receptors (SNARE) complexes (Wang et al., 2017).

Microvesicles biogenesis occurs during rearrangements of lipid and protein composition and reorganization of the actin cytoskeleton at the plasma membrane (Stahl & Raposo, 2019) (Figure 1b). Ca^{2+}-dependent proteins, such as flippases, floppases, scramblases and calpains, promote changes in the asymmetry of the phospholipid bilayer, promoting membrane budding and the release of microvesicles (Stahl & Raposo, 2019). During this process, membrane-associated cargoes are selected according to their affinity for lipids in the inner part of the plasma membrane, building highly ordered microdomains that will become the site of origin of microvesicles (Rome & Tacconi, 2024). The fission and release of microvesicles from the plasma membrane are regulated by actin/myosin interactions and ATP contraction (Figure 1b) (Colombo et al., 2014).

The term microvesicle also covers all types of EVs originating from the plasma membrane during specific cellular processes (i.e., 'migrasomes' produced during cell migration, 'large oncosomes'' or ectosomes produced from cancer cells) (Buzas, 2023).

As it is difficult to separate all these EV sub-types, we have simplified the terminology in this review, and refer to them as sEVs (for small, exosomes or small plasma membrane buddings) or lEVs (for large EVs, from the plasma membrane buddings).

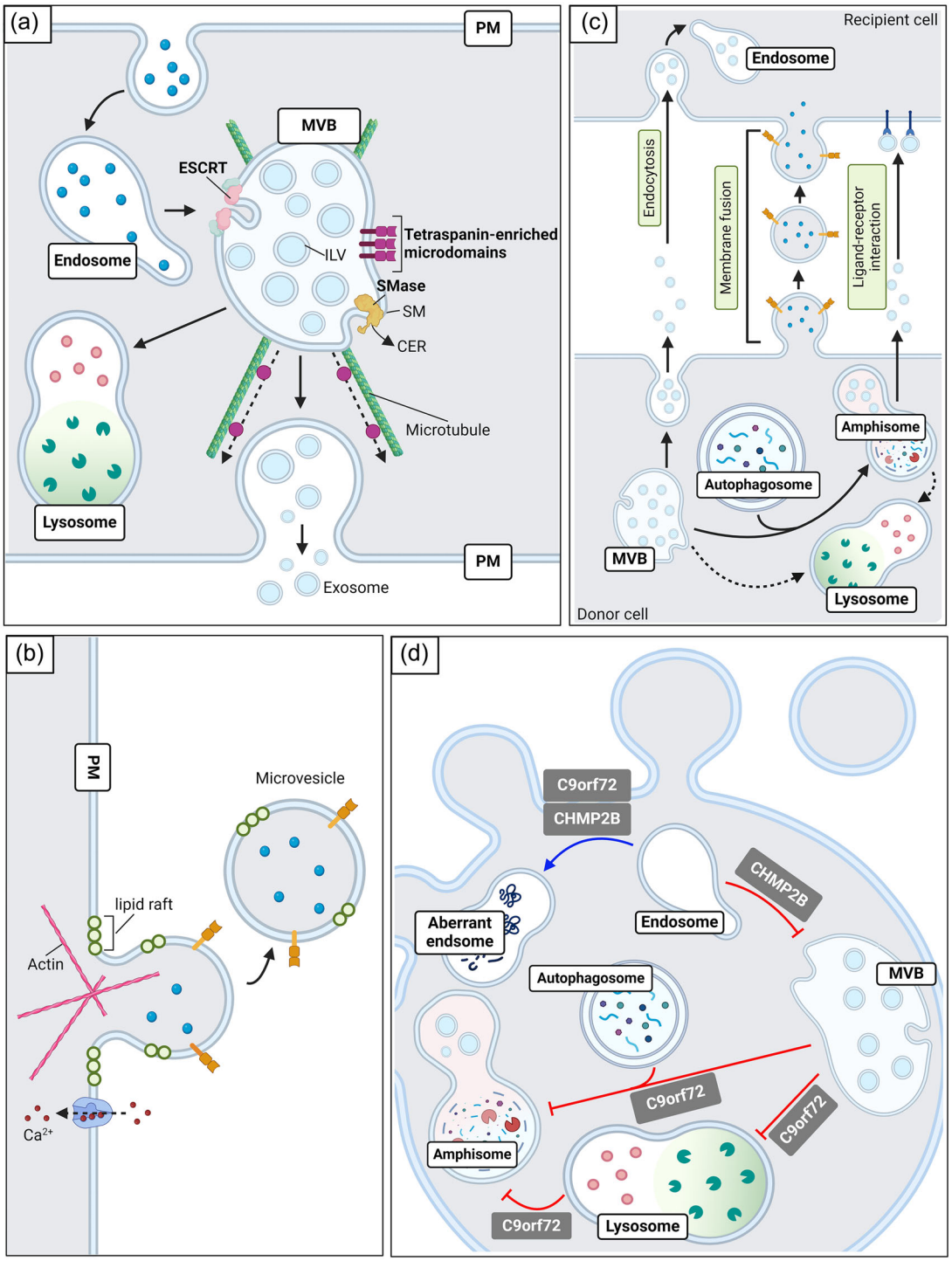

FIGURE 1 EVs biogenesis pathways and relative alterations in ALS. (a) *Exosome biogenesis*. Exosome, or small EVs, originate as intraluminal vesicles (ILVs) during the maturation of endosomes inside the multivesicular bodies (MVBs), via mechanisms involving ESCRT complexes or ESCRT-independent processes (i.e., ceramide formation or tetraspanins). (b) *Microvesicle biogenesis*. Microvesicles (or large EVs) biogenesis occurs during rearrangements of lipid and protein composition and reorganization of the actin cytoskeleton at the plasma membrane. Ca^{2+}-dependent proteins promote changes in the asymmetry of the phospholipid bilayer, promoting membrane budding and the release of microvesicles, processes regulated by actin/myosin interactions and ATP contraction. (c) *Fate of MVBs and EV interaction with recipient cells*. MVBs can fuse with lysosomes for degradation or directed to the plasma membrane (PM) via microtubule transport to export their content. At the intersection between autophagy and endolysosomal pathways, the formation of hybrid structures, called amphisomes, occurs. After their release into the extracellular environment, EVs can reach cellular targets and can be internalized via macropinocytosis, phagocytosis, endocytosis or direct membrane fusion. In addition, EVs bind to target cells via specific surface receptors, triggering various intracellular signalling pathways. EVs transfer their cargoes into recipient cells, modulating their biological functions. (d) Among the genes associated with ALS, some are involved in EV biogenesis and trafficking. Mutations in the CHMP2B gene, a component of ESCRT-III, impair the formation of ILVs during MVB formation, causing intracellular accumulation of aberrant and large endosomes and altered endosomal sorting and lysosomal degradation. Mutations in the C9orf72 gene impair autophagy, intracellular trafficking, and EV release (created with BioRender.com).

After their release into the extracellular environment, EVs can reach cellular targets, interacting with them in different ways (Figure 1c): that is, via tetraspanins, integrins, lectins, lipids and extracellular matrix components (Niel et al., 2018). Then, EVs can be internalized via macropinocytosis, phagocytosis, endocytosis or by direct membrane fusion (Niel et al., 2018) (Figure 1c). Finally, EVs transfer their cargoes into recipient cells, modulating their biological functions in various physio-pathological conditions.

3 | ALS AFFECTS EV BIOGENESIS AND COMPOSITION

More than 30 causative genes have been identified in ALS. Some of them are involved in endosomal trafficking, MVB biogenesis, RNA metabolism, RNA and protein loading into EVs and the balance between autophagy and exosome release (Figure 1d).

3.1 | EV biogenesis

The charged multivesicular body protein 2B (CHMP2B) protein is a core component of ESCRT-III (Figure 1d). Several mutations of its coding gene have been described in ALS (Ugbode & West, 2021). Mutations in the CHMP2B genes impair the formation of ILVs during the process of MVB formation, resulting in the intracellular accumulation of aberrant and large endosomes (Ugbode & West, 2021). Patients carrying mutations in CHMP2B show altered endosomal sorting and lysosomal degradation. Autophagy and intracellular trafficking are also impaired in patients with mutations in the C9orf72 gene (Farg et al., 2014). C9orf72 mutations involve a hexanucleotide repeat expansion (GGGGCC) in the non-coding region of the gene. Farg et al. demonstrated that the C9orf72 protein co-localizes with several Rab proteins (RAB1, RAB5, RAB7 and RAB11) impacting endosomal trafficking (Farg et al., 2014) and, as a consequence, EVs release (Figure 1d).

3.2 | EV composition

Mutations in the Superoxide Dismutase 1 (SOD1) gene account for 2% of ALS cases (Kubat & Picone, 2024), with great variability in its geographical distribution and clinical phenotypic heterogeneity (Huang et al., 2024). SOD1 is a cytoplasmic enzyme that catalyzes the conversion of superoxide radicals to hydrogen peroxide and oxygen. Interestingly, sEVs derived from $SOD1^{G93A}$ astrocytes had altered concentrations of two small non-coding RNAs, miR-155-5p and miR-582-3p, compared with sEVs from control astrocytes (Figure 2). The authors demonstrated that MNs exposed to $SOD1^{G93A}$ astrocyte-derived sEVs had reduced survival and neurite length and that miRNA-155-5p in sEVs contributed to this effect (Marton et al., 2023). A subsequent study reported that microglial cells overexpressing the same mutation $SOD1^{G93A}$ secreted EVs enriched in miR-155 and miR-146a, predicted to be involved in the regulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory pathway in recipient cells (Figure 2) (Vaz et al., 2019). NSC-34 neuronal cells transfected with $SOD1^{G93A}$ produce EVs enriched in miR-124 (Figure 2) (Pinto et al., 2017). Alteration of EV miRNA content has also been reported for EVs released from induced Pluripotent Stem Cell (iPSC)-derived astrocytes of ALS patients (Varcianna et al., 2019).

In addition to impacting EV biogenesis, the expanded repeat sequence in the C9orf72 mutated gene can be transcribed into RNA, forming RNA foci within the nucleus of cells. These RNA foci sequester RNA-binding proteins involved in RNA processing and transport, but also in their location into exosomes (Arnold et al., 2023; Kumar et al., 2017). Therefore, dysregulation of RNA metabolism associated with C9orf72 mutations in ALS likely affects EV composition (Arnold et al., 2023; Kumar et al., 2017). Other ALS-related mutations are associated with RNA metabolism alteration, that is, TAR-DNA-binding Protein 43 (TDP-43) is involved in RNA processing and found in the cytoplasmic inclusions characteristic of ALS, while mutations in the Fused in Sarcoma (FUS) gene are associated with familial ALS cases. FUS is an RNA-binding protein involved in multiple aspects of RNA metabolism (Weskamp & Barmada, 2018).

Interestingly, it was shown that mutated SOD1, TDP-43 and FUS can be released from cells via EVs and it is suspected that they might induce cytotoxic effects in recipient cells and propagate the disease (Figure 2) (Arnold et al., 2023; Li et al., 2023).

4 | THE 'DYING BACK' HYPOTHESIS: ROLE OF SKELETAL MUSCLE IN ALS

Research since the early 2000s has increasingly highlighted the significant contributions of non-neuronal cell types to the pathogenesis of ALS. Among the different *cell non-autonomous* pathogenetic mechanisms, recent evidence is pointing out the role of the skeletal muscle (SkM) in the progression of ALS. CNS and SkM are interconnected through a specialized chemical synapse, that is, the neuromuscular junction (NMJ). The NMJ comprises presynaptic cholinergic neurons, postsynaptic muscle fibres, and Schwann cells terminals (Lin & McArdle, 2021). This synapse is responsible for converting electrical signals originating from the

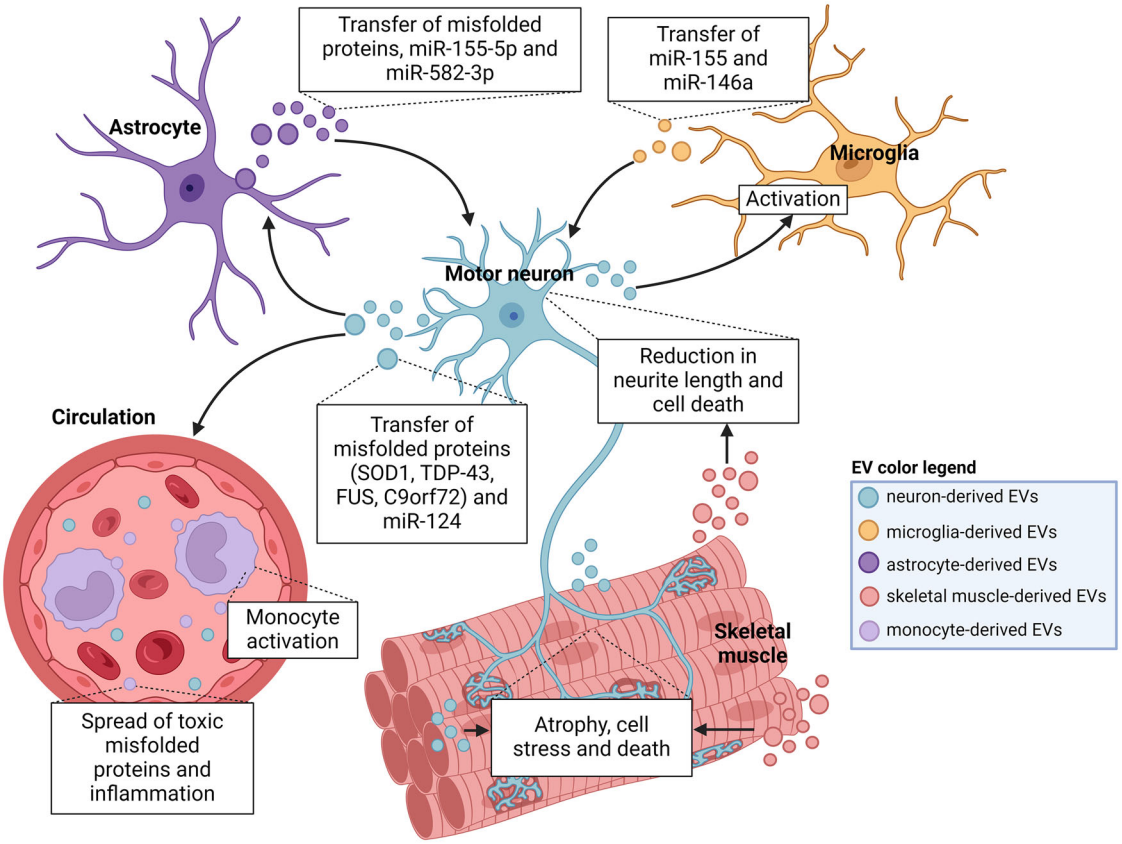

FIGURE 2 Role of EVs in the pathogenesis of ALS. During ALS pathogenesis, EVs mediate the transfer of biological factors between different cell types, inducing responses in recipient cells and contributing to the progression of the disease. Within the CNS, EVs derived from MNs drive the transmission of misfolded proteins and miRNAs to astrocytes and microglia inducing their activation, while EVs derived from reactive astrocytes and microglia drive neurodegeneration on recipient motor neurons. At the level of the NMJ, EVs from MNs induce atrophy, cell stress and death on recipient muscle cells, as well as SkM EVs induce neurite length reduction and cell death on recipient MNs. EVs crosstalk in ALS occurs also at the periphery, mediating the activation of peripheral immune cells, such as monocytes, and contributing to inflammatory processes (created with BioRender.com).

neurons into chemical signals that are sensed by the SkM 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 (Ohkawara et al., 2021). ALS is a disease characterized by multifactorial and highly complex aetiology, 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 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 SkM. In this scenario, cortical motor neurons initially damage the anterior horns of the spinal cord through glutamate-mediated excitotoxicity (Eisen, 2021). 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. The degeneration progresses retrogradely to the motor neuron cell body. Therefore, these NMJ alterations may precede neuron death and the clinical symptoms of the disease (Dadon-Nachum et al., 2011). This hypothesis is supported by the observation that the loss of lower MN was much more pronounced than upper MN. 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 (Sharma et al., 2016). The next section will focus on the emerging role of the SkM in ALS pathogenesis, which reinforces the validity of the 'dying back' hypothesis in the understanding of ALS disease. This theory positions SkM metabolism alterations as leading actors of the disease progression; therefore, any factor released from SkM could participate in this process (i.e., myokines (Lee et al., 2021) or EVs).

4.1 | Characteristics of skeletal muscle wasting in ALS from a metabolic point of view

Studies have shown that muscle fibres in individuals with ALS can undergo atrophy before significant motor neuron loss occurs. Indeed, in ALS mouse models, the specific overexpression of mutated SOD1 ($SOD1^{G93A}$) in SkM is sufficient to recapitulate the disease phenotype (Wong & Martin, 2010). In particular, these mice exhibited muscle and adipose tissue wasting, motor deficits, and a shortened lifespan. Their myofibres showed crystalline-like inclusions, disrupted sarcomeres, mitochondrial dysfunction, DNA damage and apoptotic satellite cells (SCs). ALS transgenic mice also showed loss of NMJ integrity, including reduced innervation, loss of synaptophysin and depletion of nicotinic acetylcholine receptors. Consequently, MN were characterized by inclusions in the cytoplasm and nucleus, axonopathy, mitochondrial and DNA damage, activating p53 and cleaved caspase-3, ultimately leading to cell death and loss of 40%–50% of MN (Martin & Wong, 2020). Increasing evidence indicates that skeletal muscle alterations significantly contribute to the pathogenesis of ALS, implicating toxicity in myofibres and SCs, as well as NMJ abnormalities (Martin & Wong, 2020; Scaricamazza et al., 2020; Wong & Martin, 2010). Interestingly, skeletal muscle damage and dysfunction are also reported in ALS patients (Carraro et al., 2015; Duranti & Villa, 2023). The progressive resulting atrophy and wasting share some mechanisms with other neuromuscular diseases, including age-related sarcopenia (Lepore et al., 2019). While SkM tissue exhibits increased energy demand and a fuel preference switch from glycolysis to fatty acid oxidation due to mitochondrial alterations, adipose tissue becomes hypolipidemic and has perturbed carbohydrate metabolism. Several epidemiological studies have corroborated these observations, as well as dietary studies showing that higher fat consumption decreases risk of developing ALS in humans (Ludolph et al., 2020) while, in transgenic mouse models of ALS, extends lifespan (Coughlan et al., 2016). In addition, more physically fit men have a higher risk of dying at an early age from ALS than men with less physical capacity because of their reduced ability to regenerate their SkM under physical constraints (Chapman et al., 2023; Julian et al., 2021; Vaage et al., 2024).

Additional data have demonstrated that, although MN degenerates in ALS and lose their connections to SkM fibres leading to denervation and SkM weakness and atrophy, even in the absence of denervation, SkM fibres in ALS patients may still exhibit signs of dysfunction and atrophy (Duranti & Villa, 2023). In this regard, the muscle of young presymptomatic $SOD1^{G93A}$ transgenic mice shows reduced levels of CDK5, an effector of myogenesis (Park & Vincent, 2008), and altered expression of genes involved in muscle growth and development (De Oliveira et al., 2014), supporting a possible early skeletal muscle degeneration independent from neurodegeneration.

Inflammation in the muscle tissue, independent of inflammation in the nervous system, may also contribute to muscle fibre damage and atrophy in ALS. Inflammatory mediators, that is, tumour necrosis factor alpha (TNFα) and interleukin-6 (IL-6), are important mediators of catabolic processes which promote protein degradation stimulating the ubiquitin proteasome pathway, and so lead to skeletal muscle atrophy (Ji et al., 2022).

Abnormal accumulation of proteins, such as TDP-43, within muscle fibres has been observed in ALS patients. These protein aggregates can disrupt cellular functions and contribute to muscle fibre dysfunction and degeneration (Arnold et al., 2023). Similarly, transgenic mice overexpressing $SOD1^{G37R}$ or $SOD1^{G93A}$ in skeletal muscle exhibit cytoplasmic inclusions of mutant SOD1 in some MN (Wong & Martin, 2010). Therefore, these findings give the first evidence of SkM alterations (i.e., metabolic dysfunction, inflammation and atrophy) as a potential leading cause of NMJ dismantling before MN degeneration in ALS, supporting the *dying back* hypothesis.

4.2 | The role of EVs released from skeletal muscle in ALS

4.2.1 | EV communication at the level of neuromuscular junction

MN are connected to muscle fibres through the NMJ, allowing the muscle to contract. NMJs are also composed of terminal Schwann cells and kranocytes, which contribute to their stability and maintenance. Different alterations that affect the integrity and function of the NMJs occur in ALS, including denervation, NMJ fragmentation, altered regeneration, inflammation and pre- and post-synaptic alterations (Verma et al., 2022). At the post-synaptic level, SkM and its secretome (i.e., EVs) play an important role in ensuring the integrity and regenerative capacity of the NMJ and, consequently, hinder the reinnervation of the muscle itself (Figure 3a) (Henze et al., 2024). In ALS pathogenesis, SkM is characterized by several structural and functional alterations, including proteostasis impairment and atrophy, defects in muscle regeneration and differentiation, metabolic dysfunction and secretome dysregulation (Duranti & Villa, 2023; Lambert-Smith et al., 2022; Obrador et al., 2021). EVs have been shown to mediate muscle-neuron communication, participating in the transfer of factors involved in the maintenance of NMJ (Figure 3a) (Madison & Robinson, 2019, Maggio et al., 2019). Accordingly, EVs released from C2C12 myotubes have been shown to promote motor neuronal development and branching in NSC-34 recipient cells (Madison et al., 2014). In both mouse models of ALS and patients, it has been reported that dysregulation in the profile of 'myomiRs', such as miR-206 and miR-133b, occurs in response to denervation (Maggio et al., 2019) and can be transported by EVs to other recipient cells, such as in proliferating C2C12 cells

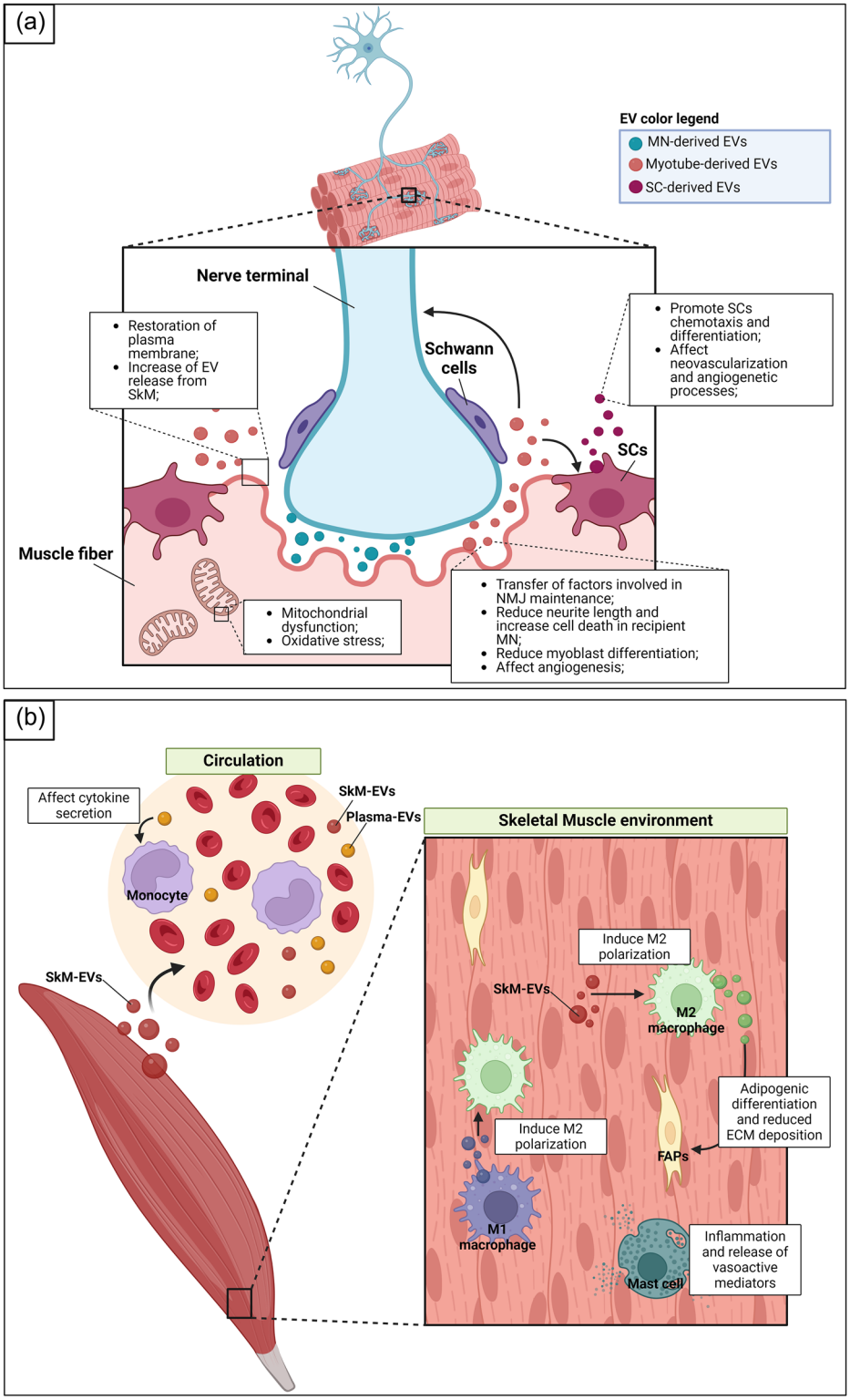

FIGURE 3 Systemic and local effects of EVs at the level of NMJ junction and skeletal muscle. (a) Role of EVs in muscle-neuron communication in NMJ dismantling in ALS. EVs can mediate muscle-neuron communication, participating in the transfer of factors involved in the maintenance of NMJ. Within the NMJ, EVs are released from motor neurons, myotubes and satellite cells (SCs). In the context of ALS, myotube-derived EVs affect SkM microenvironment, contributing to mitochondrial dysfunction and oxidative stress and impacting on myoblast differentiation and angiogenesis. SkM injury triggers repair mechanisms that induce an increased release of SkM-derived EVs, while SCs-derived EVs promote SCs chemotaxis and differentiation and affect neovascularization and angiogenic processes. Moreover, myotube-derived EVs carry neurotoxic factors affecting motor neuronal homeostasis, causing neurite length reduction and increased cell death. On the other hand, motor neuron-derived EVs can affect SkM microenvironment, exacerbating the damage. (b) EVs released from SkM can modulate immune responses at a local level within the SkM microenvironment and a systemic level in circulation in ALS. During the progression of ALS, EVs released from the SkM can mediate inflammation and regenerative processes recruiting monocytes and modulating the activation of resident macrophages. After injury, in the early phases of muscle regeneration, monocytes infiltrate within the tissue and activate towards the proinflammatory M1 phenotype. M1 macrophages promote phagocytosis of necrotic fibres and debris, sustain inflammation and support SCs activation and proliferation. With

(Continues)

FIGURE 3 (Continued)
the progression of the regenerative process, macrophages switch to the M2 anti-inflammatory phenotype, promoting the resolution of inflammation and enhancing muscle regeneration. EVs released from M2 macrophages favour fibro-adipogenic precursors (FAPs), non-myogenic stem cells master regulators of muscle regeneration, to differentiate into adipocytes. The adipogenic differentiation of FAPs prevents their differentiation into fibroblasts, therefore reducing the extracellular matrix (ECM) deposition typically occurring during muscle atrophy in ALS. On the other hand, a small fraction of SkM-derived EVs can cross microvascular barriers and reach the circulation, enriching the fraction of circulating EVs in the bloodstream and likely exerting effects on peripheral immune cells, that is, monocytes and modulating inflammatory processes at a systemic level (created with BioRender.com).

(Gasperi et al., 2017). Furthermore, SkM-derived EVs from ALS patients exert toxic effects on healthy recipient MN, reducing neurite length and increasing cell death, and on myotubes, causing atrophy, cellular stress and death (Figure 3a). The same group attributed defects in RNA processing as the putative mechanism underlying the observed neurotoxic effects, supported by proteomics analysis performed on SkM EVs (Gall et al., 2022). Another work described the differential effects of small and large EVs from SkM of ALS patients on different recipient cells. Specifically, while small EVs showed significant uptake and exerted toxic effects on recipient cells, that is, neurite debranching on MN, mild activation of astrocytes, and reduced survival of myotubes, large EVs showed very low uptake and no significant effects (Anakor et al., 2022).

These observations, together with the growing evidence of early SkM alterations in ALS (Anakor et al., 2022; Scaricamazza et al., 2021), support the potential role of EVs released from SkM in the pathogenesis of ALS by carrying neurotoxic factors that contribute to NMJ dismantling and neurodegeneration.

NMJ loss and the resulting skeletal muscle denervation is a critical early pathogenic event in both patients and animal models (Shefner et al., 2023). SkM denervation is linked to tissue injury which initiates processes of muscle regeneration and myogenesis which, in turn, can influence EVs release and composition. A crucial step in SkM repair and regeneration is the restoration of membrane structure and function to prevent myofibre degeneration (Michele, 2022). Damage to the myofibre plasma membrane is associated with increased intra- (endocytosis) and extracellular vesiculation (via EVs) to aid muscle repair (Figure 3a) (Bittel & Jaiswal, 2019). Membrane lipids reorganization, such as the enrichment in sphingolipids and the formation of specialized lipid rafts, contributes significantly to this process (Rome & Tacconi, 2024; Sastourné-Arrey et al., 2023). During tissue injury, acid sphingomyelinase (aSMase) is released in the extracellular environment by lysosomal exocytosis and converts sphingomyelin into ceramide at the plasma membrane. The enrichment in ceramides improves tissue repair by forcing the removal of injured membrane by endocytosis and EVs release (Draeger & Babiychuk, 2013; Tam et al., 2010). Furthermore, failure in ESCRT-dependent outward budding prevents plasma membrane regeneration of muscle fibres. Surface budding is initiated by the calcium-binding protein apoptosis linked gene-2 (ALG-2), which accumulates in the injured site after membrane disruption and calcium influx. ALG-2 recruits ALG-2-interacting protein X (ALIX) and the ESCRT complex promoting the shedding of damaged membranes and wound repair (Scheffer et al., 2014). Due to inadequate membrane repair, myofibres necrotize, triggering an inflammatory response, which in turn determines the regeneration of the lost myofibre by activating muscle-resident SCs. During muscle growth, maintenance and regeneration, SCs play a crucial role. In a quiescent state, they are tightly attached to muscle fibres and are hidden beneath the basal lamina in a specialized niche. Following muscle injury or denervation, SCs become activated, producing myogenic progenitors that engage, differentiate and repair damaged myofibres. SCs derived from ALS patients showed high proliferative activity but with a quiescent-like profile and low ability to differentiate in mature myotubes (Pradat et al., 2011; Scaramozza et al., 2014). Furthermore, recent evidence has shown that SCs also play a role in maintaining NMJ health. Indeed, after severing muscle-nerve connections in mice, Liu et al. (2017) found that SCs accumulated around the regenerating NMJ and that mice deficient in SCs had severely compromised muscle-nerve connections. During regeneration, myoblast differentiation triggers the release of several growth factors, including IGF-1, TGF-β1, VEGF and PDGF by EVs, which promote SC chemotaxis, differentiation and neovascularization of muscle (Murphy et al., 2018). Additionally, SC-derived EVs carry cargo miRNAs such as miR-206 and miR-1 that promote SC differentiation by altering myogenic gene expression (Figure 3a). The presence of miR-206 was also found in EVs released from mature myofibres and circulating muscle-derived EVs (Matsuzaka et al., 2016; Murphy et al., 2018).

As key features of ALS and NMJ degeneration, mitochondria alterations, reactive oxygen species (ROS) accumulation and oxidative stress within the SkM (Kubat & Picone, 2024) can affect EV release and their biological activity (Figure 3a). In a work of Hettinger et al. (2021), stress-induced premature senescence by H_2O_2 treatment of human myoblast increased EV release and the treatment of human umbilical vein endothelial cells (HUVECs) with these EVs increased senescence markers (β-galactosidase and transforming growth factor β), decreased proliferation, and impaired tube formation. Accordingly, EVs derived from H_2O_2-treated C2C12 myotubes increase myoblast proliferation and reduce their differentiation potential (Guescini et al., 2017).

4.2.2 | Skeletal muscle metabolism, EVs and ALS

SkM is responsible for glucose metabolism, energy homeostasis and motor functions and represents the main reservoir of whole-body proteins (Pedersen, 2013). An altered energy balance in SkM for prolonged periods of time may represent a risk factor for

the development of ALS. Interestingly, in SOD1^{G93A} mouse model of ALS, glycolytic fibres are more susceptible to denervation and to the loss of fast fatigable motor units suggests that the metabolic signature of glycolytic fibres may predispose them to dismantling of the NMJ (Frey et al., 2000; Hegedus et al., 2008). Furthermore, neuronal expression of the CHMP2B mutant triggers a progressive structural and functional deterioration in the NMJ. Indeed, CHMP2B mutation alters both presynaptic terminal organization and synaptic transmission that led to a switch from glycolytic fast-twitch muscle fibres to more oxidative slow-twitch muscle fibres (Waegaert et al., 2022).

The type of muscle fibres or metabolic adaptations within the SkM can modify its secretome, including EV cargo, composition and biological function. In a work conducted by Kargl et al. (2023) it was discovered that oxidative muscle produces more EVs than glycolytic ones, with a set of 297 different miRNAs mainly involved in growth and cell cycle regulation, angiogenic signaling and endothelial function. Specifically, incubation of endothelial HUVEC cells with EVs from oxidative fibres conferred more pro-angiogenic properties, in terms of tubule formation and length, and cell migration.

In light of this evidence, it is easy to hypothesize that the metabolic switch of muscle fibres towards a more oxidative phenotype in ALS could have a reparative role in terms of muscle regeneration and vascularization for the reassembly of muscle-neuron connections.

Early clinical indicators of ALS, including hypermetabolism, have provided more clues about prognostic factors that may influence the course and outcome of ALS patients. Hypermetabolism is associated with increased catabolism of carbohydrates, proteins and lipids, which leads to weight loss and muscle wasting (Maksimovic et al., 2023). Considering how metabolic alterations can affect EVs from SkM (Dini et al., 2020; Rome & Tacconi, 2024; Rome et al., 2019), muscle atrophy and imbalanced metabolic homeostasis in ALS can impair EV secretion and their biological activity. For example, Hudson et al. (2013) demonstrated that induction of atrophy in C2C12 myotubes by dexamethasone treatment reduced miR-23a levels in cells but increased in released EVs, suggesting a selective packaging of this miRNA during atrophy. Furthermore, several other miRNAs, including miR-1, miR−133, miR−206 and miR−499, involved in muscle homeostasis and metabolism were demonstrated to be associated with muscle wasting (Wang & Wang, 2016). In addition, activation of IL-1β during muscle disuse atrophy increased the levels of miR-let-7c, miR-let-7b, miR-181a and miR-124 in fibro-adipogenic progenitor cells (FAP)-derived EVs, all miRNAs involved in modulating senescence and atrophy (Parker et al., 2022).

4.2.3 | EVs as fundamental tools for the crosstalk between skeletal muscle and immunity in ALS

The characterization of the crosstalk of EVs between neuronal and non-neuronal cells has further revolutionizing the concept of ALS from a pure neurodegenerative disease to a multisystem disorder, implicating *cell non-autonomous* mechanisms that contribute synergistically to ALS pathogenesis but independently from MN (Scaricamazza et al., 2021). Microglia and astrocytes have been reported to secrete EVs containing misfolded SOD1, supporting bidirectional EVs-mediated crosstalk between MN and non-neuronal cells in the pathogenesis of ALS (Basso et al., 2013; Massenzio et al., 2018; Vaz et al., 2019). Moreover, distinct features of plasma EVs have been reported in ALS patients and mouse models, that is, characteristic size distribution, concentration and protein composition (Pasetto et al., 2021), while circulating EVs containing mutant TDP-43 affect cytokine secretion in peripheral monocytes of ALS patients (Zondler et al., 2017). Also, EVs released from SkM can act locally and even enrich the circulation by mediating systemic effects (Figure 3b). Guescini et al. demonstrated that the 5% of plasma-derived EVs were positive to alpha-sarcoglycan (SGCA) a component of the dystrophin-glycoprotein complex involved in the stability of muscle fibre membranes. These EVs were also positive for CD81, TSG101 and miR-206 (highly expressed in SkM) and are potentially involved in muscle remodelling and homeostasis (Guescini et al., 2015). After intraperitoneal injection into mice, EVs isolated from the quadriceps were also detected in different organs in addition to the SkM, including the brain, liver, heart, pancreas, spleen, gastrointestinal tract, kidney and lung (Jalabert et al., 2016). Given this, systemic and inflammatory effects related to ALS and drive by SkM-derived EVs cannot be excluded. In SOD1^{G93A} rats, mast cells infiltrate and accumulate in degenerated MN at the level of NMJ and actively contribute to neuroinflammation in ALS (Trias et al., 2017). Mast cells are constitutive immune cells within the SkM, present largely in the tendon regions and perimysium. These cells play a physiological role in muscle repair and remodelling through the release of various trophic and inflammatory factors, as well as vasoactive mediators (Gorospe et al., 1996). Together with mast cells, macrophages also participate in neuroinflammation and neuro-muscular degeneration (Figure 3b). In a disease phase-dependent manner, Van Dyke reported in SOD1^{G93A} rats an increase in inflammatory markers in SkM (i.e., CD11b, the microglial inflammatory marker CD68 and the inflammatory cytokines IL-1β and TNF-α) together with an increased expression of glial fibrillary acidic protein and nestin at the NMJ level, suggesting a possible involvement of peripheral macrophage in ALS (Dyke et al., 2016). After injury, in the early acute phases of muscle regeneration, there is an increase of monocyte infiltration within the tissue and their activation toward the proinflammatory M1 phenotype. M1 macrophages promote phagocytosis of necrotic fibres and debris, sustain inflammation and support SCs activation and proliferation (Wang & Zhou, 2022). With the progression of the regenerative process, during the intermediate-late phases, macrophages switch to the M2 anti-inflammatory phenotype which promotes the resolution of inflammation and enhances muscle regeneration by favouring the differentiation of muscle progenitor cells (MPCs) (Bernard et al., 2022; Wang & Zhou, 2022). In our previous work, we showed that EVs derived

from pro-inflammatory M1 macrophages in a hyper-glucose environment can polarize recipient naive macrophages toward M2 and alter insulin response and lipid metabolism in myotubes (Tacconi et al., 2024). In addition, anti-inflammatory polarized M2 macrophages-derived EVs favour fibro/adipogenic progenitors (FAPs), non-myogenic stem cells that master regulators of muscle regeneration, to differentiate into adipocytes (Liu et al., 2023). The adipogenic differentiation of FAPs prevents their ability to differentiate in fibroblasts and, as a consequence, reduces the abnormal deposition of extracellular matrix characteristic of muscle atrophy in ALS (Figure 3a) (Gonzalez et al., 2017). In an effort to the concept of bidirectional communication, also EVs released from SkM are able to modulate immune cell activity. For example, C2C12 myotube-derived EVs switch the pro-inflammatory phenotype of lipopolysaccharide (LPS)-treated bone marrow-derived macrophages in M2 by activating the PI3K/Akt and JAK/STAT pathways. These EVs are enriched in miR-206-3p, miR-378a-3p, miR-30d-5p and miR-21a-5p, which could be potentially responsible for their anti-inflammatory action (Yamaguchi et al., 2023).

5 | EVs AS BIOMARKERS IN ALS

Given ALS's complexity as a multisystemic disorder (Dhasmana et al., 2022), there is an urgent need to find biomarkers useful to track the disease onset, predict the clinical course of patients and finally, develop therapeutic strategies. Currently, the diagnosis of ALS consists of 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 MN (Barbo & Ravnik-Glavač, 2023). The main difficulties hindering the search for ALS-specific biomarkers are the absence of standardized procedures and methodologies, the lack of consistent patient cohorts, and the scarcity of longitudinal studies. Numerous studies are exploiting high-throughput omics techniques with the aim of characterizing alterations of different metabolites, proteins, miRNA and lipids, in the cerebrospinal fluid (CSF), blood and urine of ALS patients, in order to find an ALS-specific signature (Figure 4) (Witzel et al., 2022). Proteomics studies on CSF and blood of ALS patients have identified neurofilament proteins, whose accumulation correlates with neurodegeneration and reduced survival, as putative biomarker candidates (Rossi et al., 2018). However, these proteins are not specific to ALS but are rather common among neurodegenerative disorders (Lee et al., 2019). Several other metabolites, related to oxidative stress, neuroinflammation, hypermetabolism and muscle denervation, have been identified as altered in the body fluids of ALS patients (Barbo & Ravnik-Glavač, 2023). Being present in all biological fluids, able to cross the blood-brain barrier (BBB) and circulate in the bloodstream, reflecting the molecular signature of origin cells, EVs have emerged as a promising source of prognostic and diagnostic biomarkers for various diseases (Niel et al., 2022). In ALS, the potential of EVs as markers of disease progression is related not only to changes in their cargo, but also to their size and number. Recent work reported an increase in the size, but not in number, of plasma EVs of ALS patients compared to healthy controls (Sproviero et al., 2018). Another study, performed on the CSF of ALS patients, found an increase in leukocyte-derived EVs (Zachau et al., 2012), in contrast to a more recent study where no significant differences in the number of CSF EVs were reported (Thompson et al., 2020). To date, CSF and blood are the most studied body fluids for the identification of a specific signature of ALS carried by EVs. No misfolded SOD1 was found in CSF-derived EVs, but an increase in the INHAT repressor (NIR) has been reported as putatively involved in ALS pathogenesis (Hayashi et al., 2020). Compared to CSF, blood has the advantage of involving less invasive procedures and being more easily accessible as a source of biological markers; several studies have identified several potential candidates for ALS biomarkers by analysing circEVs. In ALS patients, only large EVs showed higher concentrations of SOD1, FUS and TDP-43, whereas no differences were reported in small EVs (Sproviero et al., 2018). Other interesting modifications found in plasma EVs of ALS and considered potential biomarkers are the decrease in heat-shock protein 90 (HSP90) and peptidylprolyl Isomerase A (PPIA) (Pasetto et al., 2021), and the increase in coronin-1a levels (Zhou et al., 2022). Furthermore, astrocyte-derived EVs isolated from plasma showed higher levels of IL-6, which is considered a valid marker of neuroinflammation, but not specific to ALS as it is shared with other neurodegenerative diseases (Pasetto et al., 2021). Regarding miRNAs, miR-4454 and miR-127-3p, which are involved in neurogenesis, MN integrity and synapse formation, as well as axon guidance and intracellular motor proteins, respectively, were found to be dysregulated in more than one study (Pregnolato et al., 2021; Sproviero et al., 2021). In addition, miR-124-3p has been identified as a potential prognostic biomarker, as it strongly correlates with ALS Functional Rating Score (ALSFRS-R), and thus with disease severity (Yelick et al., 2020). Overall, the current studies suggest that EVs of patient biofluids may be a useful tool to find putative biomarkers for ALS disease progression. Despite their potential, current studies on EVs still present conflicting results, and improvements in ALS diagnostics are needed.

6 | CONCLUSIONS AND FUTURE DIRECTIONS

ALS, formerly known as a neurodegenerative disease in which the neuronal component was initially affected, is now recognized as a complex multisystemic disorder involving several cell types, that is, CNS and peripheral immune cells, but mainly skeletal muscle, that actively contributes to disease progression (Owens, 2017). As the disease is multifactorial and not limited only to the

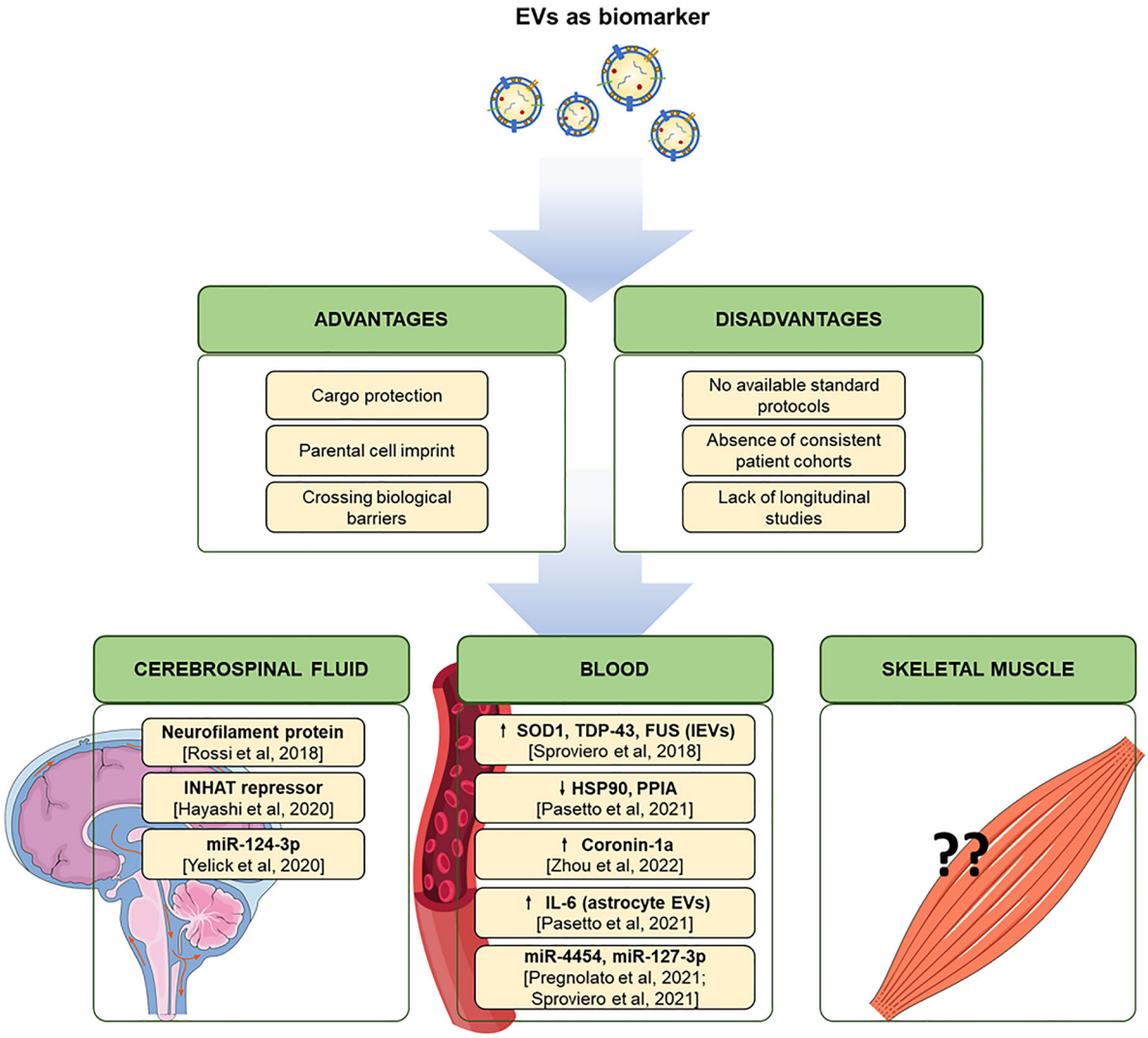

FIGURE 4 Overview of EVs as potential biomarkers in ALS. EVs show several advantages for their use as potential biomarkers for ALS diagnosis, but also some limitations. The current studies that aimed to find potential ALS biomarkers within EVs have been performed in cerebrospinal fluid (CSF) and blood. However, none of these studies have yet analysed the EVs signature in skeletal muscle.

CNS, it is currently challenging to understand the origin and progression of ALS, complicating the improvement of diagnosis and the development of targeted therapy. The reciprocal crosstalk between neuronal and non-neuronal cells, which characterizes aberrant signalling between the CNS and the periphery, is also mediated by the release of EVs (McCluskey et al., 2023), being involved in the transfer of neurotoxic factors according to a 'dying back' phenomenon (Scaricamazza et al., 2021). Although growing evidence supports the skeletal muscle is the origin of the disease, as it shows alterations in the early stages of the disease and before the onset of symptoms (Anakor et al., 2022), the crosstalk of EVs between skeletal muscle and MN is still poorly characterized. Therefore, characterizing the molecular profile of EVs and performing longitudinal functional studies to dissect the role of EV trafficking during ALS progression would be of great importance in unraveling the pathogenetic mechanisms of ALS.

Understanding the complex interplay between MN and skeletal muscle in ALS is crucial for developing effective treatments that target not only neuronal degeneration but also muscle pathology. Therapeutic approaches aimed at preserving muscle function and reducing muscle atrophy may provide additional benefits for ALS patients, improving their quality of life and slowing disease progression (Scaricamazza et al., 2021).

In addition to their role in ALS pathogenesis, EVs could be used as biomarkers to track disease progression due to their intrinsic characteristics, such as crossing biological barriers and availability in the bloodstream, which represents a more readily available source for EVs requiring minimally invasive procedures (Barbo & Ravnik-Glavač, 2023). As ALS is a multifactorial disease involving not only the CNS, circulating EVs (circEVs) may offer valuable information on early systemic alterations (Mustapic et al., 2017). Despite the aforementioned advantages, it must be considered that circEVs show high heterogeneity in terms of

cellular origin, and most of these EVs may derive from blood cells rather than neuronal cells. Furthermore, plasma proteins that interact with the EVs surface, that is, the 'protein corona', may further affect their composition and functions (Tóth et al., 2021). Therefore, it may be quite challenging to find a specific signature for ALS neurons within circEVs. However, being able to cross the BBB, it cannot be excluded that EVs of other cell types may enter the circulation and contribute to the pathological signalling that characterizes ALS. Another limitation of the current studies is the different procedures adopted to isolate and characterize EVs. This problem can be overcome by standardizing protocols for EVs isolation and downstream treatment according to the MISEV guidelines, in order to maximize the reproducibility of the obtained results (Witwer et al., 2021). Moreover, the lack of longitudinal studies represents another critical issue in the search for ALS biomarkers, as pathological alterations in ALS, as well as the molecular signature of EVs, may vary during disease progression (Barbo & Ravnik-Glavač, 2023; McCluskey et al., 2023). To the best of our knowledge, none of the current studies have yet investigated EVs released from the skeletal muscle as potential biomarkers of ALS. Given the increasing evidence of the role of the skeletal muscle in early pathogenetic events of ALS (Anakor et al., 2022; Scaricamazza et al., 2021), together with the role of SkM EVs as putative mediators of toxic signals contributing to disease (Gall et al., 2022), it may be worth investigating the molecular profile of SkM EVs to find an ALS specific signature. As skeletal muscle shows various alterations during disease progression (Anakor et al., 2022; Duranti & Villa, 2023; Scaramozza et al., 2014; Scaricamazza et al., 2021), it could be interesting to explore whether SkM EVs contribute to this impairment and to understand whether EVs are able to transfer harmful signals to the periphery. This type of information, which is still largely unexplored, could be crucial to improve research in the diagnostic field of ALS and fill current data by opening new clues towards a better understanding of the disease.

AUTHOR CONTRIBUTIONS

Conceptualization: Carolina Sbarigia, Luciana Dini, Sophie Rome, Stefano Tacconi. Investigation: Carolina Sbarigia and Stefano Tacconi. Roles/writing—original draft: Carolina Sbarigia and Stefano Tacconi. Writing—review & editing: Luciana Dini, Sophie Rome and Stefano Tacconi. Visualization: Stefano Tacconi. Figures: Stefano Tacconi.

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CONFLICT OF INTEREST STATEMENT

All the authors declare no conflict of interest.

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