# Mitochondria replacement from transplanted amniotic fluid stem cells: a promising therapy for non-neuronal defects in spinal muscular atrophy

Michela Pozzobon, Camilla Bean[*]

Spinal muscular atrophy (SMA) is a genetic disorder that primarily affects infants and leads to muscle weakness, atrophy, and paralysis. The main cause is the homozygous mutation or deletion of the SMN1 gene, resulting in inadequate levels of the survival motor neuron (SMN) protein. Approved treatments focus on restoring SMN levels through various approaches, but there is a need for "SMN-independent" therapies that target other pathological processes. Skeletal muscle is closely involved in SMA pathology, with impaired muscle function observed before motor neuron degeneration. Studies have revealed that SMN loss leads to skeletal muscle mitochondrial structural abnormalities, impaired respiration, and accumulation of reactive oxygen species. Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, have emerged as a therapeutic option due to their regenerative and immunomodulatory capabilities, including the ability to replace dysfunctional mitochondria through transcellular exchange. Amniotic fluid stem (AFS) cells are considered a favorable source for stem cell therapy due to their unique properties. By transplantation of AFS cells in a mouse model of SMN loss in the skeletal muscle we could restore mitochondrial function and correct the muscle phenotypes of SMA. Utilizing stem cell therapies to restore mitochondrial function offers promising avenues for the treatment of SMA.

**Introduction:** SMA is the most common cause of infant death that manifests from a genetic defect. Homozygous mutations of the causative gene *SMN1* lead to insufficient levels of the SMN protein. While it is not yet fully understood, the neuromuscular system is selectively more vulnerable to reduced levels of the ubiquitously expressed *SMN* gene, that cause degeneration of lower motor neurons, leading to muscle weakness, atrophy, and finally paralysis. Notably, the primate-specific paralog gene *SMN2* that originated from a duplication event is not able to fully compensate for *SMN1* loss of function. Although *SMN2* differs from *SMN1* by 1% of the sequence, a single substitution (C-to-T transition) in exon 7 causes the production of a truncated, unstable SMN protein (SMNΔ7) that is degraded for roughly 90% of the transcribed *SMN2*. The remaining 10% produces a functional SMN protein, but it is not sufficient to rescue the lack of *SMN1* (Jablonka et al., 2022). Consequently, the number of *SMN2* copies determines the wide phenotypic variability observed among SMA patients due to varying levels of compensation (Feldkötter et al., 2002). The clinical classification distinguishes the following categories: (1) severe, type I SMA or Werdnig Hoffmann disease; (2) intermediate, type II SMA; (3) mild, type III SMA, juvenile SMA or Kugelberg-Welander disease; and (4) type IV or adult SMA (Feldkötter et al., 2002).

**Therapeutic strategies for SMA:** The approved treatments aim to restore SMN levels and are referred to as "SMN-dependent" therapies. Small molecules and antisense oligonucleotides modify SMN2 splicing to promote the inclusion of exon 7 in the mRNA. Adeno-associated virus 9 vectors deliver SMN1 complementary DNA into infected cells (Jablonka et al., 2022). While these approaches have proven effective, a significant number of non-responders indicate the existence of pathological processes that cannot be reversed by enhancing SMN protein levels. The reasons for the heterogeneous clinical responses, ranging from minimal improvements to substantial motor function recovery and survival are unknown and warrant further investigation. Therefore, there is a need to explore "SMN-independent" treatments (Finkel et al., 2017). Furthermore, as the loss of neurons is irreversible, early treatment yields better responses. Combining multiple approaches has demonstrated synergistic effects and significant clinical improvements beyond individual treatment modalities. Recent advancements in genome-editing strategies using a mouse model of SMA have shown therapeutic benefits (Arbab et al., 2023). The use of a base editor in combination with the antisense oligonucleotide drug Nusinersen (Spinraza, Biogen) has extended lifespan and rescued motor function (Arbab et al., 2023). In contrast to existing treatments that require multiple doses without fully restoring SMN levels, this approach holds promise as a one-time, long-lasting therapy. Antisense oligonucleotides targeting the central nervous system overcome the limitations of genome-based editing systems in accessing motor neurons within the spinal cord. Simultaneously, the base editor enhances therapeutic efficacy by up-regulating SMN systemically. Studies have indicated that SMN up-regulation limited to the central nervous system is less effective than the systemic restoration of SMN protein levels in improving the phenotype of SMA model mice. The multifunctionality and diverse subcellular localization of SMN determine the varying threshold levels of SMN required in different tissues. For a long time, the higher susceptibility of motor neurons to SMN deficiency overshadowed the systemic nature of SMA. However, recent studies have revealed that the lack of SMN can affect other cells and tissues, particularly skeletal muscle (Nash et al., 2016), which represents the most prominent clinical manifestation across all types of SMA. Impairment of muscle growth has been observed prior to the degeneration of motor neurons (Kim et al., 2020). Interestingly, restoring SMN solely in motor neurons does not fully rescue disease phenotypes in severe mouse models of SMA. Therefore, understanding the tissue-specific pathogenesis of SMA is crucial for developing effective therapeutic approaches. Deletion of SMN1 specifically in muscle in mice leads to a severe myopathic disorder and reduced lifespan (Piccoli et al., 2012).

Furthermore, SMN can autonomously regulate myogenic differentiation by targeting MyoD and specific microRNAs that are important for muscle functions (Ikenaka et al., 2023). SMN loss results in defects in both myogenic differentiation and muscle maintenance (Kim et al., 2020). Altogether, skeletal muscle appears as an essential tissue target in the therapy of SMN.

Mesenchymal stromal stem cells of both adult (i.e., bone marrow and adipose tissue) and perinatal (i.e., amniotic fluid and umbilical cord tissue) origin play a prominent role in cell therapy due to their regenerative and immunomodulatory capabilities. In addition to their capacity for multilineage differentiation and secretion of paracrine factors, MSCs possess the ability to replace dysfunctional mitochondria through transcellular exchange. Interestingly, MSCs can also take up damaged mitochondria. Hence, there are at least three mechanisms through which MSCs exert their therapeutic actions: (1) tissue repair and regeneration, (2) release of growth factors and cytokines in response to the surrounding microenvironment, and (3) the formation of membranous channels, such as tunneling nanotubes, vesicles, and gap junctions, enabling the transfer of cargoes between MSCs and injured recipient cells. As reviewed by Liu and collaborators, the mitochondrial trafficking pathway is probably the most exciting therapeutic option for all mitochondria-related diseases (Liu et al., 2022). Importantly, amniotic fluid stem cells can also prevent neuronal alterations by participating in the communication between skeletal muscle and motor neurons through the secretion of extracellular vesicles at neuromuscular junctions (Gatti et al., 2023). Among the sources of MSCs, perinatal AFS cells have garnered attention in regenerative medicine due to their unique characteristics and potential therapeutic applications. AFS cells are considered non-controversial from an ethical standpoint, easily accessible as a source of stem cells, and deemed safe as they are not tumorigenic. Notably, AFS cells possess the ability to convert into healthy muscle stem cells and can be employed to replenish the exhausted muscle stem cell niche caused by SMA pathology (Piccoli et al., 2012). This result was the first to prove the muscle plasticity of these perinatal stem cells that originate from a fluid, corroborating the notion that AFS cells possess unique stem-differentiation properties.

**Mitochondria replacement from transplanted AFS Cells to correct muscle pathology in SMA:** Our recent research indicates a specific connection between skeletal muscle mitochondria and SMA pathology (Chemello et al., 2023). We employed a single myofiber approach to investigate the effects of SMN loss, providing a unique perspective on muscle tissue without confounding contributions from connective tissue, blood vessels, and nerves present in whole-muscle lysates (Chemello et al., 2023). Consistent with previous findings of impaired mitochondrial function, accumulation of free radicals, and increased cell death with reduced Smn1 expression (Acsadi et al., 2009), we demonstrated that skeletal muscle SMN loss in vivo leads to the accumulation of morphologically abnormal mitochondria (Chemello et al., 2023). These altered mitochondria exhibit impaired respiration, resulting in excessive production of reactive oxygen species. The mitochondrial abnormalities are accompanied by dysregulation of the autophagy-lysosome degradation system, which fails to clear defective mitochondria. Interestingly, transplantation of AFS cells corrects the myopathic phenotype in SMN knockout mice, restoring mitochondrial morphology and the

expression of mitochondrial genes (Chemello et al., 2023). Since MSCs can be isolated and expanded successfully *in vitro*, the systemic administration of MSC-isolated mitochondria has emerged as an exciting therapeutic option for multiple diseases, eliminating the inherent risks associated with whole-cell transplantation. Mitochondrial transfer from injected MSCs or even direct transplantation of MSC-isolated mitochondria has demonstrated therapeutic benefits in various mouse disease models. Examples of mitochondria-targeted therapies in the treatment of muscle disorders are recently well-reviewed (Liu et al., 2022). The transfer of mitochondria in a dexamethasone-induced model of skeletal muscle atrophy has been shown to block the AMPK/FoxO3/MuRF-1 pathway that causes muscle atrophy. Moreover, the combined delivery of MSCs with platelets or with isolated mitochondria into the tibialis anterior muscle of dystrophic α-sarcoglycan-deficient mice favors angiogenesis and myogenesis processes. Finally, mitochondrial transplantation has also been proven to significantly reduce infarct size and apoptosis, improving muscle function in a model of limb ischemia (Liu et al., 2022).

In our study (Chemello et al., 2023), we propose a similar mechanism in the SMA disease model, where transplanted amniotic fluid-derived mesenchymal stem cells were shown to contribute to muscle fiber regeneration and restoration of mitochondrial functions (Piccoli et al., 2012; Chemello et al., 2023). However, whether the correction of the muscle phenotype is due to the replenishment of the muscle stem cell niche with healthy transplanted cells containing functional mitochondria, to the direct transfer of mitochondria from AFS donors to damaged cells, or a combination of both, remains to be investigated (**Figure 1**).

**Conclusion:** Restoring mitochondrial functionality has been identified as a crucial therapeutic target for SMA. One such potential treatment, the neuroprotective drug olesoxime, was initially developed for SMA patients but failed to gain approval (Jablonka et al., 2022). This cholesterol-like molecule acts by binding two outer mitochondrial membrane proteins, the translocator protein and the voltage dependent anion channel that control the mitochondrial permeability transition pore. Olesoxime faced technical and regulatory challenges and also faced competition from the potentially more effective drug nusinersen, leading to the discontinuation of further development for olesoxime. In fact, olesoxime has been shown to provide clinical benefits for patients with type 2 or type 3 SMA, but nusinersen displays a broad spectrum of effectiveness including in the severest form of SMA. However, mitochondrial dysfunction plays a significant role in SMA pathogenesis, suggesting that restoring mitochondrial function, in addition to current gene therapies, could provide clinical benefits (Chemello et al., 2023). Considering the systemic nature of SMA and the presence of mitochondria in all tissues, preserving mitochondrial function emerges as a promising SMN-independent systemic treatment approach. AFS cells, with their immune-privileged properties enabling allogeneic transplantations and their ability to cross the endothelial barrier and be systemically injected, appear as the most suitable source of mitochondria for such therapies. A novel approach we propose for ameliorating severe cases of SMA pathology is to combine mitochondrial transfer, a natural process with no side effects, with genetic and pharmacological therapies.

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**Michela Pozzobon, Camilla Bean**[*]
Women's and Children's Health Department, University of Padova; Foundation Institute of Pediatric Research Città della Speranza, Padova, Italy (Pozzobon M)
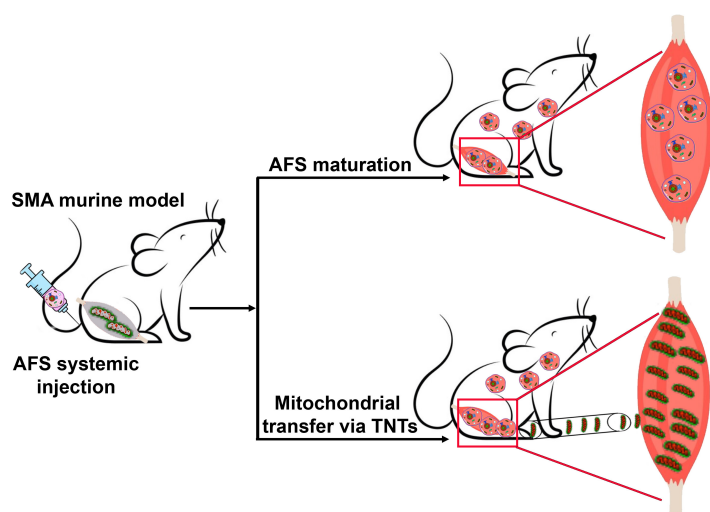Department of Medicine, University of Udine, Udine, Italy (Bean C)
**\*Correspondence to:** Camilla Bean, PhD, camilla.bean@uniud.it.
https://orcid.org/0000-0001-6491-7211 (Camilla Bean)
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**Figure 1 | AFS cells rescue the myopathic phenotype in a mouse model of SMA.**
The systemically injected AFS cells preferentially target the damaged tissues where they can act in two ways: (1) they differentiate toward healthy muscle cells by replenishing the muscle stem cell niche (Piccoli et al., 2012) and (2) they transfer healthy mitochondria via TNTs to damaged muscle cells as recently proposed (Chemello et al., 2023). Created with Adobe Photoshop 2020. AFS: Amniotic fluid stem; SMA: spinal muscular atrophy; TNTs: tunnelling nanotubes.