Bioactive Materials 29 (2023) 16–35

Contents lists available at ScienceDirect

Bioactive Materials

journal homepage: www.keaipublishing.com/en/journals/bioactive-materials

Review article

Mesenchymal stem cell secretome and extracellular vesicles for neurodegenerative diseases: Risk-benefit profile and next steps for the market access

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ARTICLE INFO

Keywords:
Exosomes
Microvesicles
Secretome
Mesenchymal stem cells
Secretome formulation
Brain regeneration

ABSTRACT

Neurodegenerative diseases represent a growing burden on healthcare systems worldwide. Mesenchymal stem cells (MSCs) have shown promise as a potential therapy due to their neuroregenerative, neuroprotective, and immunomodulatory properties, which are, however, linked to the bioactive substances they release, collectively known as secretome. This paper provides an overview of the most recent research on the safety and efficacy of MSC-derived secretome and extracellular vesicles (EVs) in clinical (if available) and preclinical models of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, Huntington's disease, acute ischemic stroke, and spinal cord injury. The article explores the biologically active substances within MSC-secretome/EVs, the mechanisms responsible for the observed therapeutic effects, and the strategies that may be used to optimize MSC-secretome/EVs production based on specific therapeutic needs. The review concludes with a critical discussion of current clinical trials and a perspective on potential future directions in translating MSC-secretome and EVs into the clinic, specifically regarding how to address the challenges associated with their pharmaceutical manufacturing, including scalability, batch-to-batch consistency, adherence to Good Manufacturing Practices (GMP) guidelines, formulation, and storage, along with quality controls, access to the market and relative costs, value for money and impact on total expenditure.

1. Introduction

Neurodegenerative diseases (NDs) encompass many acute and chronic conditions that affect the central nervous system (CNS). These illnesses are characterized by the gradual deterioration of neurons and glial cells in the brain and/or spinal cord, resulting in significant declines in specific (brain) functions, like memory, movement, and cognition [1,2]. Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Huntington's disease (HD) are all examples of neurodegenerative diseases where the degenerations extend over a decade, and for which the underlying mechanism remains elusive primarily. In acute ischemic stroke (AIS) and spinal cord injury (SCI), instead, neurodegeneration is a consequence of the disruption of blood and oxygen flow or a traumatic impact on the spine, respectively (Table 1).

Besides AIS and SCI, aging is a significant risk factor for most neurodegenerative disorders [21]; the number of people affected by NDs is therefore expected to rise due to the increasing population age. This also means that countries around the globe are facing a significant economic challenge. For example, according to some estimations, the US population aged ≥65 will increase from 53 million in 2018 to 88 million in 2050 [22]. Therefore, it is likely that the US$946 billion spent in 2016 caring for individuals with AD (which was already triple the expenditure in the year 2000 [23]) would enormously be expected to rise when the

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.bioactmat.2023.06.013
Received 3 May 2023; Received in revised form 1 June 2023; Accepted 19 June 2023

Table 1
Most common acute and chronic NDs, their clinical manifestations, and causes.

Disease	Clinical manifestations	Cause
Alzheimer's disease (AD)	Memory loss and cognitive decline.	Neuronal death that involves a vast area of the CNS, promoted by the deposition of amyloid-β (Aβ) peptides into plaques [3].
Parkinson's disease (PD)	Motor and non-motor disorders, like tremors and delayed movement. Specifically, dyskinesia with resting tremors, muscle rigidity, and cognitive dysfunction [4].	Loss of dopaminergic neurons in the substantia nigra, nigrostriatal pathway degeneration [5,6]. α-synuclein positive inclusions in cell bodies and neurites (Lewy bodies) of nigral and olfactory bulb neurons [7–9]. Further, neuroinflammation and oxidative stress are involved [10,11].
Amyotrophic lateral sclerosis (ALS)	Muscles of the limbs, throat, tongue, and respiratory system gradually deteriorate and lose strength.	Degeneration of the upper motor neurons of the corticospinal tract and the lower motor neurons that control the skeletal muscles [12]. Mechanisms are still unclear, despite many studies demonstrating the involvement of several altered signaling pathways, such as mitochondrial dysfunction, glutamate excitotoxicity, oxidative stress, and neuroinflammation [13].
Multiple sclerosis (MS)	Severe physical disabilities.	Demyelinating disease caused by CNS inflammation and immune dysfunction [14].
Huntington's disease (HD)	Motor (involuntary movement disorder and impairment of voluntary movements), cognitive (problems of attention, cognitive slowing) and neuropsychiatric (depression, irritability, and apathy) disorders.	Autosomal dominant disorder caused by the elongation of CAG repeats on the short arm of chromosome 4p16.3 in the HTT gene. As per the proposed mechanisms, the degeneration of neurons in the putamen, caudate, and cerebral cortex, and the loss of substance-P-containing medium spiny neurons can be attributed to various factors. These include the accumulation of mutant protein aggregates, which impair the ubiquitin-proteosome pathway, transcriptional dysregulation, excitotoxicity caused by the increased release of glutamate and glutamate agonist, mitochondrial dysfunction, and altered energy metabolism, as well as changes in axonal transport and synaptic dysfunction [15,16].
Acute ischemic stroke (AIS)	Aphasia, agraphia, acalculia, apraxia, visual field deficit, hemiparesis, and hemisensory loss [17].	Neurodegeneration, cell death, changes in brain volume and cognitive performance consequent to the disruption of blood and oxygen flow [18].
Spinal cord injury (SCI)	Flaccid paresis, exclusively diaphragmatic respiration, low blood pressure, bradycardia, and loss of control of the urinary outlet [19].	Commonly results from a sudden, traumatic impact on the spine that fractures or dislocates vertebrae; following this primary injury, a secondary one starts and causes progressive damage to spinal cord tissue, impeding neurological recovery due to complex and interconnected cellular, molecular and biochemical phenomena [20].

number of individuals with AD is likely to reach 115 million worldwide by 2050 [24]. A change in diagnostic and treatment methods is, therefore, crucial.

Where current treatments are mostly inadequate, stem cell therapies have emerged as a potential strategy to treat these disorders. In this regard, mesenchymal stem cells (MSCs) are particularly attractive for regenerative therapies due to their multipotentiality and pro-regenerative and immunomodulatory properties [25], which may also depend on the release of bioactive substances collectively known as secretome [26], and comprising free soluble factors and extracellular vesicles (EVs) [27].

This article provides an overview of the most recent research on the safety and efficacy of MSC-derived products, i.e., secretome and EVs, in preclinical models of NDs. The paper also explores the biologically active substances within MSC-secretome/EVs, the mechanisms responsible for the observed therapeutic effects, and the strategies that may be used to optimize MSC-secretome/EVs production based on specific therapeutic needs. The review concludes with a critical discussion of current clinical trials and a perspective on potential future directions in translating MSC-secretome and EVs into the clinic.

2. From MSCs to their secretome/EVs in NDs

As neuronal death, neurodegeneration, neuroinflammation, immune dysfunction, and oxidative stress are common features of NDs (see Table 1), it is not surprising that stem cells, particularly MSCs, have emerged as a promising treatment modality at the forefront of ND research [28,29]; this is especially significant considering the inadequacy of current treatments for most NDs [30]. Indeed, MSCs may mediate CNS repair thanks to their well-documented ability to migrate to injured tissue [31], differentiate into new cell types (multipotency) even after extended in vitro expansion [32], pro-regenerative capacity [33], and immunomodulation [34]. Multiple studies have evaluated the effects of MSCs in animal models of NDs administered by multiple delivery routes and over a wide range of doses. The safety and efficacy of MSCs undoubtedly emerge, as highlighted by recent systemic reviews and meta-analyses of the literature. For example, regarding the efficacy, transplantation of MSCs significantly improved memory loss and cognitive impairment in AD [35], rotational behavior and limb function in PD [36], improved neurobehavior in ALS [37], ameliorated the symptoms and delayed the progression of MS [38], promoted the recovery of nerve function and exerted a protective effect on the brain of cerebral AIS rats [39], and improved sensorimotor functions in SCI [40].

Due to this proven efficacy in pre-clinics, it is unsurprising that MSCs have been the broadly used adult stem cells in clinical trials on humans. However, although many clinical trials can be found when assessing the efficacy of MSCs in NDs on humans (source: http://www.clinicaltrials.org; last access 31/05/2023), only a few have been completed with published results (Table 2).

Although there have been some promising indications [43–47,50–52,57,58], the efficacy of MSCs in treating NDs has yet to be confirmed through ongoing clinical trials. However, despite the relatively limited number of studies conducted thus far, there is a consensus that MSCs are safe for use in this context. In this regard, a recent meta-analysis reviewed the adverse events following MSC administration in all populations with different diseases; the pooled analysis demonstrated that the administration was safe and closely associated with only minor adverse effects, like short-term fever, adverse events at the administration site, constipation, fatigue, and insomnia [59].

Even if cell therapy with MSC continues to be a field of substantial interest, there has been, in recent years, a shift in the paradigm of MSC-based therapies: from MSC cell therapy to MSC-secretome therapy. Indeed, while initially, the therapeutic effects of MSCs were attributed to their ability to differentiate into various cell types and replace damaged or diseased tissues [60], recent research has revealed that their therapeutic effects are mediated mainly by their secreted products, known as

Table 2
Summary of the clinical trials (completed and with results) involving MSCs in NDs. Source: http://www.clinicaltrials.org; last access 31/05/2023; search terms: mesenchymal stem cells and Alzheimer's disease or Parkinson's disease or amyotrophic lateral sclerosis or multiple sclerosis or Huntington's disease or acute ischemic stroke or spinal cord injury.

Neurological disease	Clinical trial ID number	Completion date	Study design	Treatments	Outcomes	Ref.
AD	NCT01297218	12/2011	Phase I, interventional, open-label	UC-MSCs	Administration was safe and well tolerated.	[41]
	NCT02054208	12/2019	PhaseI/II, interventional, quadruple-blind	UC-MSCs vs saline	Administration was safe and well tolerated.	[42]
ALS	NCT01771640	05/2012	Phase I, interventional, open-label	BM-MSCs	Administration was safe and well tolerated.	[43]
	NCT01759797	03/2014	Phase I, interventional, open-label	BM-MSCs	Amelioration of the forced vital capacity and ALS functional rating scale-revised.	
	NCT01051882	03/2013	Phase I/II, interventional, open-label	BM-MSCs secreting neurotrophic factors	Administration was safe and well tolerated.	[44]
	NCT01777646	09/2015	Phase II, interventional, open-label		Indications of possible clinical benefits are to be confirmed in upcoming clinical trials.	
	NCT03828123	08/2019	Phase I/II, interventional, open-label	Autologous MSCs	Administration was safe and well tolerated. The progression of the disease was slowed down.	[45]
	NCT04821479	12/2020	Phase I/II, interventional, open-label	MSCs	Administration was safe and well tolerated. Indications of medium-term clinical benefits.	[46]
MS	NCT00395200	12/2010	Phase I/II, interventional, open-label	Autologous MSCs	Administration was safe and well tolerated. Structural, functional, and physiological improvements suggestive of neuroprotection.	[47]
	NCT00813969	05/2014	Phase I, interventional, open-label	Autologous MSCs	Administration was safe and well tolerated.	[48]
	NCT02034188	03/2016	Phase I/II, interventional, open-label	UC-MSCs	Administration was safe and well tolerated.	[49]
	NCT02166021	12/2018	Phase II, interventional, double-blind	Autologous MSCs	Administration was safe and well tolerated. Short-term beneficial effects regarding the primary endpoints, especially in patients with active disease.	[50]
	NCT00781872	12/2019	Phase I/II, interventional, open-label	Autologous BM-MSCs	Administration was safe and well tolerated. Immediate immunomodulatory effects.	[51]
	NCT04823000	04/2020	Phase I/II, interventional, open-label	MSCs	Administration was safe and well tolerated. The presence of clinical benefits (especially in patients treated with more than two injections) lasted up to 4 years. Short-term immunomodulatory effects.	[52]
SCI	NCT00816803	12/2008	Phase I/II, interventional, single-blind	Autologous BM-MSCs	Administration was safe and well tolerated.	[53]
	NCT01325103	12/2012	Single-group assignment, interventional, open-label	Autologous BM-MSCs	Administration was safe and well tolerated.	[54]
	NCT02482194	03/2016	Phase I, interventional, open-label	Autologous BM-MSCs	Administration was safe and well tolerated.	[55]
	NCT01909154	03/2019	Phase I, interventional, open-label	Autologous BM-MSCs	Administration was safe and well tolerated.	[56]
	NCT03003364	02/2020	Phase I/II, interventional, quadruple-blind	WJ-MSCs	Administration was safe and well tolerated. Sensory improvement in the segments adjacent to the injury site.	[57]
	NCT03308565	10/2021	Phase I, interventional, open-label	Autologous A-MSC	Administration was safe and well tolerated. Meaningful signs of improved, rather than stabilized, neurologic status.	[58]

UC = umbilical cord; BM = bone marrow; A = adipose; WJ= Wharton Jelly.

MSC-secretome [61]. As mentioned above, the MSC-secretome comprises a diverse range of bioactive molecules, including cytokines, growth factors, and microRNAs [62–70], secreted as such or encapsulated into exosomes and other EVs. MSC-secretome and EVs have been shown to modulate various processes, including angiogenesis [66,71–74], immunomodulation [64,75,76], and tissue repair [66,77,78]. Moreover, the MSC-secretome has been found to exhibit similar therapeutic effects as MSC cell therapy [79] without the potential risks and limitations associated with direct cell transplantation [27,80–82]. It is therefore not surprising that MSC-secretome therapy is also being explored as a potential treatment for NDs: the strategy is not anymore aimed at simply replacing the lost cells (e.g., neurons) by transplanting stem cells, but by utilizing MSCs as "drug stores" that secrete neuroprotective agents which are the actual effectors of the therapeutic effects observed (Fig. 1). The preclinical and clinical safety and efficacy evidence for MSC-secretome and EVs is discussed below for each neurodegenerative disease.

3. MSC-secretome and EVs: pre-clinical and clinical safety and efficacy in neurodegenerative diseases

3.1. Alzheimer's disease (AD)

The effectiveness of MSC-secretome and EVs following intracerebral [83–86], intravenous [87–91], or intranasal [92–95] administration was assessed on transgenic animal models reporting mutations associated with AD [83,84,87–91,93–95], or injected with Aβ1-42 oligomers [85,86] (Table 3).

Researchers mainly observed amelioration in cognitive decline [83,85–91,94,95] that resulted from many different mechanisms. For example, many studies reported reduced plaque deposition and Aβ levels [84,86–89,91,93–95]; interestingly, the primary mechanism for this effect was the presence of neprilysin on the surface of EVs [84]. Neprilysin is indeed a zinc metallopeptidase that previously showed capable of breaking down Aβ1-42 peptides [96–98]. Another important effect observed was regulating the inflammatory response [83,86–89,

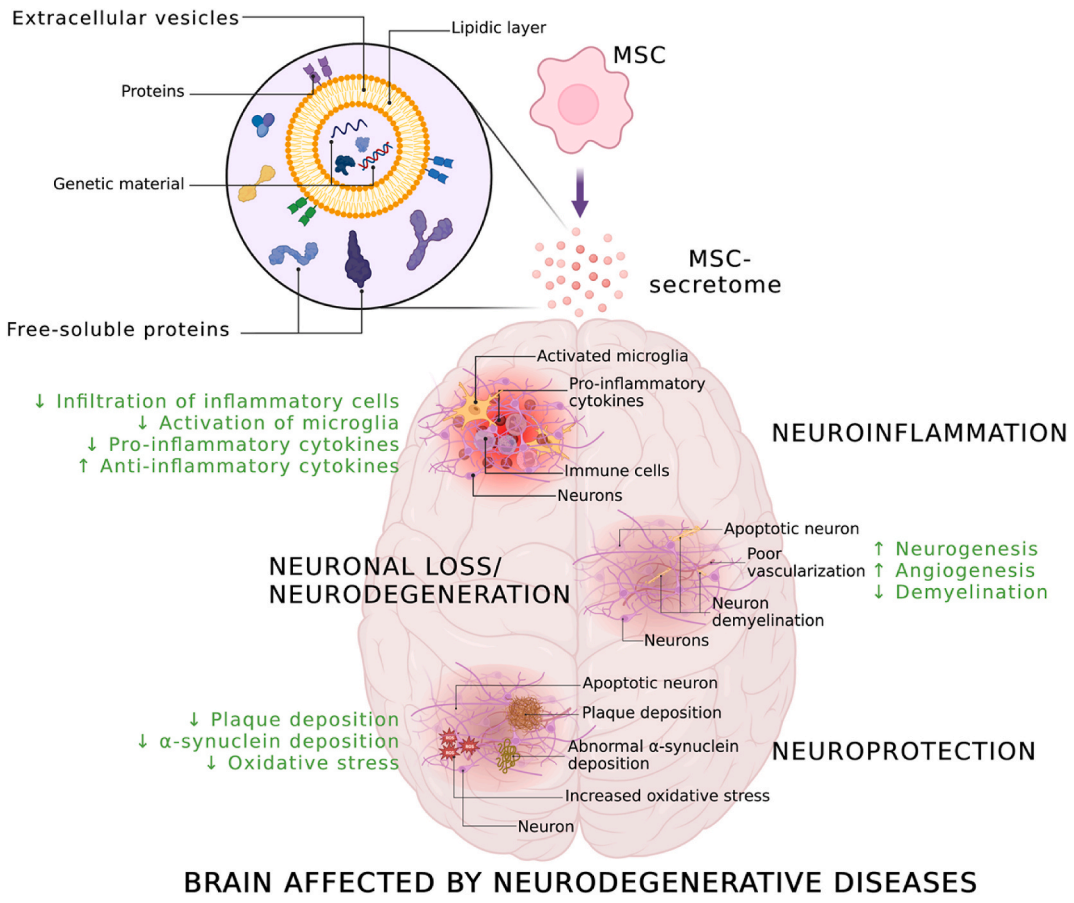

Fig. 1. MSCs as "drug stores". MSCs release secretome, which comprises free-soluble proteins and EVs enriched with genetic material. MSC-secretome and EVs exert multiple pharmacological effects; the mechanisms responsible for the anti-inflammatory, neuroregenerative, and neuroprotective effects are indicated. Created with BioRender.com.

92], specifically the downregulation of pro-inflammatory cytokines and the upregulation of anti-inflammatory ones [86,87,89]. According to other studies, the anti-inflammatory effect of MSC-secretome/EVs happened through the decrease/modulation of activated microglia [87,88,92,93], which, when chronically activated, is an important mediator of inflammation [99]. Also, EVs have been found to reduce the expression of inducible nitric oxide synthase (iNOS), which is induced by Aβ, leading to the release of high levels of nitric oxide (NO) [83]; this high NO level can cause neurotoxicity by inhibiting mitochondrial respiration and leading to neuronal cell death [100]. Finally, limited evidence was also reported regarding the rescue of synaptic dysfunction [87] or, more generally, a reduction in neuronal degeneration that resulted from neurogenesis [85,91,94] and neuroprotection [92,93].

Many researchers reported how such activities are affected by the extracellular environment. For example, exposure of MSCs to hypoxia generated EVs that showed a better effect in rescuing learning memory impairments than EVs from MSCs grown under normoxic conditions [87]. Cytokine preconditioning with tumor necrosis factor (TNF)-α and interferon (IFN)-γ up-regulated the immunomodulatory markers cyclooxygenase-2 (COX2) and indoleamine-pyrrole 2,3-dioxygenase (IDO), allowing EVs to obtain increased immunomodulatory functions [92]. Again, exposure of MSCs to brain homogenates from young AD mice did not result in a reparative effect in the MSC-secretome, but it did have an impact on impaired memory when obtained from MSCs that had been exposed to AD brain homogenates from older mice. This suggests that inflammatory molecules, absent or present in minimal amounts in the young brain, are relevant for licensing the neuroprotective effect of MSCs [94]. Finally, it is possible to target EVs to the brain to increase their potency. According to a research study, EVs were modified using a targeted rabies viral glycoprotein (RVG) peptide, which interacts specifically with the acetylcholine receptor, allowing entry into neuronal cells. The results demonstrated that RVG-EVs had a higher targeting efficiency than unmodified EVs, with concentrated fluorescence observed in the cortex and hippocampus [89].

3.2. *Parkinson's disease (PD)*

Multiple research teams have focused on utilizing MSC-secretome/EVs to develop novel therapeutic strategies for PD (Table 4). The majority of studies have evaluated their efficacy in animal models of PD using 6-hydroxydopamine (6-OHDA) [101–105] and 1-methyl-4--phenyl-1,2,3,6-tetrahydropyridine (MTPT) [106], with only one study using transgenic mice expressing human α-synuclein [107]. There have been no studies on the efficacy of MSC-secretome or EVs in humans.

The studies consistently report improved functional recovery [102,104,105,107,108], including better motor performance, reduced rotational asymmetry induced by apomorphine [101,103], and improved spatial learning ability [101]. These effects are attributed to increased dopamine levels in the striatum [103,106,108], reduced loss and apoptosis of dopaminergic neurons [101,103,107,109,110] together with the generation of new dopaminergic neurons [101,104] and neuronal terminals [102,104], and improved neuron function [111]. Proteomic characterization reveals that the secretome and EVs of MSCs harbor crucial neuroregulatory molecules that can be held responsible for the aforementioned effects. Such molecules include cystatin C, glia-derived nexin, galectin-1, pigment epithelium-derived factor, vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor, interleukin (IL)-6, and glial cell line-derived neurotrophic factor

Table 3
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of AD.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Wang et al. [83]	Adult male APP/PS1 double transgenic mice	EVs from mBM-MSCs	Intracerebral injection of EVs once per two days for two weeks	Improvement of cognitive behavior. Suppression of Aβ induced iNOS expression. Rescue impairment of CA1 synaptic transmission and long-term potentiation.
Cui et al. [87]	APP/PS1 double transgenic mice	EVs from normoxic or hypoxic mBM-MSCs	Injection via tail vein of 150 μg of EVs	Amelioration of cognitive decline. Better improvement of learning and memory capabilities by EVs released in hypoxic conditions. Rescue of synaptic dysfunction. Regulation of inflammatory responses. Reduction of plaque deposition and Aβ levels in the brain. Decrease of astrocytes and microglia activation. Down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory ones.
Ding et al. [88]	APP/PS1 double transgenic mice (7 months old)	EVs from hUC-MSCs	Injection via tail vein of 30 μg of EVs	Repair of cognitive dysfunctions. Clear Aβ deposition. Modulation of the activation of microglia in brains. Inflammatory-regulating effects.
Cui et al. [89]	APP/PS1 double transgenic mice (7 months old)	EVs from mBM-MSCs tagged or not with rabies viral glycoprotein (RVG)	Intravenous injection of 5×10^{11} EVs	Improvement of learning and memory capabilities. Reduction of plaque deposition and Aβ levels. Normalization of inflammatory cytokines levels. EVs tagged with RVG were more effective than those not tagged.
Elia et al. [84]	Adult male APP/PS1 double transgenic mice	EVs from mBM-MSCs	Intracerebral injection into the neocortex of 1×10^9 EVs	Reduction of Aβ plaque burden. Neprilysin into EVs had this action. Reduction of dystrophic neurites in both the cortex and hippocampus.
Reza-Zaldivar et al. [85]	C57BL/6 mice (7–8 weeks) injected with oligomer Aβ1-42	EVs from hMSCs	Stereotaxic intracerebral injection of 10 μg of EVs	Alleviation of cognitive impairment. Stimulation of neurogenesis in the subventricular zone.
Losurdo et al. [92]	Female 3xTg-AD triple-transgenic mice	EVs from cytokine-preconditioned hBM-MSCs	Intranasal administration of 100 μL of EVs	Induction of immunomodulatory and neuroprotective effects. Modulation of the activation of microglia cells. Increase of dendritic spine density.
Ma et al. [93]	Female APP/PS1 double transgenic mice (9 months old)	EVs from A-MSCs	Intranasal administration of 1 mg of EV protein per kg	Induction of neuroprotective effects. Increase of newborn neurons. Reduction of Aβ deposition. Decrease of microglia activation.
Santamaria et al. [94]	Male APP/PS1 double transgenic mice	Secretome from mBM-MSCs exposed in vitro to AD mouse brain homogenates of different ages	Intranasal administration once a week	Induction of persistent memory recovery. Reduction of plaques. Lower density of β-amyloid oligomers. Higher neuronal density in the cortex and hippocampus. Reduction in hippocampal shrinkage. Longer lifespan.
Chen et al. [90]	JAX-006293 transgenic mice	EVs from hWJ-MSCs	Intravenous injection of 50 μg of EVs	Improvement in cognitive function. Regulation of the neurons and astrocytes phases in the brain.
Cone et al. [95]	Male and female 5XFAD transgenic mice (6 weeks)	EVs from hBM-MSCs	Intranasal administration of 20×10^8 EVs in 5 μL	Improvement of behavior in cognitive tests. Lower Aβ plaque load in the hippocampus.
Sha et al. [86]	Sprague-Dawley rats (5–6 weeks) injected with oligomer Aβ1-42	EVs from rBM-MSCs	Stereotaxic intracerebral injection of 30 μg of EVs	Improvement in cognitive function. Reduction of Aβ deposition area, plaques, and Aβ contents. Reduction of inflammatory cytokines (interleukin (IL)-1β, IL-6, and TNF-α). Effects were mediated by miR-29c-3p carried by EVs that activated the Wnt/β-catenin pathway.
Wang et al. [91]	Male APP/PS1 double transgenic mice	EVs from hUC-MSCs	Injection via tail vein of 50 μg of EVs	Improvement of cognitive impairments. Reduction of the hippocampal Aβ aggregation. Reduction of neuronal loss. Restoration of the calcium oscillations. EVs acted through the Nrf2 signaling pathway.

m = mouse; h = human; r = rat.
BM = bone marrow; UC = umbilical cord; A = adipose; WJ = Wharton Jelly.

[102]. Evidence also suggests that MSCs secretome/EVs may reduce neuroinflammation [105,108,109,111], oxidative stress [105,108,110], and abnormal deposition of α-synuclein [107,111]. Only Xue and colleagues evidenced angiogenesis following the administration of EVs from human MSCs [106], likely linked to the presence of VEGF [112]. A single study compared the effectiveness of MSCs versus traditional anti-Parkinson drugs, such as levodopa [104]: the two treatments were effective the same in ameliorating the animal behavior; however, the human bone marrow (BM)-MSCs secretome appeared to be a modulator of the dopaminergic system, enhancing tyrosine hydroxylase (a marker for dopamine)-positive cell expression and terminals both in the substantia nigra and striatum.

The therapeutic potential of MSC-secretome and EVs was observed when administered directly into the substantia nigra and striatum [101,102,104,107,108], as well as through intravenous [103,109,110] or intraperitoneal [106] injection, thus confirming the ability of EVs, even

Table 4
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of PD.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Yao et al. [101]	Male Sprague-Dawley rats treated with 6-OHDA	rBM-MSCs secretome + neural stem cells	Implantation of 50 mg of secretome + 5×10^3 cells	Generation of dopaminergic neurons; promotion of cell survival, migration, and integration into damaged brain areas. Amelioration of functional recovery, with significant reduction of apomorphine-induced rotational asymmetry and improved spatial learning ability.
Teixeira et al. [102]	Male Wistar Han rats (10 weeks) treated with 6-OHDA	Secretome from hBM-MSCs	Injection into the substantia nigra and striatum	Increase of dopaminergic neurons and neuronal terminals. Improvement of motor performance.
Chen et al. [103]	Adult male Sprague-Dawley rats treated with 6-OHDA	EVs from hUC-MSCs	Injection via tail vein of 200 μg of EVs	Reduction of dopaminergic neuron loss and apoptosis in the substantia nigra; upregulation of the dopamine level in the striatum. Relieved apomorphine-induced asymmetric rotation.
Teixeira et al. [104]	Male Wistar Han rats (9 weeks) treated with 6-OHDA	Secretome from hBM-MSCs vs levodopa	Injection into the substantia nigra and striatum	Increase of tyrosine hydroxylase-positive cell expression and terminals. Significant amelioration of the motor outcomes.
Mahendru et al. [105]	Male Sprague-Dawley rats treated with 6-OHDA	Secretome from rBM-MSCs	Intravenous injection of 25 μg of secretome per kg	Significant modulation of inflammatory, oxidative stress and apoptotic markers. Amelioration of impaired neurobehavioral parameters.
Xue et al. [106]	BALB/c mice (8–10 weeks) treated with MTPT	EVs from hA-MSCs	Intraperitoneal injection of 200 μm/mL of EVs	Promotion of angiogenesis in the striatum and substantia nigra. Increase in dopamine production.
Yang et al. [107]	Male transgenic mice expressing A53T human α-synuclein	EVs from hBM-MSCs delivering antisense oligonucleotides targeting α-synuclein	Stereotaxic injection into the right lateral ventricle of 24 μg of EVs containing 20 μg of antisense nucleotides	Decrease of α-synuclein expression. Decrease of dopaminergic neuron degeneration. Improvement of locomotor functions.
Cai et al. [109]	Male C57BL/6 mice (6 weeks) treated with MTPT	EVs form BM-MSCs	Injection via tail vein of 200 μL of EVs	Decrease of neuron loss and injury. Reduction of the inflammatory response by inhibiting Sp1 signaling.
Li et al. [108]	Sprague-Dawley rats treated with 6-OHDA	EVs from quiescent rBM-MSCs vs EVs from rBM-MSCs during dopaminergic differentiation	Stereotaxic injection into the striatum of 15 μg of EVs	Downregulation of IL-6, IL-1β and TNF-α. Reduction of the reactive oxygen species levels in the substantia nigra. Increased expression of tyrosine hydroxylase mRNA. Rescue of the rotation behavior and climbing speed. EVs from quiescent cells were more effective.
Ma et al. [110]	Mice treated with 6-OHDA	hUC-MSCs loaded with miR-181a–2–3p	Injection via tail vein of 200 μg of EVs	Reduction of dopamine neurons apoptosis. Reduction of oxidative stress.
Peng et al. [111]	Male C57BL/6 mice (6–8 weeks) treated with MTPT	EVs from mBM-MSCs + curcumin	Intranasal administration	Reduction of α-synuclein aggregates. Promotion of neuron function recovery. Alleviation of neuroinflammation.

6-OHDA = 6-hydro-xydopamine; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
r = rat; h = human; m = mouse.
BM = bone marrow; UC = umbilical cord; A = adipose.

when administered systemically, to across the blood-brain-barrier reaching the substantia nigra [103] (Fig. 2). One study also investigated the possibility of administering EVs via intranasal administration [111].

3.3. Amyotrophic lateral sclerosis (ALS)

The application of MSC-secretome and EVs in ALS is recent, with only one study conducted on preclinical animal models [113] and two case reports after testing on humans [114,115] (Table 5).

However, all the reports consistently indicate an improvement in motor performance without any significant adverse effect [113–115]. Specifically, Crose's study reported that the patient, following the treatment with EVs from human BM-MSCs, could walk with minimal assistance for 25–50 feet after six months of no walking [114]. Similarly, Ueda and colleagues reported an improved range of motion in limbs [115]. Other improvements regarded speech and strength, the reduction of muscle spasms (and thus of the associated pain) [114], and reduced deterioration of pulmonary function [115]. These effects are likely linked to the protective effect exerted by MSC-secretome/EVs on limbal motoneurons, neuromuscular junction, and muscle and the protection from inflammation, as suggested by decreased glial cell activation [113]. Finally, improvement in sleep was also reported [114].

3.4. Multiple sclerosis (MS)

The primary experimental animal model of encephalitis used to assess the efficacy of MSC-secretome and EVs in treating MS was induced by administering oligodendrocyte glycoprotein peptide (MOG) 35–55 [116–123]. At the same time, only one example reported the development of the disease model through immunization [124] (Table 6).

Following intravenous [116–120,122–124] or intranasal [121] administration, secretome and EVs reduced the severity of the diseases, as improved motor function outcomes and symptoms were the main effects described by the researchers [118–121,123,124]. In this regard, a recent meta-analysis systematically reviewed the efficacy of MSC-EVs in preclinical rodent models of MS and reported an overall positive effect on the clinical score [125]. Also, MSC-secretome and EVs exerted anti-inflammatory and immunosuppressive properties [116,117,119–123], which markedly reduced the infiltration of inflammatory cells in the CNS [122,124] and modulated the cytokine levels [122–124]. This effect resulted from the presence in secretome and EVs of soluble immunomodulatory factors or their ability to inactivate NALP3 inflammasome and reduce NF-κB [116]. Furthermore, inhibition of antigen-specific T-cell activation [117] and reduction of microglial activation [117,123,124] were reported. More often, the administration of MSC-secretome or EVs reduced the demyelination process [117,119,

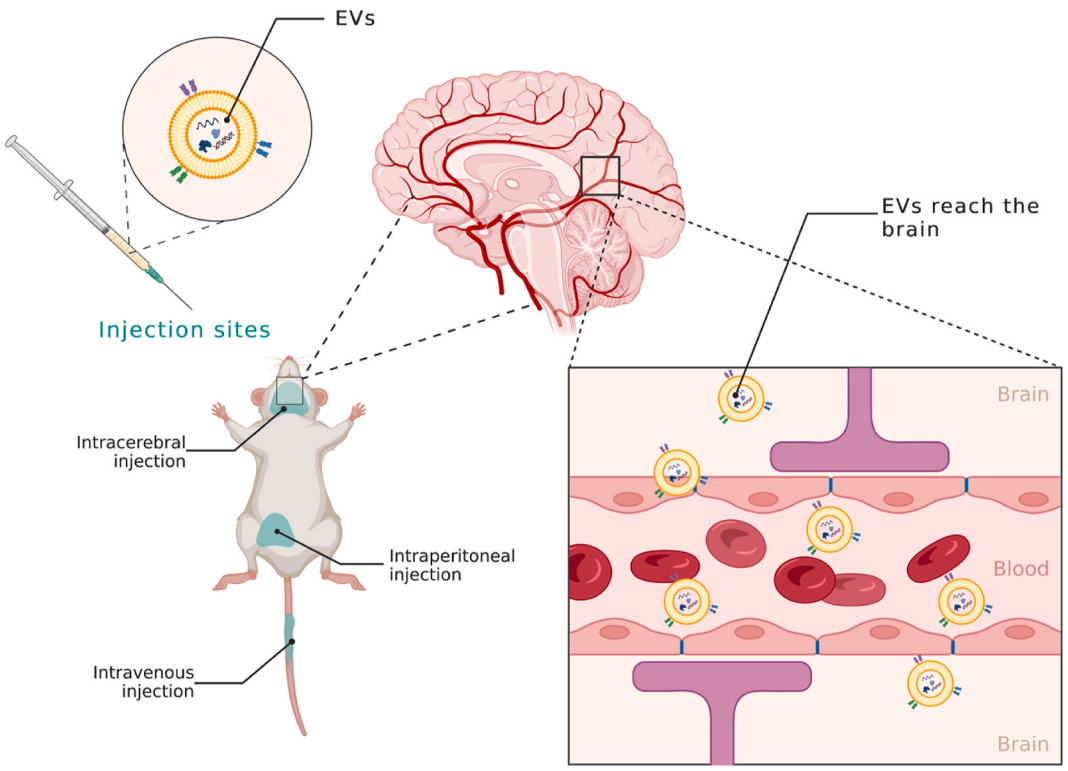

Fig. 2. Following intravenous, intracerebral, and intraperitoneal injection, MSC-EVs enter systemic circulation, cross the blood-brain barrier, and reach the brain. Created with BioRender.com.

120,124], while it was less evident an increase in myelination within the spinal cord [118,120,123]. Eventually, the immunomodulatory and remyelination effects of EVs may be increased by functionalizing their surface with LJM-3064 aptamer [119]. Finally, an increase in transforming growth factor (TGF)-β [121,124] and improvement in body weight were also reported [122].

3.5. Huntington's disease (HD)

Similarly to the previously described neurological conditions, MSC-secretome may help prevent the degeneration of neurons in the putamen, caudate and cerebral cortex observed in HD. However, to date, only Giampà and colleagues assessed the beneficial effects of the secretome from human amniotic fluid (AF)-MSC in an R6/2 mouse model of HD [126]. Following intraperitoneal injection, a protective effect in the CNS was observed. Specifically, the intervention led to a decrease in neurological dysfunction, a delay in hind paw clasping response during the tail suspension, an improvement in rotarod performance deficits, and a decrease in locomotor activity in an open field test. In addition, the study reported improvements in brain pathology, such as a reduction in striatal atrophy and the formation of striatal neuronal intranuclear inclusions, because of the intervention.

3.6. Acute ischemic stroke (AIS)

Many experimental studies investigated the effect of MSC-secretome and EVs in animal models of AIS obtained following transient [127–134] or permanent [135,136] middle cerebral artery occlusion, subcortical infarct [137] or intracerebral hemorrhage [138] (Table 7).

Following intravenous [127–130,132–138] or intraventricular injection [131], secretome/EVs were able to cross the blood-brain barrier [133] and exert their therapeutic effects, which ultimately led to improved functional recovery and neurological impairment [127–130,132–135,137,138]. Frequently, a reduction of the brain-infarct zone was observed [129,135,136], resulting from the enhanced neurogenesis [127,128,130,133–135,137,138] and angiogenesis [127,128,134,135,138]. Autophagy seems involved, even if studies contradicted its activation [131] or suppression [136]. Beyond a reduction of lactate dehydrogenase (LDH), p53, caspase 3, and NO levels [133], also an anti-inflammatory effect was often reported [135], which, according to Jiang and colleagues, occurs through the promotion of M2 microglia/macrophage polarization [136]; conversely, no effect on immune cell infiltration was reported by another study [128]. According to Lee and colleagues, the therapeutic effects of MSCs were increased when primed with normal or ischemic brain extract [135]; the enrichment of EVs with miR-17-92, instead, significantly increased neurogenesis [130]. Finally, some EVs were modified with pigment epithelial-derived factor [131], enriched with miR-30d-5p [136], or loaded with enkephalin [133]. A recent meta-analysis of the literature confirmed that EV interventions improved lesion volume with respect to control groups [139].

3.7. Spinal cord injury (SCI)

In animal models with severe [140,141] or moderate [142–146] vertebral contusion/compression or severe transection [147], MSC-secretome/EVs have been administered intraspinallly [140–142], intrathecally [143,145,147], intramuscularly [144] or intravenously [146], showing improved motor function recovery [140,142,143,147] (Table 8), as also highlighted by a recent meta-analysis [148]. Other effects observed were enhanced angiogenesis [140], a tissue protective effect [140,143], an increased axonal regeneration [141,145,146], and the attenuation of inflammation [143,144,147]. According to the study of Chudickova and colleagues, these effects were common among MSCs and their secretome; however, only secretome treatment resulted in additional enhancements of axonal sprouting and a decrease in the number of reactive astrocytes in the injury site [145].

Table 5
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of ALS and ALS patients.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Bonafede et al. [113]	SOD1 (G93A) mice	EVs from mA-MSCs	Intravenous or intranasal administration of 1 μg of EVs	Improvement of motor performance following repeated administration of EVs. Protection of lumbar motoneurons, neuromuscular junction, and muscle. Decrease of glial cell activation.
Crose [114]	Human	EVs from hBM-MSCs	Intravenous injection	Improvement of motor performance: walking with minimal assistance for 25–50 feet after six months of no walking. Improvement in speech and strength. Decrease in muscle spasms. Decrease of pain (from muscle spasms). Improvement in sleep.
Ueda et al. [115]	Human	Secretome from exfoliated deciduous teeth hMSCs	Intranasal and intravenous administration	Improvement of range of motion in limbs. Reduced deterioration of pulmonary function. No adverse effects.

m = mouse; h = human.
A = adipose; BM = bone marrow.

4. Exploring the current landscape and future possibilities of clinical trials using MSC-secretome and EVs in NDs: a forward-looking perspective

The studies described above constitute extensive evidence that supports the efficacy of MSC-secretome or MSC-EVs for treating different NDs, making them a new and promising cell-free alternative therapeutic agent. However, bringing a new medicinal product to the market involves a complex journey encompassing several stages, including molecular discovery, production process scale-up, dosage form formulation, preclinical and clinical trials, and, ultimately, regulatory approval. It is a process that takes between 10 and 15 years and requires a significant investment of hundreds of millions of dollars. These same principles also apply to MSC-secretome and EVs, but it is commonly evident to researchers active in this field that their translation into clinical practice has been slower than the standard path of medicines. It is, therefore, unsurprising that, despite the considerable evidence in preclinical animal models, clinical trial activation to evaluate the safety and efficacy of MSC-secretome and EVs in NDs is still limited (Table 9). Consequently, no regulatory-approved MSC-secretome or EV products are currently on the market.

This delay likely comes from the lack of a comprehensive and efficient path for achieving regulatory compliance of MSC-secretome or EVs, thus delaying the arrival of new potentially effective therapies in the clinical practice and amplifying the risk of losing the invested resources. Therefore, there is a need to bridge the gap between the academic laboratory scale, industrial production, and possible market distribution of MSC-secretome and EVs, once they are developed and approved. In this regard, it is desirable that since early investigations in the laboratory, fundamental aspects for the regulatory approval are taken into consideration; these include the need to define a robust and Good Manufacturing Practices (GMP)-compliant manufacturing process, with proper in-process and end-process controls that ensure the final product's pharmaceutical quality, stability, and shelf-life; the need to formulate MSC-secretome/EVs as any other drug into a pharmaceutical dosage form, and then the need to demonstrate formulation's safety and efficacy through in vitro and in vivo animal models and, finally, human clinical trials. Below will be discussed in-depth considerations about manufacturing and characterization, appropriate quality control measures, and preclinical safety and efficacy testing to ensure straightforward translation of MSC-secretome and EVs into the clinic. To this end, a hypothetic "path", reported in Fig. 3, will be taken into consideration; this scheme does not claim to be exhaustive but aims to put face-to-face with the high degrees of complexity in the clinical translation of MSC-secretome and EVs.

4.1. Scale-up and GMP manufacturing of MSC-secretome/EVs

The manufacturing process of MSC-secretome and EVs must be designed to allow the production of large quantities (scalable), possibly economically, maintaining batch-to-batch consistency and following GMP guidelines. The manufacturing of MSC-secretome/EVs can benefit from the methods currently employed in producing classical biologics, such as antibodies, proteins, or cell therapy products, that assure scalability and compliance with GMP. However, being even MSC-secretome and EVs produced by living organisms, the risks of the production process are increased compared to purely synthetic drugs, as the intrinsic biological variability leads inevitably to the heterogenicity of the final product. In other words, assuring batch-to-batch consistency may be difficult, as creating a replica of a biological product with the same characteristics is nearly impossible. Indeed, a unique relationship exists between the production process and the characteristics of the resultant final product: "the product is the process", meaning that the quality of the product, e.g., its yield, composition, and bioactivity, can be significantly influenced by both upstream and downstream processing choices [149] (Fig. 4). For instance, the culture conditions, including cell passage, cell density, and the frequency of secretome/EV harvesting, are critical upstream process steps. Therefore, these conditions must be defined early in the lab, and the upstream process should be appropriately designed to control all these parameters and the cell environment without forgetting the need to produce many cells for scalability reasons. This latter aspect may be achieved by culturing MSCs in multilayered culture flasks or, better, in bioreactors. Currently, there is no consensus on the optimal technology for MSCs production, but from a safety perspective, closed systems are preferred, even if monitoring can be more challenging than open systems [150]. Once MSC-secretome and EVs are released, technologies commonly used for biologics, such as (ultra)centrifugation, precipitation, size exclusion or affinity chromatography, depth filtration (adsorption), and tangential flow filtration (ultrafiltration), are used to collect them [151]. Even in this case, there is currently no consensus on the most appropriate isolation technique; some should be avoided as they are not scalable or may impact the integrity of EVs, such as ultracentrifugation [152]. Others may present challenges in obtaining simultaneously high yield and purity; for example, size exclusion or affinity chromatography allows highly pure preparations but with low yields, while ultrafiltration increases the final product yield but with lower purity [153].

To minimize batch-to-batch inconsistencies, numerous studies aim to develop synthetic EVs using various techniques such as top-down,

Table 6
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of MS.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Rajan et al. [116]	Male C57BL/6 mice (12 weeks old) treated with MOG35-55	Secretome or EVs from hPDL-MSCs	Injection via tail vein	Immunosuppressive action. NALP3 inflammasome inactivation. NF-κB reduction. These effects were exerted both by secretome and EVs.
Farinazzo et al. [117]	C57BL/6 mice (6–8 weeks old) treated with MOG35-55	EVs from mA-MSCs	Intravenous injection of 5 μg of EVs	Reduction of spinal cord inflammation and demyelination. Marginal inhibition of antigen-specific T cell activation. Reduction of microglial activation. Reduction of demyelination in the spinal cord.
Clark et al. [118]	Male and female C57BL/6J mice (3 months old) treated with MOG35-55	EVs from hP- MSCs	Injection via tail vein of 1 × 10^7 or 1 × 10^{10} EVs	Improvement of motor function outcomes. Improvement of symptoms. Reduction of DNA damage in oligodendroglia populations. Increase of myelination within the spinal cord.
Li et al. [124]	Female rats immunized with guinea pig spinal cord homogenate + Freund's adjuvant containing *Mycobacterium tuberculosis* H37Ra	EVs from rBM-MSCs	Injection via tail vein of 100 μg or 400 μg of EVs	Decrease of neural behavioral scores. Reduction of inflammatory cell infiltration into the CNS. Regulation of microglia polarization. Decrease of demyelination. Increase IL-10 and TGF-β levels. Reduction of TNF-α and IL-12 levels.
Shamili et al. [119]	Female C57BL/6 mice (10–13 weeks) treated with MOG35-55	EVs from mBM-MSCs conjugated with carboxylic acid-functionalized LJM-3064 aptamer	Intravenous injection of 200 μg of EVs	Reduction of the severity of the disease. Suppression of the inflammatory response. Reduction of demyelination.
Jafarinia et al. [120]	C57BL/6 mice (6–8 weeks old) treated with MOG35-55	EVs from hA-MSCs	Intravenous injection of 60 μgof EVs	Decrease of the maximum mean clinical score. Reduction of inflammation scores. Reduction of the percentage of demyelination areas.
Fathollahi et al. [121]	Female C57BL/6 mice (6–8 weeks old) treated with MOG35-55	EVs from mA-MSCs	Intranasal administration of 10 μg of EVs	Decrease the clinical symptoms. Increase in immunomodulatory responses. Increase of TGF-β levels.
Koohsari et al. [122]	Female C57BL/6 mice (6–8 weeks old) treated with MOG35-55	EVs from hUC-MSCs	Intravenous injection	Reduction of pro-inflammatory cytokines (IL-17a, TNF-α, and IFN-γ). Increase of the anti-inflammatory cytokines (IL-4 and IL-10). Decrease of leukocyte infiltration. Improvement of body weight.
Zhang et al. [123]	Female C57BL/6 mice (6–8 weeks old) treated with MOG35-55	EVs from moMSCs	Injection via tail vein of 5 × 10^{10} EVs	Improvement of neurological outcome. Increase of myelin basic protein. Decrease of neuroinflammation. Modulation of microglia activation. Modulation of cytokine levels.

MOG35-55 = oligodendrocyte glycoprotein peptide.
h = human; m = mouse; mo = monkey.
PDL = periodontal ligament; A = adipose; P = placenta; BM = bone marrow; UC = umbilical cord.

bottom-up, or biohybrid approaches, as discussed in Ref. [154]. In a top-down strategy, the parent cells are dismantled using extrusion-based, filtration, or microfluidic methods, resulting in EVs that retain the membrane protein topology [155]. Conversely, a bottom-up approach requires an in-depth understanding of the composition of natural EVs, particularly their key components, which can be chemically synthesized and then used as the building blocks for constructing larger and more intricate artificial structures. In both cases, as EVs are no longer exclusively produced by living organisms, the inherent biological variability and consequent heterogeneity of the final product are diminished. Moreover, generating artificial EVs offers practical advantages for translation due to simpler production processes, and the yield can be improved by combining cell-derived lipids with synthetic ones, as long as the resulting hybrid EVs retain their targeting and uptake properties. Nevertheless, despite the progress made thus far, substantial further research is required to achieve artificial EVs with identical or fully comparable characteristics to those derived from cells.

4.2. Formulation of MSC-secretome and EVs

As with any other active ingredient, once produced, MSC-secretome and EVs must be formulated and stored at appropriate conditions until use (Fig. 4). Some studies investigated the most appropriate storage conditions of EVs (−80 °C vs 4 °C) by assessing the size, charge, and

Table 7
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of AIS.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Xin et al. [127]	Adult male Wistar rats were subjected to transient MCAO	EVs from rBM-MSCs	Intravenous injection of 100 μg of EVs	Improvement in functional recovery. Increasing in axonal density and synaptophysin-positive areas. Increasing in the number of newly formed doublecortin (a marker of neuroblasts) and von Willebrand factor (a marker of endothelial cells) cells.
Doeppner et al. [128]	C57BL6 mice subjected to transient MCAO	EVs from hBM-MSCs	Intravenous injection	Improvement in neurological impairment. Long-term neuroprotection. Enhancement in angiogenesis and neurogenesis. Cerebral immune cell infiltration was not affected.
Chen et al. [129]	Adult male Sprague-Dawley rats subjected to transient MCAO	EVs from mini-pig A-MSCs	Intravenous injection of 100 μg of EVs	Reduction of the brain-infarct zone. Enhancement in neurological recovery.
Lee et al. [135]	Male Sprague-Dawley rats subjected to permanent MCAO	EVs from hMSCs primed with normal/ischemic brain extract	Intravenous injection of 0.2 mg of EVs per kg	Enhancement of angiogenesis. Enhancement of neurogenesis. Anti-inflammatory action. Enhancement of behavioral recovery. Reduction of the brain infarct zone. EVs from MSCs primed with normal or ischemic brain extract had significantly greater efficacy.
Xin et al. [130]	Adult male Wistar rats subjected to transient MCAO	EVs from rBM-MSCs enriched or not with miR-17-92	Intravenous injection of 100 μg of EVs	Improvement of functional recovery. Improvement of neurological function. Enhancement of oligodendrogenesis, neurogenesis, and neurite remodeling/neuronal dendrite plasticity in the ischemic boundary zone. This effect was primarily observed for EVs enriched with miR-17-92.
Otero-Ortega et al. [137]	Male Sprague-Dawley rats subjected to SCI	EVs from rA-MSCs	Intravenous injection of 100 μg of EVs	Improvement of functional recovery. Enhancement of fiber tract integrity, axonal sprouting and white matter repair markers.
Han et al. [138]	Adult male Wistar rats subjected to ICH	EVs from rBM-MSCs	Intravenous injection of 100 μg of EVs	Improvement of neurological function. Improvement of motor recovery. Enhancement of angiogenesis. Enhancement of neurogenesis.
Huang et al. [131]	Adult male Sprague-Dawley rats subjected to transient MCAO	EVs from rA-MSCs modified with pigment epithelial-derived factor	Intraventricular injection of 100 μg of EVs per kg	Amelioration of cerebral injury. Activation of autophagy. Suppression of neuronal apoptosis.
Jiang et al. [136]	Adult male Sprague-Dawley rats subjected to permanent MCAO	EVs from rA-MSCs enriched with miR-30d-5p	Intravenous injection of 80 μg of EVs	Reduction of the cerebral injury area of infarction. Suppression of autophagy. Promotion of M2 microglia/macrophage polarization.
Geng et al. [132]	Sprague-Dawley rats subjected to transient MCAO	EVs from miRNA-126-modified AD-MSCs	Intravenous injection	Improved functional recovery and significant increase of the expression of von Willebrand factor (an endothelial cell marker) and doublecortin (a neuroblasts marker). Decrease of neuron cell death and increase of cell proliferation.
Liu et al. [133]	Sprague Dawley rats (8–12 weeks) subjected to transient MCAO	EVs from rBM-MSCs loaded with enkephalin	Intravenous injection	EVs crossed the blood-brain barrier. Reduction of LDH, p53, caspase 3, and NO levels. Improvement of brain neuron density. Improvement of the neurological score.
Moon et al. [134]	Sprague-Dawley rats subjected to transient MCAO	EVs from rMSCs	Intravenous injection of 30 μg of EVs	Enhancement of neurogenesis. Enhancement of angiogenesis. Improvement of behavior.

MCAO = middle cerebral artery occlusion; ICH = intracerebral hemorrhage; SCI = subcortical infarct.
r = rat; h = human.
BM = bone marrow; A = adipose.

number of EVs and reported that the act of storing EVs can alter their size distribution, reduce their quantity and contents, and affect their cellular uptake and biodistribution [156]. Therefore, storage at −80 °C is generally recommended, even if it is the logistically most challenging and costly method. As an alternative for long-term storage, it has been proposed to freeze-drying of MSC-secretome and EVs [62,64,157–160], and using cryo/lyoprotectants, it was demonstrated that the biological activity is preserved when tested both in vitro [64,66,68,161–163] and in vivo [66,164]. Commonly used excipients suitable for the formulation of MSC-secretome and EVs may include: (i) buffers, such as TRIS, histidine, and sodium acetate, to stabilize the pH at appropriate values that prevent protein/lipid degradation; (ii) salts, such as NaCl, KCl, $MgCl_2$ to make the preparation isosmotic and thus suitable for injection; (iii) antioxidants, such as methionine, to prevent the protein/lipid oxidation; (iv) sugars/polyols, such as mannitol, trehalose, sucrose, glucose, that act as stabilizers or cryo/lyoprotectants and as such are helpful when the final product is frozen or freeze-dried; (v) for the only EV preparations, the addition of proteins, such as albumin, may be helpful to stabilize the lipidic layer [165] (Fig. 5).

4.3. Quality controls in-process and on the final product

Quality-by-design is increasingly recommended by both Food and Drug Administration (FDA) and European Medicines Agency (EMA) when manufacturing a medicinal product, and it should also be applied for MSC-secretome and EVs (Fig. 6). According to this approach, quality should be built in by design, meaning that the Quality Target Product Profile (QTPP), i.e., the quality of the final product, relies on the identification of the Quality Attributes of the drug substances and excipients that should remain within a specific range throughout the entire manufacturing process, thus becoming Critical Quality Attributes (CQAs) [166]. In our opinion, CQAs for MSC-secretome and EVs products are the amount of proteins and lipids, expression of functional markers, EV size and concentration, the absence of contaminants, no

Table 8
Summary of therapeutic efficacy and safety of MSC-secretome and EVs in preclinical animal models of SCI.

Study	Animal model	Therapeutic agent	Route and dosage	Main outcomes
Cantinieaux et al. [140]	Adult female Wistar rats subjected to severe contusion at T11	Secretome from rBM-MSCs	Intraspinal injection	Improvement of motor recovery. Enhancement of angiogenesis. Tissue protection.
Asadi-Golshan et al. [142]	Adult male Sprague-Dawley rats subjected to moderate compression at T7	Secretome from human exfoliated deciduous teeth hMSCs	Intraspinal injection	Improvement of functional recovery.
Cizkova et al. [143]	Adult male Wistar rats subjected to moderate compression at T8-T9	Secretome from rBM-MSCs	Intrathecal injection	Improvement of motor function recovery. Increasing in spared spinal cord tissue. Enhancement of GAP-43 expression. Attenuation of inflammation. Reduced levels of IL-2, IL-6, and TNF-α.
Kanekiyo et al. [141]	Adult female Sprague-Dawley rats subjected to severe contusion at T8-T9	Secretome from rBM-MSCs	Continuous injection through the cerebrospinal fluid via the fourth ventricle	Improvement of locomotion. Tissue repair. Axonal regeneration.
Khoshsirat et al. [144]	Female Wistar rats subjected to moderate contusion at T9-T10	Secretome from BM-MSCs	Intramuscular injection	Reduction of inflammation. Reduction of TGF-β1 expression. Increasing in mean spinal cord volume.
Chen et al. [147]	Adult female Sprague-Dawley rats subjected to severe transection at T8	Secretome from hBM-MSCs	Intrathecal injection	Reduction of inflammation. Improvement of functional restoration.
Chudickova et al. [145]	Male Wistar rats subjected to moderate compression at T8	hWJ-MSCs vs secretome from hWJ-MSCs	Intrathecal injection	Increasing of spared grey and white matter. Enhancement of expression of genes related to axonal growth. Only secretome treatment resulted in additional enhancements of axonal sprouting and decreased the number of reactive astrocytes in the injury site.
Tsai et al. [146]	Adult female Sprague-Dawley rats subjected to moderate contusion at T10	Secretome from rBM-MSCs	Intravenous administration of 150 μL	Improvement of behavioral recovery. Increasing of axons in the lesion site. Upregulation of Olig 2 and Heat Shock Protein 70 (HSP70) levels and autophagy-related proteins in the injured spinal cords.

r = rat; h = human.
BM = bone marrow; WJ= Wharton's Jelly.

Table 9
MSC-secretome and EVs in clinical trials for neurological diseases. Source: http://clinicaltrials.gov, last search 31 May 2023; search terms: mesenchymal stem cells secretome or mesenchymal stem cell exosome or mesenchymal stem cell extracellular vesicles and Alzheimer's disease or Parkinson's disease or amyotrophic lateral sclerosis or multiple sclerosis or Huntington's disease or acute ischemic stroke or spinal cord injury.

Disease	Clinical Trial ID Number	Study Design	Treatment	Year and Location	Status
AD	NCT04388982	Phase I/II, interventional, open-label	MSCs-EVs	2020, China	Completed, without results
PD/MSA	NCT04876326	Single-group assignment, interventional, open-label	Autologous/allogenic UC-MSCs + A-MSC-secretome	2021, Indonesia	Recruiting
AIS	NCT05008588	Phase I/II, interventional, open-label	UC-MSCs + their secretome	2022, Indonesia	Recruiting
	NCT03384433	Single-group assignment, interventional, open-label	MSCs-EVs	2019, Islamic Republic of Iran	Unknown

microbial, mycoplasma or viral contamination, low endotoxins, and batch-to-batch consistency. CQAs should also include parent cell properties (e.g., MSCs should fulfill the minimal requirement adherence to the International Society for Cellular Therapy (ISCT) criteria [167], be viable and other minimal criteria [168]) and, significantly, potency tests. Indeed, functional aspects are necessary to demonstrate that the final product maintains the biological activity, which has to be tested through standardized potency assays that reflect, likely for MSCs, the hypothesized mechanism of action [169]. Then, an in-depth understanding of the process and the process parameters is needed to identify the Critical Process Parameters (CPPs) and Critical Material Attributes (CMAs) and evaluate for their association and the impact that they may have on CQAs. Practically, in-process controls must be implemented to ensure the final product meets the previously identified CQAs. Whenever the quality of the final product is hampered, for example, by an incomplete purification, a lack of stability, or insufficient reproducibility during a specific step in the manufacturing process, it is possible to set in and solve the situation.

Unfortunately, detecting and evaluating changes in product quality for MSC-secretome and EVs is difficult due to their size and structural and compositional complexity. Indeed, the limitations of current analytical technologies hinder the detailed characterization of the physicochemical, immunochemical, or functional properties of MSC-secretome/EVs [170]. According to the most recent Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, EVs must be characterized in size and yield, using, for example, the nanoparticle tracking analysis, morphology and presence of a bilayer by electron microscopy; at least one transmembrane or cytosolic protein should be detected to differentiate EVs from cell debris or other contaminants [171,172]. In addition, other technologies like mass spectrometry may contribute to a more in-depth characterization with proteomic, lipidomic, and transcriptomic studies [173]. Finally, as identifying all the components in a drug that trigger specific pharmacological, immunological, or metabolic effects is mandatory, the challenge in the characterization of MSC-secretome and EVs may further slow down their translational process.

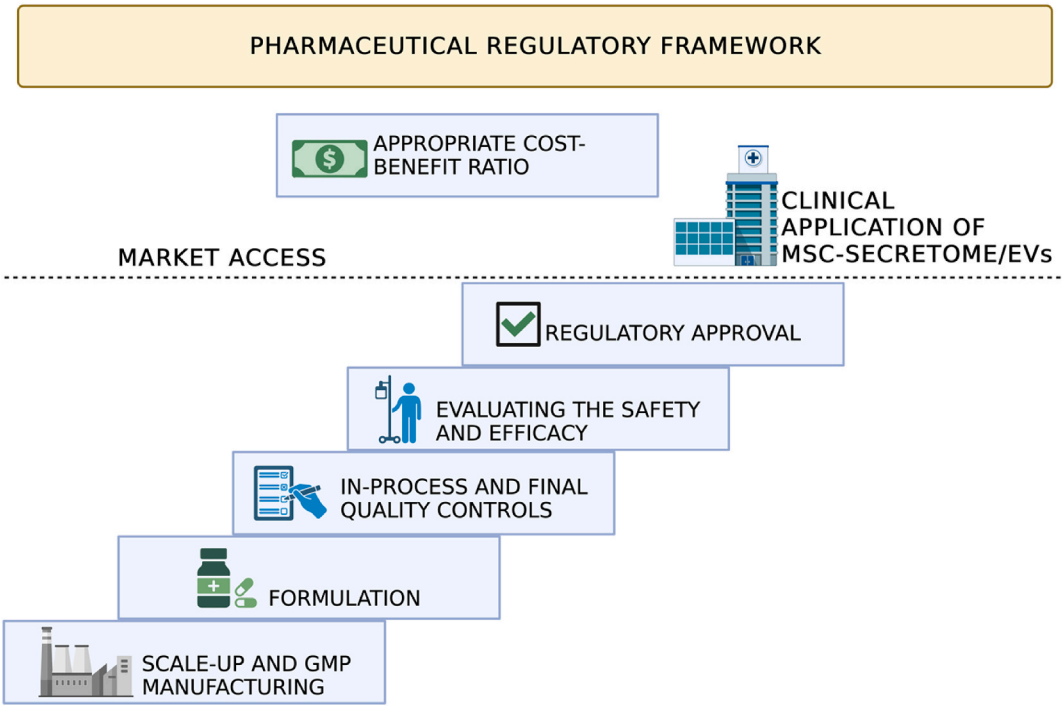

Fig. 3. The "stair" to translate MSC-secretome and EV into the clinic (and the market) is made of many steep steps, all "under" the pharmaceutical regulatory framework. Created with BioRender.com.

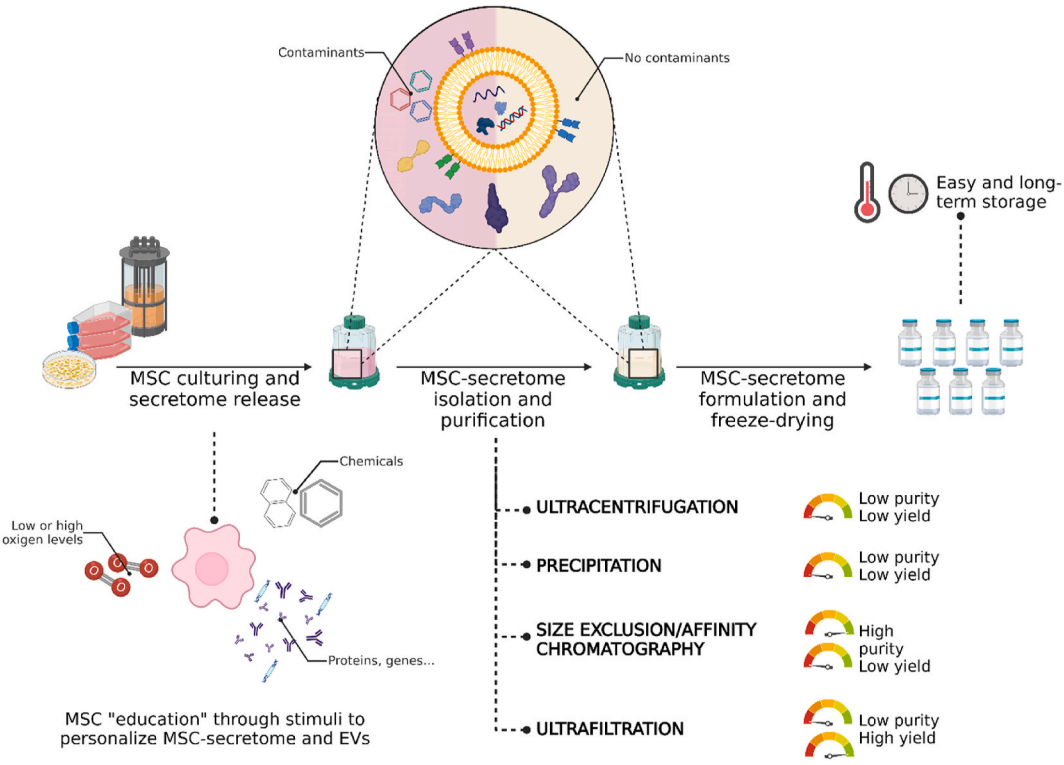

Fig. 4. Upstream and downstream processing choices when preparing medicinal products containing MSC-secretome and EVs. As "the product is the process", any minimal changes introduced may affect the qualitative and quantitative composition and biological activity of the final product. However, this can be exploited to "educate" cells to produce personalized secretome/EVs. Created with BioRender.com.

4.4. Evaluating the safety and efficacy of MSC-secretome/EVs

Safety and efficacy of MSC-secretome and EVs must be ensured in their final formulation, at first in small and large animals and then in humans, evaluating immunotoxicology and inflammatory potential based on the dose, dosage form, and route of administration. Specifically, the investigations must include pharmacokinetics and pharmacodynamic assessment, immunogenicity, biocompatibility, and overall

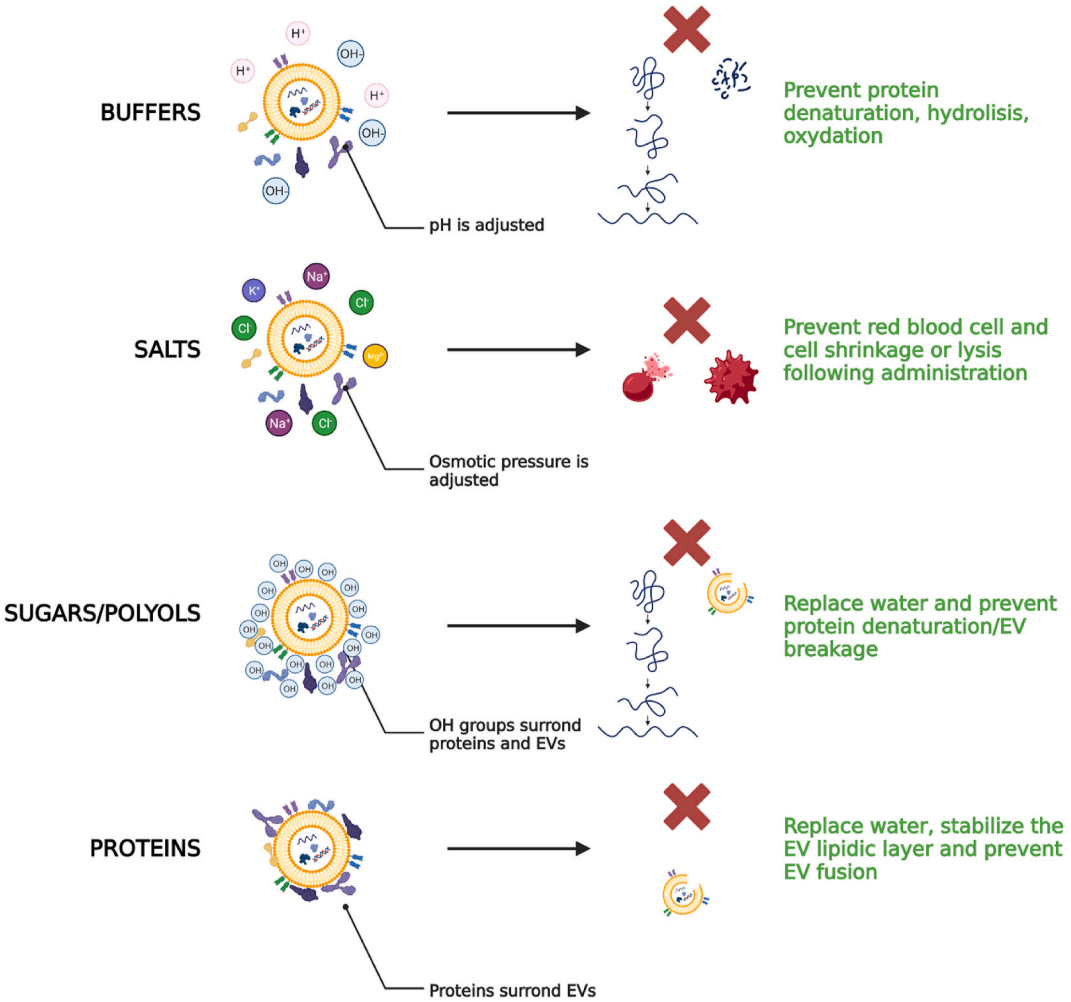

Fig. 5. Excipients suitable for the formulation of MSC-secretome and EVs. Buffers stabilize the pH to appropriate values compatible with the body administration and the product stability (e.g., to minimize hydrolysis and oxidation of proteins and lipids). Salts increase the ionic concentration making the preparation isosmotic with the body fluids. Sugars and polyols have many OH groups that surround and stabilize (by H-bonds) proteins and EVs even when the water in the formulation is subtracted following freezing or freeze-drying. Finally, proteins are adsorbed on the surface of EVs, creating a protein corona that prevents EV fusion and stabilizes the lipidic layer when the water in the formulation is subtracted following freezing or freeze-drying. Created with BioRender.com.

safety. Regarding biodistribution, many studies reported a short life of EVs administered systemically, following a non-specific accumulation of EVs in the liver, spleen, lung, and gastrointestinal tract [175]. As the large-scale pharmaceutical production and production costs impose the use of MSC-secretome and EVs in an allogeneic setting, continuous monitoring should be applied to assess immunologic and oncogenic effects, risk of microbial, mycoplasma or viral contamination, an adequate level of endotoxin and, for formulations administered intravenously, blood compatibility [170]. Once the animal model(s) pertinent to human disease has been identified, establishing a correlation between animal and human data becomes crucial. This can be accomplished by combining computational or theoretical modeling and experimental results or leveraging advanced technologies such as organs-on-chips. This approach allows for more precise predictions regarding the efficacy and safety of MSC-secretome and EVs in clinical trials, which must, in any case, be done with an appropriate design and under the control of the regulatory authorities. Even during the clinical trials, to comprehensively evaluate the efficacy and safety of MSC-secretome and EVs, it is crucial to establish their identity and purity, but achieving this goal requires conducting a systematic investigation that involves characterizing EVs and studying their interaction with the body's cells and tissues, which, again, can be a challenging task. Furthermore, guidelines from regulators regarding evaluating the safety and potency of MSC-secretome or EVs have not yet been released, with varying laboratories and companies utilizing different assays.

4.5. *Moving toward regulatory approval and the market access*

At the end of the path described above, all the data required to prepare the Common Technical Document (CTD) will be collected. The CTD consists of drug application dossiers for registering a medicinal product in the European Union, Japan, and the United States. It is specifically composed of four modules: module 2, which collects the summaries of the subsequent modules (i.e., the quality overall summary, the nonclinical overview and summary, and the clinical overview and summary); module 3, which is specifically dedicated to the quality of the final medicinal product; the module 4, which collects the non-clinical study reports; and module 5 which is dedicated to the clinical studies report (Table 10).

After carefully evaluating the CTD, if approved by regulatory authorities, MSC-secretome and EVs would finally have access to the market. At this point, the success of MSC-secretome and EVs in treating NDs would also depend on their relative costs, value for money, and impact on total expenditure. These domains, together with disease severity and the level of unmet clinical need, are usually considered by third payers (national health services, social insurance systems, private

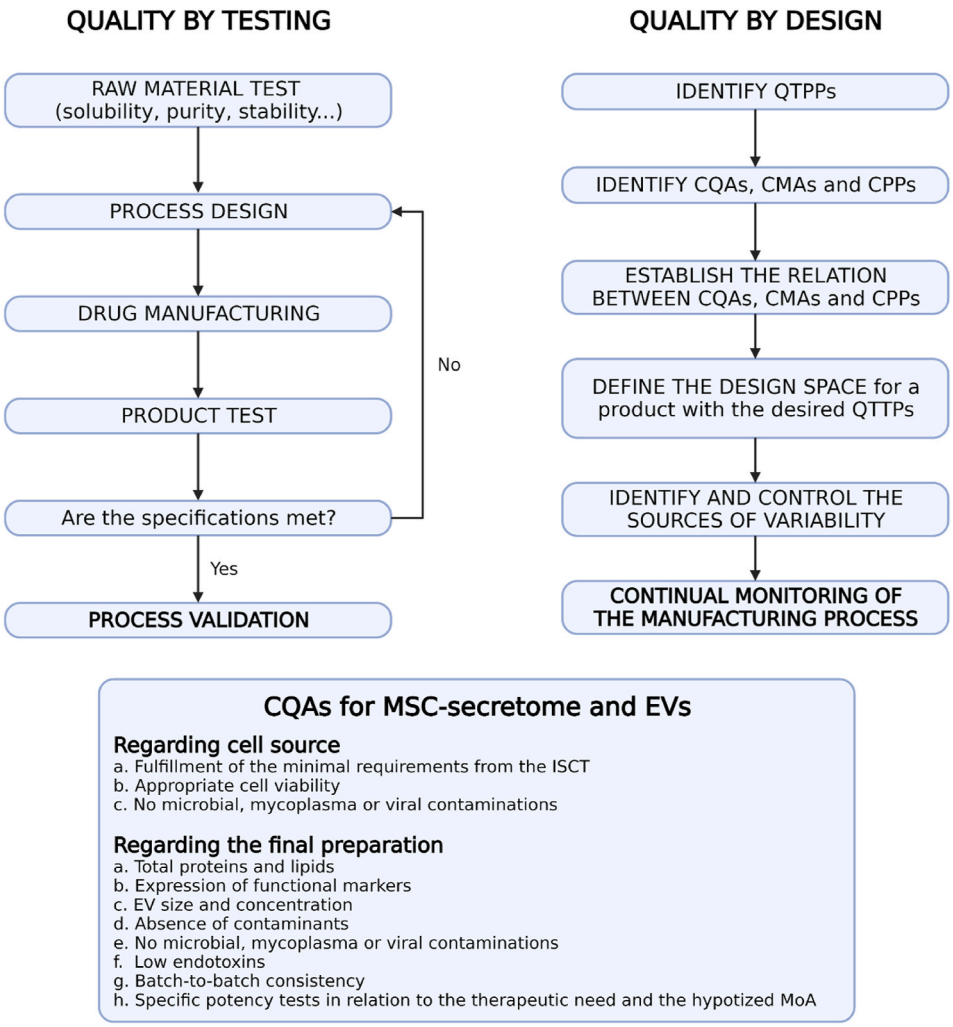

Fig. 6. Comparison between quality by testing and quality by design, and CQAs for MSC-secretome and EVs. Modified from Ref. [174]. Created with BioRender.com.

Table 10
Composition of the CTD.

	Content
Module 1	Administrative information (not formally part of the CTD).
Module 2	Quality overall summary; nonclinical overview; nonclinical summary; clinical overview; clinical summary.
Module 3	Chemical, pharmaceutical, and biological information for medicinal products containing chemical and/or biological active substances (quality).
Module 4	Non-clinical studies report (safety).
Module 5	Clinical studies report (safety and efficacy).

insurance) when they decide the reimbursement status of a new treatment [176,177]. MSC-secretome may have lower costs than stem cell therapies because appropriate facilities (e.g., the cell factories) have to be implemented only in the production site and not even in the administration site. Furthermore, pharmaceuticalizing MSC-secretome and EVs into freeze-dried and stable powder products would help make this therapy even less costly. In this regard, as MSC-secretome/EVs have been shown to reach the brain and exert their therapeutic effect also following intranasal administration [92–95,111,113,115,121], the possibility of formulating them in nasal dosage form, with reduced complexity for end-users (i.e., clinicians and health care organizations) with respect to parenteral formulations, could be relevant for market penetration. As pictured in Fig. 7, once administered intranasally, the proteins and EVs of MSC-secretome could reach the brain after being uptaken by the terminal axons of the trigeminal nerve or following the olfactory pathway, i.e., penetrating through the olfactory nerve, or the olfactory epithelium via the transcellular or paracellular pathway [178]. The residence time of the formulation in the nasal cavity should be increased using positively charged polymers that interact with the negative-charged mucus (e.g., chitosan, carbopol, polyacrylic acid, carboxymethylcellulose, and hypromellose); bile salts, fatty acids, cyclodextrins, and hydrophilic polymers, may instead be used as penetration enhancers to improve adsorption through the biological barriers [179,180]. Surfactants should be avoided to prevent the dissolution of the EV lipidic layer, with consequent loss and degradation of their cargo, and attention should be paid to the preservatives selected, as some of them may affect mucociliary clearance due to ciliostatic or ciliotoxic effects (these effects are typical of chlorbutol, hydroxybenzoate, methyl hydroxybenzoate, propyl hydroxybenzoate, chlorocresol, benzalkonium chloride, edetate, thiomersal, and phenylmercuric acetate). The typical liquid, semisolid or particulate formulations already extensively studied for intranasal delivery, as reviewed in Ref. [178], should be appropriate also for MSC-secretome and EVs, however considering that there is no need for nanoencapsulation, as EVs already act as natural nanocarriers.

The actual treatment costs are also contingent on dosage, timing, and frequency of administration of MSC-secretome and EVs for the relevant NDs, which will be determined during the clinical development

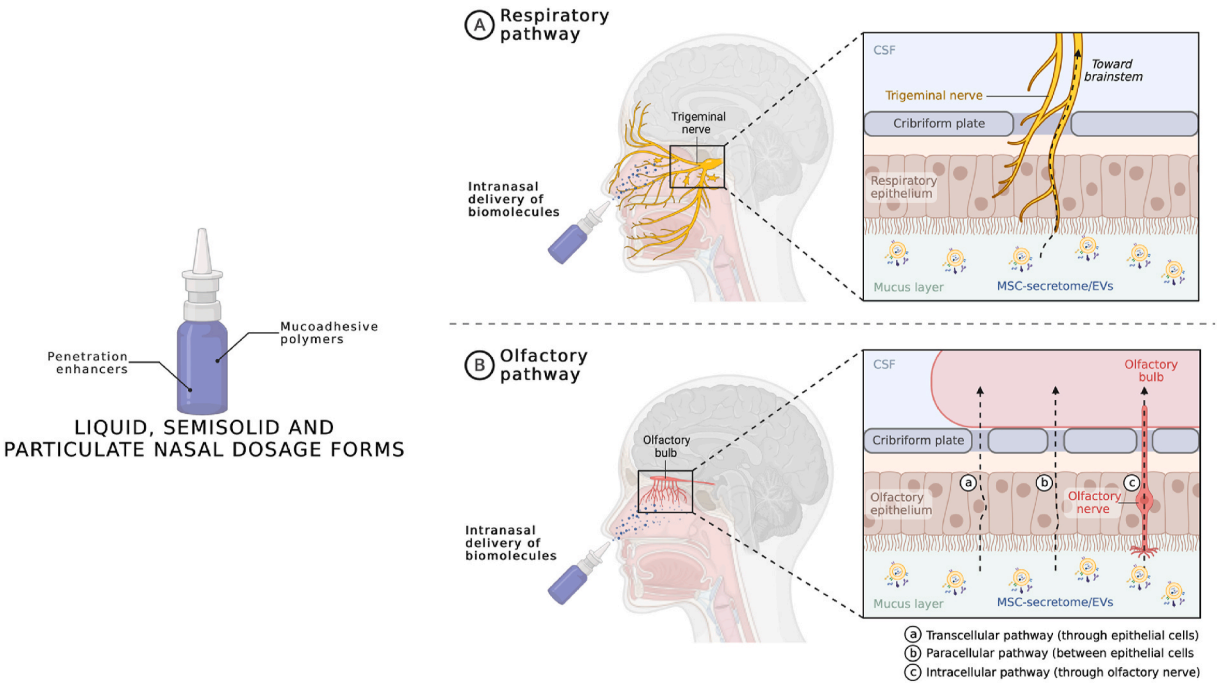

Fig. 7. Formulation of MSC-secretome/EVs as a nasal dosage form to enhance market penetration in treating NDs. Mucoadhesive excipients should be used to increase the residence time of the liquid, semisolid or particulate formulation in the nasal cavity, while penetration enhancers should be used to improve the passage of MSC-secretome/EVs through the biological barriers. CSF = cerebrospinal fluid. Created with BioRender.com.

processes in phase III clinical trials for that specific disease. More importantly, the value-for-money of treatments based on MSC-secretome and EVs will also depend on their relative risk-benefit profile. If they provide an added therapeutic value, are appropriately formulated to reduce complexity for end-users, and are less costly than competitors, they will be reimbursed. If not, payers will decide on the grounds of comparative cost-benefit analysis.

5. Conclusions

NDs are placing a significant and growing burden on healthcare systems worldwide, making the need for effective treatments urgent. According to the recent literature, MSCs offer a promising therapy due to their neuroregenerative, neuroprotective, and immunomodulatory properties, which are, however, linked to the bioactive substances they release, collectively known as secretome. In this regard, clinical and preclinical studies have shown that MSC-secretome and EVs can improve cognitive decline, plaque deposition, inflammatory response, and neuronal degeneration in AD, enhance dopamine levels, reduce loss and apoptosis of dopaminergic neurons, and improve motor performance in PD, boost motor performance, speech, strength, and sleep, and reduce glial cell activation in ALS, and decrease disease severity and improve motor function outcomes in MS. However despite, overall, MSC-secretome and EVs exhibit significant potential as a therapeutic strategy for the treatment of NDs, further research is necessary to optimize their efficacy and safety. Efforts should also be dedicated to addressing the challenges associated with their pharmaceutical manufacturing, including scalability, batch-to-batch consistency, and adherence to GMP guidelines. In addition, formulation and storage of MSC-secretome and EVs, along with quality controls, including the requirement for potency tests to demonstrate that the final product maintains its biological activity, should be considered. Finally, an appropriate cost analysis must be performed to assess the positive cost-benefit ratio for MSC-secretome and EVs with respect to the current therapies for NDs, but, unfortunately, this will be possible only after defining the dosage, timing, and frequency of administration of MSC-secretome and EVs in phase III clinical trials for that specific NDs.

Funding

This work was partially supported by Interreg V-A Italy-Switzerland 2014–2020, ATEx—Advanced Therapies Experiences. Project ID 3859153 and by the Italian Ministry of Health (Project MINSAL_INVITRO_TUMOR, CUP E85F21003590001).

Ethics approval and consent to participate

Not applicable, as it is a review.

Declaration of competing interest

Maria Luisa Torre is a co-founder and member of the advisory board of Pharmaexceed S.r.l.

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