Concise Review: Extracellular Vesicles Overcoming Limitations of Cell Therapies in Ischemic Stroke

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Key Words. Stem/progenitor cell • Stem cell transplantation • Nervous system • Neural differentiation • Mesenchymal stem cells

ABSTRACT
Despite recent advances in stroke therapy, current therapeutic concepts are still limited. Thus, additional therapeutic strategies are in order. In this sense, the transplantation of stem cells has appeared to be an attractive adjuvant tool to help boost the endogenous regenerative capacities of the brain. Although transplantation of stem cells is known to induce beneficial outcome in (preclinical) stroke research, grafted cells do not replace lost tissue directly. Rather, these transplanted cells like neural progenitor cells or mesenchymal stem cells act in an indirect manner, among which the secretion of extracellular vesicles (EVs) appears to be one key factor. Indeed, the application of EVs in preclinical stroke studies suggests a therapeutic role, which appears to be noninferior in comparison to the transplantation of stem cells themselves. In this short review, we highlight some of the recent advances in the field of EVs as a therapeutic means to counter stroke.

STATE-OF-THE-ART STROKE TREATMENT
Ischemic stroke treatment currently involves three concepts: The admission of stroke patients to stroke units, the application of thrombolytics, and the recanalization of the occluded vessel by endovascular clot removal [1–4]. With the first stroke units being introduced in the 1990s, stroke management has turned from a purely observational field toward an evidence based therapeutic field. Randomized studies not only demonstrated the utility of the thrombolytic recombinant tissue plasminogen activator to improve stroke outcome when administered intravenously within 4.5 hours after symptom onset [5], but more recently revealed the efficacy of endovascular recanalization therapy [1, 2]. Despite this great success, the majority of patients receive none of the two aforementioned treatments, partially because of narrow time windows or because of significant complication risks. This justifies the need for additional treatments, which alleviate the long-term consequences of a stroke.

POST-STROKE BRAIN REPAIR
With strategies on brain protection having failed in clinics in the 1980s and 1990s, current preclinical research strongly focuses on promoting the regenerative capacities of the ischemic brain. The physiological basis of the latter is the persistence of endogenous neurogenesis in the adult mammalian brain within so called stem cell niches, namely the subventricular zone (SVZ) of the lateral ventricles [6–8] and the subgranular zone of the dentate gyrus [9, 10]. Upon stroke, neural progenitor cells (NPCs) within the SVZ migrate toward the ischemic lesion site where they proliferate [11, 12]. Yet, the stroke-induced promotion of post-stroke neurogenesis has restricted functional relevance, as new-born cells show both low survival rates and poorly differentiate into mature neurons [13–15]. In order to use the endogenous regenerative potential of the ischemic brain, two different strategies to manipulate neurogenesis are under investigation: (a) enhancing the resistance of NPCs to delayed degeneration and (b) augmenting the number of NPCs in the ischemic brain tissue. The former can be achieved by the administration of ant apoptotic drugs [14, 16], the latter is thought to be accomplished by stimulating NPC proliferation or by transplantation of exogenous NPCs. Although transplantation of...
Effects in the ischemic brain [18, 20–22]. Especially due to their preclinical models.

Studies using stereotactic transplantation are excluded.

Act in an indirect manner, very likely by releasing trophic and anti-inflammatory factors that promote the survival, remodeling, and plasticity of the ischemic brain tissue [17–19].

Considering the paracrine nature of stem cell-mediated beneficial effects, the choice of stem cell source might not be essential for achieving recovery-promoting effects of cellular therapeutics. As a matter of fact, in addition to NPCs stem cells derived from various adult tissues have been found to promote restorative effects in the ischemic brain [18, 20–22]. Especially due to their broad availability, their simple handling and their low side effects, bone marrow-derived mesenchymal stem cells (MSCs) became an attractive cell source to treat ischemic stroke in a number of different preclinical models.

**Table 1. Preclinical studies and clinical trials on systemic post-stroke delivery of MSCs and NPCs**

| Species | Cell type | Delivery timing | Key results | References |
|---------|-----------|----------------|-------------|------------|
| Mouse  | Umbilical cord MSCs | Within 30 minutes | Reduction of brain injury & modulation of TGF expression | [23] |
| Rat    | Adipose-derived MSCs | Within 24 hours | Reduction of brain injury/improved motor coordination | [24] |
| Rat    | Adipose-derived MSCs (i.ventr./i.v./i.a.) | Within 24 hours | Reduction of brain injury/improved motor coordination | [25] |
| Rat    | BM-derived MSCs | Up to 1 month | Increased angiogenesis and better neurological recovery | [26] |
| Rat    | Placenta-derived MSCs | 24 hours versus 8 + 24 hours | Increased neurological recovery | [27] |
| Rat    | BM-derived MSCs (i.a.) | d2 and d7 | Increased angiogenesis and homing/no effect on neurological recovery | [28] |
| Rat    | BM-derived MSCs | 3 hours | Reduction of brain injury/improved functional outcome | [29] |
| Rat    | BM-derived MSCs | 24 hours | Increased angiogenesis | [30] |
| Rat    | NPCs (i.a./i.v./i.c.) | 24 hours | Migration and distribution patterns depend on delivery routes | [31] |
| Mouse  | NPCs | d7 | Reduced brain injury/improved neurological recovery | [32] |
| Mouse  | NPCs | 6 hours | Improved neurological recovery | [33] |
| Mouse  | NPCs | Up to 1 month | Reduced brain injury/increased tissue regeneration/improved functional recovery | [34] |
| Mouse  | NPCs (i.v./i.a./i.s./i.ventr./i.cort.) | 6 hours (i.v.) | Sustained reduction of brain injury after systemic transplantation | [35] |
| Rat    | NPCs | 24 hours | Reduced tissue injury and better neurological score | [36] |
| Human Phase II | Adipose-derived MSCs | Within 2 weeks | Recruiting patients | [37] |
| Human Phase II | BM-derived MSCs (i.a.) | Between 5–9 days | No safety concerns/no better outcome after 6 months | [38] |
| Human | BM-derived MSCs | Within 1 week after randomization | No safety concerns/better outcome for some scores | [39] |
| Human | BM-derived MSCs | 36–133 days post-stroke | No safety concerns within 1 year | [40] |
| Human | BM-derived MSCs | 3–12 months post-stroke | No safety concerns within 24 weeks | [41] |
| Human | BM-derived MSCs | 3–24 months post-stroke | No safety concerns within 24 weeks/improved Barthel index | [42] |

This list is not intended to be complete. It reflects a selection of representative studies where MSCs or NPCs have been applied systemically after stroke, that is, intravenously (if not stated otherwise) or intraarterially. Studies using stereotactic transplantation are excluded.

Abbreviations: BM, bone marrow; i.a., intraarterial delivery; i.c., intracisternal delivery; i.cort., intracortical delivery; i.v., intravenous delivery; i.ventr., intraventricular; MSCs, mesenchymal stem cells; NPCs, neural progenitor cells; TGF, transforming growth factor.

Preclinical transplantation studies in a plethora of stroke models using MSCs or NPCs have shown beneficial effects (Table 1) in a large number of different readouts [23, 26–36, 43–45]. NPCs, either administered intracerebrally or systemically, mediate neuroprotection and enhance neurological recovery via stimulation of endogenous angiogenesis and neurogenesis. The mechanisms involved in the process of NPC-induced brain protection and brain regeneration greatly depend on both cell delivery routes and cell delivery timing [34, 35]. For example, acute NPC transplantation reduced neuronal injury and infarct volume, while transplantation at later stages rather modifies post-stroke brain regeneration and neuronal plasticity.

Likewise, the transplantation of MSCs, which have been administered systemically in the majority of studies, revealed promising effects in experimental stroke models. MSC transplantation was found to reduce neuronal injury and infarct volume, increase angiogenesis and neurogenesis, and improve neurological recovery. Although a majority of studies has been performed on BM-derived MSCs, some studies imply the application of adipose-derived MSCs which might appear to be an attractive cell type as well [24, 25], since the latter is easy to obtain. Due to their beneficial effects in the preclinical models, controlled randomized clinical trials (Table 1) using MSCs (and to a lesser extent NPCs as well) for stroke treatment have been started [38–40, 46]. Although patient recruitment is so far low, which precluded more
endosomes are called multivesicular bodies (MVBs) or multivesicular bodies of origin [60, 66, 67]. Apart from lipids and proteins, metabolites are recovered in prepared EV fractions [68–70]. Whereas early studies proposing a paracrine mode of action of administered MSCs claimed that soluble factors, such as growth factors or cytokines, mediate the stem cells’ beneficial therapeutic effects [47]; more recent data qualified extracellular vesicles (EVs) as the critical agents [56]. Indeed, MSC-derived EVs (MSC-EVs) mediating therapeutic activities have been documented in a variety of different preclinical models and in a GVHD patient as well [49, 56–59]. EVs are released by almost all cell types and are detected as membrane-surrounded vesicles in all body fluids [60]. According to their origin, different EV types can be discriminated [61]. Exosomes are derivatives of the late endosomal compartment and have diameters of 70–150 nm. They correspond to intraluminal vesicles (ILVs) that are formed by the inward budding of the limiting membrane of sorting and late endosomes. The ILV containing endosomes are called multivesicular bodies (MVBs) or multivesicular endosomes. At the example of maturing reticulocytes, it has been shown that MVBs can fuse with the plasma membrane and release their ILVs as exosomes into the extracellular compartment [62–64]. In contrast, microvesicles (MVs), which have diameters of 100–1,000 nm, are formed as bud offs of the plasma membrane; together with apoptotic bodies which have said sizes of 500 nm to several micrometers, exosomes and MVs form the most prominent EV subtypes [65]. EVs contain specific molecular signatures reflecting their cell of origin [60, 66, 67]. Apart from lipids and proteins, metabolites and nucleic acids are recovered in prepared EV fractions [68–70]. A proportion of EVs might contain molecules that cells cannot metabolize, which are released into the extracellular environment for further processing. Other EVs seem to be assembled in a tailored manner to act as intercellular communication vehicles mediating complex signal exchanges between cells within and between different organs [60, 61, 71].

**Structure of Extracellular Vesicles and Biological Properties**

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**Preclinical Studies Using EVs in Animal Models Associated with Ischemia**

More recent studies identified the therapeutic efficacy of EVs in experimental conditions mimicking peripheral limb, heart or brain ischemia, that is, in models of peripheral occlusive artery disease, myocardial infarction and stroke (Table 3). For myocardial ischemia, the therapeutic efficacy of EVs has been shown in a large number of in vitro and in vivo studies [49, 101–111]. Thus, EVs from various cell sources including MSCs and embryonic stem cells, promoted cellular survival, reduction of infarct size, and stimulated myocardial remodeling and angiogenesis. Of note, these EV actions were associated with functional recovery evaluated by ejection fraction. To the best of the authors’ knowledge, six different studies have examined effects of EVs in ischemic stroke models, most in rats and one in mice [112–114, 116–118]. In the first rat study,

Positive therapeutic effects of MSC-EVs were reported for the first time in 2009; the group of Giovanni Cammussi described EV-mediated therapeutic activities in a kidney failure model [59]. In 2010, the group of Sai Kiang Lim and Dominque de Kleijn discovered cardioprotective activities in their MSC-EV fractions [49]. We were the first group who applied MSC-EVs to a human patient in an individual treatment attempt. We applied an allogeneic MSC-EV fraction to a steroid refractory graft-versus-host disease patient, who failed to react on several second side strategies. Remarkably, the clinical symptoms declined significantly during and after the 2-week MSC-EV therapy, without revealing any side effects [57]. Meanwhile, EVs have been applied to several preclinical diseases models unrelated to ischemia, with some of them mentioned in Table 2.

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Chopp and colleagues [113] intravenously applied MSC-EVs in a model of transient intraluminal middle cerebral artery occlusion. EVs were administered via tail vein injection at 24 hours post-stroke. The authors observed a significant reduction of brain injury and neurological impairment that was associated with enhanced post-ischemic neurogenesis. In the hitherto only mouse study, we studied effects of MSC-derived EVs in transient intraluminal middle cerebral artery occlusion. Using the polyethylene glycol (PEG) method EVs were enriched from MSC conditioned media. MSCs were raised from BM samples of two healthy bone marrow donors; as serum supplement 10% human platelet lysate was used [119, 120]. MSC-EVs were administered at days 1, 3, and 5 post-stroke. The treatment enhanced neurological recovery and increased endogenous neurogenesis and angiogenesis, at the same time reversing stroke-induced peripheral immunosuppression. In a head-to-head comparison, the therapeutic potential of MSC-EVs was comparable to that of the transplanted MSCs from which the MSC-EVs were derived [112].

A more recent rat study examined the effects of MSCs combined with MSC-EVs [114], demonstrating that combined MSC

| Disease condition                  | In vitro/in vivo | EV source                                           | Key results                                                                 | References |
|-----------------------------------|------------------|----------------------------------------------------|-----------------------------------------------------------------------------|------------|
| Amyotrophic lateral sclerosis     | In vitro         | Adipose-derived stem cells                         | Alleviation of SOD1 and mitochondrial dysfunction                           | [73]       |
| Hepatitis C                       | In vitro         | Umbilical MSCs                                      | Antiviral activity by microRNA transport                                   | [74]       |
| Cancer therapy                    | In vivo (mice)   | Modified melanoma cells                             | Suppression of tumor growth                                                | [75]       |
| Osteochondral disease             | In vivo (rats)   | Embryonic MSCs                                      | Increased cartilage repair                                                 | [76]       |
| Head and neck cancer cells        | In vitro         | (Ir)radiated head and neck cancer cells             | Increased survival of irradiated tumor cells                               | [77]       |
| Chemotherapy-induced POF          | In vitro/in vivo (mice) | Amniotic fluid stem cells                     | Prevention of ovarian follicular atresia                                  | [78]       |
| Diabetic nephropathy              | In vivo (rats)   | Human urine-derived stem cells                      | Increased cell survival/vascular regeneration                              | [79]       |
| Osteoporosis                      | In vitro/in vivo (rats) | Human-induced pluripotent stem cell-derived MSCs | Enhanced bone regeneration                                                | [80]       |
| Endothelial regeneration          | In vitro         | EPCs                                               | Increased re-endothelialization                                            | [81]       |
| Myasthenia gravis                 | In vivo (rats)   | Atorvastatin-modified BM-derived DCs               | Suppression of immune responses                                            | [82]       |
| Traumatic brain injury            | In vivo (mice)   | MSCs                                               | Reduced inflammation and cognitive impairment                              | [83]       |
| Hepatocellular carcinoma          | In vitro/in vivo (rats) | Modified adipose tissue-derived MSCs | Increased sensitivity to chemotherapy                                       | [84]       |
| Experimental colitis              | In vivo (rats)   | MSCs                                               | Attenuation of inflammation                                                | [85]       |
| Gastric cancer                    | In vitro         | MSCs                                               | Increased drug resistance                                                  | [86]       |
| Arthritis                         | In vivo (mice)   | Bovine milk                                         | Diminished cartilage pathology/reduced inflammation                       | [87]       |
| Parkinson’s disease               | In vitro         | Dental pulp stem cells                              | Reduced apoptosis                                                         | [88]       |
| Carrageenan-induced inflammation  | In vivo (mice)   | Human dental pulp stem cells                        | Suppressed inflammation                                                    | [89]       |
| Skin burn                         | In vitro/in vivo (rats) | Human umbilical cord MSCs                       | Increased angiogenesis in wounded tissue                                   | [90]       |
| Cutaneous wounds                  | In vivo (rats)   | Human induced pluripotent stem cell-derived MSCs   | Promotion of collagen synthesis and angiogenesis                          | [91]       |
| Traumatic brain injury            | In vivo (rats)   | MSCs                                               | Enhanced neurological recovery/increased angiogenesis and neurogenesis     | [92]       |
| HIV infection                     | In vitro         | Breast milk                                         | Inhibition of infection of monocyte-derived DCs                           | [93]       |
| Endotoxin-induced lung injury     | In vivo (mice)   | MSCs                                               | Reduced inflammatory response                                              | [94]       |
| Cisplatin-induced kidney injury   | In vitro/in vivo (rats) | Human umbilical cord MSCs                       | Reduced cell injury/increased cell proliferation                           | [95]       |
| Brain tumor                       | In vivo (rats)   | MSCs                                               | Reduced glioma growth                                                     | [96]       |
| Liver fibrosis                    | In vitro         | Human umbilical cord MSCs                          | Reduced liver fibrosis                                                    | [97]       |
| Sepsis                            | In vivo (rats)   | DCs                                                | Decreased release of cytokines/reduced mortality                          | [98]       |
| Arthritis                         | In vivo (mice)   | Modified DCs                                       | Anti-inflammatory actions                                                  | [99]       |

This list is not intended to be complete. It reflects a selection of studies based on their influences on the development of this field.

Abbreviations: ALS, amyotrophic lateral sclerosis; BM, bone marrow; CTx, chemotherapy; DCs, dendritic cells; EPCs, Endothelial progenitor cells; HIV, human immunodeficiency virus; MSCs, mesenchymal stem cells; POF, premature ovarian failure; SOD1, superoxide dismutase.
and MSC-EV delivery was superior in terms of brain protection and neurological recovery when compared with MSC transplantation or EV injection only. These studies raised the question of how therapeutic effects of EVs may be boosted by loading naive EVs with biologically active molecules such as noncoding RNAs, which by means of EVs may safely be transported to target tissues [121]. In rats exposed to transient middle cerebral artery occlusion, increased neural plasticity and neurological recovery were noted after delivery of EVs obtained from miR-133b overexpressing MSCs when compared with EVs obtained from naive MSCs [117]. In vitro experiments using oxygen-glucose-deprivation suggested that the enhanced action of miR-133b containing EVs may be due to stimulation of secondary EV release from astrocytes [117]. In another study, EVs harvested from MSCs transfected with a miR-17-92 cluster plasmid induced better neurological recovery when compared with EVs derived from naive MSCs [116]. These observations stress the heterogeneity of EV actions depending on the loading of EVs with survival and plasticity promoting molecules.

### CLINICAL STUDIES USING EVS IN HUMANS

Despite an increasing body of evidence demonstrating that EVs might serve as biomarkers for stroke outcome [122], there is currently no study in which EVs (and especially MSC-EVs) have therapeutically been administered to human stroke patients. According to the promising data obtained in a variety of different animal models and the very promising result of the individual treatment attempt of a GvHD patient with MSC-EVs, a number of groups now try to translate EVs into the clinics. As EVs are novel biological agents and MSC-EVs are not considered as Advanced Therapy Medicinal Products (ATMP), they provide a new class of biologicals, for whose production no concrete rules have been defined by the FDA or any other national regulatory agency, yet. To this end, experts in the field have summarized in an International Society of Extracellular Vesicles (ISEV) position paper the different therapeutic EV-application fields, discussed their regulatory status and recommended requirements to be fulfilled to translate EVs as therapeutic agents into the clinics [56].

### Table 3. Therapeutic application of EVs in preclinical disease models associated with ischemia

| Disease condition | In vitro/in vivo | EV source/EV isolation | Key results | References |
|-------------------|-----------------|------------------------|-------------|------------|
| Limb ischemia     | In vivo (mice)  | Human-induced pluripotent stem cell-derived MSCs/UC | Promotion of angiogenesis | [100] |
| Myocardial ischemia | In vitro | MSCs/Exo-Quick | Enhanced survival of cardiomyocytes | [101] |
| Myocardial ischemia | In vivo (rats) | MSCs/Exo-Quick | Increased angiogenesis/reduced inflammation | [102] |
| Myocardial ischemia | In vivo (rats) | Umbilical cord MSCs/UC | Improved systolic function | [103] |
| Myocardial ischemia | In vitro/in vivo (mice) | Cardiac fibroblast-derived iPSCs/UC | Increased myocardial survival | [104] |
| Myocardial ischemia | In vivo (rats) | Embryonic stem cells/UC | Increased myocardial regeneration | [105] |
| Myocardial ischemia | In vivo (rats) | Plasma from rats and humans/UC | Reduction of infarct size | [106] |
| Myocardial ischemia | In vitro (mice) | GATA-4 overexpressing MSCs/UC | Cardioprotection | [107] |
| Myocardial ischemia | In vitro (mice) | MSCs/HPLC | Increased angiogenesis/systolic function | [108] |
| Myocardial ischemia | In vitro (mice) | Cardiac progenitor cells/UC | Increased survival of cardiomyocytes | [109] |
| Myocardial ischemia | In vivo (mice) | Human embryonic stem cell-derived MSCs/HPLC | Cardioprotection | [110] |
| Stroke | In vivo (mice) | Adipose derived MSCs/UC | Enhanced neurological recovery angiogenesis and neurogenesis | [111] |
| Stroke | In vivo (mice) | miR-133b-overexpressing MSCs/UC | Secondary EV release by astrocytes/neuroplasticity | [112] |
| Stroke | In vivo (mice) | Embryonic stem cells/UC | Reduction of post-stroke inflammation/neuroplasticity | [113] |

*EVs administered in a prophylactic manner, that is, prior to ischemia.

*EVs were given as coronary perfusates from rats exposed ischemic pre-conditioning.

Abbreviations: HPLC, high performance liquid chromatography; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; UC, ultracentrifugation.

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Despite their different origin and their different proposed sizes, EV subtypes could not be discriminated during isolation until now. Thus, the ISEV agreed in 2014 to name fractions proposed to contain exosomes, MVs, apoptotic bodies and/or other EV types appropriately as EV fractions [123]. Since EV fractions contain a heterogeneous mix of different EV types, care has to be taken, of how EVs are purified and characterized. As such, the application of differential centrifugation (i.e., ultracentrifugation) is hampered by a low EV output due to restricted sample volumes in comparison to other techniques like size exclusion chromatography [124]. In this sense, the recently identified observation of low density lipoprotein contamination after EV enrichment might pose a problem for the evaluation of past and future work when dealing with mechanistic approaches [125]. On the contrary, for pure therapeutic applications, contaminations might be tolerated. Despite a plethora of different enrichment techniques available, ultracentrifugation, however, remains to be the gold standard for EV enrichment, albeit other techniques such as PEG isolation provide some advantages (own unpublished observation). Consequently, the ISEV has released consensus recommendations on EV purification and characterization [123]. Still, several studies do not follow these recommendations, making it difficult to compare research outcomes. To increase the reliability of the data and to promote standardization in the field the EV-TRACK consortium was formed which defines several criteria to score EV-based studies that will hopefully be followed in the future [126]. Furthermore, caution has to be taken when interpreting studies from both the stem cell and the EV field. Comorbidities and comedications, for instance, might modulate experimental outcomes. As such recommendations—especially from the cardiotherapeutic field—have been made in order to overcome typical pitfalls of cell-based therapies [127–129]. The latter emphasize the necessity of selecting the appropriate cell type or components of the secretome depending on the endpoint chosen and the definition of the application mode, including the amount of applications, the application timing and the delivery routes, to name but a few.

As EVs lack nuclei they cannot self-replicate and thus in contrast to cells do not contain any endogenous tumorigenic potential. In addition, EVs are easier to handle and, due to their small size, they can be sterilized by filtration [56]. Thus, EV-based therapeutics provide several advantages over cellular therapeutics, resulting in a competition between several research groups to produce MSC-EVs for the clinical setting. There are several challenges connected to this issue. On the one hand, large volumes have to be processed under good medical practice compliant conditions to obtain sufficient material to treat a patient. Then, as MSCs provide a heterogeneous cell entity, MSC-EV fractions may show varying therapeutic activities as well. Indeed, the authors detected significant differences in the cytokine profile of independent MSC-EV preparations during their own research activities [57].

**CONCLUSION**

The application of stem cell derived EVs, especially that of MSC-EVs, offers a great opportunity for adjuvant stroke treatment. For now, EVs appear to be safe in mammals and potentially also in man, thus avoiding putative side effects that are inherent to stem cell transplantation such as malignant stem cell transformation. Besides, tissue engineering techniques allow the usage of EVs as potent carriers for bioactive molecules, which may be used for overcoming tissue barriers such as the blood-brain barrier for targeting distinct cell populations [56]. Yet, fundamental questions as to their exact mode of action and their optimal enrichment, characterization, and storage have to be answered to optimize them for the clinical setting [56].

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**AUTHOR CONTRIBUTIONS**

T.R.D., M.B., D.M.H., and B.G.: manuscript writing, final approval of the manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.
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