

Skeletal Muscle Preservation During Nerve Regeneration

Cell-Based, Extracellular Vesicle,
and Biomaterial Approaches

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ISBN: 978-91-6850-013-3 (print)

ISBN: 978-91-6850-014-0 (pdf)

ISSN: 0346-6612

Umeå University Medical Dissertations New Series no. 2426

Cover image was designed by Maria Vittoria Giraudo and generated by AI.

Electronic version available at: <http://umu.diva-portal.org/>

Printed by: Scandinavian Print Group, Hågersten, 2026

*Ai miei nonni e a Cris,
sempre con me*

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Abstract

Peripheral nerve injuries often result in prolonged denervation of skeletal muscle, leading to muscle atrophy and impaired functional recovery. Although surgical repair can restore nerve continuity, successful outcomes depend on both axonal regeneration and preservation of the target muscle during the denervation period. This work investigates strategies to support the regenerative environment and maintain skeletal muscle following nerve injury, focusing on secretory mechanisms and biomaterial-based approaches.

Adipose-derived stem cells (ASCs) were studied as a source of regenerative secretome. Human ASCs were isolated and expanded under xeno-free, Good Manufacturing Practice (GMP)-compatible conditions. The influence of culture conditions and a short stimulation protocol on ASC properties and secretory activity was evaluated. Both affected the composition of the secretome, resulting in enhanced angiogenic activity under defined conditions. To examine cell-free signalling, extracellular vesicles (EVs) were isolated from denervated skeletal muscle tissue. Denervation increased EV release and altered their molecular profile. However, muscle-derived EVs did not impair neurite outgrowth *in vitro*, indicating that EVs from denervated muscle do not negatively affect axonal growth under the conditions tested. Finally, a nerve-derived extracellular matrix hydrogel was evaluated in a rat sciatic nerve repair model. When used as a filler in a synthetic nerve conduit, the hydrogel supported axonal regeneration and contributed to preservation of skeletal muscle structure following reinnervation.

Together, these findings highlight the importance of the regenerative environment in peripheral nerve repair and support the development of strategies that modulate secretory activity to improve muscle preservation and functional recovery.

Sammanfattning på svenska

När en nerv skadas måste kroppen lösa två problem samtidigt. Nerven behöver växa tillbaka och muskeln som den styr måste hålla sig frisk medan den väntar på nervens återväxt. Detta är ett problem eftersom nerver växer långsamt och muskeln kan förtvina innan kontakten återställs. Till följd av detta är det många som inte återhämtar sig helt efter nervskador.

Den här avhandlingen har undersökt olika sätt att stödja läkningen. Istället för att enbart fokusera på att reparera nerven så studerades de "signaler" som celler och vävnader skickar till varandra under återhämtningen. Dessa signaler består av små molekyler och mycket små partiklar som frisätts i omgivande vävnad och hjälper till att styra hur kroppen reagerar på en skada.

Tre olika strategier har studerats för att främja återhämtning: den första använde stamceller från fettvävnad eftersom de frisätter signaler som kan stödja läkning. Genom att anpassa hur dessa celler odlas kan deras förmåga att hjälpa vävnader förbättras. Den andra studerade små partiklar som frisätts från muskel som tappat kontakt med nervsystemet för att förstå hur vävnaden kommunicerar under återhämtning. Slutligen användes ett mjukt material framställt från nervvävnad för att skapa en bättre miljö för den växande nerven.

Tillsammans visar fynden att återhämtning inte bara beror på nerven, utan på hela miljön runt omkring. Genom att förbättra dessa signaler och förutsättningar kan framtida behandlingar hjälpa både nerver och muskler att återhämta sig bättre efter skada.

Riassunto in Italiano

Quando un nervo viene lesionato, il corpo deve affrontare simultaneamente due problemi: il nervo deve rigenerarsi e il muscolo, controllato dal nervo, deve mantenersi in salute mentre attende la ricrescita del nervo stesso. Questo rappresenta un ostacolo a livello clinico, poiché, mentre i nervi crescono lentamente, il muscolo può andare incontro ad atrofia prima che la connessione venga ristabilita. E questo spesso causa un recupero parziale delle funzioni muscolari.

Questa tesi esamina diversi approcci per favorire il processo di guarigione. Non si concentra esclusivamente sulla riparazione del nervo ma esamina i “segnali” che cellule e tessuti si scambiano durante il recupero. Questi segnali consistono in piccole molecole e minuscole particelle rilasciate nei tessuti circostanti, che contribuiscono a regolare la risposta dell’organismo alla lesione.

Sono state analizzate tre diverse strategie per favorire il recupero: la prima ha utilizzato, estraendole da tessuto adiposo, cellule staminali che rilasciano segnali in grado di favorire la guarigione. Modificando le condizioni di coltura di queste cellule è possibile migliorare la capacità di guarigione dei tessuti. La seconda strategia ha esaminato delle piccole particelle rilasciate dai muscoli che hanno perso la connessione con il sistema nervoso, al fine di comprendere come i tessuti utilizzano queste particelle per comunicare durante la convalescenza. Infine, è stato impiegato un materiale gelatinoso derivato dal tessuto nervoso per creare un ambiente più favorevole alla crescita del nervo.

Nel complesso i risultati dimostrano che il recupero non dipende soltanto dal nervo, ma dall’intero ambiente circostante. Perfezionando la conoscenza di questi segnali e migliorando le condizioni dei tessuti danneggiati, le future cure potrebbero favorire una simultanea rigenerazione dei nervi e dei muscoli dopo una lesione.

Abbreviations

CNS	Central nervous system
PNS	Peripheral nervous system
PNIs	Peripheral nerve injuries
NMJ	Neuromuscular junction
MHC	Myosin heavy chain
MSCs	Mesenchymal stem cells
ASCs	Adipose-derived stem cells
EVs	Extracellular vesicles
uPA	Urokinase plasminogen activator
ECM	Extracellular matrix
IGF-1	Insulin-like growth factor 1
VEGF	Vascular endothelial growth factor
HGF	Hepatocyte growth factor
FBS	Fetal bovine serum
GMP	Good manufacturing practice
PLT	Platelet lysate
XV or XSBM	PRIME-XV MSC Expansion XSBM medium
MyomiRs	Muscle-specific microRNAs
NGCs	Nerve guidance conduits
PCL	Polycaprolactone
dECM	Decellularized extracellular matrix
DTI	Diffusion tensor imaging
CM	Conditioned medium
GFs	Growth factors
FSK	Forskolin
FA	Fractional anisotropy

List of Papers

- I. **Lauvrud AT, Giraud MV, Wiberg R, Wiberg M, Kingham PJ, Brohlin M.**
“The Influence of Xeno-Free Culture Conditions on the Angiogenic and Adipogenic Differentiation Properties of Adipose Tissue-Derived Stem Cells.”
Regen Ther. (2024); Oct10; 26:901-910. DOI: 10.1016/j.reth.2024.09.013

- II. **Giraud MV, Lauvrud AT, Wiberg R, Brohlin M, Andersson G, Kingham PJ.**
“Forskolin Enhances Urokinase Plasminogen Activator Secretion and Angiogenic Activity of Xeno-Free Cultures of Human Adipose Tissue-Derived Stem Cells.”
Adv Biol. (2025) ;9(10):e00466. DOI:10.1002/adbi.202400466

- III. **Giraud MV, Novikova LN, Novikov LN, Andersson G.**
“Extracellular Vesicles Isolated from Denervated Skeletal Muscle Tissue and their Effects on Neurite Outgrowth.”
Manuscript. (2026).

- IV. **Giraud MV, Novikova LN, Kuna VK, Kelk P, Brohlin M, Novikov LN, Kingham PJ, Andersson G.**
“Use of a Nerve-Derived Extracellular Matrix Hydrogel for Regeneration in Synthetic Nerve Conduits: A Longitudinal Diffusion MRI and Histological Study on Outcome.”
Manuscript. (2026).

Author's Contributions

- I. The author carried out part of the experimental work in the study such as some qRT-PCR analyses and standard characterisation of the cells such as chondrogenic differentiation. The author participated in the analysis and representation of the data obtained. The author contributed to the writing of the article.
- II. The author was extensively involved in the planning and design of the study. The author carried out experimental work in the laboratory, including cell culture experiments and molecular analyses, excluding the characterisation of the adipose-derived stem cells. The author performed the analysis and representation of the obtained data and produced the figures for the study. The author wrote the article and was involved in revisions and additional experimental work during the revision process.
- III. The author had a leading role in the planning and design of the study. The execution, analysis and representation of the experiments were conducted by the author. The author was present during the surgical procedures and collected the samples from the animals. The author carried out all subsequent experimental work including extracellular vesicles isolation, vesicle characterisation, and cell culture experiments. The author performed the analysis of the obtained data and produced the figures for the manuscript. The author wrote the manuscript and was involved in revisions of the manuscript.
- IV. The author carried out the analysis of the skeletal muscle samples, including histological processing and quantitative analysis of muscle tissue. The author participated in the analysis and representation of the data, produced the figures for the study and contributed to the writing of the manuscript and revisions of the manuscript.

Introduction

1. Preface: Modulating the Regenerative Environment

Peripheral nerve injury (PNI) affects a functional unit in which nerves and skeletal muscle are closely interconnected. The maintenance of this system relies on coordinated interactions among several cell types, including neurons, Schwann cells, and muscle fibres, as well as on electrical signalling and the surrounding extracellular environment. The extracellular microenvironment, composed of matrix components, soluble factors, and extracellular vesicles (EVs) supports tissue structure and intercellular communication. Following injury, this system is disrupted at multiple levels. Loss of neural input alters signalling in both nerve and muscle, leading to progressive muscle atrophy and changes in the local environment that can limit regeneration. As a result, functional recovery depends not only on the ability of axons to regrow, but also on the condition of the surrounding tissue in which regeneration occurs.

This has led to increasing interest in therapeutic strategies that target the extracellular and secretory environment rather than focusing purely on individual cell types. By influencing paracrine signalling, vesicle-mediated communication, and extracellular matrix (ECM) composition, it may be possible to support both nerve regeneration and preservation of skeletal muscle during the period following injury.

This work explores this concept through three complementary approaches. First, the secretome of adipose-derived stem cells (ASCs) is investigated as a source of bioactive factors that may influence the regenerative environment. Second, EVs derived from denervated skeletal muscle are examined as mediators of intercellular communication. Third, a nerve-derived ECM hydrogel is evaluated as a biomaterial strategy to provide structural and biological support within regenerating nerves. Together, these approaches address different aspects of the extracellular environment with the aim of improving muscle preservation and functional recovery after peripheral nerve injury.

To provide context for these strategies, the structural and functional organisation of peripheral nerves and skeletal muscle is outlined below.

2. The Nerve-Muscle Unit in Peripheral Nerve Injury

2.1 Structural and Functional Organisation of Peripheral Nerves

The **peripheral nervous system (PNS)** provides the anatomical and functional connection between the central nervous system (CNS) and peripheral tissues. The CNS consists of the brain and spinal cord, where neuronal cell bodies are organised into grey matter, and myelinated axonal tracts form white matter. Motor and sensory pathways exit and enter the spinal cord through distinct anatomical structures that give rise to peripheral nerves¹.

Motor axons innervate skeletal muscle fibres, and they originate from motor neuron cell bodies located in the anterior horn of the spinal cord grey matter. Each axon represents a continuous cellular extension of a single neuron and can project long distances from the spinal cord to its peripheral target. These axons exit the spinal cord through the ventral root². **Sensory axons**, whose cell bodies reside in the dorsal root ganglion, enter the spinal cord through the dorsal root. These neurons are pseudounipolar, with a single axonal process that extends both centrally into the spinal cord and peripherally towards target tissues. The dorsal root ganglia contain the somata of primary sensory neurons that convey afferent information such as proprioception, nociception, and mechanosensation³. The dorsal and ventral roots merge to form a spinal nerve, which then gives rise to peripheral nerves containing bundles of axons that transmit motor, sensory, and autonomic signals to and from target tissues. Autonomic fibres regulate vascular tone, sweat glands, and other involuntary functions within peripheral tissues. These different fibre types coexist within the same nerve trunk, enabling coordinated communication between the CNS and peripheral organs, including skeletal muscle⁴.

Structurally, peripheral nerves are arranged in a hierarchical organisation of connective tissue layers. Individual axons are surrounded by Schwann

cells and embedded within the endoneurium, a delicate connective tissue matrix containing capillaries that support metabolic exchange. Groups of axons form fascicles that are enclosed by the perineurium, a multilayered cellular sheath that provides mechanical stability and contributes to the blood-nerve barrier. Resident macrophages are also present within the nerve, where they participate in immune surveillance, debris clearance, and regulation of inflammatory responses⁵. The outer layer, the epineurium, surrounds the entire nerve and contains larger blood vessels and collagen fibres deposited by fibroblasts that confer tensile strength and protection against external mechanical forces (Fig. 1)⁴.

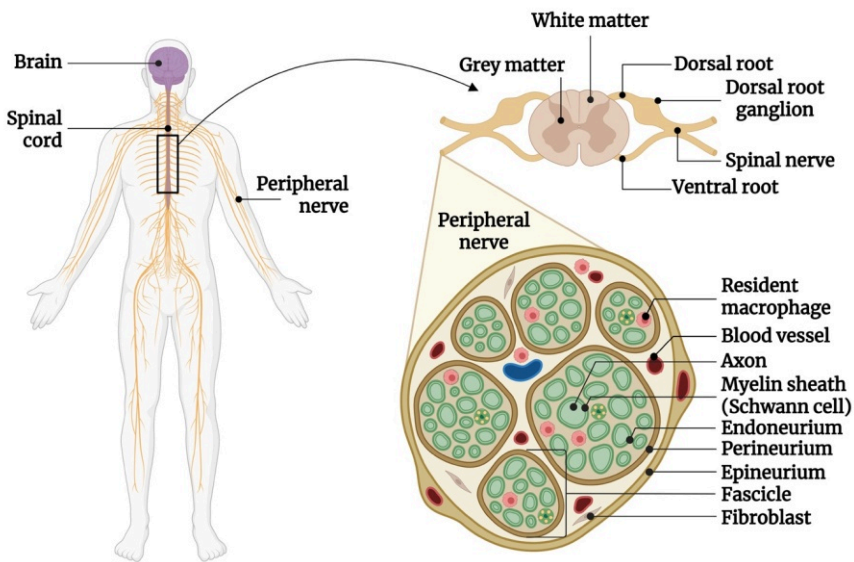


Figure 1. PNS overview and peripheral nerve structure.

Schwann cells are glial cells essential for peripheral nerve function. In large-diameter fibres, Schwann cells form myelin sheaths around axons that enable rapid saltatory conduction of action potentials. In smaller fibres, non-myelinating Schwann cells provide structural and metabolic support. Beyond their role in conduction, Schwann cells actively regulate axonal maintenance, ECM composition, and trophic signalling within the nerve microenvironment. This close association between axons and Schwann cells ensures coordinated structural and functional stability along the length of the nerve^{6,7}.

Peripheral nerves are therefore not simply conduits for electrical impulses but organised biological structures in which axons, glial cells, and connective tissue components interact to maintain signal transmission and tissue homeostasis.

2.2 Motor Innervation and Neuromuscular Dependency

Skeletal muscle contraction depends on the transmission of signals from motor neurons to muscle fibres at specialised synapses known as neuromuscular junctions (NMJs). As motor axons branch within the muscle, each neuron establishes connections with a distinct group of fibres, forming a **motor unit**. The size and recruitment properties of motor units determine the balance between fine motor control and force generation within a muscle⁸.

At the NMJ, neuronal electrical activity is converted into a contractile response. Arrival of an action potential at the presynaptic terminal leads to calcium-dependent release of acetylcholine into the synaptic cleft. Acetylcholine binds to nicotinic receptors on the postsynaptic membrane, generating depolarization of the muscle fibre and triggering excitation-contraction coupling. This process links motor neuron firing directly to calcium release within the muscle fibre and activation of the contractile apparatus⁹. Although the NMJ is structurally specialised to ensure reliable transmission, its stability is not static¹⁰. Maintenance of synaptic integrity requires continuous signalling between motor neurons, muscle fibres, and perisynaptic Schwann cells¹¹. Loss of innervation disrupts this balance and leads to progressive structural and functional alterations at the synapse (Fig. 2)¹².

Motor innervation also plays a central role in regulating muscle phenotype and metabolic properties. Patterns of motor neuron activity influence fibre type specification by shaping transcriptional programmes that control contractile protein expression, mitochondrial content, and metabolic enzyme profiles^{13, 14}. The interaction between motor neurons and muscle fibres is bidirectional. Muscle fibres provide **retrograde support** to motor neurons through trophic and activity-dependent signals, while motor neurons regulate muscle structure and phenotype through anterograde input¹⁵. This reciprocal relationship is essential for maintaining neuromuscular stability. Disruption of this communication,

such as during denervation or nerve injury, affects both synaptic integrity and muscle homeostasis¹⁶.

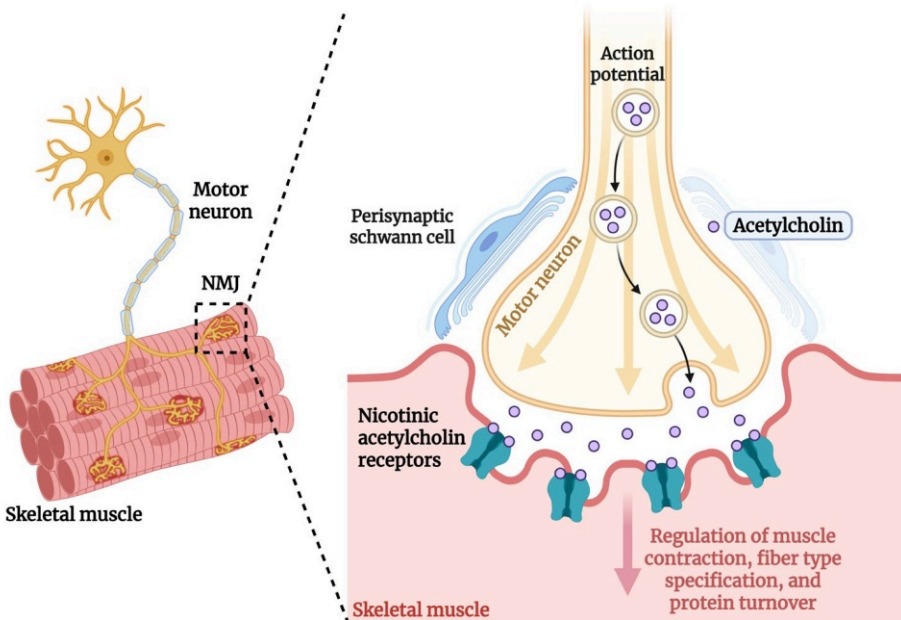


Figure 2. Motor neuron innervation and neuromuscular junction (NMJ) structure.

2.3 Healthy Muscle Structure and Neural Maintenance

Skeletal muscle is a highly organised tissue composed of multiple interacting cell types that work together to maintain contractile function, metabolic stability, and structural integrity. The principal cellular unit of skeletal muscle is the muscle fibre, also known as a myofibre. A muscle fibre is a long, multinucleated syncytial cell responsible for generating force¹⁷.

Within each muscle fibre are numerous myofibrils, which are the contractile structures responsible for muscle contraction. These myofibrils are bundled within the fibre, while muscle fibres themselves are grouped into fascicles. Fascicles are surrounded by perimysium, and

several fascicles together form the whole muscle, which is enclosed by epimysium¹⁸.

The sarcomere is the basic functional unit of the myofibril. Sarcomeres contain four major myofilament systems: thick filaments, thin filaments, titin, and nebulin. Thick filaments are primarily composed of myosin, a motor protein with ATPase activity that produces force through cyclic interactions with actin. Thin filaments consist primarily of actin, along with regulatory proteins troponin and tropomyosin, which regulate contraction in a calcium-dependent manner. Titin contributes to passive elasticity and structural alignment of the sarcomere. Nebulin helps stabilise thin filament length (Fig. 3)¹⁷.

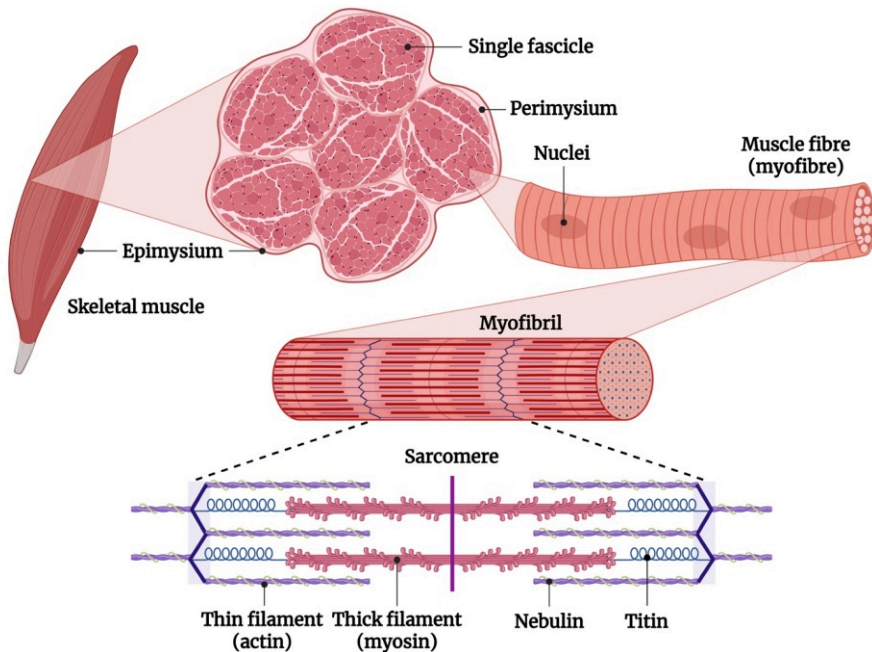


Figure 3. Skeletal muscle organisation and sarcomere structure.

Together, these proteins ensure structural stability and efficient force transmission. Sarcomeres are arranged in series to form the myofibrils, and multiple myofibrils are packed within each muscle fibre. Muscle fibres are enclosed by the sarcolemma, the specialised cell membrane of muscle fibre¹⁸.

Muscle fibres can also be classified according to their contractile speed and metabolic properties, which are largely determined by the expression of different myosin heavy chain (MHC) isoforms. **Type I (slow twitch) fibres** have high mitochondrial density, strong oxidative capacity, and high resistance to fatigue. **Type II (fast twitch) fibres** contract more rapidly and rely more on glycolytic metabolism, though their properties can vary among subtypes. Type II fibres can be further subdivided according to ATPase activity (IIA, IIB, IIC) or by the specific myosin heavy chain (MHC) isoforms they express (MHCIIA, MHCIIIB, and MHCIIIX/MHCIIID). In addition, hybrid fibres, which co-express more than one MHC isoforms, are commonly observed.

In general, Type I and Type IIA fibres tend to have smaller diameters, whereas Type IIX and Type IIB fibres exhibit intermediate to larger diameters. Most skeletal muscles contain a mixture of fibre types, and the relative proportion of these fibres largely determines the muscle's metabolic characteristics and force-generating capacity. Importantly, fibre-type identity is strongly influenced by motor neuron activity patterns, highlighting the critical role of neural input in regulating muscle fibre specialisation^{19,20}.

Satellite cells serve as the resident muscle stem cell population. Located between the sarcolemma and the basal lamina, they remain largely quiescent under physiological conditions. Upon activation, they proliferate and differentiate to support growth and repair. Their maintenance and activation state are influenced by neural input and by local signals within the muscle microenvironment^{21,22}.

The **ECM** provides mechanical support and coordinates force transmission between fibres and connective tissue. Fibroblasts regulate ECM synthesis and remodelling, maintaining an appropriate balance between collagen deposition and degradation. In healthy muscle, ECM turnover is tightly controlled to preserve compliance and structural organisation²³.

Skeletal muscle is highly vascularised. **Capillary networks** formed by endothelial cells ensure oxygen and nutrient delivery in proportion to metabolic demand²⁴. Oxidative fibres generally exhibit higher capillary density than glycolytic fibres²⁵. Resident immune cells, including

macrophages, contribute to tissue surveillance and regulation of local inflammatory signalling²⁶.

Motor neurons integrate and regulate this cellular network. Continuous neural input maintains muscle fibre phenotype, contractile protein expression, metabolic profile, and NMJ structure¹³. Through coordinated interactions among muscle fibres, satellite cells, fibroblasts, vascular cells, immune cells, and motor neurons, skeletal muscle functions as a dynamic and adaptable tissue.

2.4 Peripheral Nerve Injuries (PNIs)

Peripheral nerve injuries (PNIs) occur when there is a disruption of the structural integrity or functional capacity of nerves outside the CNS. Common mechanisms include traction, compression, laceration, ischaemia, and crush injury, often in association with fractures, joint dislocations, or damage to surrounding soft tissues²⁷. PNIs affect individuals across all age groups but are most commonly observed in young and middle-aged adults with an incidence of 1-3% in trauma populations^{28,29}. This reflects the increased exposure of young adults to high-energy trauma such as traffic accidents, occupational injuries, and sports-related incidents. In the paediatric population, the prevalence of PNIs among trauma cases ranges from 0.1% to 6%, with older age and higher injury severity scores associated with increased risk³⁰. Beyond their clinical presentation, PNIs initiate a series of biological processes within the injured nerve that determine the potential for recovery.

2.4.1 Response of Peripheral Nerves to Injury

PNI triggers a coordinated biological response consisting of an initial degenerative phase followed by axonal regeneration. In the distal nerve segment, Wallerian degeneration is characterised by axonal fragmentation, myelin breakdown, and Schwann cell dedifferentiation³¹. Schwann cells downregulate myelin-associated genes and adopt a repair phenotype, contributing to myelin clearance and the regenerative process³². Resident and infiltrating macrophages play a central role in debris removal and in regulating the local inflammatory response³³. This phase is essential for eliminating inhibitory components and preparing the tissue for regeneration.

Following degeneration, injured neurons undergo a phenotypic shift towards a regenerative state, upregulating growth-associated genes and extending axons from the proximal stump. **Regenerating axons** form growth cones that are guided through the distal nerve by Schwann cells, which provide structural support³⁴. In parallel, angiogenesis contributes to nerve repair by restoring blood supply and supporting Schwann cell migration, thereby facilitating axonal extension³⁵.

Finally, ECM remodelling further supports regeneration by maintaining structural integrity and regulating growth factor (GF) availability. Key components, including laminin, fibronectin, and type IV collagen, facilitate cell adhesion and migration³⁶. Despite these coordinated processes, recovery is often incomplete, and clinical intervention is frequently required.

2.4.2 Clinical Management and Repair Strategies of PNI

Because spontaneous regeneration is often insufficient to fully restore function, clinical management aims to support or reconstruct the injured nerve to enable axonal regrowth towards the target tissue. Although precise global incidence rates are difficult to establish, PNIs represent a significant clinical problem, frequently requiring prolonged rehabilitation and specialised surgical management³⁷.

The **clinical management of PNIs** ranges from clinical and electrophysiological monitoring to surgical reconstruction. The choice of treatment strategy is guided by the mechanism and extent of injury, anatomical location, gap length, timing of intervention, and patient-related factors³⁸. Injuries without structural disruption may recover with observation, whereas complete transections or segmental defects commonly require operative repair. In such cases, the primary objective of surgery is to restore continuity between the proximal and distal nerve segments to permit axonal regeneration towards the target tissue. Repair approaches include direct end-to-end nerve repair, reconstruction using grafts or conduits, and nerve transfer procedures.

When nerve continuity can be restored without excessive tension, direct end-to-end repair remains the preferred option. This approach involves precise microsurgical alignment of the proximal and distal nerve ends to

support axonal regeneration across the repair site. Direct repair yields the most favourable outcomes in clean lacerations and when performed early, before retraction of nerve ends, scar formation, or secondary tissue changes compromise the regenerative environment³⁹.

In injuries associated with segmental nerve loss, reconstruction using grafts is required. **Autologous nerve grafts** are still regarded as the clinical gold standard due to their preserved endoneurial architecture and Schwann cell support⁴⁰. However, donor-site morbidity, limited graft availability, and poor outcomes with substantial defects have driven the development of alternative strategies. These include processed nerve allografts and synthetic nerve guidance conduits, which aim to provide structural support for axonal growth. Despite these advances, regeneration across longer gaps remains inconsistent, and functional recovery often remains incomplete⁴¹.

Nerve transfer procedures are employed when proximal reconstruction is not feasible, such as in high-level or proximal injuries including severe brachial plexus lesions⁴². By redirecting a functioning donor nerve or fascicle to a denervated target, nerve transfers shorten the distance required for axonal regrowth and can improve the likelihood of timely reinnervation of critical muscles. Nevertheless, nerve transfers require partial sacrifice of donor function and do not eliminate the biological constraints associated with delayed regeneration⁴³.

Across all surgical repair strategies, a common and often underappreciated limitation is the progressive deterioration of **denervated skeletal muscle**, during the prolonged period required for axonal regeneration. While surgical techniques focus on restoring axonal continuity, successful functional recovery ultimately depends on the ability of regenerating axons to reconnect with viable muscle fibres. Prolonged denervation leads to muscle atrophy, fibrotic remodelling, altered ECM composition, and changes in the local signalling environment, all of which can compromise reinnervation even when nerve repair is anatomically successful⁴⁴.

2.5 Denervation-Induced Skeletal Muscle Changes

Skeletal muscle integrity is tightly dependent on continuous neural input. Following peripheral nerve injury, loss of motor innervation initiates a progressive degenerative process within the target muscle that

compromises both structure and function. Even when axonal regeneration occurs, prolonged denervation leads to changes that limit the capacity for recovery.

2.5.1 Short-Term Changes in Denervated Muscle

Denervation leads to rapid disruption of the NMJs, one of the earliest detectable events following nerve injury. Loss of presynaptic input results in withdrawal of the motor terminal and fragmentation of postsynaptic acetylcholine receptor clusters. Junctional folds become disorganised, and the specialised synaptic architecture begins to deteriorate. Acetylcholine receptors are redistributed beyond the synaptic region, and fetal-type receptor subunits are re-expressed. These changes impair synaptic transmission and initiate transcriptional programmes associated with atrophy^{45,46}.

Microvascular changes are also observed. Alterations in perfusion and capillary structure occur, and capillary density begins to decline after an initial adaptive phase⁴⁷.

Within days of denervation, muscle fibres undergo a reduction in cross-sectional area. Although both slow- and fast-twitch fibres are affected, the rate and extent of atrophy vary between fibre types and between different muscles. This early atrophy reflects both decreased protein synthesis and increased protein degradation. Activation of proteolytic systems, including the ubiquitin-proteasome pathway and autophagy-related mechanisms, contributes to loss of myofibrillar proteins⁴⁸. In parallel, reduced contractile activity leads to mitochondrial dysfunction, increased production of reactive oxygen species, and activation of redox-sensitive signalling pathways which further promote proteolysis and inflammatory gene expression^{49,50}.

During this early phase, overall sarcomeric alignment remains largely preserved despite progressive thinning of myofibrils. Fibre membranes remain intact, and the basal lamina is maintained⁵¹. Satellite cells may show transient activation; however, in the absence of reinnervation, this response is not sufficient to restore fibre size (Fig.4)⁵².

2.5.2 Long-Term Changes in Denervated Muscle

With **prolonged denervation**, skeletal muscle undergoes progressive and often irreversible remodelling. Continued absence of neural input leads to marked fibre atrophy, extensive loss of contractile proteins, and increasing disorganisation of sarcomeres. Myofibrils become fragmented, and fibres undergo segmental degeneration^{53,54}. Mitochondrial content and structural integrity decline over time, contributing to impaired oxidative metabolism and sustained oxidative stress⁵⁵.

Proteolytic activity remains elevated, with sustained involvement of the ubiquitin-proteasome and autophagy-lysosome systems. Over time, some fibres undergo apoptosis or necrosis, resulting in a reduction in total fibre number^{56,57}. Denervated fibres may also display nuclear clustering and centralization, reflecting ongoing structural instability^{58,59}.

Vascular remodelling becomes pronounced with prolonged denervation. Capillary density decreases, and microvascular organisation deteriorates. Reduced perfusion contributes to impaired oxygen and nutrient delivery, which further accelerates fibre degeneration and limits regenerative capacity^{60,61}.

Concomitantly, ECM deposition increases. Myofibroblasts produce excess collagen and other matrix components, leading to fibrosis^{62,63}. Progressive interstitial and perimysial fibrosis disrupt normal muscle architecture, reduces elasticity, and interferes with effective force transmission. Dense connective tissue accumulation can also hinder regenerating axons from forming functional neuromuscular junctions^{45,64}.

Adipose infiltration frequently develops in chronically denervated muscle. Expansion and differentiation of fibro-adipogenic progenitor cells contribute to this fatty replacement during prolonged denervation. This compositional shift reflects the transition from functional muscle towards structurally compromised tissue (Fig. 4)^{65,66}

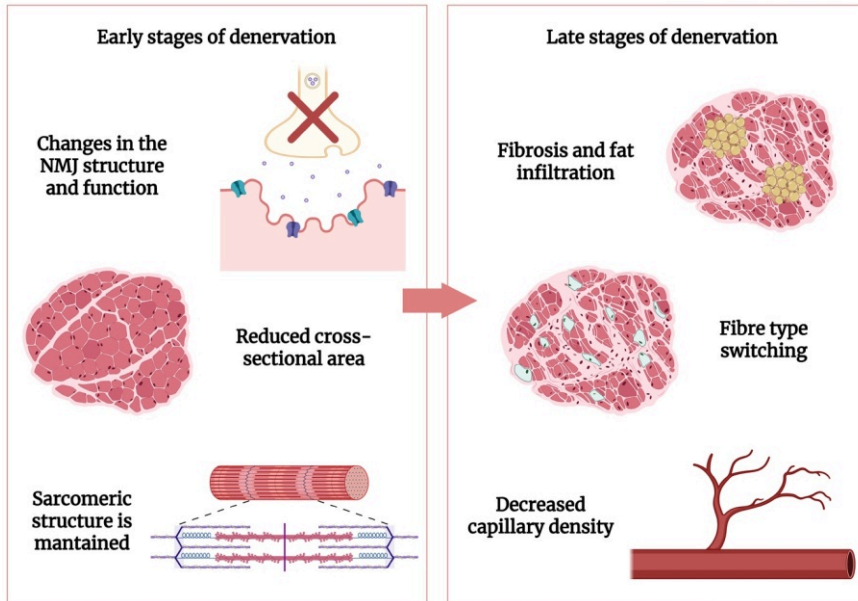


Figure 4. Skeletal muscle changes following denervation.

Importantly, the degenerative process is strongly time dependent. While early denervated muscle remains receptive to reinnervation, prolonged denervation leads to skeletal muscle atrophy. Even if regenerating axons successfully reach the target muscle and reform structurally intact neuromuscular junctions, effective neuromuscular transmission may fail to be restored. Thus, functional outcome after peripheral nerve injury is determined not only by axonal regeneration but also by the condition of the denervated muscle at the time of reinnervation⁶⁷.

Together, these changes show that denervation affects not only muscle fibres themselves but also the surrounding extracellular and secretory environment. As denervation persists, this environment becomes progressively altered through changes in inflammatory signalling, vascular support, and ECM composition, reducing the tissue's capacity to support successful reinnervation.

3. The Regenerative Challenge in the Nerve-Muscle Microenvironment

PNI disrupts not only the structural continuity of the nerve but also the local microenvironment that supports both nerve regeneration and muscle maintenance. Following injury, coordinated changes occur in the distal nerve segment and the denervated muscle, including inflammation, vascular alterations, ECM remodelling, and changes in the secretory activity of resident and infiltrating cells. These processes collectively define a dynamic microenvironment that evolves over time and critically influences regenerative outcomes.

Successful recovery depends on the preservation of this regenerative environment. However, while axonal regeneration progresses slowly, the denervated muscle undergoes progressive degeneration. In parallel, the distal nerve environment also undergoes structural and cellular changes that can limit axonal guidance and growth. The temporal mismatch between nerve regeneration and target tissue degeneration represents a major barrier to recovery⁶⁸.

This interdependence highlights that peripheral nerve injury should be considered as a disorder of the nerve-muscle unit rather than of the nerve alone. The condition of the target muscle at the time of reinnervation is a critical determinant of outcome. Even when regenerating axons successfully reach the muscle, an altered microenvironment may prevent effective neuromuscular junction formation and restoration of contractile function.

4. Paracrine Signalling and the Therapeutic Secretome

Given the central role of the microenvironment, therapeutic strategies have increasingly focused on modulating key processes such as inflammation, angiogenesis, and ECM remodelling. These processes regulate both nerve regeneration and muscle preservation and represent important targets for intervention.

Paracrine signalling is a key component of this regulation and is more broadly encompassed by the concept of the **secretome**, which includes soluble factors, EVs, and matrix-associated signalling molecules. Secreted signalling occurs through several interconnected mechanisms. Soluble factors, including cytokines and GFs, mediate communication between cells, while EVs transport proteins, lipids, and nucleic acids. In parallel, the ECM acts as a resource for signalling molecules, binding and releasing GFs in a spatially and temporally regulated manner. Together, these components form a complex signalling network that contributes to regulation of the regenerative environment following nerve injury⁶⁹. Targeting the secretome therefore represents an approach to influence multiple processes simultaneously.

Among emerging strategies, cell-based and cell-derived therapies have attracted particular interest due to their capacity to modulate the secretome and thereby influence several aspects of the tissue microenvironment at once.

4.1 Cell-Based Therapies for Muscle Preservation and Regeneration

Cell-based therapies have emerged as a promising strategy for preserving skeletal muscle structure and function following denervation. In contrast to therapeutic approaches that primarily address one molecular pathway, transplanted cells may influence multiple components of the tissue microenvironment through direct cell-cell interactions and the release of soluble factors. This multifaceted activity is particularly relevant in denervated muscle, where degeneration involves coordinated changes across several cell populations including muscle fibres, fibroblasts, immune cells, endothelial cells, and resident progenitor cells⁷⁰.

Early cell-based approaches for muscle atrophy focused on myogenic cells, such as satellite cells and myoblasts, because of their ability to directly contribute to muscle fibre formation. Upon activation, these cells proliferate, differentiate into myoblasts, and ultimately fuse with existing muscle fibres or form new ones. While satellite cells and myoblasts demonstrate strong myogenic potential, their clinical application has been limited by several practical challenges, including difficulties in cell

isolation, limited expansion capacity in vitro, poor survival after transplantation, and inefficient integration into host tissue⁷¹.

An alternative cell type that has gained significant attention in regenerative medicine is the **mesenchymal stromal cell (MSC)**. MSCs can be isolated from a variety of tissues including bone marrow, adipose tissue, dental pulp, skin, and neonatal tissues such as umbilical cord or placenta. These cells are characterised by their capacity for plastic adherence in culture, multipotent differentiation potential, and a defined pattern of surface marker expression. In addition to their differentiation capacity, MSCs exhibit strong immunomodulatory, anti-fibrotic, and pro-angiogenic properties (Fig. 5)^{72,73}.

In the context of muscle atrophy, MSC-based therapies have been shown to support muscle regeneration and alleviate atrophy^{74,75}. Experimental studies have demonstrated that transplantation of bone marrow-derived MSCs into sites of nerve injury can improve subsequent muscle contractile function⁷⁶. However, tracking studies have consistently demonstrated that transplanted MSCs show limited long-term engraftment within host tissues. In many cases, the majority of transplanted cells become undetectable within a relatively short time after transplantation^{77,78}.

Despite this limited persistence, structural and functional improvements are frequently observed. This apparent discrepancy has led to increasing recognition that MSCs act primarily through **paracrine mechanisms**⁷⁹. Consistent with this concept, several studies have demonstrated that cell-derived products such as conditioned media (CM) obtained from MSC cultures can reproduce many of the beneficial effects observed with cell transplantation⁸⁰. In vitro, CM derived from umbilical cord MSCs has been reported to suppress the expression of atrophy-associated proteins and to reduce reactive oxygen species generation in models of skeletal muscle injury⁸¹.

Rather than serving as structural building blocks of the regenerating tissue, MSCs appear to function as temporary regulators of the local environment, modifying the behaviour of surrounding cells through the secretion of bioactive molecules⁸². This mode of action does not rely on long-term engraftment or differentiation of the transplanted cells, processes that have often limited the success of cell transplantation approaches in clinical trials^{83,84}.

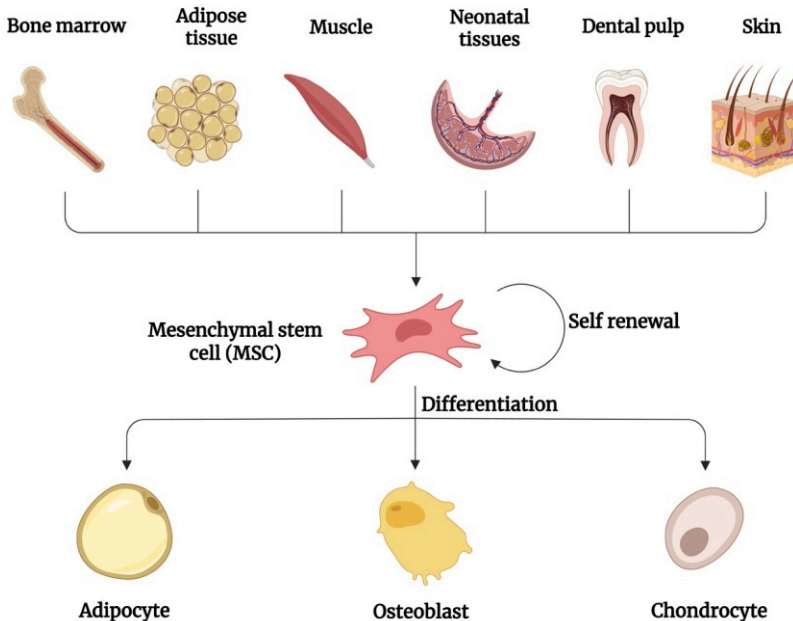


Figure 5. MSC sources and differentiation.

4.1.1 Adipose-Derived Stem Cells (ASCs) as a Therapeutic Resource

Adipose-derived stem cells (ASCs) are a population of mesenchymal stromal cells isolated from adipose tissue - typically from the stromal vascular fraction - and have become an important focus in regenerative medicine. Compared with other types of MSCs, adipose tissue yields a substantially higher number of progenitor cells per unit volume. In addition, adipose tissue can be obtained through relatively minimally invasive procedures such as liposuction, making it an accessible and abundant cell source for both research and clinical applications⁸⁵.

ASCs fulfil the minimal criteria commonly used to define MSCs. These criteria include adherence to plastic under standard culture conditions, the ability to differentiate towards adipogenic, osteogenic, and chondrogenic lineages in vitro, and the expression of a characteristic pattern of cell surface markers⁸⁶. ASCs **typically express markers** such as CD34, CD73, CD90, and CD105, while lacking expression of

haematopoietic and endothelial markers including CD45 (haematopoietic cells) and CD31 (endothelial cells)⁸⁷.

Additional markers are sometimes used in research settings to further characterise ASC subpopulations. One such marker is **CD146**, which is commonly associated with perivascular cells including pericytes. ASC subpopulations expressing CD146 are frequently interpreted as having a perivascular origin and have been linked in experimental studies to enhanced angiogenic and trophic activity⁸⁸. However, CD146 is not part of the minimal MSC definition and is therefore not universally required for clinical product characterisation⁸⁹.

In vitro, ASCs demonstrate adipogenic, osteogenic, and chondrogenic differentiation potential. Although this multipotency initially suggested that their therapeutic effects might rely on direct differentiation into target tissues, accumulating evidence indicates that their primary activity in vivo is mediated through **paracrine signalling** rather than structural engraftment⁷⁹.

4.1.2 Regenerative Potential of the ASCs Secretome

ASCs secrete a wide range of cytokines, GFs, and EVs capable of influencing multiple biological processes. These secreted mediators can reduce inflammation, promote angiogenesis, modulate ECM turnover, and enhance the survival of stressed or injured cells in various models of tissue injury⁹⁰.

Angiogenesis represents a central component of tissue regeneration and muscle preservation. Adequate vascularisation ensures oxygen and nutrient supply to regenerating tissues and supports the survival of muscle fibres. ASCs also contribute to angiogenesis through the secretion of GFs that regulate endothelial cell proliferation, migration, and capillary network formation⁹¹.

Among these mediators, **hepatocyte growth factor (HGF)** plays an important role. Its primary signalling function is mediated through binding to the c-MET receptor which is expressed on multiple cell types including endothelial cells, activating pathways that promote cell proliferation, migration, and ECM remodelling⁹². In skeletal muscle, HGF has also been shown to stimulate muscle regeneration and support

functional remodelling of injured muscle tissue in experimental models^{93,94}.

Another key factor involved in vascular regulation is **vascular endothelial growth factor (VEGF)**. VEGF is widely recognised for its central role in promoting vascular sprouting and endothelial cell survival. In addition to its angiogenic activity, VEGF has also been shown to influence skeletal muscle regeneration by promoting activation and proliferation of myogenic progenitor cells in experimental models of ischaemic muscle injury^{95,96}.

Insulin-like growth factor 1 (IGF-1) represents another trophic mediator secreted by ASCs. IGF-1 activates intracellular signalling pathways including PI3K/Akt and MAPK cascades in both endothelial and muscle fibres. These pathways promote cell survival, protein synthesis, and angiogenic responses^{97,98}. In skeletal muscle denervation, experimental studies have shown that increased IGF-1 can attenuate skeletal muscle atrophy^{99,100}.

Among the molecules secreted by ASCs, **urokinase plasminogen activator (uPA)** has been identified as an important mediator of tissue remodelling. uPA is a serine protease that converts plasminogen into plasmin, an enzyme capable of degrading ECM components such as fibrin, fibronectin, and laminin¹⁰¹. Through this proteolytic activity, uPA contributes to ECM turnover and facilitates the migration of inflammatory and progenitor cells during tissue regeneration¹⁰².

The activity of uPA is tightly regulated and occurs in coordination with other components of the plasminogen activation system, including tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)¹⁰³. Experimental studies using Plau knockout models have demonstrated that uPA plays a particularly important role in skeletal muscle regeneration compared with tPA and PAI-1. uPA-dependent matrix remodelling facilitates macrophage infiltration into damaged muscle tissue, a process required for efficient clearance of degenerating muscle fibres and subsequent regeneration^{104,105}. In contrast, increased levels of PAI-1 have been associated with impaired muscle regeneration¹⁰⁶.

4.1.3 Modulation of the ASC Properties

The functional properties of ASCs can be influenced by environmental conditions. Changes in oxygen concentration, biochemical stimulation, and mechanical cues can alter gene expression patterns and modify the composition of the ASC secretome^{107,108}. For example, hypoxic culture conditions have been shown to increase the secretion of angiogenic factors¹⁰⁹.

Chemical stimulation of ASC cultures represents another strategy to modulate their biological activity. The addition of GFs or signalling molecules to the culture medium can direct ASCs towards particular phenotypic states and alter their secretory profile. In the context of peripheral nerve injury, several studies have investigated protocols that induce ASCs to acquire a Schwann cell-like phenotype^{110,111}.

Schwann cells play an essential role in peripheral nerve regeneration by producing neurotrophic factors and guiding axonal growth. ASCs that have been stimulated towards a Schwann cell-like phenotype have been reported to display increased secretion of neurotrophic and angiogenic factors¹¹².

In the context of skeletal muscle, the secretome derived from stimulated MSCs has demonstrated promising effects. For example, indirect co-culture of Schwann-like differentiated ASCs and myoblasts enhanced myoblast proliferation and improved regenerative responses *in vitro*¹¹³. Similarly, extracellular vesicles as another component of MSCs secretome derived from skin precursor-derived Schwann cells alleviated denervation-induced skeletal muscle atrophy¹¹⁴.

Despite these encouraging findings, the optimal combination of factors and timing required to induce such phenotypic changes remains under investigation. Many differentiation protocols involve prolonged culture periods and complex combinations of signalling molecules, which may limit their practical applicability^{115,116}.

From a translational perspective, safety and regulatory considerations must also be addressed. Conversion of research-based differentiation protocols into procedures compatible with **Good Manufacturing Practice (GMP)** standards requires careful evaluation of reagents, culture conditions, and potential risks for patients. One study has

explored ASC differentiation in the presence of human platelet lysate, a supplement compatible with GMP requirements, suggesting that clinically compliant protocols may be feasible¹¹⁷. However, studies combining ASC stimulation strategies with GMP-compatible culture systems remain relatively limited.

4.1.4 Clinical Translation and Good Manufacturing Practice Considerations

The translation of adipose-derived stromal/stem cells (ASCs) into clinical therapies requires compliance with regulatory frameworks governing **advanced therapy medicinal products (ATMPs)**. Manufacturing processes must therefore be standardised, reproducible, and performed under GMP conditions¹¹⁸. These requirements include controlled tissue procurement procedures, validated cell isolation methods, standardised expansion protocols, and full traceability of reagents used during production. Comprehensive quality control testing is also required throughout the manufacturing process¹¹⁹.

One of the major challenges in ASC manufacturing is the selection of appropriate culture supplementation for in vitro expansion. The composition of the culture medium can significantly influence both the safety profile and biological function of ASCs.

Traditionally, **fetal bovine serum (FBS)** has been widely used as a supplement to support cell growth and proliferation¹²⁰. However, the use of FBS raises several safety and regulatory concerns in clinical applications. Because FBS is derived from bovine blood, it may introduce xenogeneic proteins, pathogens, and batch-to-batch variability¹²¹. These factors may trigger immune reactions in patients and reduce reproducibility between production batches¹²². For this reason, regulatory agencies such as the European Medicines Agency recommend avoiding animal-derived components whenever possible in clinical-grade manufacturing processes¹²¹.

Human platelet lysate (PLT) has emerged as a widely used human-derived alternative to FBS. PLT supports robust ASC proliferation and maintains efficient cell expansion under xeno-free conditions¹²³. However, variability between platelet donors and differences in preparation protocols can influence the concentration of platelet-derived

GFs and cytokines present in the lysate, potentially affecting reproducibility¹²⁴. To reduce donor-specific variability, platelet units are often pooled during PLT production. While pooling may improve batch consistency, it also introduces additional considerations regarding donor screening, traceability, and regulatory compliance¹²⁵.

Another strategy involves the use of chemically defined **xeno-free or serum-free culture media**. These systems eliminate undefined biological components and therefore improve standardisation of the manufacturing process. Serum-free media have been investigated for many years and aim to support cell growth and proliferation without the addition of serum or other animal-derived supplements^{126,127}.

Although these media reduce the risk of contamination and improve regulatory compliance, their effects on ASC phenotype, differentiation potential, and secretory activity must be carefully evaluated during process development. Changes in culture conditions can influence the biological properties of ASCs, including the composition of their secretome, which may ultimately affect therapeutic efficacy¹²⁸.

Overall, the development of robust xeno-free culture systems, together with optimised isolation procedures and reproducible downstream processing protocols, represents a critical step towards the safe and reliable clinical translation of ASC-based therapies.

4.2 Extracellular Vesicles (EVs)

EVs are membrane-enclosed particles released by cells into the extracellular space. They are now widely studied as mediators of intercellular communication in both physiological and pathological conditions¹²⁹. EVs contain proteins, lipids, and nucleic acids derived from their cells of origin, and their molecular composition reflects the state of the releasing cell. Once taken up by recipient cells, EVs can influence gene expression, metabolism, and signalling pathways. Through these mechanisms, EVs contribute to processes such as inflammation, angiogenesis, and tissue repair^{130,131}.

EVs are commonly categorized according to size and presumed biogenesis. Small EVs, often referred to as **exosomes**, are generally described as vesicles below approximately 150-200 nm in diameter and are associated with the endosomal pathway¹³². Larger vesicles, frequently

termed **microvesicles**, are thought to originate from outward budding of the plasma membrane¹³³. However, clear separation of vesicle populations based solely on biogenesis remains technically difficult. Isolated EV preparations often contain heterogeneous populations, and definitive assignment to a specific formation pathway is rarely possible. For this reason, current recommendations emphasise describing EVs using operational criteria such as size, density, and the presence of characteristic protein markers rather than strict biogenetic labels¹³⁴.

EV cargo loading is not random. Multiple studies have demonstrated selective enrichment of specific microRNAs, RNA-binding proteins, and signalling molecules within EVs, suggesting active sorting mechanisms^{135,136}. In addition to their internal cargo, EVs display membrane-associated molecules such as tetraspanins, adhesion proteins, and other surface antigens, which can influence their stability, biodistribution, and interactions with recipient cells. Tetraspanins (CD9, CD63, CD81), ALIX, and TSG101 are commonly enriched in small EVs and serve as operational markers, although none are exclusive (Fig. 6)¹³⁴.

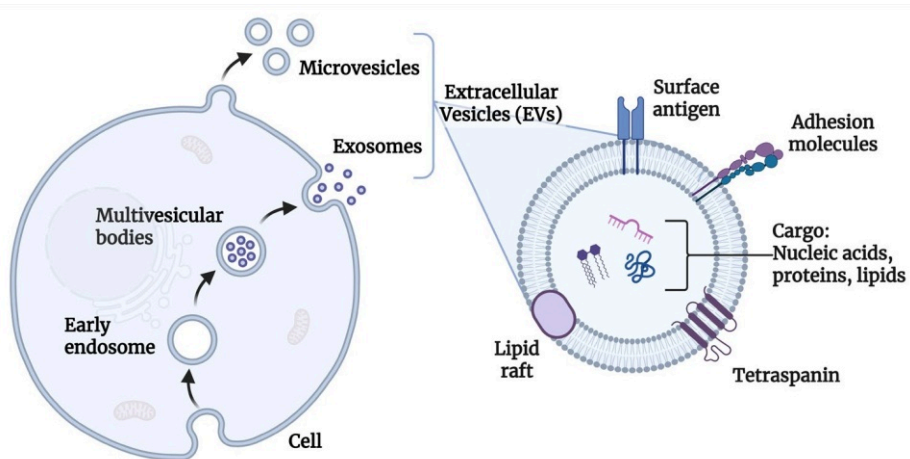


Figure 6. Schematic of EV biogenesis, structure and composition.

Once released, EVs can be internalised by recipient cells through clathrin-mediated endocytosis, macropinocytosis, phagocytosis, or direct membrane fusion. Functional studies have demonstrated that transferred miRNAs can repress target mRNAs in recipient cells, and EV-associated proteins can activate intracellular signalling cascades. These findings

establish EVs as active mediators of intercellular regulation rather than byproducts of cellular turnover¹³⁷⁻¹⁴⁰.

4.2.1 Methodological Considerations in EVs Isolation

EVs can be isolated from multiple biological sources, including conditioned cell culture media, biofluids, and solid tissues. While these approaches are sometimes treated as interchangeable, the biological meaning of the resulting vesicle populations differs substantially depending on the source material. Therefore, isolation strategy is not methodologically neutral: it shapes both vesicle composition and the conclusions that can be drawn from downstream functional or profiling studies.

Conditioned media (CM) from cultured cells is widely used in EV research due to its accessibility and experimental controllability. In vitro systems allow manipulation of environmental variables, genetic background, and signalling pathways. In skeletal muscle research, EVs are frequently studied using primary human or animal myoblasts as well as immortalized muscle fibre lines, which allow investigation of muscle-derived vesicle signalling under controlled conditions^{141,142}. However, vesicles released under culture conditions reflect a simplified cellular environment that lacks cellular diversity, ECM architecture, and mechanical and metabolic inputs of native muscle tissue. Furthermore, culture parameters, including serum supplementation, oxygen tension, and cell confluency, can influence EV release rates and alter EV cargo composition. For example, serum-derived particles can contaminate preparations if depletion is incomplete, and hypoxia can shift the abundance of angiogenic and stress-associated factors. As a result, EVs derived from CM represent a controlled but reductionist model of vesicle biology¹⁴³⁻¹⁴⁵.

Biofluid-derived EVs, particularly from plasma, serum, urine, and cerebrospinal fluid, have been widely explored for biomarker discovery. Circulating EVs integrate signals from multiple tissues and may provide insight into systemic physiological or pathological states. In skeletal muscle research, circulating vesicles carrying muscle-associated molecules such as miRNAs have been investigated as potential biomarkers of muscle damage, denervation, and neuromuscular diseases^{146,147}. However, the EV fraction in plasma or serum contains diverse non-vesicular components that may co-isolate depending on the

method used, which can further complicate interpretation. Changes detected in circulating EV populations reflect contributions from several organs simultaneously, making mechanistic interpretation challenging without complementary experimental strategies^{148,149}.

In contrast, isolation of **EVs directly from solid tissues** allows examination of vesicle populations within a defined microenvironment. Tissue-derived EVs reflect integrated signalling among resident cell populations embedded within an intact ECM and exposed to physiological mechanical and metabolic signals^{150,151}. This approach offers spatial specificity but introduces additional technical complexity. Numerous protocols have been developed to isolate EVs from solid tissue, including skeletal muscle^{152,153}. Often this includes mechanical disruption and enzymatic digestion, which may release intracellular material or generate vesicle-like particles. This creates a trade-off between yield and purity: harsher dissociation can increase particle recovery while also increasing contamination by intracellular fragments and stress-induced vesicles¹⁵⁴. Moreover, tissue-derived EV preparations represent mixed populations originating from multiple cell types, and distinguishing their cellular source remains difficult in the absence of labelling approaches.

Taken together, comparisons across CM-, biofluid-, and tissue-derived EV studies require explicit reporting of source material and isolation methodology, as well as careful interpretation of which biological level is being captured. A comprehensive understanding of EV biology therefore depends on the relationship between biological source, isolation strategy, and downstream readouts¹⁵⁵.

4.2.2 Skeletal Muscle as a Source and Target of EV-Mediated Communication

Skeletal muscle is not only a contractile organ but also a **secretory tissue** that releases a broad range of signalling molecules, including cytokines and EVs. In this context, muscle functions as both a producer of EV-mediated signals and a recipient of vesicle-dependent regulation originating from other tissues. This bidirectional signalling is relevant for muscle adaptation, regeneration after injury, and coordination of systemic metabolic responses.

Muscle-derived EVs are involved in communication at several levels, including between muscle fibres, by muscle-specific transferring microRNAs (myomiRs), proteins, and lipids to recipient muscle fibres and other cell types. Studies have shown that myomiRs, including miR-1, miR-133a, miR-133b, and miR-206, regulate myogenic differentiation and muscle regeneration^{156,157}. Another study showed that the lipid composition of muscle-associated EVs can modulate inflammatory responses and satellite cell activation¹⁵⁸. Overall, these studies highlight the involvement of muscle-derived EVs in local muscle communication and their interaction with different cellular components of the muscle tissue.

Communication mediated by EVs also extends to **nerve-muscle interactions**. Although neurotransmission, such as acetylcholine signalling, represents the primary mode of communication at the NMJ, muscle-derived EVs provide an additional mechanism for transferring regulatory molecules that influence neuromuscular signalling. EVs released by muscle fibres can mediate communication with motor neurons by transferring regulatory molecules involved in synaptic stability and regeneration¹⁵⁹. One of the first studies done on EVs from skeletal muscle in the nerve-muscle context showed that EVs derived from myotubes promote motor neuron development and neurite branching¹⁶⁰. The **retrograde action** of EVs at the NMJ has also been investigated in the context of amyotrophic lateral sclerosis (ALS). In ALS models, dysregulation of myomiRs, including miR-206 and miR-133b, occurs in response to denervation, and miR-206 affected formation and maturation of NMJs¹⁶¹. Additionally, muscle-derived EVs isolated from ALS patients have been reported to present toxic effects on healthy motor neurons, reducing neurite length and increasing cell death¹⁶². Together, these findings indicate that skeletal muscle-derived EVs can influence NMJ integrity and motor neuron function, acting as mediators of both adaptive and pathological signalling in neuromuscular disease.

Skeletal muscle EVs can also influence gene expression and metabolic pathways in distant tissues. Evidence has shown that EVs released from skeletal muscle can enter the circulation and be taken up by recipient cells in target organs¹⁶³. In the pancreas, myomiR such as miR-16, miR-133a and miR-206 has been shown to influence beta cell viability and function in a diabetic model^{164,165}. Muscle-derived proteins and myomiRs from EVs have also been studied in the context of muscle-cardiovascular system

crosstalk, where they have been reported to enhance cardiac function, reduce fibrosis, and promote angiogenesis¹⁶⁶.

Together, these observations indicate that EVs represent an important mechanism of communication between skeletal muscle, other cells, and organs in both physiological and pathological conditions.

4.2.3 Denervation-Induced Remodelling of EV-Mediated Communication

As described above, **denervation** is associated with altered protein turnover, inflammatory activation, vascular remodelling, and satellite cell responses¹⁶⁷. These changes arise as a consequence of the loss of neural input to skeletal muscle, which disrupts the normal regulatory signals required to maintain muscle structure and metabolic balance.

Given that **EV cargo** mirrors the physiological state of the releasing cell, EVs released from denervated muscle contain molecular signatures reflecting atrophy, metabolic stress, and inflammatory activation. Such vesicles could therefore transmit information about the denervated state to neighbouring muscle-resident cells or to distant tissues.

In recent years, emerging evidence has indicated that EV are affected by denervation and its cargo can change in response to the **absence of innervation**. For example, increased levels of miR-206 have been detected in vesicles released from denervated muscle fibres¹⁶⁸. In another study, changes in EV-associated microRNA cargo were observed depending on the innervation status of cultured muscle fibres¹⁶⁹. Together, these findings indicate that neuronal signals can influence the molecular composition of vesicles released by muscle fibres. However, these studies were conducted primarily in cell culture systems, which only partially reproduce the complex cellular environment of skeletal muscle tissue.

More recently, researchers have begun to investigate EVs isolated directly from denervated skeletal muscle tissue. For instance, EV-associated miR-21a-3p derived from **solid denervated muscle tissue** has been reported to negatively influence kidney fibrosis in a cross-tissue signalling model¹⁷⁰. In addition, one study showed that EVs isolated from denervated skeletal muscle significantly improved the accuracy of motor

axon regeneration in a peripheral nerve injury model, whereas vesicles derived from healthy muscle had no detectable effect¹⁷¹.

Despite increasing evidence that EVs contribute to intercellular communication following denervation, their functional effects are likely to depend on the local tissue environment in which they act. Secreted factors and vesicle-mediated signals do not operate in isolation but are influenced by their spatial distribution, retention, and interaction with surrounding cells and matrix components. In this context, the extracellular environment not only provides structural support but also regulates the availability and activity of signalling molecules. This highlights the importance of considering how biological signals are maintained and organised within the injury site.

These considerations have contributed to growing interest in biomaterial-based strategies for peripheral nerve repair. In this context, biomaterials are not only used to restore structural continuity but also to influence how secreted factors and EVs are retained and distributed within the injury site. By providing a defined physical and biochemical framework, they may help recreate aspects of the extracellular environment that support and regulate regenerative signalling.

4.3 Extracellular Matrix and Biomaterial-Based Modulation of Regeneration

Although PNI repair strategies have advanced considerably, recovery in **large defects** or proximal injuries is often incomplete. Clinical and experimental evidence shows that functional outcome decreases significantly as gap length increases, highlighting the importance of improving the regenerative environment within the defect.

Autologous nerve grafting remains the clinical gold standard for bridging critical-sized gaps. However, given the clear limitations with autologous grafts, including donor-site morbidity, limited availability of graft material, mismatch in diameter or fascicular structure, and longer operative times, these drawbacks have motivated the development of biomaterial-based strategies designed to reproduce key structural and biological features of native nerve tissue while avoiding the disadvantages of autografts^{172,173}.

Biomaterial-assisted approaches aim to stabilise the injury site, guide axonal growth, and influence the local immune and cellular response. Over the past decades, strategies have evolved from simple hollow tubes to more complex constructs incorporating bioactive matrices, GFs, and cells (Fig. 7). Current research focuses not only on providing mechanical support but also on recreating aspects of the native extracellular microenvironment that regulate regeneration¹⁷⁴.

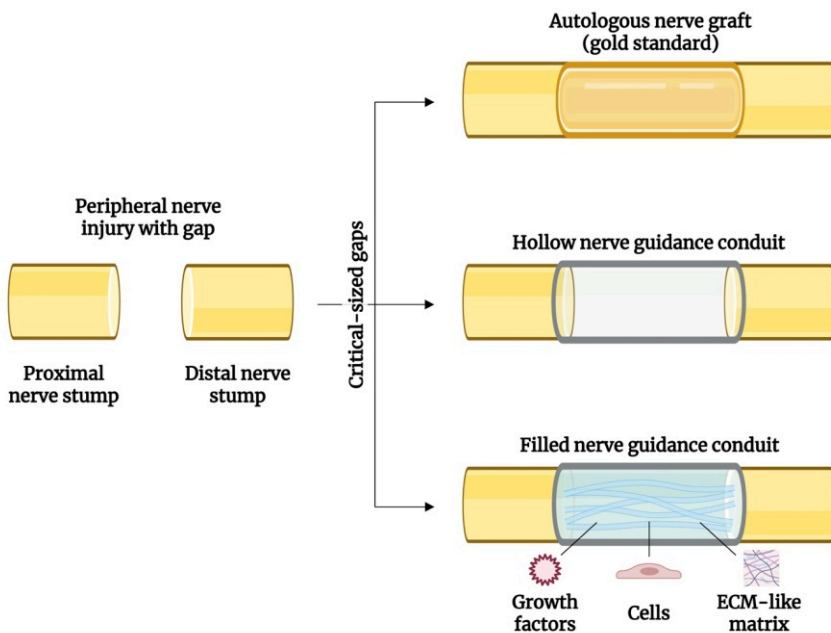


Figure 7. Strategies for peripheral nerve repair across critical gaps.

4.3.1 Nerve guidance conduits (NGCs)

Nerve guidance conduits (NGCs) were developed as an alternative to autografts for bridging nerve defects. These tubular structures connect the proximal and distal nerve stumps and create a protected channel in which regeneration can occur.

Clinically approved conduits composed of collagen, polyglycolic acid, or other biodegradable polymers are currently used for short sensory nerve gaps^{173,175,176}. Clinical studies indicate that outcomes in small defects can approach those of autografts. However, their performance decreases with

increasing gap length¹⁷⁷. In larger defects, hollow conduits often show reduced axonal regeneration, lower fibre density, and thinner myelin compared to autografts. Experimental models consistently demonstrate that empty conduits lack sufficient biological support to sustain robust regeneration across critical-length defects¹⁷⁸.

Material properties influence conduit performance. Synthetic polymers such as **polycaprolactone (PCL)** are widely investigated due to their mechanical strength, slow degradation rate, and processability^{179,180}. Electrospinning techniques allow fabrication of aligned nanofibrous PCL scaffolds that resemble the longitudinal organisation of native nerve tissue. Several studies report that aligned fibres improve Schwann cell orientation and axonal directionality compared to random structures^{181,182}. However, PCL is biologically inert and hydrophobic, which limits cell attachment and interaction unless surface modified. Coating with ECM proteins or functionalization with peptides can improve cell adhesion, but the material itself does not provide intrinsic biological signals¹⁸³.

Structural guidance alone is insufficient, particularly in long-gap injuries. Successful regeneration requires not only physical continuity but also biochemical and cellular support within the lumen.

4.3.2 Decellularized Nerve-Derived Extracellular Matrix Hydrogels (dECM)

To support cellular infiltration and tissue regeneration within hollow conduits, research has increasingly focused on the use of **hydrogel fillers**. Hydrogels are three-dimensional polymer networks with high water content that resemble the hydrated structure of soft tissues. Their porous architecture allows cell infiltration, nutrient diffusion, and matrix remodelling. Both synthetic and naturally derived hydrogels have been investigated for nerve repair.^{184,185}

Decellularized extracellular matrix (dECM) derived from peripheral nerve tissue has gained attention because it preserves components of the native neural ECM. The decellularization process removes cellular material while maintaining structural proteins such as collagen, laminin, and fibronectin, as well as glycosaminoglycans. These molecules have shown to regulate Schwann cell behaviour and axonal

growth¹⁸⁶. In addition to their structural role, ECM components bind GFs and other signalling molecules, allowing their local retention and controlled release¹⁸⁷. This creates a microenvironment in which bioactive signals are not only present but spatially organised. In this context, dECM hydrogels may influence the distribution and activity of soluble factors and EVs within the repair site. Additionally, such hydrogels can function as platforms for delivering or retaining biologically active components, including cells, cells-derived products or EVs¹⁸⁸.

Despite promising results, challenges remain. Variability in decellularization protocols can affect ECM composition and reproducibility¹⁸⁹. Mechanical strength may be insufficient for large defects without reinforcement. Furthermore, functional recovery equivalent to autografts across critical-length defects has not yet been consistently demonstrated¹⁹⁰. Therefore, while dECM hydrogels represent an important step towards biologically active scaffolds, further optimisation is required to improve outcomes in clinically relevant injuries.

5. Rationale and Scope of the Thesis

Despite advances in the understanding and clinical management of PNIs, functional recovery remains limited, particularly in cases involving prolonged denervation or large nerve defects. While current strategies primarily aim to restore axonal continuity, successful recovery also depends on the condition of the surrounding tissue environment and the ability of the target muscle to respond to reinnervation.

In this context, increasing attention has been directed towards extracellular and secretory mechanisms that influence the regenerative environment within the nerve-muscle unit. Paracrine signalling, EV-mediated communication, and biomaterial-based approaches have emerged as potential strategies to modulate these processes. However, their combined therapeutic potential and relevance across different stages of regeneration remain to be fully clarified.

Therefore, this work investigates ASC-derived secretome, EV-mediated signalling, and an ECM-based hydrogel as complementary approaches to modulate regenerative processes relevant to peripheral nerve regeneration and the maintenance of denervated skeletal muscle.

Aims

The overall aim of the thesis was to investigate and test secretory mechanisms and strategies that support regeneration in the context of the nerve-muscle unit. We hypothesized that ASC-derived secretome, EV-mediated signalling, and a biomaterial-based approach influence peripheral nerve regeneration and maintenance of denervated skeletal muscle following injury.

The specific aims of this work were:

- To study how adipose-derived stem cells and their secretome modulate regenerative and angiogenic properties in clinically compatible, xeno-free culture conditions (Paper I).
- To evaluate how targeted stimulation affects the regenerative and angiogenic properties of adipose-derived stem cell secretome (Paper II).
- To investigate EV-mediated signalling from denervated skeletal muscle and determine whether muscle-derived vesicles influence neurite growth (Paper III).
- To evaluate whether a nerve-derived ECM hydrogel can support axonal continuity across a critical-length nerve defect and thereby preserve denervated skeletal muscle in vivo (Paper IV).



Materials and Methods

Ethical approvals and biological material (Papers I-IV)

Human adipose tissue (Papers I-II). Human subcutaneous adipose tissue was obtained as surplus material from elective liposuction procedures at Umeå University Hospital following written informed consent (seven female donors; mean age 49.5 years; mean body mass index 27.5; approval 2013-276-31 M).

Animal experiments (Papers III-IV). All animal procedures were conducted in accordance with applicable EU and Swedish regulations and were approved by the Animal Review Board at the Court of Appeal of Northern Norrland in Umeå. For Paper III, adult female Sprague-Dawley rats were used (age: 10 weeks old; n = 10; ethical permit DNR A12-25). For the peripheral nerve regeneration study (Paper IV), adult female Sprague-Dawley rats (12-16 weeks old; n = 18) were used (ethical permits DNR A15-20 and A31-19). Porcine vagus nerves (n = 20) were harvested from discarded internal organs used for research and educational purposes at Umeå University (ethical permit DNR 6.7.18-5352/17).

Human ASCs: Isolation, Expansion, and Characterisation

Isolation of stromal vascular fraction and ASC expansion (Papers I-II)

Adipose tissue was processed to obtain stromal vascular fraction (SVF) containing ASCs. Samples were digested with collagenase NB4 prepared in phosphate-buffered saline (PBS; 0.3 U/mL per mL aspirated tissue) for 1 h at 37 °C, followed by centrifugation at 300 x g for 5 min. The fat layer and enzyme-containing supernatant were removed, and the cell pellet was resuspended in PBS. Red blood cells were lysed using ammonium-

chloride-potassium (ACK) lysing buffer, followed by centrifugation and resuspension in growth medium.

Cells were cultured in one of three expansion conditions: (i) MEM- α + 10% (v/v) fetal bovine serum (FBS), (ii) MEM- α + 2% (v/v) human platelet lysate (PLTGold® Human Platelet Lysate), or (iii) PRIME-XV MSC Expansion XFSM medium (XV or XFSM). All media were supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen). Cells in the FBS and PLT groups were expanded in polystyrene flasks, whereas XFSM cultures were maintained in Corning® CellBIND® flasks. Primary cultures were seeded at $\geq 10,000$ cells/cm² and incubated at 37 °C. After 24 h, non-adherent cells were removed by rinsing with PBS and fresh medium was added. At 90% confluence, adherent cells were detached with TrypLE Express and reseeded at 2,000 cells/cm². Cultures were expanded until the desired passage and harvested for downstream assays. Where applicable, cells were cryopreserved in Synth-a-Freeze® Cryopreservation Medium and stored at -80 °C for later use. Cell proliferation was assessed by cumulative population doubling calculated at each passage.

Flow Cytometry (Papers I-II)

ASC phenotype was assessed at early and late passages by flow cytometry. Cells were stained with PE-conjugated antibodies against CD73, CD90, CD105, and CD146. Matched isotype controls (mouse IgG1, κ) were included for each sample. For each donor, $\geq 10,000$ events were acquired on a BD Accuri™ C6 flow cytometer and analysed per marker.

Multilineage Differentiation (Papers I-II)

Adipogenic differentiation. ASCs were seeded in 24-well CellBIND™ plates (55,000 cells/well) and differentiated upon reaching confluence. Adipogenic medium consisted of low-glucose DMEM + 10% FBS + 1% pen/strep supplemented with dexamethasone (1 μ M), IBMX (0.5 mM), indomethacin (100 nM), and insulin (10 ng/mL). Medium was changed every 2 days. From days 7 and 15, dexamethasone, IBMX, and indomethacin were omitted as described in the original protocol. In

selected experiments, adipogenic differentiation was also performed with 2% PLT supplementation.

Osteogenic differentiation. ASCs were seeded in 24-well plates (55,000 cells/well) in DMEM + 10% FBS + 1% pen/strep. Upon confluence, cells were exposed to regular growth medium with the addition of dexamethasone (1 μ M), 2-phosphate-L-ascorbic acid (0.2 mM) and glycerol 3-phosphate (10 mM) for 4 weeks. The osteogenic medium was replaced three times/week.

Chondrogenic differentiation. Chondrogenesis was performed using a pellet culture approach. Briefly, ASCs (250,000 cells) were suspended in ChondroMAX Differentiation Media, centrifuged at 200 x g for 5 min, resuspended in 0.5 mL fresh medium, and centrifuged again to promote pellet formation. Tubes were incubated upright at 37 °C in 5% CO₂ with loosened caps for gas exchange. Medium was changed every other day.

ASCs Culture and Conditioned Media (CM) Collection (Papers I-II)

ASCs were cultured to generate CM for downstream analyses. All CM were either centrifuged (300 x g, 5 min) and filtered through 0.8 μ m Millex®-AA filters before analysis or only centrifuged (300 x g, 5 min).

In Paper I, ASCs were seeded at 100,000 cells per well in 24-well plates and cultured for 24 h in either 10% FBS, 2% PLT, or XSFM. To minimise background from growth supplements, cells were washed twice with PBS and incubated in MEM- α alone. After 48 h, CM were collected to later assess angiogenic properties.

For stimulation experiments (Paper II), ASCs expanded in XSFM were seeded at 4,000 cells/cm² and cultured overnight. Cells were then stimulated for 72 h with either single factors or combinations of forskolin (10 μ M), basic fibroblast growth factor (bFGF; 10 ng/mL), neuregulin1- β 1 (200 ng/mL), and platelet-derived growth factor-AA (PDGF-AA; 5 ng/mL). Unstimulated controls were maintained in XSFM alone. After 72 h stimulation, CM were collected. In a separate set of experiments, ASCs

pooled from six donors were treated in parallel with the ERK1/2 inhibitor PD98059 (10 μ M), added to both stimulated and unstimulated conditions. After 72 h, CM were collected.

Molecular and biochemical analysis

RNA Extraction and RT-qPCR (Papers I-II)

Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. cDNA was synthesized from 10 ng/ μ L RNA using the iScript cDNA synthesis kit. RT-qPCR was performed using a Myra Liquid Handling System and a Mic qPCR cycler with SsoFast™ EvaGreen® Supermix, using 1 ng cDNA per reaction. Each sample was analysed in quadruplicate. RPL13a served as the housekeeping gene, and relative expression was calculated by the $\Delta\Delta$ Ct method. Primer sequences and annealing conditions are provided in Table 1 (Papers I-II).

ELISA (Papers I-II)

In Paper I, adiponectin in CM collected from adipogenic differentiated ASCs (day 21) was quantified using a sandwich ELISA. In Paper II, secreted uPA (PLAU) in ASCs CM was measured using the Human PLAU/uPA ELISA kit, and intracellular uPA was measured from cell lysates using the Human uPA Quantikine ELISA. Samples from five donors were analysed in duplicate. Absorbance was read at 450 nm using a microplate reader.

Protein arrays (Papers II-III)

All protein lysates were prepared and processed for protein array analysis following the manufacturer's instructions. Total protein concentration was measured using the DC protein assay. In both arrays, samples were pooled within each experimental group for a final protein concentration of 100 μ g. For ASC samples (Paper II), protein lysates from unstimulated and stimulated ASCs were collected after 1 h of stimulation and lysed using the lysis buffer supplied with the Human Phospho-Kinase Array and then added to the provided membranes. Instead, EV samples isolated from control and denervated muscles (Paper IV) were lysed in RIPA

buffer. Samples were added to the Proteome Profiler Rat XL Cytokine Array membranes. For both arrays, signals were detected using the Odyssey Fc imaging system. Array procedures were done following the manufacturer's instructions.

Table 1. Primer sequences for RT-qPCR and annealing temperatures used (°C)

Factor	Forward primer 5'-3'	Reverse primer 5'-3'	Temp
<i>BDNF</i>	AGAGGCTTGACATCATCATTGGC TG	CAAAGGCACTTGACTACTGAG CATC	65°C
<i>GDNF</i>	CACCAGATAAACAAATGGCAGTG C	CGACAGGTCATCATTCAAAGG CG	65°C
<i>NGF</i>	ATACAGGCGGAACCACTCAG	GTCCACAGTAATGTTGCGGGT C	64°C
<i>PLAU</i>	GCTTGTCCAAGAGTGCATGGT	CAGGGTCGGTTCTCGATGG	64°C
<i>AP2</i>	GGTGGTGGAATGCGTCATG	CAACGTCCCTTGGCTTATGC	65.0
<i>PPARG</i>	TACTGTCGGTTTCAGAAATGCC	GTCAGCGGACTCTGGATTGAG	62.6
<i>ANG</i>	TGGCAACAAGCGCAGCATCAAG	GCAAGTGGTGACTGGAAAGA AG	68.2
<i>ANGPT1</i>	CTTGACCGTGAATCTGGAGC	AGCAAGACATAACAGGGTGAG	59.7
<i>HGF</i>	CAATAGTCAATTTAGACCATCCC	CGTGTGGAATCCCATTTACAA	62.4
<i>IGF1</i>	TGTGGAGACAGGGGCTTTTA	ATCCACGATGCCTGTCTGA	61.5
<i>VEGFA</i>	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCACGAAG	61.4
<i>IL1B</i>	TTTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG	65.2
<i>TNF</i>	GTGACAAGCCTGTAGCCCAT	TATCTCTCAGCTCCACGCCA	62.1
<i>IL10</i>	GGAGAACCTGAAGACCCTC	ACTCACTCATGGCTTTGTAGAT	56.8
<i>MCP1</i>	AATAGGAAGATCTCAGTGCA	TCAAGTCTTCGGAGTTGGG	57.3
<i>TGFB1</i>	GGCCAGATCCTGTCCAAGC	GTGGGTTTCCACCATTAGCAC	63.3
<i>RPL13a</i>	AAGTACCAGGCAGTGACAG	CCTGTTTCCGTAGCCTCATG	58°C

Western Blotting (Papers II-III)

Samples were lysed in RIPA buffer supplemented with protease inhibitors, pooled within each experimental group, and processed for western blotting. Total protein concentration was measured using the DC protein assay. For protein denaturation, 10 µg of total ASC lysate protein or 3.5 µg of total EV lysate protein were mixed 1:1 with 2x Laemmli buffer containing 50 mM DTT.

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Proteins were resolved on 12% or 4-20% pre-cast gels at 100 V for 1.5 h and transferred to 0.2 μ m nitrocellulose membranes at 40 V for 1 h using a tank transfer system or using a Trans-Blot Turbo system.

Membranes were blocked for 1 h in TBST containing either 5% BSA or 5% non-fat milk. Primary antibodies were incubated overnight at 4 °C using anti-phospho-ERK1/2 (1:2000) and anti-beta tubulin (1:1000) for ASC samples or anti-CD81 (1:200), anti-CD63 (1:500), and anti-CD9 (1:1000) for EV samples. After washing in TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:2000) for 1 h at room temperature.

Chemiluminescent signal was developed using ECL and imaged using the Odyssey Fc system. Where required, membranes were stripped using stripping buffer for 10 min prior to re-probing.

In Vitro Functional Assays

HUVEC tube formation assay (Papers I-II)

HUVEC tube formation assays were performed on polymerized ECM in 48-well plates using 175 μ L matrix per well. ECM gel (ECM gel from Engelbreth-Holm-Swarm murine sarcoma) or ECMatrix was polymerized for 30 min at 37 °C prior to cell seeding. HUVECs were seeded at 25,000 cells per well in ASC-CM or corresponding controls and incubated for either 2 h (Paper I) or 4 h (Paper II). Tube-like structures were imaged using an Olympus IX71 microscope equipped with a ColorViewII camera.

Image analysis was performed using Image-Pro® Plus to quantify total network length (Paper I) and number of closed networks (Papers I-II). Data were expressed as the mean of four images per donor, with triplicate wells per donor.

Migration Assay (Paper II)

Ibidi 2-well culture inserts were placed into 24-well plates. HUVECs were seeded at 27,000 cells/100 μ L per insert well in endothelial basal medium and cultured for 48 h. Human dermal fibroblasts were seeded at 10,000 cells/80 μ L per insert well in basal medium and left to settle overnight. Inserts were removed, cells were washed with dulbecco's phosphate-buffered saline (DPBS), and wells were filled with donor-CM or XSBM controls. In selected conditions, the uPA inhibitor BC-11 hydrobromide (50 μ M) was added. Images were acquired at 0 h and 8 h, and wound area was measured in ImageJ to calculate percent closure.

Nerve-Derived Extracellular Matrix Biomaterial

Decellularization of Porcine Nerve Tissue (Paper IV)

Decellularization of porcine nerve tissue was performed as previously described for the P2 procedure⁹¹. Briefly, porcine vagus nerves (~30 cm) were collected in ice-cold PBS, trimmed of connective tissue, snap-frozen, thawed, and sectioned into 10 cm segments. Decellularization was achieved using detergent-based extraction cycles consisting of Triton X-100 and sodium deoxycholate under agitation, followed by enzymatic removal of residual nucleic acids using Benzonase® endonuclease.

Hydrogel Preparation (Paper IV)

Decellularized nerve tissue was minced, freeze-dried, and digested in pepsin (20 mg/mL) for 55-60 h until complete solubilization. The digest was neutralized to pH 7.4, adjusted to physiological ionic strength using PBS, and stored at -85 °C. On the day of the experiments, the hydrogel was diluted in DMEM to final concentrations of 12 mg/mL and let solidified at 37 °C.

Fabrication and Loading of Polycaprolactone (PCL) Conduits (Paper IV)

Tubular conduits were fabricated from biodegradable polycaprolactone (PCL), molded to form thin-walled tubes (14 mm length) by heating the PCL to 80 °C, surface-treated with NaOH, and sterilized by UV exposure. Liquid P2 hydrogel (12 mg/mL) was loaded into conduits, and gelled at 37 °C.

In Vivo Models of Sciatic Nerve Injury

Sciatic Nerve Injury Models and Tissue Collection (Papers III-IV)

All surgical procedures were performed under inhalation anaesthesia with 2% isoflurane. Animals received peroperative buprenorphine (0.025 mg/kg s.c.), and postoperative care included flunixin-meglumine (2.5 mg/kg s.c.), 2 mL sterile PBS. In Paper IV, benzylpenicillin (60 mg s.c.) was also given postoperatively. Animals were housed individually on a 12 h light/dark cycle with food and water available ad libitum. Procedures were performed under an operating microscope.

In Paper III, animals were randomized to receive either (i) a complete sciatic nerve transection distal to the sciatic notch using straight microscissors (den 2w; n = 4), (ii) a sham operation involving nerve exposure without transection (sham 2w; n = 4), or (iii) no surgery (control; n = 2). To prevent spontaneous regeneration in transected animals, Spongostan® was placed between the proximal and distal nerve stumps in denervated-operated rats. In Paper IV, animals were randomized to receive either (i) an empty polycaprolactone (PCL) conduit (PCL; n = 6), (ii) a PCL conduit pre-filled with P2 hydrogel (P2 gel; n = 8), or (iii) a 10 mm autologous reverse nerve graft (ARNG; n = 4). For conduit groups, a 14 mm conduit was positioned to telescope 2 mm of each nerve stump into the lumen and secured with 10-0 epineural sutures, resulting in a 10 mm interstump gap. In all animals, wounds were closed in layers. Animals were sacrificed 14 days (Paper III) or 84 days (Paper IV) days postoperatively by sodium pentobarbital overdose (240 mg/kg i.p.) or by exsanguination via cardiac access. In Paper III, gastrocnemius and soleus muscles were dissected and placed in cold DPBS for same-day processing.

Tissue Processing, Histology and Imaging

Tissue Fixation, Cryoprotection, and Sectioning of Tissue Sample (Papers IV)

Conduits were removed and fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Samples were cryoprotected in 10% and 20% sucrose for 2-3 days, frozen in liquid propane, and sectioned longitudinally at 12 µm using a cryomicrotome. Sections were mounted onto SuperFrost® Plus slides, dried at 37 °C for 1 h, and stored at -20 °C. For skeletal muscle, gastrocnemius muscles were rapidly frozen in propane cooled with liquid nitrogen, stored at -80 °C, embedded in OCT oriented for cross-sectioning, and cryosectioned at 10 µm -22 °C. Sections were mounted on glass slides, air-dried, and stored at -20 °C prior to staining.

Immunohistochemistry and Histological Staining (Papers I-IV)

All cellular samples were fixed in 4% PFA for 20-30 minutes at room temperature and subsequently washed twice with PBS or DPBS. For adipogenic differentiation of ASCs (Papers I-II), lipid accumulation was assessed after 21 days using Oil Red O staining. For quantitative analysis, the dye was eluted with 60% isopropanol, and absorbance was measured at 492 nm using a BioTek Synergy microplate reader. For osteogenic differentiation (Papers I-II), samples were stained with 1% (w/v) Alizarin Red. For chondrogenic differentiation (Papers I-II), pellets were fixed after 21 days, cryosectioned (12 µm), and stained with 1% (w/v) Toluidine Blue. For immunostaining (Papers III-IV), slides or cell plate wells were blocked in serum-containing buffer supplemented with BSA, sodium azide, and Triton X-100. Primary antibodies were incubated for 2 h at room temperature or overnight at +4 °C, followed by secondary antibodies Alexa Fluor® 568 goat anti-mouse or Alexa Fluor® 488 goat anti-rabbit secondary antibodies (both 1:1000 or 1:2000) for 1 h in the dark. The following primary antibodies were used for immunohistochemical analyses. NG108-15 cells (Paper III) were incubated with a rabbit anti-βIII-tubulin antibody (1:200) to assess neurite outgrowth. Muscle

cryosections (Paper IV) were incubated with mouse anti-myosin heavy chain (slow isoform; 1:50) and rabbit anti-laminin (1:300). For the detection of regenerative axons and glial cells (Paper IV), sections were stained with mouse monoclonal anti-neurofilament 200 (1:400) and rabbit polyclonal anti-S100 (1:1000). All samples were mounted using ProLong mounting medium containing DAPI. Specificity controls were performed by omitting primary antibodies.

Image Acquisition and Quantification (Papers I-IV)

Images were acquired using a Nikon Eclipse E80i microscope with a Nikon DS-U2 camera or Olympus IX71 microscope with a Nikon DS-R1 camera (Papers I-IV). Neurite outgrowth in NG108-15 cells (Paper III) was analysed using ImageJ. Neurites were traced manually, and total neurite length and the number of neurites per cell were quantified. Only neurites exceeding the diameter of the cell soma were included in the analysis. Axonal regeneration (Paper IV) was evaluated in two randomly selected longitudinal sections per animal from the central region of the tissue cable connecting the proximal and distal nerve stumps within the conduit. Regions of interest (ROIs) were defined based on the experimental group: in conduit groups, proximal and distal ROIs corresponded to the edges of the PCL tube, whereas in ARNG, ROIs were defined at the level of the epineural sutures at the proximal and distal nerve ends. Individual images were stitched using Adobe Photoshop to generate composite images spanning the full ROI. Neurofilament-labelled axons crossing a line drawn perpendicular to the longitudinal axis of the conduit were quantified in three 10 μm -wide sampling regions spaced 150-200 μm apart.

For muscle morphometry (Paper IV), whole muscle cross-sections were scanned using an Axioscan Z1 slide scanner. A Fiji macro was used to define 10 standardised ROIs (1000 x 1000 pixels) in the central muscle region; four ROIs without major artifacts were randomly selected for analysis. Fibre borders were segmented in CellProfiler (v4.2.8) using laminin staining, enabling quantification of fibre number and cross-sectional area. Fibre type classification was based on slow myosin heavy

chain staining intensity, and the DAPI channel was used to distinguish nuclei located inside versus outside the fibre boundary.

Diffusion Tensor Imaging (DTI) and Data Analysis (Paper IV)

At 6 and 12 weeks after repair, animals were scanned using a 9.4 T Bruker BioSpec 94/20 USR system running ParaVision® 6.1. Anesthesia was maintained with 2% isoflurane with respiratory monitoring. Four diffusion EPI spin-echo scans were acquired using a multi-shell scheme ($b = 1000, 1500, 2000, 2500 \text{ s/mm}^2$) with 32, 32, 64, and 64 directions, respectively. Imaging parameters included TR 2000 ms and isotropic voxel size 0.4 mm. Diffusion data were reconstructed and analysed in DSI Studio (Build 13 Dec 2018) using generalized q-sampling imaging (GQI; diffusion sampling length ratio 0.6). Standard diffusion parameters (fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), radial diffusivity (RD), and quantitative anisotropy (QA)) were derived. ROIs (0.5 mm^3) were placed at the proximal stump entering the conduit, mid-conduit, and distal stump. Mid-conduit ROIs were also used as seed regions for tractography to visualize fibres passing through the conduit. Tracking used a 0.2 mm step size and 40° angular threshold; tracts shorter than 8 mm were excluded.

Extracellular Vesicles (EVs) from Skeletal Muscle: Isolation and Characterisation

Isolation of EVs from Skeletal Muscle (Paper III)

Gastrocnemius and soleus muscles were gently minced into 1-2 mm fragments and enzymatically digested for 30 min at 37°C in DMEM containing 1% pen/strep, collagenase D (2 mg/mL), and DNase I (40 U/mL). The suspension was filtered through a $70 \mu\text{m}$ strainer and centrifuged at $300 \times g$ for 5 min and $2,000 \times g$ for 20 min to remove cells and debris. Supernatants were stored at -80°C until EV isolation. After thawing overnight at 4°C , samples were centrifuged at $10,000 \times g$ for 20 min and ultracentrifuged at $118,000 \times g$ (average) for 2.5 h (Optima XPN-

100, SW32Ti rotor), with all steps performed at 4 °C. EV pellets were resuspended in 15 mM HEPES, aliquoted, and stored at -80 °C.

Nanoparticle Tracking Analysis (Paper III)

EV size distribution and concentration were measured using a NanoSight NS300 with NTA 3.1 software. Samples were diluted 1:250 in DPBS. Measurements were acquired at camera level 7 and detection threshold 2, recording five videos of 60 s per sample.

Transmission Electron Microscopy (Paper III)

For negative staining TEM, EV suspensions (3.5 μ L) were applied to glow-discharged 200-mesh formvar/carbon copper grids for 1 min, washed twice in Milli-Q water, and stained with 1.5% uranyl acetate for 30 s. Grids were examined using a FEI Talos L 120C TEM operated at 120 kV with a Ceta 4k x 4k CMOS detector, and images were acquired using Velox software.

EVs In Vitro Functional Assays

Neurite Outgrowth Assay and Co-Treatment with P2 Hydrogel (Paper III)

NG108-15 neuroblastoma-glioma hybrid cells were expanded in DMEM supplemented with 10% FBS and 1% pen/strep at 37 °C in 5% CO₂. At ~80% confluence, cells were passaged with 0.05% trypsin-EDTA and seeded at 10,000 cells/cm² in 24-well plates containing cell culture inserts. After 24 h, medium was replaced with DMEM + 1% pen/strep containing EVs at 10,000 particles per cell (standard dose). Dose-response experiments used 50,000 or 100,000 particles per cell. DMEM + 1% pen/strep served as control medium. P2 hydrogel (12 mg/mL; 25 μ L) was cast into inserts (1.0 μ m PET membrane) and gelled at 37 °C for 15 min before being placed into wells containing NG108-15 cells. Treatments were performed in quadruplicate and incubated for 48 h prior to imaging.

Metabolic Activity Assay (Paper III)

Alamar Blue reagent (10% v/v) was added during the final 4 h of the 48 h treatment period. Absorbance was measured at 570 nm with background correction at 600 nm using a microplate reader.

Statistical analysis

Statistical Analysis (Papers I-IV)

Statistical analyses were performed using GraphPad Prism (GraphPad Software). For Papers I-II, group comparisons were performed using t-tests or one-way ANOVA with Bonferroni post hoc testing, depending on the experimental design. For Paper III, data were assessed for normality using the Shapiro-Wilk test; one-way ANOVA with Tukey post hoc testing and/or two-way ANOVA were applied as appropriate. For Paper IV, non-parametric tests were applied based on sample size and data distribution. Group differences were evaluated using the Kruskal-Wallis test, followed by post hoc multiple comparisons with Dunn's test or Mann-Whitney tests with Bonferroni correction. For Papers I-IV, significance thresholds were defined as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$; "ns" indicates not significant.

Results and Discussion

This work examined secretory mechanisms and their role in modulating regenerative processes involved in nerve repair and muscle preservation following peripheral nerve injury. Papers I and II focus on modulation of ASCs-derived secretome under clinically compatible conditions and defined stimulation protocols. Paper III investigates EVs from denervated skeletal muscle and their effects on neurite growth. Paper IV evaluates a nerve-derived ECM hydrogel to support axonal regeneration and preserve skeletal muscle *in vivo*.

Paper I

A summary of the following published paper:

The Influence of Xeno-Free Culture Conditions on the Angiogenic and Adipogenic Differentiation Properties of Adipose Tissue-Derived Stem Cells.

Lauvrud AT, Giraud MV, Wiberg R, Wiberg M, Kingham PJ, Brohlin M.

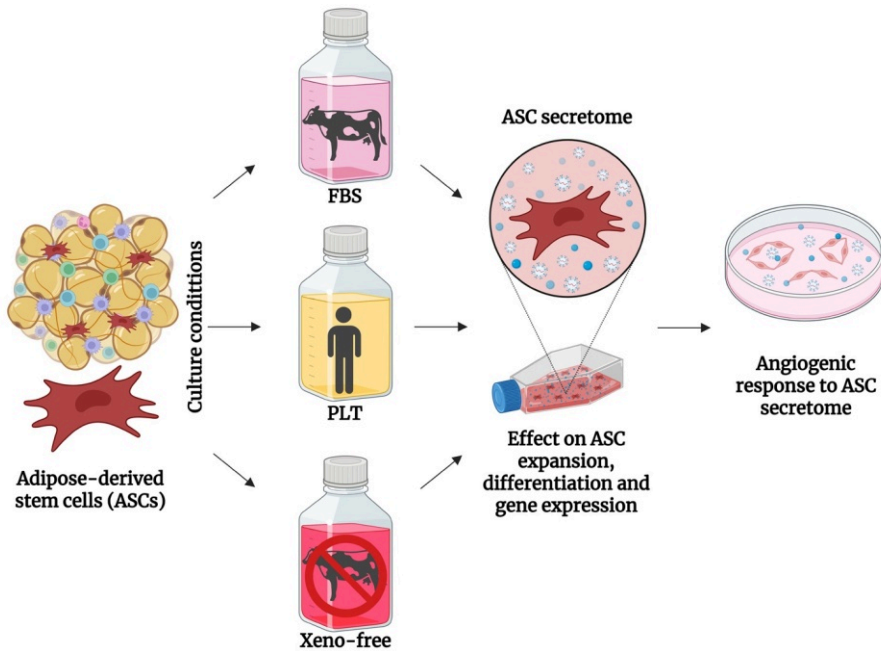


Figure 8. Schematic representation of the experimental design of Paper I. ASCs were cultured under different conditions (fetal bovine serum (FBS), platelet lysate (PLT), or xeno-free (XV)). The effects of these culture conditions on ASC expansion, differentiation, and gene expression were assessed. In parallel, the angiogenic response to the ASC secretome was evaluated.

Paper I addressed the first specific aim of the thesis by investigating how ASCs and their secretome modulate regenerative and angiogenic properties in clinically compatible, xeno-free culture conditions (Fig. 8).

This question is central to the overall hypothesis of the thesis, as the muscle-nerve unit depends on angiogenesis to support the preservation and recovery of denervated muscle. This angiogenic response can be influenced by the ASC secretome. Thus, culture systems must not only comply with GMP standards but also preserve the angiogenic potential of ASCs and their secretome.

A critical prerequisite for clinical translation of stem cell-based therapies is the use of standardised, reproducible, and safe culture conditions. Mesenchymal stromal cells, including ASCs, are currently being evaluated in a large number of clinical trials^{192,193}. To meet regulatory and safety requirements, the replacement of FBS with xeno-free alternatives has become increasingly important¹²¹. The use of chemically defined or human-derived supplements minimises the risk of zoonotic contamination and reduces batch-to-batch variability, which are known limitations of FBS-based systems^{194,195}. However, such changes may also influence ASC phenotype and function, including their secretory profile, with potential consequences for their regenerative capacity and relevance within the muscle-nerve regenerative context.

In the first study, we compared the effects of three culture conditions - 10% (v/v) FBS, 2% (v/v) human platelet lysate (PLT), and the GMP-compliant PRIME-XV MSC Expansion XSFM (XV) - on ASC expansion, phenotype, and functional properties. In clinical manufacturing, the expansion phase represents a critical step, as sufficient cell yields must be achieved while maintaining cellular identity and function under the defined culture conditions¹⁹⁶. Therefore, we compared a defined GMP-grade medium (XV) with FBS- and PLT-supplemented media. ASCs were cultured for up to 90 days, allowing assessment of long-term proliferative behaviour. Morphological changes were accompanied by a significantly higher proliferation rate in the XV group, consistent with previous reports describing enhanced growth of mesenchymal stromal cells under chemically defined, xeno-free conditions (Paper I: Fig. 1A-B)^{197,198}.

Because culture conditions can shift stromal cell subpopulations, immunophenotyping is an important analysis when considering translation and batch consistency¹⁹⁹. Phenotypic characterisation by flow

cytometry demonstrated that ASCs expanded under all three conditions retained expression of canonical mesenchymal stem cell markers, including CD73, CD90, and CD105, confirming preservation of MSC identity. However, differences were observed in the expression of CD146 at both early and late passages. CD146 expression was significantly higher in ASCs expanded in XV medium (Paper I: Fig. 2A-B). This highlighted that stem cell markers varied depending on the culture medium, a finding that aligns with previous studies showing that xeno-free conditions can influence the proportion of CD146-positive subpopulations, although reported trends differ between cell sources and experimental systems^{200,201}. Additionally, CD146 has been associated with pericyte-like characteristics and vascular support functions, and its expression has been correlated with enhanced angiogenic capacity in mesenchymal stromal cell populations. An ability that we have later explore in our study²⁰².

Since vascular support is a key component of tissue maintenance and repair, we next assessed whether the different expansion conditions altered the ability of ASC-derived factors to support endothelial network formation *in vitro*. ASCs expanded in XV medium exhibited significantly elevated expression of *HGF*, with levels approximately 90-fold higher than those observed in FBS-expanded cells (Paper I: Fig. 5B). HGF is a well-established pro-angiogenic factor known to stimulate endothelial cell proliferation and migration, often acting in concert with VEGF signalling to promote vascular remodelling and ECM interactions^{203,204}. Consistent with these molecular findings, XV-expanded ASCs demonstrated superior performance in *in vitro* angiogenesis assays compared to both FBS- and PLT-expanded cells. In contrast, ASCs cultured in FBS showed the weakest angiogenic activity, in line with previous reports highlighting limitations of FBS in supporting pro-angiogenic properties of mesenchymal stromal cells (Paper I: Fig. 6A-B)²⁰⁵.

The enhanced angiogenic profile observed in XV-expanded ASCs may be linked to the increased proportion of CD146-positive cells within this group. Previous studies have reported reduced CD146 expression in FBS-expanded cells, which may partially explain the lower angiogenic activity observed under these conditions²⁰⁰.

Expression of *IGF1* was highest in ASCs cultured in PLT medium, with levels approximately 30-fold greater than in FBS-expanded cells (Paper I: Fig. 5A). IGF1 is known to support neural survival, proliferation of muscle cells, as well as endothelial cell viability and stabilisation of vascular structures. This last role of IGF-1 potentially explains the intermediate angiogenic performance observed in the PLT group (Paper I: Fig. 6A-B). In contrast, IGF1 expression in XV-expanded ASCs was lower than in FBS-expanded cells, suggesting that angiogenic signalling under xeno-free conditions may rely predominantly on HGF-driven mechanisms rather than IGF1-mediated pathways (Paper I: Fig. 5B). These observations illustrate how distinct culture environments can shift the balance of angiogenic signalling networks. This concept is important to consider when evaluating the ability of ASC secretome to support regeneration.

Previous studies comparing PLT and FBS have reported variable effects on angiogenesis, with some demonstrating enhanced tube formation under PLT conditions, while others found no significant differences between the two supplements^{206,207}. In a study conducted with Wharton's Jelly-derived MSCs, xeno-free medium enhanced angiogenic activity compared to FBS-expanded cells, with HGF identified as a key mediator of this effect²⁰⁵.

In addition to the observed effects on growth factor (GF) expression and angiogenic response, our study also revealed differences in adipogenic differentiation capacity. ASCs cultured in XV medium demonstrated an enhanced ability to differentiate into adipocytes compared to cells expanded in FBS or PLT (Paper I: Fig. 3A-C; Fig. 4A-C)^{127,201}. These findings suggest that culture conditions influence not only proliferative and paracrine properties but also lineage commitment, which could have implications for other areas of regenerative medicine, including soft tissue regeneration and fat grafting.

Several limitations should be considered when interpreting these results. First, angiogenic activity was assessed using in vitro endothelial assays, which do not fully capture the complexity of vascular regulation in vivo. Second, analysis of the ASC secretome was limited to selected GFs, and broader changes in gene expression or protein composition may not have

been detected. Third, variability in responses reported in the literature, particularly for PLT-expanded cells, highlights the influence of donor variability and experimental conditions. Finally, this study did not directly assess the effects of ASC-derived factors on nerve regeneration or muscle preservation, and therefore conclusions regarding functional outcomes remain indirect.

Overall, Paper I demonstrates that xeno-free, GMP-compatible culture conditions are not only a regulatory requirement but also a key determinant of ASC secretome composition and function. By enhancing angiogenic signalling and altering ASC subpopulation dynamics, these conditions promote a more regenerative secretory profile that may contribute to modulation of the muscle-nerve microenvironment. These findings provide a foundation for the subsequent studies in the thesis, which further explore how different components of the secretome can be harnessed to influence regeneration within the muscle-nerve unit.

Paper II

A summary of the following published paper:

Forskolin Enhances Urokinase Plasminogen Activator Secretion and Angiogenic Activity of Xeno-Free Cultures of Human Adipose Tissue-Derived Stem Cells.

Giraudo MV, Lauvrud AT, Wiberg R, Brohlin M, Andersson G, Kingham PJ.

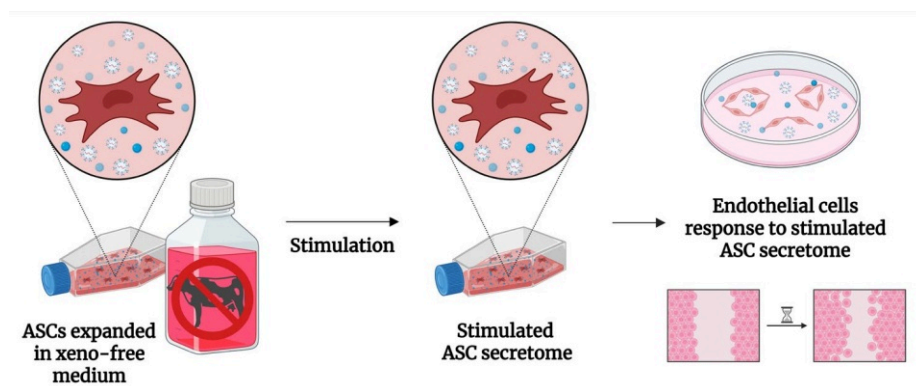


Figure 9. Schematic representation of the experimental workflow of Paper II. ASCs were expanded in xeno-free medium (XV) and subsequently stimulated. The secretome from stimulated ASCs was collected and used to assess endothelial cell response.

The findings from Paper I established a methodological and conceptual basis for subsequent studies by identifying a GMP-compliant, xeno-free culture medium (XV/XSFM) that supports efficient ASC expansion while preserving differentiation capacity. The ability of ASCs cultured in XV medium to generate high cell numbers without compromising functional properties represents an important advantage for translational applications. In addition, CM derived from ASCs expanded in XV was shown to promote angiogenic responses, indicating that ASC paracrine signalling may contribute to regenerative processes. These results motivated further investigation into whether ASC function could be enhanced through targeted stimulation strategies under xeno-free conditions.

Paper II addressed the second specific aim of this work by evaluating whether targeted stimulation under xeno-free conditions can further modulate the regenerative and angiogenic properties of the ASC secretome (Fig. 9). Previous work from our group and others, performed largely under FBS-based conditions, demonstrated that chemical stimulation protocols involving specific GFs and small molecules could enhance the regenerative properties of ASCs, including their ability to support neurite outgrowth and Schwann cell-like phenotypes^{110,208}. Building on these findings, we sought to determine whether combining xeno-free culture conditions with short-term chemical stimulation could further augment ASC regenerative potential. Forskolin (FSK), a compound known to elevate intracellular cAMP levels, was used either alone or in combination with GFs such as bFGF, PDGF, and NRG1^{208,209}. In contrast to previously published multi-step differentiation protocols, this approach was designed to be short, cost-efficient, and more compatible with clinical manufacturing workflows. This represents a departure from more extensive protocols that are time-consuming and resource-intensive^{115,210}.

To explore the molecular effects of these stimulation conditions, we performed a broad PCR-based screening of genes associated with regeneration. Treatment with FSK for 72 h, either alone or in combination with GFs, resulted in increased expression of *IGF1* and *PLAU* (Paper II: Fig. 1A). This observation is notable given the established roles of IGF1 in muscle growth and nerve regeneration, and of urokinase plasminogen activator (uPA) in ECM remodelling and cell migration^{211,212}. These processes are essential for restructuring the injury environment following denervation.

Given the growing interest in cell-free therapeutic approaches, we next examined whether these transcriptional changes were reflected at the level of the ASC secretome. CM were analysed for the presence of IGF1 and uPA. While IGF1 was not detectable in the CM, a marked increase in uPA levels was observed following FSK stimulation (Paper II: Fig. 1B). This represents, to our knowledge, the first report of uPA secretion by chemically stimulated ASCs cultured under xeno-free conditions,

suggesting that the combination of defined media and stimulation protocols can selectively shape secretome composition.

uPA has previously been detected in MSC secretome, although the signalling mechanisms regulating its production under xeno-free and chemically stimulated conditions remain poorly defined. To investigate the pathways involved, we performed a phospho-kinase array analysis. This revealed activation of the ERK1/2 signalling pathway in FSK-treated ASCs, consistent with the known ability of forskolin to elevate cAMP levels and activate protein kinase A, leading to downstream ERK1/2 phosphorylation (Paper II: Fig. S1)^{213,214}. Western blot analysis confirmed ERK1/2 activation, with slightly lower activation observed in cells treated with GFs alone (Paper II: Fig. 2A).

To further examine the role of ERK1/2 signalling in uPA production, we used the ERK1/2 inhibitor PD98059²¹⁵. Inhibition of ERK1/2 resulted in an approximately 50% reduction in uPA secretion in FSK-stimulated ASCs, supporting a role for this pathway in regulating uPA release (Paper II: Fig. 2C). However, *PLAU* transcript levels were not significantly altered by ERK1/2 inhibition, suggesting that transcriptional regulation of *PLAU* may involve additional signalling pathways beyond ERK1/2 (Paper II: Fig. 2B).

Based on the enhanced angiogenic properties observed in XV-expanded ASCs in Paper I, we next assessed whether chemical stimulation further influenced the angiogenic activity of ASC-CM. In vitro angiogenesis assays were performed using HUVECs. CM from FSK- and GF-stimulated ASCs induced an increase in capillary-like network formation, consistent with previous reports demonstrating that ASC secretome can promote angiogenesis^{216,217}. Notably, statistically significant enhancement was observed only when ASCs were treated with the combination of FSK and GFs, indicating that maximal angiogenic effects require combined stimulation (Paper II: Fig. 3A-B). The angiogenic responses observed in Paper I and in this study suggest that xeno-free culture conditions provide a baseline for angiogenic activity, which may be further enhanced through targeted chemical stimulation.

The contribution of uPA to endothelial cell migration was examined using transwell migration assays. HUVEC migration was significantly increased in response to ASC-CM, with the strongest effect observed for media derived from ASCs treated with both FSK and GFs. Importantly, addition of the uPA inhibitor BC-11 reduced HUVEC migration by 60-80%, demonstrating a central role for uPA in mediating this response (Paper II: Fig. 4A-B). The presence of BC-11 did not affect HUVEC viability, as assessed using a live/dead cell viability assay (Paper II: Fig. S4). These findings are consistent with previous studies identifying uPA as a key regulator of endothelial cell migration and vascular remodelling²¹⁸. Together, these results indicate that modulation of ASC secretome composition can directly influence endothelial behaviour through specific proteolytic pathways.

CM from both stimulated and unstimulated ASCs were also tested in fibroblast migration assays. In contrast to the effects observed in HUVECs, ASC-CM did not significantly alter fibroblast migration compared with control media (Paper II: Fig. S3A-B). This suggests that the pro-migratory effects of the ASC-CM are cell-type dependent, preferentially influencing endothelial cells rather than inducing a generalized migratory response. However, the mechanisms underlying this differential sensitivity were not explored in the present study. While others have reported effects of ASC secretome on fibroblast migration, cells responses are known to depend strongly on culture conditions and stimulation parameters, as also demonstrated in Paper I²¹⁹.

Several limitations should be considered. First, although this study provides mechanistic insight into uPA regulation through ERK1/2 signalling, the broader regulatory network controlling ASC secretome composition under stimulation conditions remains incompletely defined. Second, the functional assays focused primarily on endothelial responses and therefore do not capture how the stimulated secretome may influence other key cell types involved in regeneration, such as neurons, Schwann cells, or muscle fibres. In addition, the study design does not address the temporal stability of the stimulated phenotype, and it remains unclear whether the observed effects are transient or can be sustained in a therapeutic context.

Overall, Paper II demonstrates that ASC function can be actively directed towards specific regenerative pathways through short-term, clinically compatible stimulation strategies. In particular, the identification of uPA as a key mediator of endothelial migration highlights the possibility of selectively enhancing components of the regenerative environment involved in ECM remodelling and vascular dynamics. These findings extend the results from Paper I by showing that ASC-based approaches are not only dependent on baseline culture conditions but can be further refined to achieve targeted functional outcomes. In this context, modulation of the secretome contribute to shaping conditions that support both structural repair and cellular interactions required after denervation.

Paper III

A summary of the following unpublished manuscript:

Extracellular Vesicles Isolated from Denervated Skeletal Muscle Tissue and their Effects on Neurite Outgrowth.

Giraud MV, Novikova LN, Novikov LN, Andersson G.

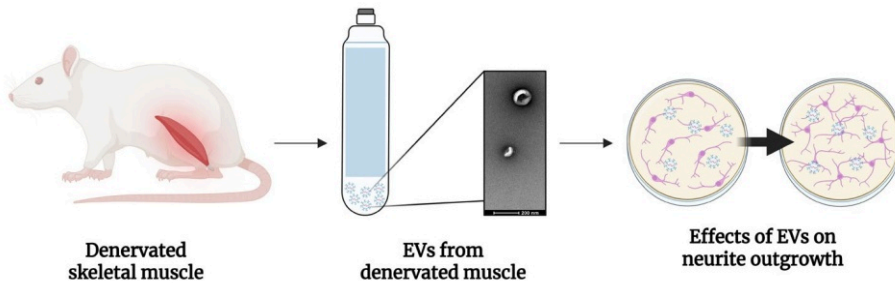


Figure 10. Schematic representation of the experimental workflow of Paper III. EVs were isolated from denervated skeletal muscle and characterised. The effects of these EVs on neurite outgrowth were subsequently evaluated.

The results from Papers I and II demonstrated that ASCs secretome can be modulated by altering the molecular environment, either through defined culture conditions or targeted stimulation protocols. These studies highlighted the importance of soluble and vesicle-associated factors in influencing cellular behaviour and shaping the local regenerative environment. Denervated skeletal muscle itself undergoes profound molecular and cellular changes that actively influence the regenerative process²²⁰. Rather than being a passive target, denervated muscle releases signalling factors that may affect nerve growth, guidance, and survival²²¹. In Paper III, we addressed this by investigating the third specific aim: to study EV-mediated signalling from denervated skeletal muscle and determining whether muscle-derived EVs influence neurite growth (Fig. 10).

In this study, EVs were isolated from rat calf skeletal muscle tissue two weeks after sciatic nerve transection and compared with EVs from control and two weeks sham-operated muscles. The isolation protocol was

adapted based on previous published work¹⁵⁴. Transmission electron microscopy confirmed the presence of vesicle-like structures across all experimental groups, and nanoparticle tracking analysis demonstrated comparable size distributions consistent with the exosomal size range (Paper III: Fig. 1A-B). When EV yield was normalized to tissue weight, denervated muscles released a significantly higher number of particles compared to control muscles (Paper III: Fig. 1C). This increase suggests that muscle denervation alters vesicle release dynamics at the tissue level.

The elevated EV release observed following denervation is likely not restricted to muscle fibres alone but reflect contributions from multiple cell populations within the muscle, including fibro-adipogenic progenitors, endothelial cells, immune cells, and satellite cells, all of which are known to be activated during denervation-induced remodelling^{167,222}. Similar increases in EV release following nerve injury have been reported in other systems, supporting the concept that EV-mediated signalling is a general response to tissue perturbation^{170,223}.

A technical limitation of this study was the inability to detect classical exosome markers such as CD9, CD63, and CD81 by western blotting (Paper III: Fig. S1A-C)²²⁴. This likely reflects the low protein yield of the EVs we isolated directly from solid muscle tissue. Moreover, increasing evidence suggests that exosome marker expression profiles established for cell culture systems may not fully translate to vesicles isolated from tissues, particularly under pathological conditions such as denervation¹⁵¹. For these reasons, despite vesicle size and morphology being consistent with the exosomal range, the isolated particles are referred to here as EVs rather than exosomes¹³⁴.

EVs mediate intercellular communication by transporting bioactive molecules, such as proteins, lipids, and regulatory RNAs, thereby influencing recipient cell behaviour^{225,226}. Analysis of EV-associated protein cargo revealed selective denervation-dependent changes rather than broad alterations. Among the proteins examined, IGFBP-5 levels were increased, whereas VEGF-A levels were reduced in EVs derived from denervated muscle (Paper III: Fig. 2A-B). These changes are consistent with known pathological features of denervation^{49,227}. Associations

between IGFBP-5 expression, ECM regulation, and muscle wasting have been reported in denervation and other catabolic conditions, whereas reduced VEGF-A signalling has been linked to impaired vascular and neural support²²⁸⁻²³¹. Given the semi-quantitative nature of the cytokine array and the limited protein yield obtained from tissue-derived EVs, these findings should be interpreted with caution and should be validated using more sensitive quantitative methods.

To evaluate whether EVs released from denervated skeletal muscle could influence neuronal growth in a regeneration-relevant context, we assessed their effects on neurite outgrowth *in vitro*. This experiment was designed to simulate a model of peripheral nerve repair *in vivo*, previously done by our group. In previous work, a decellularized nerve-derived hydrogel (P2 gel) was shown to support and maintain axonal regeneration in a rat sciatic nerve injury model¹⁹¹. In the present study, we aimed to test whether EVs derived from denervated muscle would negatively affect this model when assessed *in vitro*. EVs were added to the culture medium, while the P2 gel was placed in a transwell insert, allowing soluble signals from the gel to influence the neurons without direct contact. Considering the detrimental changes that occur in muscle in the absence of neuronal input, we hypothesized that EVs derived from denervated muscle would impair neurite outgrowth in the presence or absence of P2 gel.

Using NG108-15 neuronal cells, neurite growth was maintained, including in cultures treated with EVs from denervated muscle. Importantly, EVs isolated from sham-operated, or denervated muscle did not reduce neurite length or branching when added to the culture medium. Increasing the EV concentration also did not result in reduced neurite outgrowth (Paper III: Fig. 3A-B; Fig. S2A-B). A recent study reported that EVs isolated from acutely denervated skeletal muscle enhanced axonal outgrowth. Contrary to our initial hypothesis, the study reported that EVs derived from denervated muscle promoted neurite outgrowth *in vitro* and supported nerve regeneration *in vivo*²²³. Similarly, another study demonstrated that EVs derived from denervated skeletal muscle could guide axonal growth in a nerve repair model more effectively than EVs from healthy muscle¹⁷¹. In the presence of the P2 gel, neurite growth was maintained, including in cultures treated with EVs from denervated

muscle (Paper III: Fig. 3A-B; Fig. S2A-B). These findings are consistent with previous work showing that decellularized nerve-derived matrices support neurite outgrowth in vitro^{187,232}.

Metabolic activity assays demonstrated a reduction in metabolic activity in NG108-15 cells cultured within the P2 gel, independent of EV concentration (Paper III: Fig. 3C; Fig. S2C-D). However, brightfield images confirmed viable cells, suggesting that the observed reduction reflects altered cellular metabolism rather than cytotoxicity (Paper III: Fig. S3A). Such changes in metabolic activity are commonly observed in matrix-rich culture systems and may be related to altered proliferation or differentiation rather than reduced cell survival^{185,186}.

Several limitations should be acknowledged. First, the functional effects of muscle-derived EVs were assessed exclusively using one in vitro neuronal cell line. Second, EV cargo analysis was limited in scope, and other bioactive components, including microRNAs or lipid mediators, were not examined. Third, the cellular origin of the isolated EVs could not be determined. Finally, interactions between EVs and other components of the regenerative environment, such as Schwann cells or immune cells, were not addressed in this study.

In Papers I and II, we demonstrated that the ASC secretome can be modulated through defined culture conditions and targeted stimulation, resulting in altered angiogenic and migratory responses relevant to muscle and nerve regeneration. In the present study, we extended this approach by examining EVs derived from denervated skeletal muscle as part of the secretory environment in the muscle-nerve unit.

Paper IV

A summary of the following unpublished manuscript:

Use of a Nerve-Derived Extracellular Matrix Hydrogel for Regeneration in Synthetic Nerve Conduits: A Longitudinal Diffusion MRI and Histological Study on Outcome.

Giraud MV, Novikova LN, Kuna VK, Kelk P, Brohlin M, Novikov LN, Kingham PJ, Andersson G.

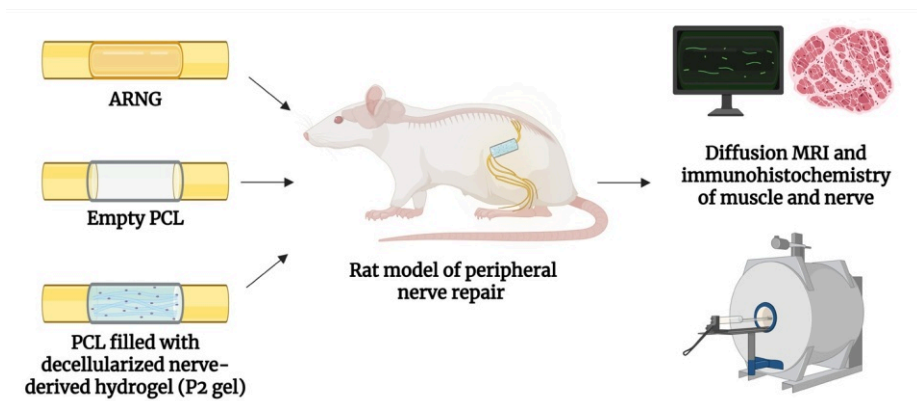


Figure 11. Schematic representation of the experimental design of Paper IV. Autologous reverse nerve grafts (ARNG), empty polycaprolactone (PCL) conduits, and PCL conduits filled with decellularized nerve-derived hydrogel (P2 gel) were tested in a rat sciatic nerve repair model. Regeneration outcomes were evaluated using diffusion MRI and immunohistochemical analysis of muscle and nerve tissue.

Paper IV addressed the fourth aim by evaluating whether a nerve-derived ECM hydrogel can support axonal continuity across a critical-length nerve defect and thereby preserve denervated skeletal muscle in vivo (Fig. 11). While Papers I-III focused on modulation of the regenerative environment through paracrine signalling and EV-mediated mechanisms, this study examined whether the P2 gel, as a decellularized ECM biomaterial with inherent bioactive properties, can support axonal growth. Although biomaterials with ECM supplementation have shown encouraging effects on axonal growth, their ability to sustain regeneration across critical-length defects and preserve denervated target muscle in

vivo remains incompletely understood¹⁷⁷. By combining a clinically relevant repair model with longitudinal diffusion MRI and terminal histological analysis, this work aimed to clarify how different repair strategies support axonal continuity over time and whether this continuity is sufficient to preserve denervated muscle structure.

A central motivation for using diffusion tensor imaging (DTI) was the need for a non-invasive method capable of monitoring regeneration dynamics within the repair site over time. Histological analysis, while definitive, is inherently limited to single time points and requires large animal cohorts. In contrast, diffusion MRI provides repeated measures of tissue organisation within the same construct, offering insight into the temporal evolution of regeneration. Fractional anisotropy (FA) was used as the primary imaging metric because it reflects the degree of directional water diffusion associated with aligned tissue structures^{233,234}. Importantly, FA does not report axon number, but rather the coherence and organisation of diffusion-restricting structures, making biological interpretation context dependent.

Three repair strategies were compared: empty synthetic poly(ϵ -caprolactone) (PCL) conduits, PCL conduits filled with P2 hydrogel, and autologous reverse nerve grafts (ARNG), which served as the clinical gold standard control. Regeneration was evaluated longitudinally using high-field DTI at 6 and 12 weeks post-injury, followed by terminal histological analysis of axons within the construct and morphological assessment of the gastrocnemius muscle.

At six weeks post-injury, FA values were consistently higher in ARNG than in either synthetic conduit group. This finding is best explained by the preserved endoneurial architecture of the graft, which creates a highly anisotropic diffusion environment independent of newly regenerated axons²³⁵. In this context, high early FA likely reflects scaffold-driven anisotropy rather than advanced regeneration. In contrast, the low FA values observed within both empty and hydrogel-filled PCL conduits at this time point reflect the absence of intrinsic alignment and the presence of a hydrated, immature intraluminal environment populated by early cellular infiltration and nascent axonal extensions²³⁶. Despite being low,

FA values within conduits were above background, indicating that the lumen was not simply fluid-filled but contained anisotropic structures compatible with early regenerative processes (Paper IV: Fig. 1A-B; Table S1)²³⁷. One animal showed near-zero FA throughout the conduit and was later confirmed histologically to lack regenerating axons. This was accompanied by severe muscle atrophy, highlighting the sensitivity of FA to regeneration failure and supporting its utility as an early indicator of unsuccessful nerve repair (Paper IV: Fig. S1A-C; Fig. S2A)²³⁵.

By twelve weeks post-injury, FA values increased within both conduit groups, indicating progressive structural organisation over time²³⁸. This pattern is consistent with increasing microstructural alignment within the conduit. Regional analysis identified the mid-conduit region as particularly informative. The P2 hydrogel-filled conduits maintained higher FA values distally, consistent with improved axonal continuity towards the distal stump. Complementary diffusion metrics supported this interpretation: quantitative anisotropy (QA) increased, while mean and radial diffusivity decreased over time, reflecting greater diffusion restriction and tissue compaction. Axial diffusivity remained stable or increased modestly distally, consistent with the presence of longitudinally aligned structures (Paper IV: Fig. 2A-B; Table S2)²³⁹.

In ARNG, although not statistically significant, the FA appeared to decrease between six and twelve weeks (Paper IV: Fig 1A-B; Fig 2A-B; Table S1; Table S2). Rather than indicating impaired regeneration, this likely reflects remodelling of the graft. Autologous grafts initially exhibit high anisotropy due to intact native architecture; as regeneration progresses, degradation of the original scaffold, axonal pruning, revascularisation, and increasing microstructural complexity may reduce diffusion coherence^{235,240}. The accompanying changes in QA and diffusivity metrics support this interpretation. These findings highlight that diffusion metrics must be interpreted in relation to the specific repair environment.

Histological analysis at twelve weeks validated the imaging findings. Proximal axon densities were comparable across groups, indicating similar regenerative entry into the repair site. At the mid-conduit region,

ARNG exhibited the highest axon density, while both synthetic conduit groups showed reduced counts, consistent with axonal attrition within conduits. Distally, P2 hydrogel-filled conduits demonstrated axon densities comparable to autologous grafts and higher than empty PCL conduits (Paper IV: Fig. 3A-B). This suggests that the hydrogel primarily supports axonal persistence across the conduit rather than uniformly increasing axon number. The retained ECM components within the decellularized nerve matrix may provide structural and molecular signals absent in empty conduits.

Preservation of target muscle structure is a key determinant of functional recovery after peripheral nerve injury. Analysis of gastrocnemius muscle morphology at twelve weeks revealed no significant differences in muscle weight, fibre number, cross-sectional area, or centrally nucleated fibres among animals with successful regeneration (Paper IV: Fig. 4A-B). These findings indicate that once reinnervation occurs within an appropriate time frame, gross muscle architecture can be maintained irrespective of repair strategy. In contrast, the one animal lacking axonal regeneration, as seen in both DTI and conduit histology examination, exhibited pronounced muscle atrophy, underscoring the dependence of muscle preservation on successful nerve continuity (Paper IV: Fig. S1A-C; Fig. S2A).

In addition to providing structural guidance, P2 hydrogel also function as a biologically active compartment within the conduit. Decellularized matrices retain not only structural proteins but also bound GFs and bioactive molecules that can be released over time. In this context, the hydrogel act as a local reservoir of signalling molecules that contribute to modulation of the regenerative environment. Furthermore, the hydrated and porous nature of the hydrogel may facilitate the diffusion and retention of soluble factors, EVs, and potentially infiltrating host cells within the repair site. The hydrogel functions not only as a passive scaffold but also as a dynamic interface that supports the exchange of biological signals between regenerating axons, Schwann cells, and surrounding tissues.

Several limitations should be considered. Group sizes were modest and functional assessments were not included. Second, the twelve-week endpoint may not capture even longer-term differences in muscle quality and nerve regeneration. Finally, subtypes of muscle fibres were not investigated in this work.

In summary, incorporation of P2 gel into synthetic PCL conduits supports axonal regeneration across a critical-length sciatic nerve defect and preserves downstream muscle architecture at twelve weeks. Longitudinal diffusion MRI identified distinct regeneration patterns and highlighted the mid-conduit region as a critical determinant of repair success. These findings extend the earlier work presented in the thesis by linking structural regeneration to preservation of denervated muscle *in vivo* and support continued development of cell-free strategies for nerve-muscle repair.

General Discussion

The central premise of this work is that the secretome represents a unifying and multifaceted regulator of the muscle-nerve unit. This is particularly relevant in the context of PNIs, where both nerve regeneration and the prevention of denervation-induced muscle atrophy are critical for functional recovery. Rather than viewing these processes as separate challenges, this work approaches them as interconnected biological events that can be modulated through secreted factors derived from cells, tissues, and biomaterials. Across the studies included in the thesis, different components of the secretome were explored with the shared objective of understanding how they influence regeneration processes such as angiogenesis, nerve restoration and the preservation of muscle integrity following nerve injuries.

A foundational step in this work was to ensure that ASCs, as a clinically relevant source of regenerative secretome, could be expanded under conditions suitable for therapeutic translation. This was addressed by comparing different culture supplements and their effects on ASC behaviour and secretory function. The findings demonstrated that xeno-free conditions not only supported cell expansion but also enhanced the

angiogenic potential of the ASC-derived secretome. Improved vascularisation is essential for maintaining the metabolic and structural integrity of denervated skeletal muscle, thereby limiting atrophy.

Building on this, the thesis explored strategies to further modulate and enhance the regenerative properties of the ASC secretome. Through biochemical stimulation, specific factors within the secretome were upregulated, most notably uPA. The observed increase in uPA secretion and its functional effects on endothelial cell migration and angiogenesis suggest that targeted manipulation of the secretome can amplify its regenerative potential. Although this work does not directly model the muscle-nerve unit, it introduces a mechanistic link between secreted proteases and the vascular and remodelling processes that support both nerve regeneration and muscle preservation. In this context, uPA emerges as a potential mediator that could influence ECM remodelling, cell migration, and ultimately the regenerative microenvironment following denervation.

In parallel to the cell-derived secretome, this work investigated tissue-derived EVs as mediators of signalling within the muscle-nerve unit, directly addressing the aim of determining whether EVs from denervated skeletal muscle influence neurite growth. Denervation is known to induce significant molecular and cellular changes in muscle, leading to the release of signalling factors that affect nerve regeneration. In this context, EVs were explored as carriers of such signals. EVs isolated from denervated skeletal muscle showed an increased release compared to control, indicating that denervation alters vesicle dynamics at the tissue level. However, no effects on neurite outgrowth were observed *in vitro*. Importantly, this finding should not be interpreted as a lack of biological relevance but rather suggests that EV-mediated signalling from denervated muscle alone is not sufficient to directly modulate neuronal growth under the tested conditions.

To further explore the role of the secretome in the nerve-muscle unit, the thesis incorporated a biomaterial-based approach using a decellularized nerve-derived hydrogel (P2 gel). This hydrogel provides both structural support and a biologically active matrix. P2 gel can retain and present

ECM components and signalling molecules relevant to regeneration. Initially, P2 gel was used in an in vitro model with neural cells to further evaluate the effects of EVs derived from denervated skeletal muscle. However, no significant effects on neurite outgrowth were observed in this system. This approach was intended to approximate certain aspects of the regenerative environment present in vivo. When evaluated in vivo in a rat sciatic nerve repair model, P2 gel supported axonal continuity and contributed to the preservation of denervated skeletal muscle, as demonstrated by DTI, axonal, and skeletal muscle analysis.

Taken together, the different approaches presented in the thesis converge on a single conceptual framework: that the secretome, in its various forms, plays a central role in coordinating the regeneration of the muscle-nerve unit. Whether derived from stem cells, injured tissues, or engineered biomaterials, secreted factors shape the regenerative microenvironment by influencing angiogenesis, cellular communication, ECM remodelling, and tissue integration. Importantly, this work demonstrates that these components should not be studied in isolation, but rather as parts of an integrated system.

Concluding Remarks and Future Perspectives

In conclusion, this work shows that different secretory mechanisms can influence processes relevant to peripheral nerve regeneration and denervated muscle maintenance. ASCs cultured under clinically compatible, xeno-free conditions exhibit a secretome with regenerative and angiogenic potential, which can be further modulated through targeted stimulation. In addition, denervation was shown to influence EVs derived from skeletal muscle, and we investigated their role for EV-mediated signalling in nerve-muscle communication. Finally, a nerve-derived ECM hydrogel demonstrated the ability to support axonal continuity and contribute to preservation of denervated skeletal muscle *in vivo*. Together, these findings address the aims of the thesis by demonstrating that stem cell-derived secretome, EV-mediated signalling, and a biomaterial-based strategy, as secretome components, contribute in distinct ways to regeneration in the nerve-muscle unit.

Looking forward, future studies should aim to better define the key components within the secretome that drive these effects. Identifying critical factors in more physiologically relevant models will be important. Further characterisation of the ASC secretome is also needed, including the isolation of exosomes from stimulated ASCs and direct comparisons between EV fractions and soluble GFs. In addition, investigating the interaction between the ASC-derived secretome or exosomes and the P2 hydrogel may provide insight into how this biomaterial modulates the retention and release of secreted factors. This understanding will be important for optimising combined secretome-based strategies incorporating biomaterial delivery systems. These approaches should then be evaluated for their therapeutic potential alongside the development of advanced *in vitro* models and *in vivo* validation to support clinical translation.

Acknowledgements

I would like to start by thanking everyone whom I have encountered in this long journey. I am deeply appreciative of your support and help throughout these years.

First, I would like to thank **Gustav Andersson**, my supervisor, for the support and guidance over these 4 years. I am truly grateful for the opportunity you gave me. We had many engaging discussions and enjoyable meetings over the years, especially those featuring AI-generated songs and images. I also wish to thank my co-supervisors, **Maria Brohlin** and **Paul Kingham**, for your input, feedback, and guidance across all the projects.

I would like to thank **Jonas von Hofsten**, my examiner, for your advice and help throughout these 4 years. I am extremely grateful to **Isabel Goicolea**, my reference person. With your strength and integrity, you helped me in one of the most difficult times. You inspire me and I am thankful for your help.

I would like to extend special appreciation to **Liudmila Novikova** for your input and support over these years. I will dearly cherish our nice conversations during experiments. I thank **Lev Novikov**, for your knowledge and suggestions across the different projects. To **Peyman Kelk** and **Mikko Lammi**, with whom it all started. Many thanks for introducing me to research by supporting me in my Master projects and throughout these years. I would also like to extend my appreciation to **Anna-Karin Olofsson**, laboratory technician, for your help in navigating the lab and your contribution to the projects.

Special thanks to my lab mates. **Luis**, you were there from the very beginning of my journey in this department. Now, at the very end of it, I can really understand the discussions and talks we had about research. I am honoured to have had you as my office mate and tutor. **Ashang**, also known as egg gamer. You are the definition of scientist, your curiosity inspired me and your scientific and non-scientific support meant the

world. **Nick, Phani, and Yashar**, thank you for the good moments we have shared.

I want to thank the rest of the **NRCT** group for your support and help with the projects.

Special thanks to all **administration** and **IT staff** members at MTB!

Thanks to **Sara Wilson**, for creating a better environment in the department and being an inspirational scientist. I appreciated your practical help and exchanges of ideas over these years.

During my time as PhD representative, we had many discussions about the importance of having a support network. I have then realized how important it is to have a solid and helpful community around you, especially when things don't go as planned. Thanks to **LuzMa**, you taught me the word “embrace”, or better, you taught me how to embrace. We share the love of a simple but full life. I am truly grateful to have met such a colourful and determined person. In friendship, Commander. **Feryal**, my Turkish animated partner in crime. Your energy follows you around and it never leaves the room. Thank you for sharing these years with me in the highs and the lows. To our old couple relationship. **Vanessa**, I do not even have to write much because, as my telepathic friend, you already know what I am thinking. I will tell you just in case the connection is a bit weak today. I could not think of another person to share this journey with, until the last minute, we did it together. Merci. **Chloe**, thanks for your contagious laugh that I could hear from my office, our long talks about life and values, and your deep friendship. **Iwan**, thank you for the good memes, listening to my presentation 100 times, the help with my work, and the infinite laughs (the one with glasses? If you know, you know).

I am grateful to everyone who has crossed my path in these years. Special thanks to **Ratish, Itzel, Eva, Mario, Chai, Karsten, Ben, Sara, Maud, Fanny, Amol, Meghana, Bianca, Lauri, Manish, Dipanjan, Diana, Linkun, Noor, Nils, Emanuel, Mikael, Giada, Sabrina, Tessy, Xhensila, Abraha, Taiyeba, Rashmi, Jingyu, Roshni**,

Luisa, Vanessa, Yuchen, Elin, Irene, and Qiongxuan for all the fikas, lunches, parties, and good memories we have shared.

Nu till min svenska familj och vänner. Tack **Petra** och **Jan** för alla roliga och trevliga stunder, och jag har alltid känt mig välkommen i ert hem. Tack till **Mickan, Lizette, Camilla, Jennifer, Hannes, Linus, Elis, Zeld, Mira, Sebastian, Dennis, Anton, Nils, Sara** och **Elias**. Jag önskar er alla det bästa.

Mamma e **Papà**, non c'è stato un momento in cui non avete creduto in me. Grazie per il sostegno in tutti questi anni: non sarei dove sono oggi se non fosse per voi, letteralmente. Fin da piccola mi avete incoraggiata ad andare più a fondo nelle mie passioni: mamma con l'amore per la montagna, l'arte e la cultura; papà con l'amore per la musica, la storia e lo sport.

Cami, da dove iniziare... il mio braccio destro, la mia roccia e la sorella migliore che si possa avere. Grazie per avermi convinta a venire in Svezia, è stata la scelta migliore della mia vita ed è grazie a te. **Giovi**, il fratellone maggiore, grazie per esserti preso cura di me in questi anni. Le tue parole di supporto mi hanno aiutata nei momenti più difficili. **Giorgia**, lo stesso vale per te: ci sei sempre stata anche nelle difficoltà. Grazie. Un bacio a **Tommaso** ed **Edoardo**, ancora così piccoli ma già così importanti per la nostra famiglia. Un grazie anche al mio bestie, **Jonathan**, per i bei momenti insieme. Un sentito ringraziamento ai "cuginetti" **Marghe, Amos, Noah** ed a **Zio Millo** e **Zia Cate**. Grazie per essere sempre stati i miei tifosi numero uno e per il vostro affetto. **Grazie a tutto il resto della mia famiglia.**

Un sentito ringraziamento alla famiglia **Giacobini-Arnér**, che fin da piccola mi ha avvicinata alla cultura svedese. La mia più sincera gratitudine ad **Ezio Giacobini** ed a **Christine Nu Viet Vu** per avermi seguita e sostenuta nel mio percorso accademico.

Un mega giga grazie ai miei amici per il sostegno, da vicino e a distanza: **Fus, Giulia, Marghe, Virgi, Meme, Desde, Berg, Vitto** e **Ken**.

Grazie di cuore. Ed ai miei amici storici, compagni di mille avventure, che ci sono sempre stati: **Linda, Maddi, Anto, Fatu e Leila.**

Last but not least, **Mathias**, my supporter number one. You have truly believed in me, at moments in these years more than what I have believed in myself. From the first time I have met you, you showed me kindness and unconditional love. You have taught me that being dynamic is a strength, to not be afraid to change path and, for how shallow it might sound, to follow my dreams and passions. I am forever grateful.

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