Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE CHIRURGICHE

Ciclo 35

Settore Concorsuale: 06/E3 - NEUROCHIRURGIA E CHIRURGIA MAXXILLO FACCIALE

Settore Scientifico Disciplinare: MED/29 - CHIRURGIA MAXILLOFACCIALE

VALIDATION OF A NEW METHOD FOR HUMAN NERVE DECELLULARIZATION: TOWARD A NEW TOOL IN PERIPHERAL NERVE RECONSTRUCTIVE SURGERY.

Presentata da: Federico Bolognesi

Coordinatore Dottorato

Bianca Maria Piraccini

Supervisore

Achille Tarsitano

Esame finale anno 2023

ABSTRACT

Defects of the peripheral nervous system are extremely frequent in trauma and surgeries and have high socioeconomic costs. In case of peripheral nerve injury, the first approach is primary neurorrhaphy, which is direct nerve repair with epineural microsutures of the two stumps. However, this is not feasible in case of stump retraction or in case of tissue loss (gap > 2 cm), where the main surgical options are autologous grafts, allogenic grafts, or nerve conduits. While the gold standard is the autograft, it has disadvantages related to its harvesting, with an inevitable donor site morbidity and functional deficit. Fresh nerve allografts have therefore become a viable alternative option, but they require immunosuppression, which is often contraindicated. Acellular Nerve Allografts (ANA) represent a valid alternative in order to overcome the limits of autologous nerve graft and fresh allografts: in fact, Acellular Nerve Allografts (ANAs) do not need immunosuppression and appear to be safe and effective based on recent studies. Commercial ANAs available on the market have some limitations such as high cost, shipping limitations, U.S. donor selection criteria that differ from those in Europe and the requirement of γ-ray sterilization after production.

The purpose of this study is to propose and develop an innovative method of nerve decellularization (*Rizzoli method*), conforming to cleanroom requirements in order to perform the direct tissue manipulation step and the nerve decellularization process within five hours, so as to accelerate the detachment of myelin and cellular debris, without detrimental effects on nerve architecture and without disrupting the asepsis chain. New ANAs should not require terminal sterilization if microbiologically negative at the end of handling.
In this study, the safety and the efficacy of the new method are evaluated in vitro and in vivo by histological, immunohistochemical, and histomorphometric studies in rabbits and humans.

The new method is rapid, safe, and inexpensive if compared with available commercial ANAs. Besides, the present study shows that the method, previously optimized in vitro and in vivo on animal model presented by our group, can be applied on human nerve samples, obtaining similar, and sometimes even better results compared with the control technique, the Hudson one.

This work represents the first step in providing a novel, safe, and inexpensive tool for use by European tissue banks to democratize the use of nerve tissue transplantation for nerve injury reconstruction.

SUMMARY

1. INTRODUCTION

1.1 The Peripheral Nervous System: organization and main cellular components

The central nervous system (CNS) includes the brain and the spinal cord. The peripheral nervous system (PNS) involves all the other nerves. Through the PNS it is possible to communicate with the outside world and have control of many body mechanisms.

A distinction can also be made between the voluntary and involuntary nervous system. The voluntary nervous system (somatic nervous system) controls all the movements that we are aware of and can consciously influence, such as moving arms, legs and other parts of the body.

The involuntary nervous system (vegetative or autonomic nervous system) regulates the processes in the body that we cannot consciously influence and is made up of 3 systems:

1. Sympathetic Nervous System.
2. Parasympathetic Nervous System.
3. Enteric Nervous System.

The peripheral nervous system is composed of neurons, glial cells and stromal cells. Glial cells are small and numerous, are unable to generate action potentials, but they maintain the mitosis ability. They surround cell bodies into the ganglion and control the levels of CO_2, O_2, nutrients, and neurotransmitters around neurons.

Among the glial cells, we also find Schwann cells, that surround every axon in PNS. They are responsible for peripheral axons myelinisation, and have a part in reconstruction after injury.

Every neuron is formed of different structures (*Figure 1*):

- *Cell Body*: composed of cytoplasm and nucleus.
- *Dendrites*: they receive signals and transfer them on to the cell body.
- *Axon*: it receives the signals from the cell body and conveys it to periphery.
- *Myelin*: responsible for electrical isolation and conduction speed.

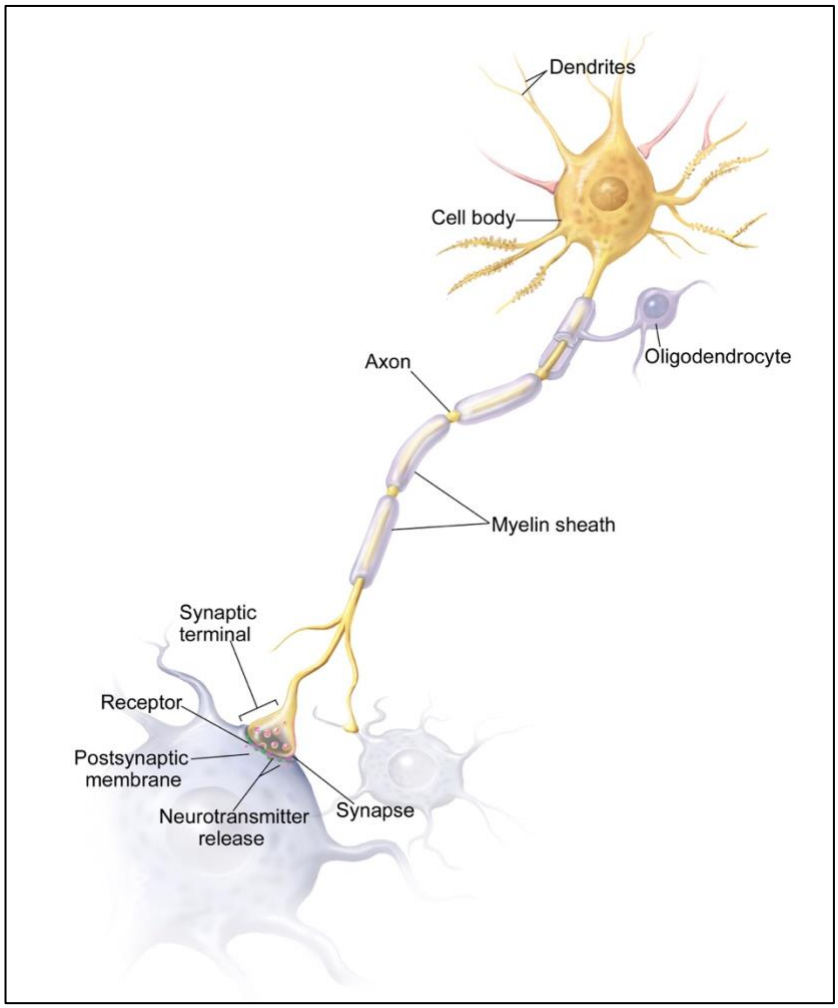

FIGURE 1: *Morphological representation of a neuron. Terese Winslow, 2001 ©*

Peripheral nerves have multiple layers of connective tissue surrounding axons, with the endoneurium surrounding individual axons, the perineurium binding axons into fascicles and the epineurium binding the fascicles into a nerve.
The PNS is made up of sensory and motor nerves. Sensory nerve fibers in the peripheral nerves are the peripheral axonal processes of neurons in the dorsal root ganglion. The motor axons are the processes of anterior horn cells of the spinal cord.

After a serious injury of the cell body, since the nervous tissue cannot be reconstructed, there is an improvement of the axoplasmic flow and glial cells mitosis.

1.2 PNS reconstruction

In the PNS, long-distance axon regeneration and substantial functional recovery can occur in the adult. Both extracellular molecules and the intrinsic growth capacity of the neuron influence regenerative success. This is different from the CNS, where axon regeneration is extremely limited after injury [1].

After PNS injury, axons promptly regenerate. The distal portion of the axon, which is disconnected from the cell body, undergoes the so-called Wallerian degeneration. This active process results in fragmentation and disintegration of the axon. Debris are removed by glial cells, mostly macrophages. Proximal axons can then regenerate and re-innervate their targets, allowing recovery of function.

1.2.1 History

Wallerian degeneration is named after Augustus Volney Waller. Waller experimented on frogs in 1850, by severing their glossopharyngeal and hypoglossal nerves. He then observed the distal nerves from the site of injury, which were separated from their cell bodies in the brain stem [2].

Waller described the disintegration of myelin, which he referred to as “medulla”, into separate particles of various sizes. The degenerated axons formed droplets that could be stained, thus allowing studies of the course of individual nerve fibres.

1.2.2 Main Principles

Regeneration and repair processes go on at multiple levels following nerve injury, including the nerve cell body, the segment between the neuron and the injury site

(proximal stump), the injury site itself, the segment between the injury site and the end organ (distal stump), and the end of the nerve [3].
The success of regeneration from the proximal stump depends on the distance from the injury site [3].
Wallerian degeneration starts 24–48 hours after peripheral nerve injury and both the distal axons and surrounding myelin degenerate [1]. The proximal axonal segment also degenerates back to the adjacent node of Ranvier, where the axon will regrow (*Figure 2*) [4].

Following various forms of axon injury, this rapid degeneration process begins with degradation of axoplasm and axolemma with development of axonal and myelin debris that is subsequently removed by Schwann cells and invading macrophages. In recent years it became apparent that Wallerian degeneration starts from an active process intrinsic to the axon that shares some principles with apoptosis [5].

Schwann cells phagocytise axonal and myelin debris until they create empty endoneurial tubes. Macrophages are recruited and release growth factors, which stimulate Schwann cells and fibroblast proliferation. Schwann cells fill the empty endoneurial tubes in organized longitudinal columns called *bands of Bungner* [6]. This environment situation is a critical point for the success of axonal regeneration.

Axonal regeneration occurs from the most distal node of Ranvier left intact in the proximal stump. As many as 50–100 nodal sprouts appear, mature into a growth cone, and elongate responding to signals from local tissue and denervated motor and sensory receptors (neurotrophic and neurotropic factors) [7].

Proteases are also released from the growth cone to support axonal regeneration. Numerous axonal extensions elongate from the growth cone until they connect with a receptor. Axonal pruning then occurs with the remaining neurites. If a receptor or endoneurial tube is not reached, growth cone branches continue to grow in a disorganized manner producing a neuroma, which can manifest clinically as a painful lump [2].

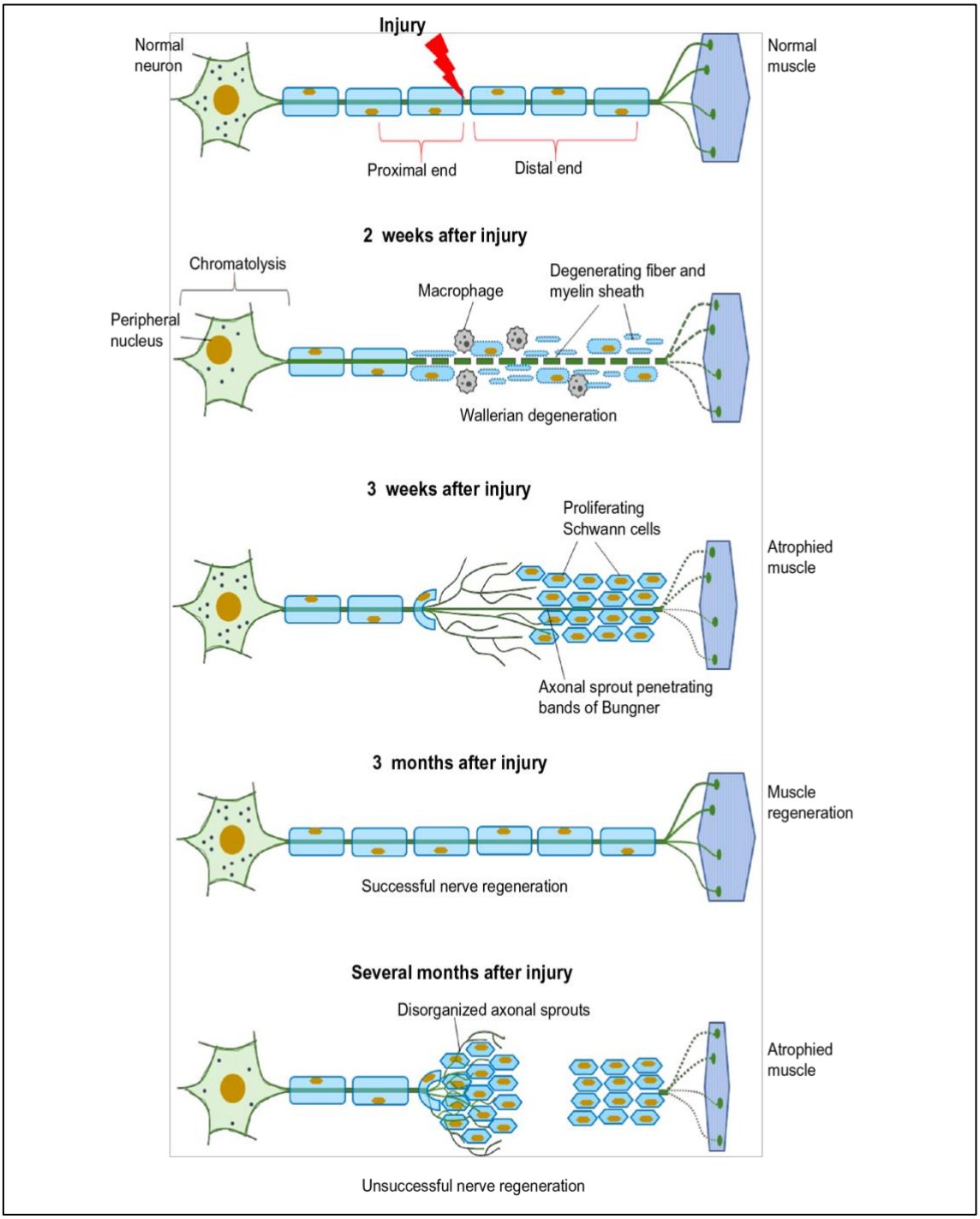

FIGURE 2: *Cellular responses to nerve injury: nerve degeneration and regeneration. [4]*

1. Wallerian Degeneration

Wallerian degeneration is formed by 2 steps: axonal degeneration and myelin clearance.

Axonal degeneration

Axonal injuries initially lead to acute axonal degeneration (AAD), which is rapid separation of the proximal and distal stumps within 30 minutes of injury [8]. Although most injury responses include a calcium influx signalling to promote resealing of severed parts, research has shown that this AAD process is calcium–independent. The process needs about 24 hours in the PNS [9].

Granular disintegration of the axonal cytoskeleton and inner organelles occurs after axolemma degradation. Early changes include accumulation of mitochondria in the paranodal regions at the site of injury. Endoplasmic reticulum degrades and mitochondria swell up and eventually disintegrate. The depolymerisation of microtubules occurs and is soon followed by degradation of the neurofilaments and other cytoskeleton components. The disintegration is dependent on ubiquitin and calpain proteases (caused by influx of calcium ion), suggesting that axonal degeneration is an active process and not a passive one as previously misunderstood [10].

Myelin clearance

Myelin is a phospholipid membrane that wraps around nerves, to provide them insulation. It is produced by Schwann cells. An important step is the increase of permeability of the blood-tissue barrier throughout the distal stump [9].
The response of Schwann cells to axonal injury is rapid. The time period of response is estimated to be prior to the onset of axonal degeneration. Probably the main character of this rapid activation are neuroregulins. They activate *ErbB2 receptors* in the Schwann cell microvilli, which results in the activation of the mitogen-activated protein kinase (MAPK) [11].

The sensing is followed by decreased synthesis of myelin lipids and eventually stops within 48 hours. The myelin sheaths separate from the axons at the Schmidt-Lanterman incisures first and then rapidly deteriorate and shorten to form bead-like structures. Schwann cells continue to clear up the myelin debris by degrading their own myelin, phagocytise extracellular myelin and attract macrophages to myelin debris for further phagocytosis. [9] However, macrophages are not attracted to the region in the first few days; therefore, Schwann cells take a major role in myelin cleaning until then. Schwann cells have been observed to recruit macrophages by release of cytokines and chemokines after sensing of axonal injury. The recruitment of macrophages helps to improve the clearing rate of myelin debris. The resident macrophages present in the nerves release further chemokines and cytokines to attract further macrophages. The degenerating nerve also produces macrophage chemotactic molecules. These signalling molecules together cause an influx of macrophages, which peaks during the third week after injury. Macrophages are facilitated by opsonins, which label debris for removal. The three major groups found in serum include complement, pentraxins, and antibodies. However, only complement has shown to help in myelin debris phagocytosis [12].

2. Regeneration

Regeneration is rapid in PNS, allowing for rates of up to 1 millimetre a day of regrowth [13]. It is supported by Schwann cells through growth factors release.

In healthy nerves, nerve growth factor (NGF) is produced in very small amounts. However, upon injury, NGF mRNA expression increases by five to seven-fold within a period of 14 days. Nerve fibroblasts and Schwann cells play an important role in increased expression of NGF mRNA [14]. Macrophages also stimulate Schwann cells and fibroblasts to produce NGF via macrophage-derived interleukin-1 [15].

Other neurotrophic molecules produced by Schwann cells and fibroblasts together include brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, ciliary neurotrophic factor, leukemia inhibitory factor, insulin-like growth factor, and fibroblast growth factor. These factors together create a favourable environment for axonal growth and regeneration [9].

Apart from growth factors, Schwann cells also provide structural guidance to further enhance regeneration. During their proliferation phase, Schwann cells begin to form a line of cells called Bands of Bungner within the basal laminar tube. Axons have been observed to regenerate in close association to these cells. [16] Schwann cells up-regulate the production of cell surface adhesion molecule *Ninjurin* further promoting growth [17]. These lines of cells guide the axon regeneration in proper direction.

1.3 Peripheral nerve lesions

1.3.1 Epidemiology and aetiology

Peripheral nerve injuries represent a serious problem for society, affecting ≈2.8% of all trauma cases, often resulting in debilitating motor and sensory impairments, and subsequent impaired quality of life for the patient [18-20].
Despite application of contemporary therapies, the 360,000 people in the United States with upper extremity peripheral nerve injury incur 4,916,000 days of lost productivity annually [21].

The most common causes of PNS injury are:

a. Compression Injury

They commonly occur in locations where nerves pass through narrow anatomical structures. The most common sites in the upper limb are the carpal tunnel and the cubital tunnel.

They often belong to neurapraxia (Grade I) injuries, defined by focal demyelination at the site of compression without axonal or connective tissue damage.

They can be divided in acute and chronic compressions: the acute can be caused by external compression, like hanging one arm over a chair, typically they present with transient symptoms like paraesthesia, numbness and wrist drop. Complete recovery may require weeks or years. On the other side, the chronic damage, like Carpal Tunnel Syndrome, causes a progressive loss of function and gain of importance of the symptoms, they begin with paraesthesia and distal numbness (like acute) but they often progress over time to muscle weakness and, eventually, muscle wasting, based on axonal damage [22].

An important theory in the aetiopathogenesis of nerve compression syndromes, called *Double crash* [23], pertains to the increased susceptibility of a nerve to develop a compressive neuropathy distally, when a proximal compressive lesion of the same nerve is found.

b. Crush and Transection Injuries

Crush injuries usually occur from acute traumatic compression of a nerve from a blunt object, without a complete transection of the nerve. They can represent any of the schemes described by Seddon or Sunderland, and they probably represent mixed injuries [24-25].

In contrast, *Transection* injuries are also known as Neurotmesis (Grade V), with complete transection of the nerve. They are commonly due to a laceration from a knife, gunshot, glass shard or other sharp objects.

c. Other causes

Other causes that often occur are:

- *Stretching:* for example, in child birth, motorbike accidents.
- *Disease:* for example, degenerative disease or tumour.
- *Burns:* caused by fire, caustic, chemicals agents.

1.3.2 Classification of injury

Classification of nerve injuries is useful for understanding their pathological basis, making decisions on management, and predicting the prognosis for recovery.

There are 2 most important classifications of injury, today used in the clinical activity: the "*Seddon classification*" (1943) and the "*Sunderland classification*" (1951).

➢ SEDDON CLASSIFICATION FOR SURGICAL DISORDERS OF THE PERIPHERAL NERVES

Seddon Classification for Surgical disorders of the peripheral nerves distinguishes three classes of injury [26] (*Figure 3*):

- **CLASS 1st – Neurapraxia**
  This disorder is described by Seddon as a "Transient Block". It is a loss of nerve conduction caused by an injury, a compression or an ischemia. Paralysis of muscles innervated by the nerve is complete but some sensation may be preserved.

  Neurapraxia injury is characterized by a damage of Schwann cell's myelin coat, with axonal continuity and nerve sheath preserved. Conduction is absent or reduced, although it is sustained proximally and distally to the injury.

  There is *restitutio ad integrum*, usually within some weeks or a few months from the trauma, providing the cause. Recovery does not follow a proximal to distal progression as occurs with axon regeneration.

- **CLASS 2nd - Axonotmesis**
  Seddon defines axonotmesis as a "Lesion in continuity". It involves loss of the relative continuity of the axon and its covering of myelin, but preservation of the connective tissue framework of the nerve (the encapsulating tissue, the epineurium and perineurium are preserved).

  The most common causes are linked to stretch injury and can either dislocate joins or fracture a limb.

  In axonotmesis, EMG changes (2 to 3 weeks after injury) in the denervated muscles include:

  - Fibrillation potentials (FP).
  - Positive sharp waves.

- NCV (nerve conduction study) shows loss of nerve conduction in the distal segment (3 to 4 days after injury).

Prognosis: The prognosis is usually good in terms of recovery. Rate of recovery depends on the distance from the site of injury, and axonal regeneration can go up to 1 inch per month. Complete recovery can take anywhere from 6 months to a year [27].

- **CLASS 3rd – Neurotmesis**

  It is a "*Division of a Nerve*", with complete interruption of a nerve. In the site of damage, it is possible to find some swelling, called "*Neuroma*", at the end of the proximal stump. It is a mass of scar tissue and new nerve fibres. There is another swelling at the end of the peripheral stump, called "*Glioma*" composed of Schwann cells and fibrous tissue, smaller than the Neuroma.

  This class is characterized by a complete motor and sensory paralysis that causes muscles degenerations and responds to electrical stimulation with a characteristic denervation reaction.

  Neurotmesis most often occurs in the upper limb [2] accounting for 73.5% of all peripheral nerve injury cases, the Ulnar nerve being the most often injured.
  Most frequently Neurotmesis is caused by Trauma, that could be civilian [2] or military. Civilian trauma is most commonly caused by motor vehicle accidents. Other causes are:
  - Lacerations by glass, knives, guns or fractures.
  - Sports injury, occasionally.

  Stretch injuries are the most common types and are considered to be a closed injury, [28] they are usually the result of dislocation.

  Military trauma mostly results in open injuries from bombs. Other mechanisms are less common but include ischemia, thermal and electric

shock, radiation, adverse reactions to certain chemotherapy medications, percussion and vibration.

Patients with Neurotmesis have poor chances of *restitutio ad integrum* of their functionality, only a surgical procedure could give them some chances of regaining functions.

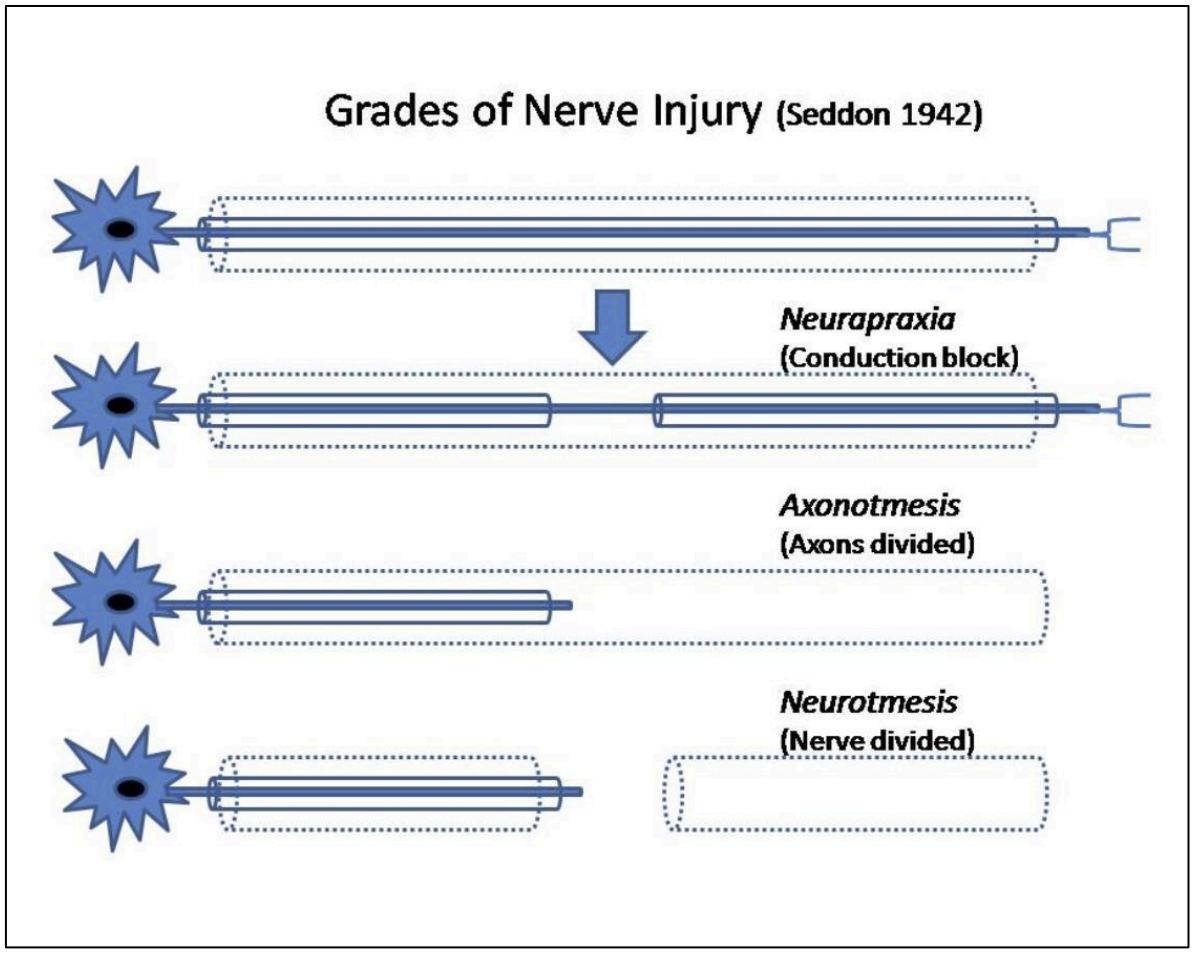

FIGURE 3: *Diagram outlining the grades of injury described in Seddon's classification. [29]*

Limit of Seddon's Classification

Seddon's classification does not distinguish between all grades of intraneural damage. Lesions classified as axonotmesis have been observed to have variable recovery. This is because variable degrees of damage to the connective tissue layers of the nerve, including the endoneurium and perineurium, as well as disruption of the axons are possible without loss of continuity of the nerve trunk.

➢ SUNDERLAND CLASSIFICATION

In 1951, Sunderland expanded Seddon classification in five degrees of peripheral nerve injury [29-30]:

- **1st degree:** It includes Seddon's Neurapraxia.
- **2nd degree:** it corresponds to Seddon's Axonotmesis.
- **3rd degree:** it is included in Seddon's Neurotmesis; Sunderland's third-degree is a nerve fibre interruption. In third-degree injury, there is a lesion of the endoneurium, but the epineurium and perineurium remain intact. Recovery from a third-degree injury is possible, but surgical intervention may be required.
- **4th degree:** the perineurium is disrupted. This distinction is useful exploring a nerve-damaged by blunt trauma. Surgical repair is required.
- **5th degree:** Fifth-degree lesion is a complete transection of the nerve. Recovery is not possible without an appropriate surgical treatment.

Classifications Overview

It is important to recognize between cases of block of the conduction (signal of non-degenerative lesions) and injury causing axon degeneration, because this difference plays an important role in the prognosis, althought this can be difficult to diagnose early after an injury. The most important element influencing the management is the difference between lesions where continuity of the nerve is preserved (neurapraxia and axonotmesis) and lesions where nerve is completely damaged and recovery will not occur without surgical repair (neurotmesis). In cases of loss of continuity, the classifications do not distinguish between tidy lacerations and injuries where a length of nerve is disrupted [31].

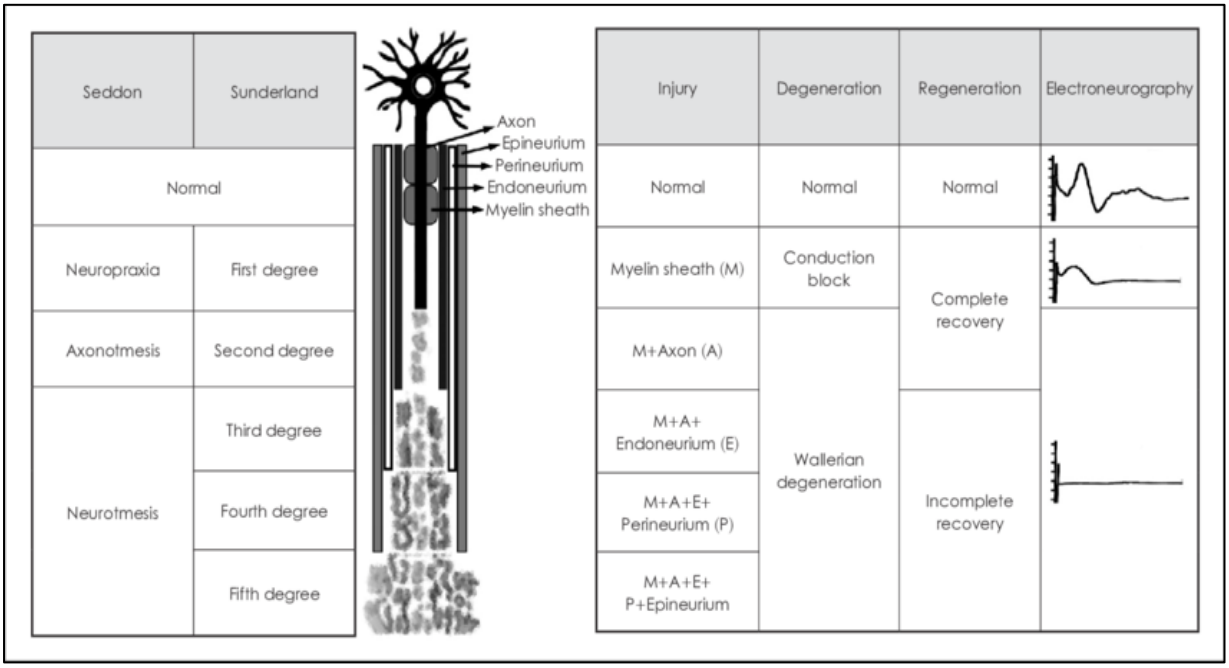

FIGURE 4: *Overview of Sunderland and Seddon Classifications. [31]*

1.3.3 Clinical assessment

Clinical assessment is the first and one of the most important step of the workflow in the diagnosis of nerve injury, above all in blunt or sharp trauma. High energy and open injuries tend to be associated with more severe nerve injuries.
It is important to examine if the neurological deficit was present immediately after the injury or had a late onset. Besides, it is important to investigate associated skeleton or soft tissue damage.
Examination of nerve function includes motor power, sensation and autonomic function.

1.3.4 Diagnosis

Diagnosis starts with the medical history and objective observation: it is important to test the patient's muscle strength, reflexes, and/or their ability to detect discriminating variations in sensation, pressure, temperature, and pain in the affected area. Clinical evaluation is usually followed by diagnostic procedures, such as Ultrasounds (US), Magnetic Resonance Imaging (MRI), Computed

Tomography (CT), Nerve Conduction (NC) studies and Electromyography (EMG) [30].

1.3.4.1. Tests

The most used techniques are [30]:

- **2-POINT DISCRIMINATION:** It is a measure of tactile recognition. It investigates the ability of patient to discern the sensation in two points in the skin. It is a non-invasive test for nerve dysfunction. The minimum width, which can be perceived as separate, is measured either using a calibrated device or a U-shaped piece of wire. Normal two-point discrimination is approximately 4 mm on the pulps of the fingers

- **MONOFILAMENT TEST:** A Semmes-Weinstein monofilament test is used to measure loss of sensation. Test consists in some filaments of different measures placed on the skin. After a force is applied to blend filaments, the test allows a measure of the minimum pressure which can be perceived. In the position where damage is located, the patient cannot detect some filaments. It is a quick and non-invasive way to test nerve injury.

Filament	**Interpretation**	**Force (Grams)**
1.65 – 2.83 (Green)	Normal	0.008 – 0.08
3.22 – 3.61 (Blue)	Diminished light touch	1.72 – 0.217
3.84 – 4.31 (Purple)	Diminished Protective Sensation	0.445 – 2.35
4.56 (Red)	Loss of protective sensation	4.19
6.65 (Red)	Deep pressure sensation	279.4

- **SENSITIVITY TEST:** this test uses heat, cold and pain to investigate sensitivity, using a warm instrument, a cold cube or a needle prick. Normally people can feel each sensation. A lack of any of these is a signal of nerve injury.

- **MEDICAL RESEARCH COUNCIL (MRC) GRADING SYSTEM:** it analyses muscular strength on scale from 0 to 5. The patient should be asked to perform the desired movement while the examiner resists this movement and reports the result using this table:

Grade	Clinical features
M0	*Complete paralysis*
M1	Flicker of muscle activity
M2	Power insufficient to overcome gravity
M3	Movement against gravity throughout the range of movement of the joint
M4	Movement against resistance
M4+	Strong movement, but normal
M5	Normal, full power

1.3.4.2 Investigation procedures

- **NEUROPHYSIOLOGY:** it may give additional information on a nerve injury. Also an interrupted nerve can conduct signal for some days. Two weeks could be necessary before conduction stops completely. Therefore, neurophysiology is not applicable in open injuries or other injuries requiring very early surgical exploration. In general neurophysiology can distinguish Neurapraxia, with no axon degeneration, and those with distal degeneration as axonotmesis and neurotmesis. Neurophysiology studies are based on:

  - **EMG (Electromyography):** The examination works by placing a sensing electrode into the muscle and measuring the difference in electrical activity between muscle at rest and muscle contraction.

  - **Motor and sensory NCS (Nerve Conduction Studies):** measures how electrical signals are conducted through the nerves. The exam consists in 2 electrodes, one stimulates above the injury and one records below the

injury. An electrical signal passes through the stimulating electrode and time is measured by the second one. A decrease in nerve conduction is linked to possible nerve damage [32-33].

- **MRI, CT and US:** they provide a detailed view of the area in the body where the nerve damage is suspected. The images produced by these examinations can be used to investigate any possible structural damages to the nerves, including scarring from an injury, nerve tumours, or pressure from the surrounding structures. It is difficult to obtain consistent results using standard clinical MRI scanners. In the zone of injury, signals are affected by oedema and haemorrhage in the surrounding tissues. MRI has proved effective in imaging peripheral nerve tumours. The resolution of the modern ultrasound scanners have improved in the last years and now is greater than MRI, especially for examination of nerves damaged by closed trauma. It is able to confirm continuity of a nerve, or diagnose rupture or entrapment, for example, in a fracture. However, ultrasound is operator dependent and requires experience for optimal interpretation [30].

1.3.5 Prognosis

The outcomes after surgical repair of a damaged peripheral nerve depend on several factors:

- **Age**: Recovery of a nerve after surgical repair depends mainly on the age of the patient. Young children can recover close-to-normal nerve function. In contrast, a patient over 60 years old with a cut nerve in the hand would expect to recover only protective sensation; that is, the ability to distinguish hot/cold or sharp/dull.

- **The mechanism of injury**: Sharp injuries, such as a knife wound, usually damage only a short segment of the nerve, which could be treated with direct neurorrhaphy. In contrast, nerves divided by stretch or crush may be damaged over long segments. These nerve injuries are more difficult to treat

and generally have a poorer outcome. In addition, associated injuries, such as injury to bone, muscle and skin, can make nerve recovery more difficult.

- **The level of injury**: After a nerve is repaired, the regenerating nerve endings must grow all the way to their target. For example, a nerve injured at the wrist that normally provides sensation to the thumb must grow to the end of the thumb in order to provide sensation. The return of function decreases with increased distance over which a nerve must grow.

- **Smoking**

- **Timing:** Primary repair is the optimal approach for peripheral nerve injuries taking place within the first couple of days. Secondary repair takes place one week or more after the injury [34]. Partial injuries (15% of injuries) as a consequence of stretch or contusions are commonly managed with secondary repair [35-36]. For complete injuries the method of repair depends on what is found during exploration. If the epineurium is found to be neatly divided, then primary repair without tension is usually undertaken but if the ends are ragged or in case of a gap, then a nerve graft may be required [37].

1.3.6 Therapy

One of the main differences between the central nervous system and the peripheral nervous system is the possibility of regeneration of the latter.

Despite the physiological regeneration of PNS, it is not always possible to recover full activity of the nerves. If nerve is totally or partially damaged, a surgical approach is strictly recommended.

Indications for nerve injury surgery are:

- *Closed nerve injury*: If there is no evidence of recovery, either clinically or from electrodiagnostic studies, at 3 months after injury, surgery is recommended; if there is evidence of recovery as indicated by motor unit potentials (MUPs), patients should be assessed to determine the progression of recovery and the possible requirement for surgery

- *Open nerve injury* (i.e., laceration): Surgical exploration is recommended as soon as possible; all lacerations with a reported loss of sensation or motor weakness should be surgically explored

- *Crush nerve injury*: Surgical exploration of the nerve may be delayed for as long as several weeks; however, if, after 3 months, there is no evidence of reinnervation, either clinically or from electrodiagnostic studies (the absence of MUPs signals the absence of reinnervation), surgical reconstruction with nerve repair, transfer, or grafting is indicated.

In contaminated or crush nerve injuries, delayed reconstruction may be indicated [38], especially for the high risk of infection.
Regarding the surgical technique, there are different options related to the type of injury and nerve damage:

- *Primary end-to-end neurorrhaphy*, which is a direct nerve repair with epineural micro-sutures, is still the gold standard surgical treatment for severe axonotmesis and neurotmesis injuries. Epineural repair is performed when a tension free coaptation in a well-vascularised bed can be achieved; it is also necessary that the gap between the two ends of the nerve is relatively short [39]. In contaminated wounds, primary repair should not be undertaken; however, nerve ends should be approximated and marked using colored stitches during initial debridement to prevent the retraction and to ease dissection of the nerve stumps in the course of second surgery [40]. When tension free primary repair is not possible because nerve ends will retract due to their elasticity or in the case of greater defects or longer gaps

between the cut ends, neurorrhaphy will cause excessive tension at the repair site that will impair microvascular flow in the nerve tissue and lead to excessive scarring at the repair site [41]. In these situations, primary neurorrhaphy should not be performed, and a suitable alternative should be considered [42], such as nerve graft or conduits.

- *Nerve grafting* is usually performed when nerve tissue defect is longer than 2 cm, after all the additional procedures for approximation of the nerve stumps without tension [43].

There are three main options (*Figure 5*):

- Autologous grafts
- Allogeneic grafts
- Nerve conduits

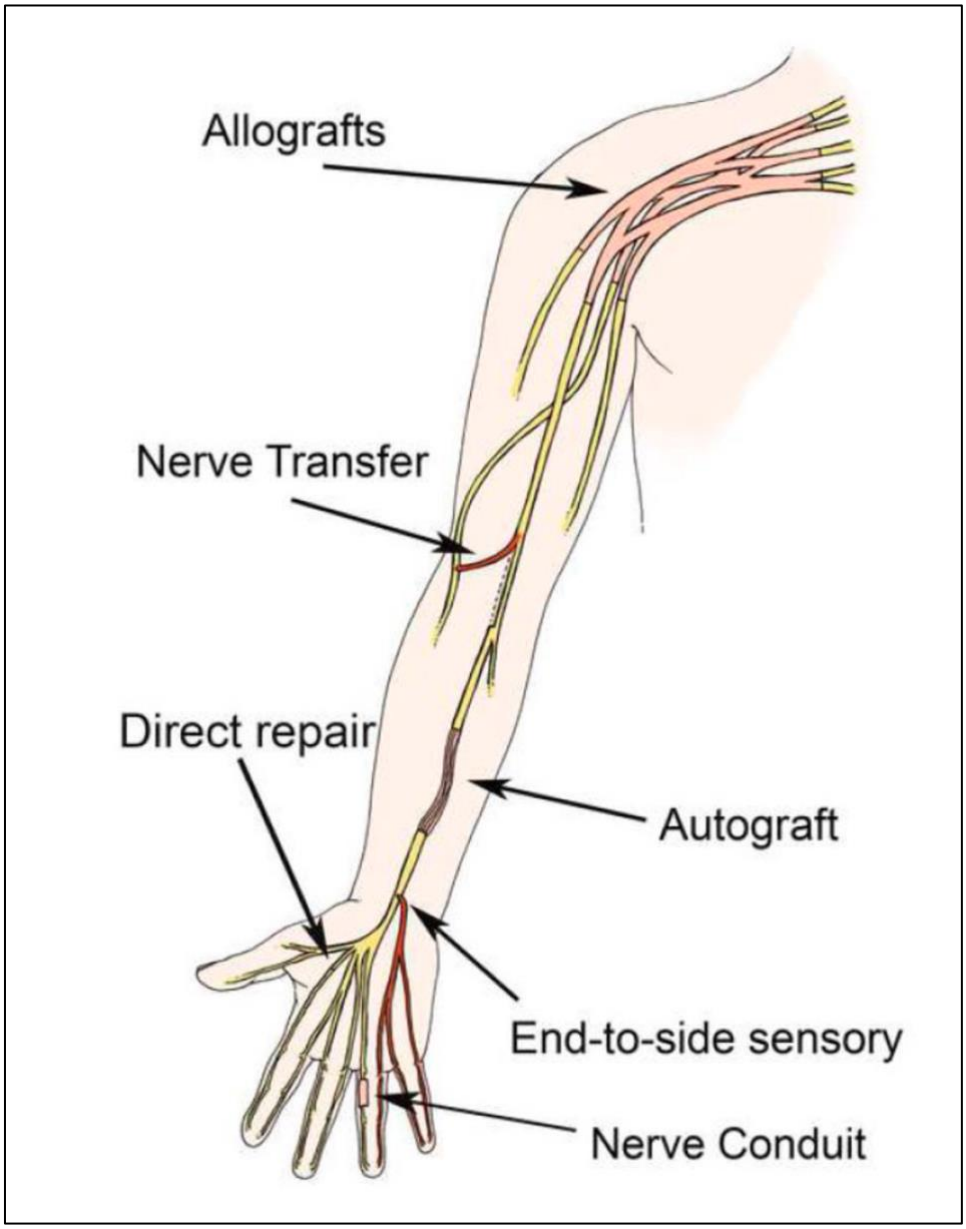

FIGURE 5: *Summary of the various options for nerve repair [38]*

Information regarding the mechanism of injury or onset of symptoms will guide towards the most suitable treatment modality.

Patients presenting with open injuries and neurological deficit require early exploration. In case of concomitant vascular injuries, urgent exploration is mandatory.

The use of Sunderland's classification of nerve injury is absolutely essential in the management of nerve injuries. First, second, and third-degree injuries will recover without the need for surgery, where fourth and fifth degree will require surgical operation.

Initial management of closed injuries is expectant, with baseline electrodiagnostic evaluation at six weeks. Fibrillations will appear approximately four to six weeks after injury, while motor unit potentials (MUPs) and nascent units take several weeks to appear.

1.3.6.1 Autologous grafts

Autologous nerve grafting has long been considered the "gold standard" for repair of irreducible nerve gaps. Autologous grafts act as immunogenically inert scaffolds, providing appropriate neurotrophic factors and viable Schwann cells (SCs) for axonal regeneration.
The choice of autologous is dependent on several factors:

- Size of the nerve gap
- Location of proposed nerve repair
- Associated donor-site morbidity.

Although the Sural nerve is the most commonly used autograft, there are many other suitable nerves that can be used as interposition grafts including [44]:

- Medial and Lateral Cutaneous nerves of the forearm,
- Dorsal cutaneous branch of the ulnar nerve,
- Superficial and deep peroneal nerves,
- Intercostal nerves,
- Posterior and lateral cutaneous nerves of the thigh
- Great auricular nerve.

As with all repair techniques, when utilizing an interposition graft it is critical to avoid any tension at the repair site, even minimal tension can negatively impact the functional outcome [45].

If grafting is performed within a few months of injury, the distal nerve will still contain viable SCs. The proximal portion of the nerve will always contain viable SCs.

It is obvious that the distal end of the sensory nerve is not reinnervated but is turned end-to-side to a normal adjacent sensory nerve so that even minimum collateral sprouting from the end-to-side repair will provide some sensation to the area [46].

It is possible to describe pros and cons:

- Pros:
    - Non-immunogenic reaction
    - Bridge nerve gap

- Cons:
    - Sensory loss at the donor site
    - Scarring
    - Neuroma formation
    - Second incision
    - Limited supply
    - Inferior to tension free primary repair

1.3.6.2 Allogenic Grafts

Nerve allograft is a technique used to bridge a peripheral nerve lesion with tissues derived from a different individual of the same species. An allograft nerve tissue is as a support for guidance and a source for viable donor-derived Schwann cells that would facilitate the connection of axons at the proximal and distal ends to achieve reinnervation of target tissue or organs [47].

Like all tissue allotransplantation, nerve allografts require systemic immunosuppression; the associated morbidity of immunomodulatory therapy limits the widespread application of nerve allografting.

Therefore, the systemic immunosuppressive therapy is not a desirable treatment due to increased risk of infection, decrease of healing rate, and it occasionally results in tumour formation and other systemic effects.

In case of fresh allografts, once adequate host Schwann cells migration has occurred into the nerve allograft at approximately 24 months, systemic immunosuppression can be withdrawn. Furthermore, despite the morbidity of immunosuppressive therapy, the commonly utilized immunosuppressive agent FK-506 (*Tacrolimus*) has been demonstrated to augment neuroregeneration [38]. Nonetheless, fresh allografts are not recommended because patients require a long systemic immunosuppression treatment. Depressing the immune system allows Schwann cells (SCs) and exogenous axons to be tolerated, but leaves patients vulnerable to infectious and neoplastic risks.

- Pros:
    - Readily accessible,
    - Unlimited supply,
    - Bridges a nerve gap,
    - Avoids donor site morbidity.

- Cons:
    - Potential side effects of host immunosuppression,
    - Immune rejection,
    - Risk of cross contamination.

1.3.6.3 Acellular Nerve Allografts (ANAs)

Researchers have made efforts to develop valid alternatives to autologous grafts to overcome allograft cons, especially in cases of multiple nerve injuries.
Acellular nerve allografts (ANAs) are a recent established option for peripheral nerve reconstruction (PNR) of segmental deficits or large nerve gaps that cannot approximate the nerve endings directly and represent a valid alternative to fresh nerve allografts because they can support peripheral nerve regeneration without needing immunosuppression.
The advantage of these clinically available grafts over hollow nerve conduits is that the internal nerve structure including endoneurial tubes, basal lamina, and laminin remain intact, facilitating axonal regeneration [48]. A recent level III study demonstrated functional recovery for injuries with gaps between 5 and 50 mm [49]. Besides, nerve allografts have the advantage of being readily available and can provide a virtually limitless source of nerve tissue.
The process of decellularization can be various, using chemical detergent, enzyme degradation, and irradiation resulting in a graft with no requirements for immunosuppression [48].

It is possible to identify some main methods of decellularization, currently used in the clinical practice:

- "Cold preserved'' nerve allografts: *cells are reduced, but not eliminated*.
  The objective is to create a method intended to provide a low cost and passive method of decellularization while maintaining the basal lamina tubes and the extracellular matrix. Early storage methods used various solutions to reduce antigenicity, including Ringers and Locke's solution. The immunologic properties of cold-preserved allografts have been extensively studied in rat, mouse, and sheep models. These studies show that the immunogenicity de- clines with time [50].
  Cold-preserved allografts provided insight into graft immunology and regeneration, but their success in supporting axon regeneration is inferior to regeneration through freeze- thawed allografts and the detergent techniques. [51,52] When they retain some viable cells, their implantation requires immunosuppression using cyclosporine A and/or FK506, although

at lower dosages, to support axon regeneration. [53,54] Therefore completely acellular grafts are theoretically advantageous in this regard.

- Freezing and freeze-thaw techniques: *cells are killed, not removed*. Freezing techniques for preparation of acellular allografts were developed earlier than the use of detergent methods. The two primary methods include deep-freezing to - 70°C and repeated freeze-thaw cycles [55]. The precise effects of ice crystallization are unknown but laminin staining of freeze-thaw allografts demonstrated that the basal lamina was preserved [56]. Freeze-thawed allograft studies provided important insights into the role of the basal lamina in axon regeneration which is now well recognized. These studies preceded the development of detergent-based allografts in which the basal lamina tubes were better conserved and associated with significantly better axon regeneration [56].

- Chemical detergents: *cellular content eliminated.* Allografts prepared with chemical detergents are now commercially available for clinical use in humans. The technique of preparing acellular nerve grafts using chemical detergents was pioneered by *Johnson et al.*, using minced human nerves agitated with detergents *Triton X-100* and *sodium deoxycholate* [57] and refined by *Sondell* [58]. In vivo studies of 1-cm acellular grafts demonstrated that they were well integrated in the host, displayed immune tolerance, and supported axon regeneration [58]. Later, *Hudson* and colleagues optimized a detergent protocol, in their systematic morphological examination of a sciatic acellular graft, using a five-point histological grading scale with several morphologic criteria of preservation of the extracellular matrix [59]. The integrity of the basal lamina was superior with the graft prepared with a new combination of three detergents, *Triton X-200, sulfobetaine-16,* and *sulfobetaine-10*, than with the two detergents used previously by Sondell. These optimized acellular grafts displayed stronger regeneration potential than the previous Sondell protocol [56,57]. Hudson's axon densities were significantly higher than Sondell's and the freeze-thaw treated allografts. Hudson's refined protocol balanced

the need for cellular removal and extracellular matrix maintenance. The Michigan group adapted the Sondell protocol, using five solutions including Triton X-100 detergent. Hudson's detergent-based method, combined with treatment with chondroitinase ABC to remove regeneration-inhibiting chondroitin-6-sulfate, led to AxoGen's commercialization of their Avance clinical allograft [60]. However, the number of fibres supported by the allograft was only 30% of the one supported by autografts [55]. *Figure 6* shows a schematic representation of the five primary methods for decellularized allograft.

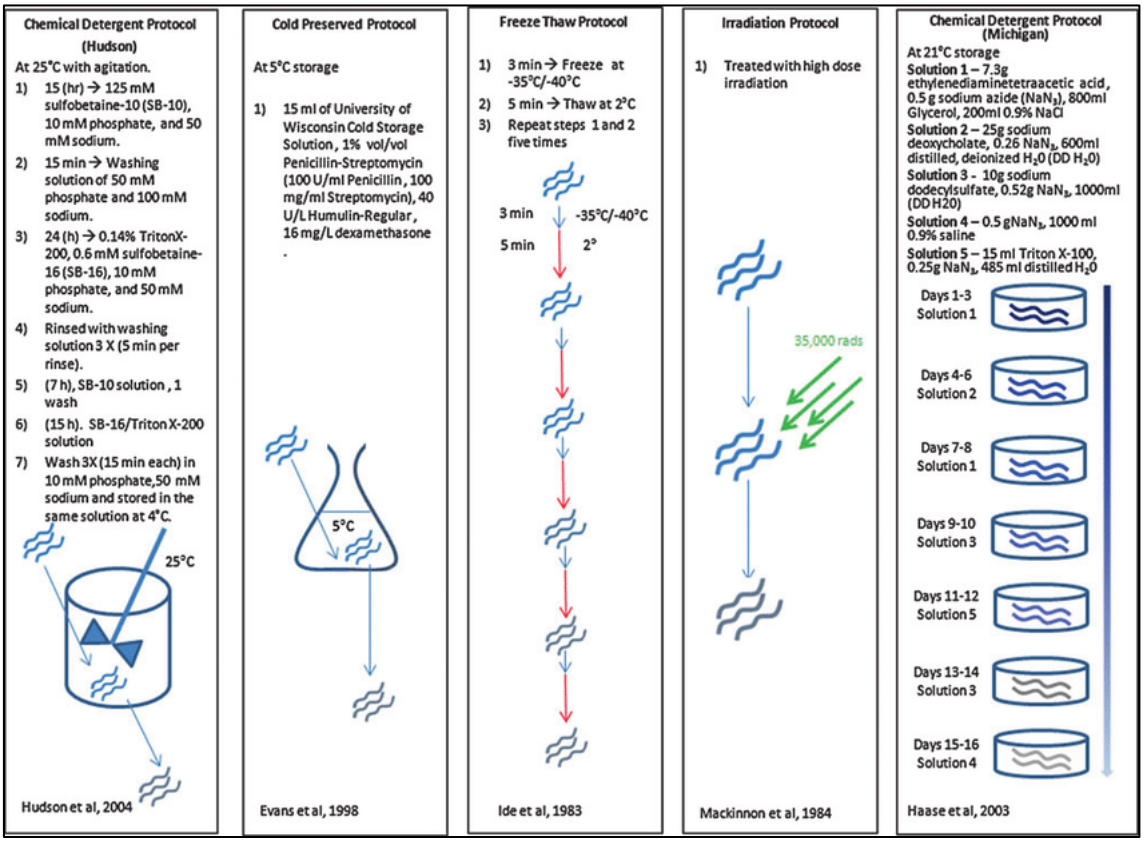

FIGURE 6: *(Above) Schematic representation of the five primary methods for decellularized allografts in the treatment of peripheral nerve defects. (Below) Summary of methods used for decellularization of peripheral nerve [55].*

SUMMARY OF METHODS USED FOR DECELLULARIZATION OF PERIPHERAL NERVE

Outcome measure	*Cold preserve*	*Freeze-thaw*	*Detergent (Hudson)*	*Detergent (Michigan)*	*Irradiation*
Complexity of protocol	Low	Low	High	High	Moderate (dependent on availability of equipment)
Time to prepare graft	21 days or longer	~10 min	~65 h, including intermediate steps	16 days	Immediately after treatment
Cells removed	No	No	Yes	No	No
Cells killed	Only at very long duration of storage	Yes	Yes	Yes	No
Immunologically inert	Only at very long duration of storage	Yes, following macrophage clearance	Yes	Greatly reduced	No
Regeneration capacity advantage	Inferior to freeze-thaw and detergent methods	Better than cold preservation but inferior to detergent methods	Superior to either cold preservation or freeze-thaw methods	Partial recovery of reinnervated muscle force but unable to compare with other protocols	Poor with no direct comparisons

1.3.6.4 Nerve conduits

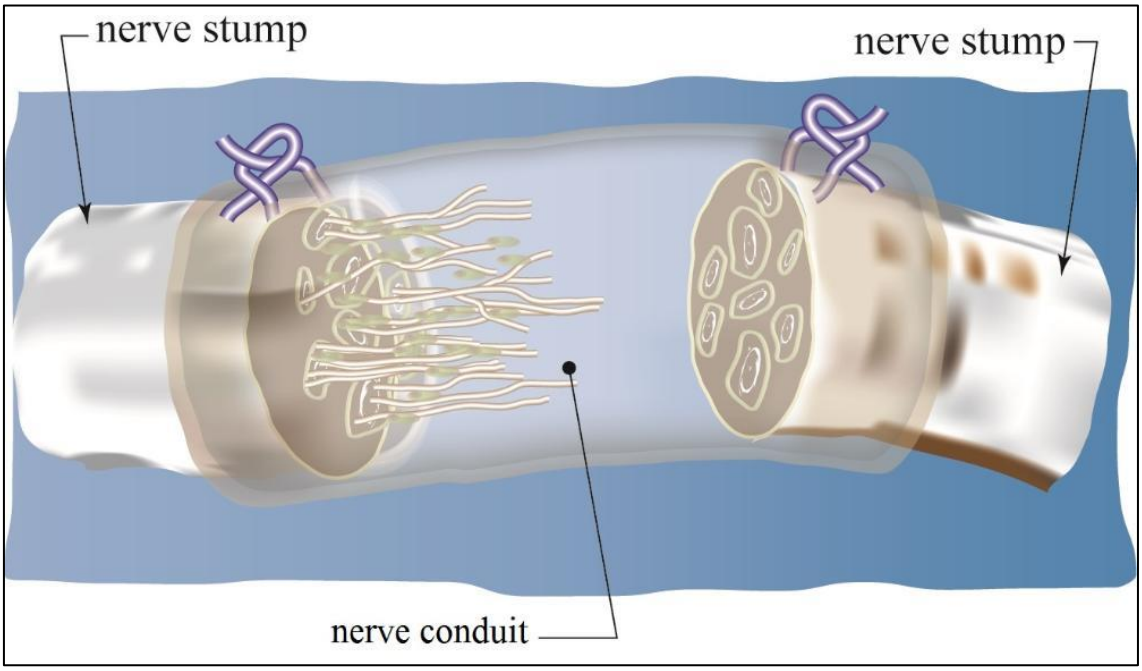

FIGURE 7: *Nerve Conduit, image by Aleo BME, Inc. ©Copyright 2015-2017*

A lot of research has been done to the development of a viable synthetic or biologic nerve conduit (*Figure 7*), and currently several commercially available options exist. Most of authors limit their use of nerve conduits to the repair of noncritical small diameter sensory nerves with a gap less than 3cm and as a nerve repair wrap. It is sure that the concentration of neurotrophic factors is critical to advancing nerve regeneration, and if the volume of a conduit increases beyond a critical size, regeneration is inhibited unless the length of the conduit is dramatically shortened. As surgeons achieve clinical success with short-gap, small diameter nerve injuries, the clinical front is being extended to include larger diameter nerves at longer gaps, and we are beginning to see the failed results of such procedures [55,61].

- Pros:
    - Readily available,
    - Avoids donor site morbidity,
    - Bridges a nerve gap,
    - Barrier to scar tissue infiltration,
    - May allow for accumulation of local neurotrophic factors.

- Cons:
    - Variable outcomes,
    - Lack of laminin scaffold and Schwann cells,
    - Limits its use to short nerve gaps

1.4 Enhancement of acellular nerve allografts

Despite their multiple demonstrated advantages, a significant concern of plain ANAs is the absence of SCs and a lower tendency to be recolonized by host SCs, thus making them a suboptimal treatment option compared to autografts [62]. This has led to studies investigating the ability to enhance plain ANA properties in order to increase their neuroregenerative potential. In the current literature, several methods to enhance ANAs have been described. The methods can be classified into the following 3 categories: cellular, biochemical/biological, and physical.

1.4.1 Cellular enhancement

Regarding **cellular enhancement**, some main cell lines can be identified:

- ***SCs:* Schwann Cells**
- ***BMSCs:* Bone Marrow-Derived Stem Cells**
- ***ASDCs:* Adipose-Derived Stem Cells**
- ***SKP-SCs*: Skin derived Schwann cell precursors**
- *Others:*
    - *Fetal-Derived Stem Cells*
    - *Hair Follicle Stem Cells (HFSCs)*
    - *Dental Pulp Stem Cells (DPSCs)*
    - *Wharton's Jelly MSCs (WJMSCs)*
    - *Umbilical Cord-Derived MSCs (UC-MSCs)*
    - *Amniotic Tissue-Derived Stem Cells (ATDSCs)*
    - *Embryonic Stem Cells (ESCs)*
    - *Neural Stem Cells (NSCs)*

- *Schwann Cells*

The isolation and culture of primary SCs was first accomplished in the mid 1970's [63]. Autologous transplantation of primary SCs has, therefore, been used to enhance nerve regeneration in animal models of peripheral nerve injury [64]. SCs isolated from nerve tissue and cultured in vitro retain the ability to express trophic factors to support axonal regeneration and to myelinate axons following regeneration [64].

Clinical translation of SC transplantation requires surgical harvest of a peripheral nerve, effective isolation, and extended expansion time before transplantation is possible.

Harvested nerves contain a vast number of contaminating cells including fibroblasts, which replicate more readily in culture than the primary SCs. As a result, cultures that are not purely SCs can be overrun with fibroblasts and there is some evidence to suggest that transplantation of activated fibroblasts could actually harm peripheral nerve regeneration through the production of scar [64]. Even with optimization studied in some protocols, the expansion of SCs requires 6–10 weeks from the time of harvest.

Thus, the combined barrier of donor site morbidity and difficulty of effective SC isolation and culture has significantly prevented clinical translation despite decades of concerted interest and effort [64].

- *Stem Cells*

The difficulty associated with the isolation and culture of primary SCs has led researchers to search for an alternative source of cells to support axonal regeneration across a tissue engineered construct. Stem cells are a plausible choice because of their ability to differentiate into multiple cell types and self-renew in culture [64].

It is possible to define 2 main categories of stem cells used in acellular nerve allograft:

a. ES – Embryonic stem cells

   They can be isolated from the fertilized oocyte and they are defined as *totipotent* stem cells; they can also be isolated from cells taken from the blastocyst, in this case they are called *pluripotent* as these cells appear to be forming the three germ layers during embryogenesis.

b. Adult stem cells

   Fully developed adult tissues and organs contain niches of *multipotent* adult stem cells; these cells have been isolated from a wide range of adult tissues such as brain, heart, lungs, kidney, adipose tissue, dermis, and spleen.

The limitations associated with primary SCs culture, the difficulty to isolate/purify, the insufficient number, slow expansion, and donor site morbidity to patients, can be overcome by the use of ES cells. ES cells are readily available, can be expanded quickly and indefinitely in culture, and can be prepared in mass prior to clinical use [65, 66].

Transplantation of native pluripotent ES cells to treat peripheral nerve injury is not a viable option due to the propensity for ES cells to excessively proliferate in vivo [67, 68]. To overcome this obstacle, ES cells can be induced in vitro using defined culture protocols to become progenitor cells that are still multipotent, but are more limited in their differentiation and proliferation potential.

Although stem cell-based therapies could be an interesting option for future treatment of nerve-based injuries, they are linked to serious disadvantages that must be considered [55]:

- *Teratoma formation:* one of the most potentially negative consequences of stem cell-based treatment in any system is the tumorigenic capability of

multipotent precursors. They are involved in benign or malignant tumours referred to as teratomas when injected in an undifferentiated state. There are stem cell types that have not been shown to induce teratoma formation. The SKPs have been shown to possess no tumorigenic capabilities, most probably because they are in a pre-differentiated state.

- *Mislocalization and/or misdifferentiation*: One important consideration is that the cells appropriately differentiate into the preferred cell type and that they reside within the nerve graft itself.

- *Host immune system attack*: One potentially serious danger of administering exogenous cell therapies within acellular nerve grafts is cell death caused by host immune system attack.

MSCs – Mesenchymal stem cells

An important type of cells consists of *Mesenchymal stem cells (MSC)*; these are self-renewing multipotent adult precursors [64]. They originate from the mesoderm germ layer and they give rise to connective tissue, skeletal muscle cells, and cells of the vascular system.

Their multipotency, ease of isolation and expansion in vitro make them an attractive candidate as a component for tissue engineering applications. Under normal developmental paradigms and cellular environments, MSCs are able to differentiate into tissues of mesodermal origin, for example, muscle, bone, cartilage, fat, and tendon.

However, recent data suggest that under specific cell culture conditions, MSCs have the potential to transdifferentiate into many cell lineages (other than mesodermal). With appropriate stimuli and environmental conditions, MSCs have been shown to differentiate into sweat glands, myocardium, endothelial cells, astrocytes, and neurons [64].

It has been demonstrated that cocktails of growth factors and cytokines can promote a SC phenotype in cultured MSC. Subsequent studies have demonstrated,

however, that removal of these in vitro signals results in reversion of the cultured cells to a myofibroblast phenotype that is consistent with the MSC germ line origin [64]. This would suggest that the switch is temporary and artificial, not allowing for long-term maintenance of nerve by SCs derived from MSCs.

Bone Marrow stem cells (BMSC) belong to this group of cells. These cells have found an important role in the cellular enhancement in acellular nerve allografts.

ASCs – Adipose derived stem cells

Another important group of cells is *Adipose derived stem cells* (ASCs). ASCs can be easily isolated from liposuction waste and can exhibit the potential for chondrogenic, osteogenic, adipogenic, and myogenic differentiation. They have many similarities to MSC; they arise from the same mesodermal germ layer, express an ~90 % similarity in cell surface markers, and have multipotent differentiation potential [64]. ASCs, however, have the advantage of increased abundance in the body in comparison to MSCs and this makes them easier to isolate and culture.

Isolated ASCs, when treated with a mixture of glial growth factors (GGF-2, bFGF, PDGF and forskolin), adopt a spindle-like morphology similar to SCs. Analysis of the protein expression from these induced cells reveals expression of the glial markers, GFAP, S100 and p75, indicative of transdifferentiation into a SC like phenotype [64].

SCs derived from ASCs also share the same concerns as those derived from MSCs. Their utility depends on the controversial process of transdifferentiation and is subject to the same instabilities in phenotype.

Limited ability of ASCs to survive would suggest that prior regenerative benefit would be due to production of growth factors and not a functional replacement of lost SCs.

Skin derived Schwann cell precursors

The proliferation/immune concerns associated with ES cell derived cells, and the transdifferentiation/stability concerns associated with MSCs and ASCs are significant barriers to translation. Ideally, an autologous source of adult progenitor cells from the neural crest (ectoderm) lineage that can be readily expanded and induced to form true SCs would be optimal for the development of tissue engineered peripheral nerve.

Neural crest progenitor cells have been identified in two locations in adult tissue; the gut and the skin. The gut is not a convenient source of autologous progenitor cells for tissue engineering.
SKP-SCs located in the dermis are an available source for somatic multipotent cells. In addition to the proliferative capacity, SKP-SCs can differentiate into a wide range of cell types [69].

SKP-SCs cultured in neuregulin-1β express the same markers with SCs. In addition, both undifferentiated and differentiated SKP-SCs showed accelerated nerve regeneration.

Treatment with SKP-SCs significantly increases the average number of axons and reduces the percentage of myelin debris. Several studies have demonstrated the superior results of SKP-SCs on de-myelination and crush injury as well as acute and chronic transection injury [69].

MECHANISM OF ACTION

The impact of stem cells transplantation in PNI mainly depends on their capacity in differentiation phenotype, ability in enhancing neurotrophic action, and promotion of myelin formation (*Figure 8*).

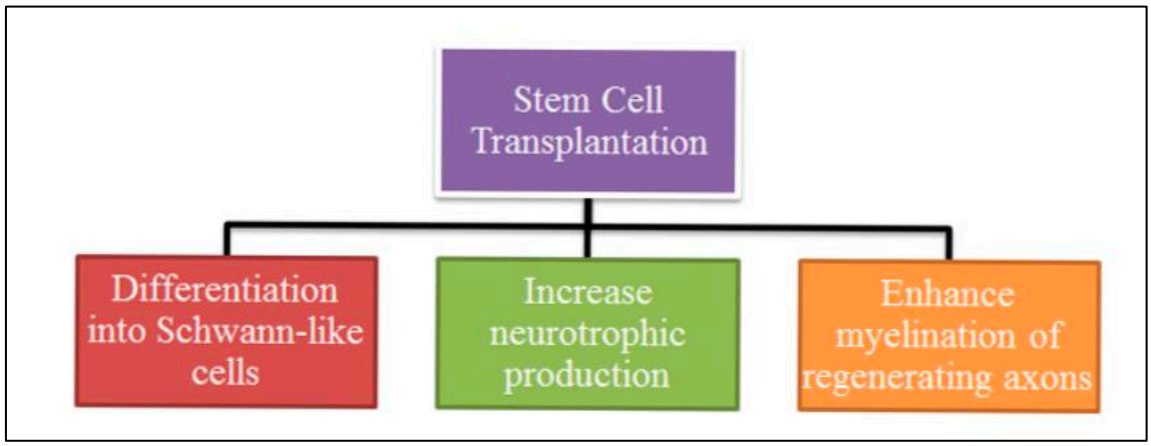

FIGURE 8: *Mechanism of stem cell transplantation for peripheral nerve injury regeneration. [69]*

Although nerve tissue originates from the ectodermic layer, mesoderm-tissues-extracted stem cells (BMSC and ASC) secrete massive amounts of bioactive molecules, such as BDNF, NGF, GDNF, NT3 (neurotrophin 3), and VEGF49 - 51 and, thanks to this paracrine biological activity, they are more suitable to reduce inflammation into the graft implantation site; limit formation of scar-like tissues; recruit endogenous SCs; and support local vascularization.

Differentiation Type of Stem Cells

The self-renewal ability of stem cells makes it possible to send numerous cleavage cells to the site of injury. Stem cells continue to proliferate after migrating into the injured nerve tissue and further differentiate into the necessary cell type under appropriate microenvironmental conditions.

The representative protocol of MSC induction is exposure to growth factors β-mercaptoethanol (β-ME) and alltransretinoic acid (RA), the cytokines forskolin (FSK), basic fibroblast growth factor (bFGF), and platelet-derived growth factor

(PDGF) sequentially. After transplantation of differentiated stem cell, damaged axons regenerate and achieve better remyelination [69].

Neurotrophic Action Enhancement

Stem cells also produce a microenvironment conducive to neural cell survival and neurogenesis by secreting neurotrophic molecules. They support the differentiation, maturation, and proliferation of SCs and can enhance neurotrophic action.

MSCs synthesize and release a variety of growth factors, such as nerve growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*), *GDNF*, neurotrophin-3 (*NT-3*), *VEGF*, and ciliary-derived neurotrophic factor (*CDNF*).

SKP-SCs increase *BDNF, NGF*, and *NT-3* compared with single SCs in culture.

ADSCs also up-regulate protein expression of BDNF, glial growth factor, neuregulin-1, VEGF, HGF, and insulin-like growth factor.

Overexpressed neurotrophic factors facilitate peripheral nerve regeneration even beyond the nerve-damaged region [69].

Myelin Promotion

Myelination is another important factor that determines the quality of regeneration and functional recovery in PNI. Several somatic stem cell types exhibit the ability to myelinate neuronal cells in the form of SC-like cells in vitro. SCs play a critical role in the recovery of myelin sheath structure and function by synthesizing a large amount of myelin proteins. Similar to SCs, stem cells differentiated into SC-like cells also show the ability to sustain myelination in regenerated nerves in vivo.

Stem cells delivery

Stem cells can be delivered through numerous ways [69]:

- Stem cells can be suspended in a medium that can be directly microinjected into the nerve ending. The microinjection process can be traumatic for both the stem cells and the delicate intra-neural architecture, leading to abnormal cell distribution.
- Another method is to suspend the stem cells in *fibrin matrix and inject* the matrix around the repair sites. In repairs with a *conduit*, stem cells can be injected in the conduit lumen or on the conduit matrix.
- *Tse et al*. describes a method for inkjet printing Schwann cells with phenotypic analysis over seven days. Glial cell viabilities of >90% were detected immediately after printing [70].
- *Three-dimensional printing* aims at creating tissues with multiple cell types within a scaffold for mimicking native tissue, which is a progressive step towards peripheral nerve printing.

Methods	***Application***	***Advantage and Disadvantage***
Micro Injection		Traumatic both to the stem cells and delicate intra-neural architecture, ab-normal cell distribution
Conduit	Natural or artificial conduits	Difficult for cell delivery
Conduit + ECM	Collagen, Fibrin	Good cell distribution, lack of 3-D construction
Conduit + Internal		Beneficial for axonal guidance
3D print		Customization, good cell distribution

A recent Systematic Review was performed in relation to all animal and human studies on peripheral nerve regeneration through cell-enhanced ANAs into a peripheral nerve defect. A meta-analysis was carried out which compared cell-enhanced ANAs against plain ANAs and then against autologous nerve allografts [71]. The results obtained by the cell-enhanced groups of ANAs were comparable to autografts in most of the studies, although autografts remained the gold standard. Skin-derived precursors (SKPs) played an important and promising role in term of nerve regeneration process because they appeared to be associated with a consistently valid enhancing effect in the neuroregenerative process. Authors revealed that the adjunct of cells to ANAs, compared to plain ANAs, improved neuroregeneration with a $P < .00001$ in all performed meta-analyses. On the other hand, recellularization of ANAs did not demonstrate to be superior to the autologous gold standard. This finding is aligned with previous literature assigning to host SC senescence the reason for failure or limited efficacy of ANAs, particularly in lengthy gaps. The mechanism of ANAs repopulation based on the host's SC migration is therefore slow and of limited efficacy when compared to an already colonized graft.
Thus, through further research, enhanced ANAs might become a valid alternative to autografts.

1.4.2 Biochemical/biological enhancement

ANAs are becoming valid alternatives to fresh nerve allografts because they can support peripheral nerve regeneration, bypassing the need for immunosuppression. However, plain ANAs seems to be a suboptimal option compared with autografts owing to their absence of SCs and relatively low tendency to be recolonized by host SCs. The lack of efficacy of plain ANAs is the reason investigators have begun studies that attempt to enhance this tool to increase the neuroregenerative properties of ANAs. In addition to the cellular enhancement previously described, many studies tried to demonstrate the effects on nerve regeneration when ANA enrichment was obtained through biological, chemical, and physical modification instead of cells. The group of biological and pharmacological factors constituted the greatest number of studies. The enhancing factor was most commonly a growth or other biologic factor, such as VEGF, nerve growth factor, GDNF, Kruppel-like factor, PRP/PPP, CNTF, and human adenovirus type 5 (dE1/E3) expressing

hepatocyte growth factor. Other studies explored a drug (etifoxine) or a supplement (ginkgo biloba extract).
Below is the list of the main biochemical/biological factors and biomaterials for ANA enhancement:

- ***NGF:* Nerve Growth Factor**
- ***GDNF:* Glial Cell-Derived Neurotrophic Factor**
- ***VEGF:* Vascular Endothelial Growth Factor**
- ***HGF:* Hepatocyte Growth Factor**
- ***PRP/PPP: Platelet Rich Plasma/ Platelet Poor Plasma***
- *Others:*
  - *Ginkgo biloba extract*: EGb 761
  - Etifoxine
  - **Graphene oxide**
  - KLF_7

➢ *NGF:* Nerve Growth Factor

NGF plays an important role in the differentiation, maintenance, and survival of sensory and sympathetic neurons during development and adulthood [72-73]. NGF induces the nerve regeneration along the basal tubes of acellular nerves by direct action on the growth cones [74]. Several studies have demonstrated that NGF loading into the lumen of various biological and synthetic nerve grafts enhances peripheral nerve regeneration [75-81].
For instance, Yu et al demonstrated that axonal diameter, axon number, and myelin thickness were significant better with NGF-treated acellular grafting than with acellular grafting alone and acellular grafting with fibrin glue, with no significant differences between NGF-treated acellular grafting and autografting [82].
Ovalle et al asserted that NGF can augment axonal ingrowth as well as preferentially induce smaller caliber axons consistent with sensory fibers into acellular, chondroitinase-treated nerve grafts in an in vivo sciatic nerve injury model [83].

- *GDNF:* Glial Cell-Derived Neurotrophic Factor

The Glial cell line-derived neurotrophic factor (GDNF) is one of the most potent trophic factors that have been identified for midbrain dopamine (DA) neurons. Null mutations for trophic factor genes have been used frequently for studies of the role of these important proteins in brain development.

The GDNF family of neurotrophic factors consists of 4 members: GDNF, neurturin (NTN), persephin (PSP), and artemin (ART). There is considerable evidence that GDNF promotes the survival of several motoneuronal populations after injury.

Glial cell line-derived neurotrophic factor (GDNF), which are essential for peripheral nervous system development, have been shown to promote axon regeneration and enhance functional recovery [84-86]. However, the challenge for achieving a clinically suitable application for GDNF is its localized and sustained release to the nerve injury site. Current investigational methods of GDNF local delivery include viral transfected Schwann cells [87-88], and catheter/mini-osmotic pump systems [89]. While viral transduction of primary cells generates local release, regulation of GDNF release is difficult to manage and can result in excess and toxic GDNF release. In addition, clinical translation may be a significant regulatory challenge and these methods are not currently approved for clinical use. Osmotic pump delivery systems, despite providing sustained and localized release, can hinder recovery due to risk of infection and even nerve compression secondary to capsular fibrosis [3]. A sustained and tunable delivery from a biodegradable and biocompatible system is therefore preferred to effectively delivery GDNF to the injured nerve. Tajdaran et al demonstrated that GDNF local administration from a local drug delivery system (DDS) for glial cell line-derived neurotrophic factor (GDNF), enhanced nerve regeneration and made the ANAs as effective as isografts in supporting nerve regeneration [90].

- *VEGF:* Vascular Endothelial Growth Factor

VEGF is a signal protein that stimulates vasculogenesis (de novo formation of vessels) and angiogenesis (growth of vessels from pre-existing vasculature)[91], and it has been proved to increase vascularization in a variety of tissues, including bone [92-94] and nerve [95]. VEGF also has important roles in nerve function not only enhancing local blood flow but also with direct stimulation of axon regeneration.

Enhancement in nerve allograft revascularization has been demonstrated in experimental studies after a short term local administration of vascular endothelial growth factor (VEGF). However, little is known about the direct effects of VEGF or the enhancement of revascularization of nerve allografts in motor nerve recovery [96-97]. VEGF has been demonstrated to be a key regulator in the angiogenesis and axon regeneration and possibly improving functional recovery.

Sondell et al. demonstrated that allografts treated with VEGF had significantly increased vascularization and invasion of Schwann cells [98-99] as well as Hobson et al [100]. demonstrated that the amount of axon sprouts growing into autografts were proportional to the dose of VEGF delivered to the autografts. On the other hand, Giusti et al demonstrated that addition of VEGF to allografts, did not show any changes in revascularization and did not improve functional motor recovery in the long-term, but when allograft revascularization was inhibited with silicone conduits, a significant decrease in motor recovery was noticed, emphasizing the importance of a healthy local tissue to support nerve revascularization [101]. The performance of autografts and allografts were similar, making the allografts used in their study a feasible autograft substitute. They concluded that nerve revascularization is an important step after nerve transplantation in order to achieve a good recovery, and early nerve revascularization does not, necessarily, reflect an improvement in motor function.

- *HGF:* Hepatocyte Growth Factor

HGF is a pleiotropic cytokine, originally discovered [102] and purified as a potent mitogen for mature hepatocytes in primary culture [103-104]. The factor was later found to be involved in multiple biological functions such as organ regeneration, angiogenesis, tumor invasion and development and function of the nervous system [105-107]. HGF has also been shown to be a neurotrophic factor in motor, sensory and parasympathetic neurons in vitro [108]. It promoted both the survival of neurons and the regeneration of injured nerves [109] and may also function as a target-derived axonal chemoattractant, guiding axons to their target tissue [110]. HGF and its receptor c-Met have been found in the developing and mature central nervous system [111], but few reports have described their expression in the peripheral nervous system [112]. Recently, sciatic nerve ligation was found to elevate c-Met RNA level in the distal segment of nerves, with delayed elevation in proximal segments [113], and exogenous application of HGF as a recombinant protein was effective in preventing the downregulation of choline acetyltransferase activity that follows nerve transection [114].
Li et al demonstrated that HGF-enhanced acellular grafting gave better recovery than acellular grafting alone. Neovascularization was greater with HGF-enhanced acellular grafting than with autografting and acellular grafting alone [115].

- *PRP/PPP: Platelet Rich Plasma/ Platelet Poor Plasma*

Platelet-rich plasma (PRP) is an autologous cell therapy containing many bioactive factors that are involved in wound healing and tissue repair, which has several advantages compared with other products and techniques [116]. It can be prepared from autologous blood isolated by puncture as an entirely safe procedure. The clinical use of PRP causes no adverse events or postoperative complication. PRP provides a high level of a natural variety of growth factors, including platelet-derived growth factor (PDGF), transforming growth factor-b (TGF-b), insulin-like growth factor-1, fibroblast growth factor, epithelial growth factor, and vascular endothelial growth factor, among others [117].

The presence of these growth factors with high concentrations in PRP is directly responsible for increasing cell proliferation, raising collagen production, initiating angiogenesis, and inducing cell differentiation involved in tissue regeneration [118]. PRP related products are used in various surgical fields to accelerate the healing process after muscle, ligament, joint, and tendon injuries. Zeng et al demonstrated that PRP had the potential to stimulate cell proliferation, induced the synthesis of neurotrophic factors, and significantly increased migration of SCs, which indicated that PRP may also provide beneficial therapeutic effects for peripheral nerve regeneration after a nerve injury by supplying growth factors [119]. They also discovered that ANAs loaded with PRP as tissue-engineered scaffolds can enhance nerve regeneration and functional recovery after the repair of large nerve gaps nearly as well as autografts [120].

- Graphene Oxide (*GO)*

Graphene is a new type of two-dimensional nanomaterials constituted by single-layer sheet carbon atoms, and its thickness is only the diameter size of one carbon atom [121]. Graphene has very good transmittancy, minimum resistivity and ultrastrong conductivity, the electrons on the surface can efficiently migrate [122], and the connections among each carbon atom are extremely flexible [123-126]. But due to that the surface of graphene lacks functional groups, it is difficult to dissolve in solvent and easy to agglomerate. Graphene oxidized (GO) is a special derivative of graphene, its surface is rich in functional groups containing oxygen, such as epoxy group-CH(O)CH-, hydroxy-OH, and carboxy-COOH, which can change van der Waals force among sheet layers of GO and have good biocompatibility and aqueous solution stability, the existence of functional groups is good for the modification of chemical functionalization, these factors make GO have received extensive attention, and be gradually applied to various fields, it also has more applications in aspects of cell imaging and drug delivery in the field of biomedicine [127-130]. GO has also good conductivity and absorbability. For this reason Wang et al. demonstrated that combining GO nanomaterial with allogenic sciatic nerve decellularized scaffold (ANA+GO) in order to

facilitate nerve regeneration, the sciatic nerve action potentials, the thickness of myelin sheath, the diameter of axon and the dominated muscle rehabilitation level of the nanomaterial group were significantly higher than the ANA group and the self-rotating group (Autograft) [131].

1.4.3 Physical stimulations

Regarding **physical stimulation** in order to improve axonal regeneration, we can distinguish between:

- **Ultrashort waves**
- **Electrical stimulation**
- Low-energy laser

➢ Ultrashort waves

The peripheral nerve would regenerate after being injured and transected, but the velocity is very slow (about 1 mm a day). It is a focus that how to promote its regeneration, prolongation, and function restore. Besides neurotrophic factors and chemical medication, many physical factors have also been promoting nerve regeneration action [132-135]. Former studied showed that low dose ultrashortwave (USW) can promote injured peripheral nerve regeneration by expanding blood vessel, ameliorating blood circle, and nutrition of nerve and peripheral tissue, and intensifying tissue metabolizability and nerve system function [136]. Zhang et al found that USW therapy could promote Schwann cell proliferation and could be beneficial to formation and maturation of myelin sheath. Except myelin sheath thickness, the difference between USW group and autograft group was not significant. They demonstrated that low dose USW could accelerate nerve regeneration velocity after operation and promote Schwann cells proliferation by upregulating of VEGF mRNA expression of spinal cord and muscle [137].

- Electrical stimulation

In pioneering work, Gordon and colleagues showed that both the speed of reinnervation of target muscles and the sensorimotor precision with which they were reinnervated was enhanced if the proximal stump of the cut nerve was stimulated electrically at the time of surgical repair [138]. Since such stimulation resulted in the up regulation of the mRNA for both brain derived neurotrophic factor (BDNF) and its receptor, trkB, in motoneurons and dorsal root ganglion cells [139], they postulated that the enhancement of axon regeneration produced by electrical stimulation was the result of an enhancement of a cell autonomous regulation of axon growth. In particular, they speculated that electrical stimulation promoted an increase in trkB signaling originating from BDNF of neuronal origin. The simplest explanation of these findings is that the increase in neuronal BDNF and trkB which is thought to accompany electrical stimulation forms the basis for a cell autonomous regulation of axon growth [140].

Acellular grafts were used as a means of evaluating the source of the ligand. In these grafts, Schwann cells have been destroyed, leaving behind endoneurial tubes and their associated extracellular matrix [141-142]. These grafts contain neither neurotrophins nor the cellular machinery with which to synthesize them. Therefore, neurotrophins of Schwann cell origin would not be expected to contribute significantly to axon regeneration during the first week following nerve repair.

English et al demonstrated that electrical stimulation produces a potent enhancement of the regeneration of axons in cut peripheral nerves which is independent of neurotrophin production by cells in their surrounding environment but is dependent on stimulation of trkB and its ligands in the regenerating axons themselves [143].

A recent Systematic Review was performed of all animal and human studies on peripheral nerve regeneration using noncellular enrichment of ANAs into a peripheral nerve defect. Noncellular enrichment methods can be grouped into biological and/or pharmacological factors, physical stimulation and biomaterials. A meta-analysis was also carried out which compared cell-enhanced ANAs and plain ANAs and autologous nerve allografts, which still represent the reference standard [144]. Across all reports, a consistent result was the inferior neuroregenerative performance achieved by plain ANAs compared with autografts and isografts and enhanced ANAs. In most of the studies, the results obtained with the enhanced groups of ANAs were comparable to those obtained with autografts. The biological and physical enhancement of ANAs, compared with plain ANAs, demonstrated an overall effect of improved neuroregeneration ($P < 0.01$) in all performed meta-analysis attempts. In contrast, biological or physical enhancement of ANAs did not demonstrate superiority to the autologous reference standard. In only one study, the ANA group enhanced with graphene oxidized (GO), showed superior function even compared with the autograft-based arm of that study [131].

2. EXPERIMENTAL PART

In case of PNI, the first approach is primary neurorrhaphy, which is direct nerve repair with epineural microsutures of two stumps. However, this is not feasible in case of stump retraction or in case of tissue loss (gap > 2 cm) [145], where the main surgical options are autologous grafts, allogeneic grafts, or nerve conduits [146]. Considerable efforts have been made in recent years to develop solutions that take into account different types of nerve repair [147-149]. When larger nerve gaps exist (20 mm or more in humans), the current clinical gold standard for peripheral nerve reconstruction is an autologous sensitive nerve graft (autograft); although it offers a cell-rich material through which axons can regenerate, its use is not ideal because of limited availability and mismatch between donor nerve size and recipient site [150-151]. In addition, nerve harvesting and subsequent donor site morbidity result in functional loss, as well as an increased risk of neuroma formation, paresthesias, and higher facility costs associated with a second surgical site. Fresh nerve allografts have therefore become a viable alternative option, but they require immunosuppression, which is often contraindicated, for example, in cases of nerve loss after tumor resections. Acellular nerve allografts (ANAs), rather than fresh allografts, do not require immunosuppression and appear to be safe and effective based on recent clinical trials [152-153].

Commercial ANAs are already available on the market (Avance® Nerve Graft, Axogen, Alachua, FL) and they are ready for transplantation [60].

Although based on a small subset of patients, these commercialized ANAs up to 5 mm in diameter appear to be able to support successful nerve regeneration. However, although these grafts represent a good surgical option, they still have some limitations (mainly related to their high cost, shipping limitations, and U.S. donor selection criteria that differ from those in Europe). In addition, commercially available ANAs require γ-ray sterilization after production, the effects of which on tissue integrity are still controversial [154-158].

To avoid post-production sterilizations, preparation methods should be performed in aseptic environments, such as Class A clean rooms. On the other hand, the decellularization methods reported in the literature are very time-consuming and therefore suboptimal to be transferred to clean rooms; for example, Hudson's

method, has a duration of 3 days and has a number of disadvantages, such as increased risk of contamination and technical incompatibility with clean room requirements [155; 159-160].
The purpose of this study was to develop an innovative strategy, conforming to cleanroom requirements, to perform the direct tissue manipulation step and the nerve decellularization process within five hours, so as to accelerate the detachment of myelin and cellular debris, without detrimental effects on nerve architecture and without disrupting the asepsis chain. Therefore, new ANAs should not require terminal sterilization if microbiologically negative at the end of handling. In this study, the safety and efficacy of ANAs were evaluated in vitro and in vivo by histological, immunohistochemical, and histomorphometric studies in rabbits and humans.
In addition, the new method avoids the high costs of commercial products, due to the reduction of shipping costs and the price defined by the regional fee schedule of the Musculoskeletal Tissue Bank (BTM) of Rizzoli Orthopedic Institute of Bologna (*Figure 9*).

At the same time, all harvested nerves are subjected to European donor selection criteria, which are different from those in the United States [161]. Another goal of the study is to create storage in the ANA Tissue Bank ready for use in this country to ensure better availability and reduce costs.

DOI - 15 Rev.11

TARIFFARIO BTM REGIONE EMILIA-ROMAGNA

stabilisce i rimborsi massimi per le varie tipologie di tessuto muscolo-scheletrico.

Applicazione dal 01/07/2017

TENDINI E FASCE				
Cod	**Tipo**	**Tessuto**	**Dimensione**	**Prezzo (€)**
	cong	menisco mediale / laterale		856,00
	cong	menischi con piatto tibiale		2.536,00
	cong	menisco mediale / laterale con bratta ossea		1.436,00
	cong	pes anserinus tendine multiplo sartorio, gracile, semitendinoso		1.276,00
	cong	tendine d'Achille		1.276,00
	cong	emitendine d'Achille		916,00
	cong	tendine rotuleo intero		1.680,00
	cong	emitendine rotuleo		1.260,00
	cong	tendine rotuleo intero e tendine quadricipite		1.790,00
	cong	emitendine rotuleo ed emitendine quadricipite		1.486,00
	cong	tendine quadricipite con emirotula		1.050,00
	cong	tendine tibiale anteriore/posteriore		956,00
	cong	altro tendine/nervo		866,00
	cong	tendine peroneo breve/lungo		780,00
	cong	tendine Gracile / Semitendinoso		500,00
	cong	estensore / flessore alluce		500,00
	cong	fascia lata	< 50 cm^2	600,00
	cong	fascia lata	51 - 100 cm^2	716,00
	cong	fascia lata	101 - 150 cm^2	866,00
	cong	fascia lata	> 150 cm^2	1.016,00

FIGURE 9: *Emilia-Romagna BTM Tariff.*

2.1 Prior experimental part: THE ANIMAL MODEL

The need to create a new decellularization method that would compensate for the shortcomings of existing ones led to the development of an innovative method based on an animal model. At an early stage, studies involved the development of an in vitro method on rabbit nerve; subsequently, the analysis was continued with in vivo experimentation on an animal model. All studies were conducted at Rizzoli Orthopedic Institute of Bologna and were performed by the Authors [156], in order to set the basis for validation of the method on human nerve.

2.1.1 In vitro study of the new method on animal model

The in vitro study to develop the innovative decellularization process was conducted using tibial and femoral nerve segments taken from euthanized rabbits. Different decellularization treatments were tested using various detergents in combination with ultrasound, including the Hudson method, which was used as a control [159].
Commercial nerve grafts (AvanceVR Nerve Graft, AxoGen, Alachua, FL) were used for histological comparison to verify the histological quality of nerves treated with the new technique.

After several experiments, considering various solutions, sonication times and resting times, the final protocol, called the *"Rizzoli Method"*, was defined. After harvesting, the nerves were:

- Immersed in PBS containing SB-10 125 mM, 0,2% Triton X-100 and Pen Strep 1% (called *HS, Harvesting Solution*), incubated for 48h at room temperature in an orbital shaker, and frozen.

- The day of the manipulation, the nerves were transferred to a Class A glove box to simulate sterile condition, thawed; moreover, the microbiology swabs sterility assays were performed.

- The nerves were rinsed three times with PBS (30 min), and immersed in 0.25% SDS in OBS (*Decellularization Solution DS*) for 180 minutes.

During this phase, ultrasounds were applied, for 5 min, every 30 min: sonication cycles were performed with the nerves soaked in 30 mL of DS sealed inside sterile 50 mL tubes, which, in turn, were immersed in a Bath Sonicator; ultrasound frequency was 40 Hz. During the incubation, the tubes were kept in agitation on a radial shaker.

- At the end of the process, the ANAs were rinsed three times with PBS for 30 min and frozen for a long-term preservation. Microbiology swab sterility assays were taken after the completion of the decellularization process, and no growth was detected.

The most important objectives of the decellularization technique were:

- The absence of immunogenic donor cell residues
- The efficacy of the model, which means axon removal, along with basal lamina and interstitial endoneurium preservation.

This evaluation of nerve decellularization protocol was done using light microscopy (LM), transmission electron microscopy (TEM), and immunohistochemistry. After staining of the tissue with hematoxylin–eosin (H&E) and S-100, LM was applied for observation of general morphology and to investigate Schwann cell removal.
We compared the test group of nerves processed with Rizzoli method (both Triton/SB-10/sonication experiment and Triton/SDS/sonication experiment) with untreated intact nerves and nerves processed with the Hudson method (control).
Figure 10 shows the best morphological and immunohistochemical results. The untreated nerves [Fig. 10(a-e)] showed intense fluorescence around the axons with positivity for the cellular marker S-100, indicating the presence of Schwann cells, as well as fluorescence of 4',6-diamidino-2-phenylindole (DAPI) nuclei, used to detect DNA remnants after decellularization process [Fig. 10(a,b)]; LM of semithin cross-sections stained in toluidine blue [Fig. 10(c)] and TEM observation [Fig. 10(d,e)] demonstrated the normal arrangement of unmyelinated and myelinated axons. In the nerve treated with the Hudson protocol, Schwann cells were completely undetectable [Fig. 10(f)] and DAPI positivity was negligible [Fig. 10(g)].

In nerves treated with Rizzoli method, Triton/SB-10/sonication [Fig. 10(m)] and Triton/SDS/sonication [Fig. 10(r)], some Schwann cell remnants and some DNA remnants were present [Fig. 10(n,s)]. Semithin cross sections stained with toluidine blue showed that the demyelination process was not uniform and that the peripheral area of nerves was better decellularized than the central area [Fig. 10(h,o,t)]. Decellularization with Triton/SDS/sonication resulted in a cleaner peripheral area than the other two protocols.

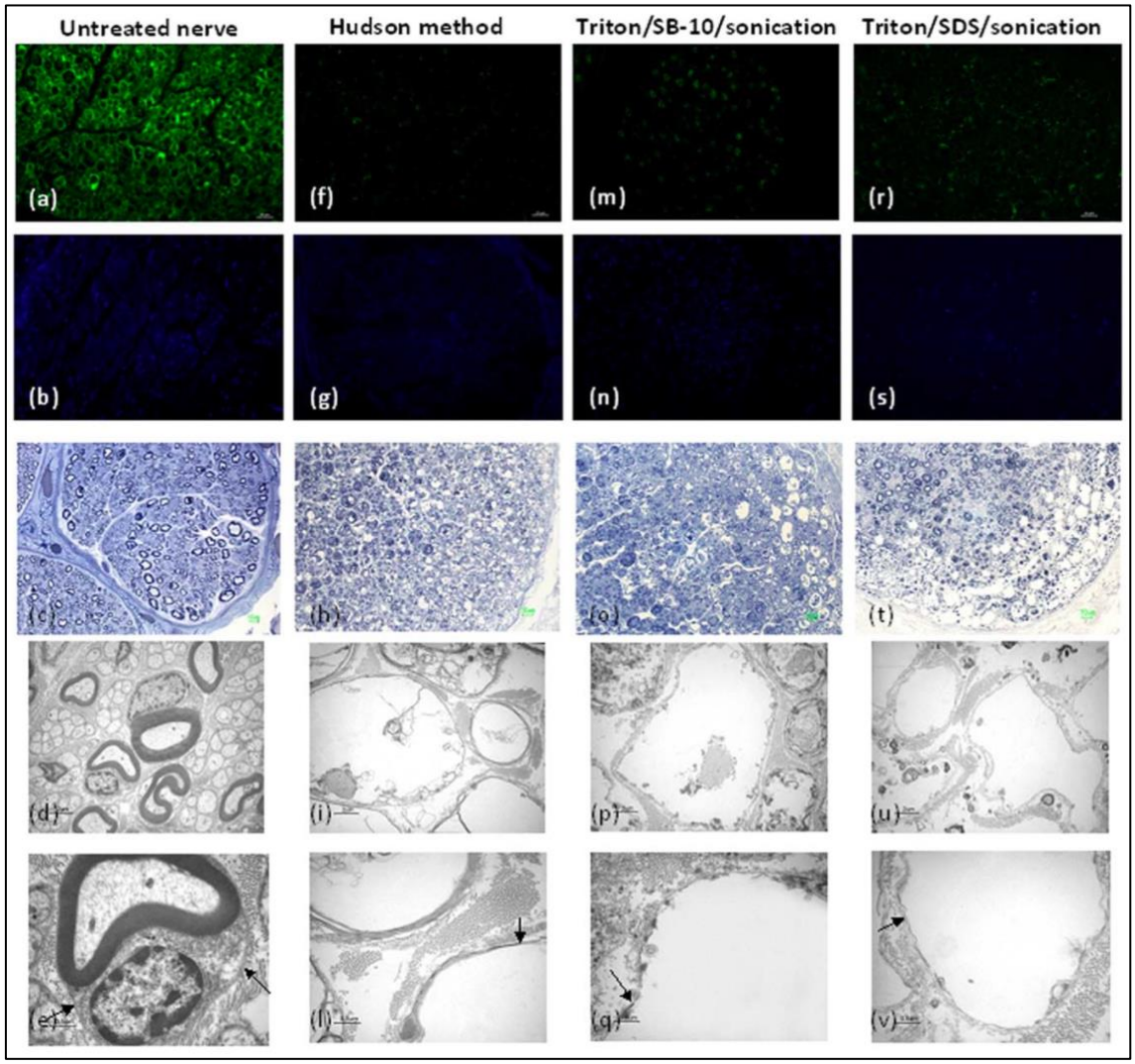

FIGURE 10: *Comparison between several decellularization models.*
A–E: Untreated rabbit femoral nerve. ***A****: Protein S-100 immunostaining;* ***B****: DAPI;* ***C****: LM of semithin cross-section (toluidine blue);* ***D****: TEM of ultrathin cross-section;* ***E****: TEM at higher magnification.*
F–L: Hudson treatment. ***F:*** *Protein S-100 immunostaining;* ***G****: DAPI;* ***H****: LM of semithin cross-section (toluidine blue);* ***I****: TEM of ultrathin cross-section;* ***L****: TEM at higher magnification*
M–Q: Triton/SB-10/sonication treatment. ***M:*** *Protein S-100 immunostaining;* ***N****: DAPI;* ***O****: LM of semithin cross-section (toluidine blue);* ***P****: TEM of ultrathin cross-section;* ***Q****: TEM at higher magnification.*
R-V: Triton/SDS/Sonication treatment. ***R:*** *Protein S-100 immunostaining;* ***S****: DAPI;* ***T****: LM of semithin cross-section (toluidine blue);* ***U****: TEM of ultrathin cross-section;* ***V****: TEM at higher magnification.*

With TEM analysis, observation of the outer zones showed that the ECM was almost entirely retained in all three cases, as was the basal lamina; some remnants of axon cytoplasm were present in the Hudson and Triton/SB-10/sonication Rizzoli protocol [Fig. 10 (i,l,p,q)]. All methods tested detached myelin and cellular swelling, although some remnants of myelin leaflets, axon cytoplasm, and DNA were present. Very few remnants were detectable after the use of Rizzoli method Triton-100/SDS/sonication. In addition, microbiology was consistently negative in all samples.

Figure 11 shows the results obtained with the new method compared with commercial grafting. S-100 staining demonstrated the absence of Schwann cells: no fluorescence was present in either case [Fig. 11(a,f)]; DAPI fluorescence was almost completely undetectable, demonstrating the absence of DNA remnants [Fig. 11(b)], as in commercial grafting [Fig. 11(g)]. No cellular remnants were evident in the semithin cross sections stained with toluidine blue, and unlike the other treatments, the new method induced a more homogeneous removal of myelin, both at the periphery and in the core of each nerve fascicle [Fig. 11(c)]. TEM observations confirmed the absence of residual cells and myelin; the basal lamina and ECM were preserved [Fig. 11(d,e)]. The results obtained with this new method are similar to those obtained with the already established and commercialized nerve graft [Fig.11 (h,i,l)].

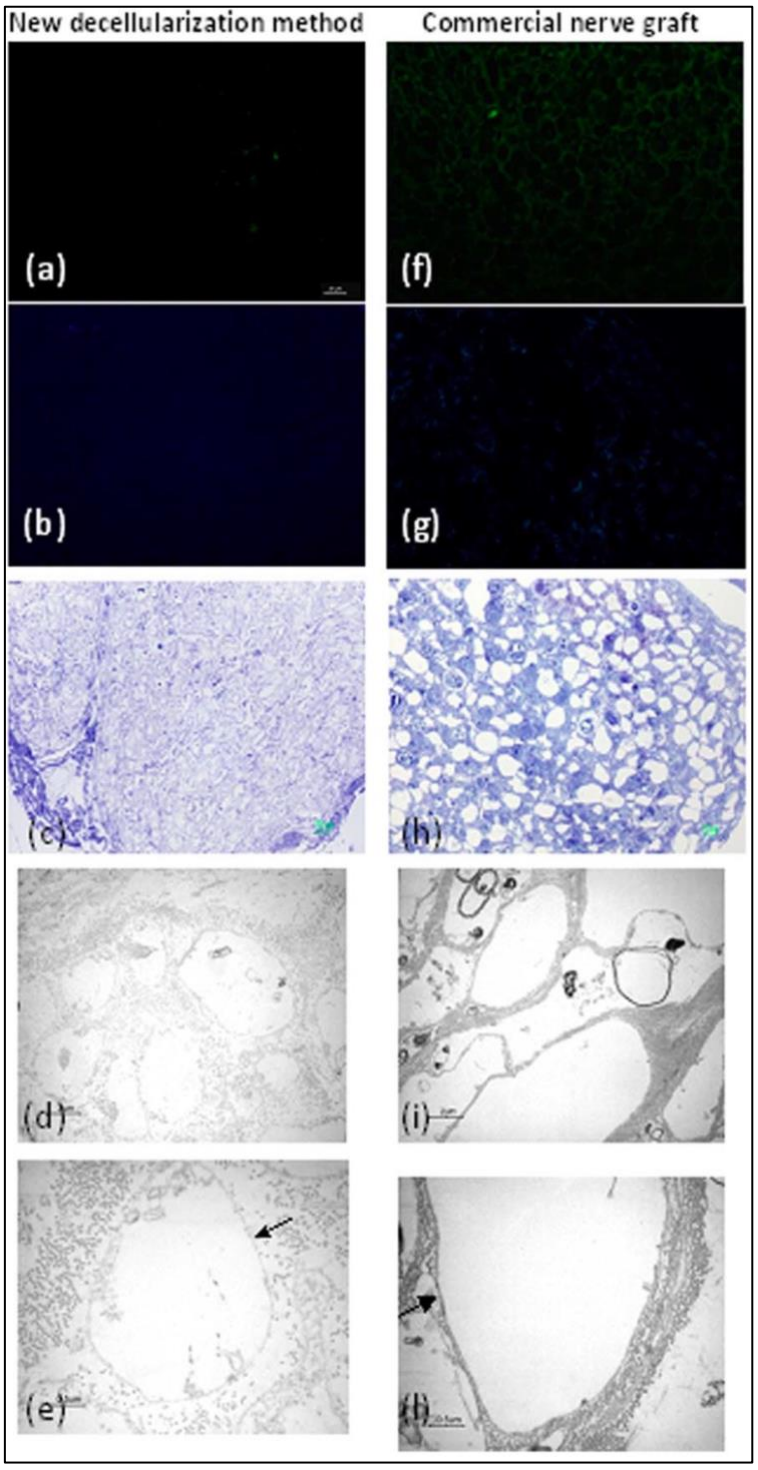

FIGURE 11:
A-E: Novel decellularization technique (Triton/SB-10/SDS) in rabbit femoral nerve. ***A****, S-100 immunostaining;* ***B****, DAPI;* ***C****: LM of semithin cross-section: (toluidine blue);* ***D****: TEM of ultrathin cross-section;* ***E****: TEM at higher magnification.*
F-L: Commercial nerve graft (AvanceVR Nerve Graft, AxoGen). ***F****, S-100 immunostaining;* ***G****, DAPI;* ***H****: LM of semithin cross-section: (toluidine blue);* ***I****: TEM of ultrathin cross-section;* ***L****: TEM at higher magnification.*

By analysing the results of this study, it is possible to observe the good results obtained with the histological and immunohistochemical evaluations [Fig. 10], compared with the control sample and the commercialized nerve graft [Fig. 11 (h,i,l)]. Although Schwann cell residues were present, as well as some DNA residues, the results encouraged the in vitro experimentation.

Among the molecules tested, SDS gave the best results; therefore, this detergent was chosen for subsequent experiments. According to other authors, the addition of SDS to a decellularization protocol can make the difference between complete and incomplete removal of cells [162]. In addition, the ultrastructure of the nerve ECM was almost entirely preserved from the disruption previously described by other studies [163-164]. An additional series of experiments was performed to understand the effect of sonication on tissue integrity and the potential for removal of cells and debris; cycles of 5 min achieved the best morphological results. Shorter cycles were unable to purify the tissue, while longer cycles had detrimental effects on the integrity of the nerve structure.

The new technique is designed to limit graft manipulation to a single 4-hour cleanroom session, without disrupting the asepsis chain; thus, ANAs do not appear to require terminal sterilization, based on negative microbiological swabs resulting at the end of nerve processing. The decellularization process must preserve the structural components of the ECM to provide biomechanically and bioactively sufficient scaffolds, which can support and promote nerve regeneration and function [165].

In this study, we modified the previously published decellularization methods [56;159;166] in order to develop a new standardized decellularization protocol with the introduction of ultrasonic energy and mechanical agitation in combination with chemical treatment and freeze-thaw. In addition, this new method was designed to make it as compatible as possible with the requirements of cleanroom production, limiting the duration of the protocol to a single day of handling in a sterile environment and reducing tissue handling time below the 5 hours of a common aseptic work session. In fact, according to Azhim et al. [165] this expedient reduced the cost of the entire process and avoided post-production sterilization techniques, such as gamma irradiation, whose effects on ANA integrity are still controversial [162-163].

2.1.2 In vivo study of the new method on animal model

Following the good results collected in the *in vitro* study, the Authors moved on to the *in vivo* experimentation on six rabbits.

The experimental surgical protocol was approved by the local Ethics Committee (Authorization 2675 of 26/11/2015) and OPBA (Responsible Body for Animal Welfare of the Rizzoli Orthopaedic Institute) and authorized by the Ministry of Health (Decree No.667/2015-PR of 13/07/2015). The study was conducted in accordance with Legislative Decree 26/2014 (Implementation of Directive 2010/63/EU).

After premedication with intramuscular injection of 44 mg/ kg ketamine (Imalgene 1000, Merial Italy S.p.A, Assago - Milano, Italy) and 3 mg/kg xylazine (Rompun 25 mL, Bayer S.p.A., Italy), a general administration of ketamine was given. p.A, Italy), general anesthesia was induced and it was maintained in spontaneous ventilation by face mask and administration of a gas mixture (O2/air: 60%/40%) and sevoflurane (Sevoflurane, Baxter S.p.A, Rome, Italy). The surgical procedure was performed under sterile conditions after shaving and washing the right hind limb of the rabbit with antiseptic solution. A longitudinal skin incision was made in the postero-lateral part of the right limb; the tibial nerve was exposed, using a blunt dissection, between the biceps femoris and semitendinosus muscles; the muscles were retracted and the tibial nerve was isolated. A 10-mm defect was created unilaterally in the medial-distal segment of the tibial nerve, using the microsurgical technique and in three out of six rabbits newly produced ANAs were used to repair the defects. Decellularized tibial nerve segments were used. In three comparison rabbits, autografts were applied, which is the gold standard. A 9–0 nylon monofilaments end-to-end neurorrhaphy was performed to fix the grafts.

The recovery of all the animals was rapid and none of them showed any visible neuromuscular dysfunction. There were no signs of inflammation or infection at the operated site, and the wound was completely healed within a week. At final evaluation before euthanasia, complete sensory recovery and plantar flexion of the foot was noted in all cases.

After 12 weeks, animals were euthanized by intravenous injection of 1 mL of TanaxVR (Hoechest AG, Franfurt-am-Main, Germany) under deep sedation with ketamine and xylazine; tibial nerves and surrounding tissues were harvested and observed macroscopically. Then, the implanted segment, the 1-cm pre-graft

portion, and the distal portion of each nerve were retrieved and paraffin-embedded (*Figure 12*): the occurrence of adverse/immune reactions around the grafts, the morphology of axons, and the presence of Schwann cells were analyzed by LM and TEM analysis, with the same procedures applied in vitro. S-100 antigen immunostaining was also evaluated on samples taken to detect the presence of Schwann cells.

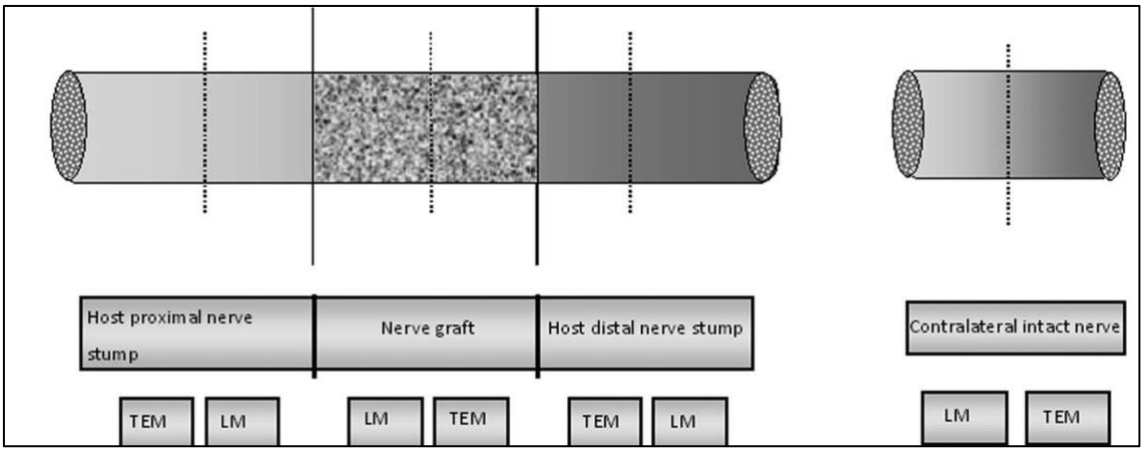

FIGURE 12: *Diagram displaying nerve sectioning for the morphological and immunohistochemical analysis[156].*

The morphologic features of the regenerated ANAs were analyzed and showed in *Figure 13*: hematoxylin-eosin-stained sections revealed neither infection nor immune reaction, as evidenced by the absence of granulocytes and lymphocytes [Fig. 13(a,d,g)]. A discrete remyelination process was noted by the presence of S-100-positive Schwann cells in the analyzed fragments [Fig. 13(b,e,h)]. Contralateral intact nerves showed a regular nerve arrangement [Fig. 13(l)] and homogeneous S-100 fluorescence [Fig. 13(m)].

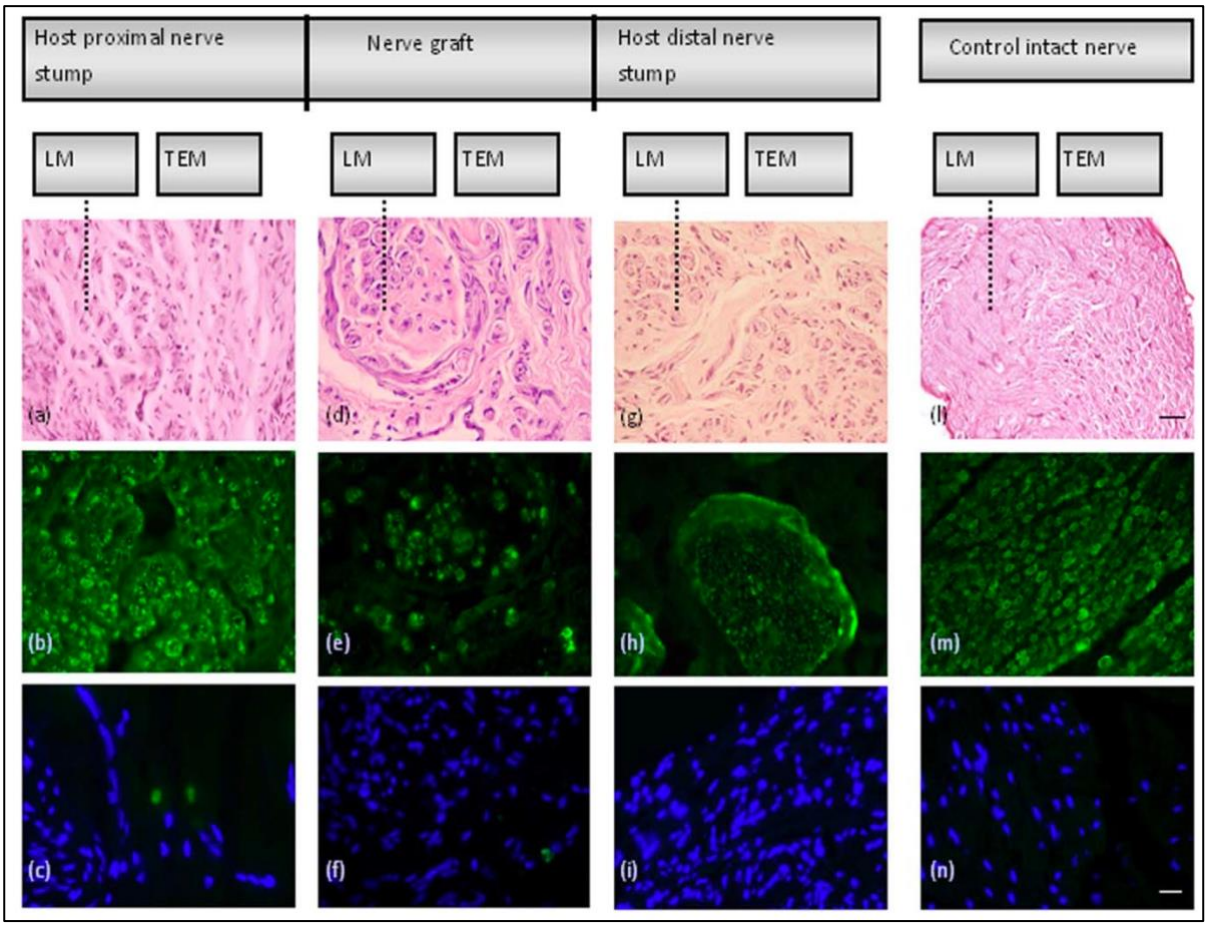

FIGURE 13: *In vivo study on rabbit tibial nerve. a–c: proximal nerve stump; d–f: nerve graft; g–i: distal nerve stump; l–n: contralateral intact nerve for comparison. Numerous small fascicles repopulated the graft portion (d), the proximal (a), and distal part (g) (hematoxylin–eosin). A discrete remyelination process is present (b, e, h) (S-100 immunostaining).*

In *Figure 14*, semithin sections stained with toluidine blue and thin cross sections of the proximal stump of the host nerve [Fig. 14(a)] showed smaller axons than those of the contralateral intact nerve [Fig. 14(o)]. A regular array of neurofilaments and neurotubules was observed both in the proximal stump [Fig. 14(c,d)] and in the intact nerve [Fig. 14(q,r)]. Semithin sections of the graft showed abundant and diffuse axons arranged in microfascicles, a typical sign of nerve regeneration [Fig. 14(e)].

TEM analysis showed a small number of myelinated axons for each fascicle, surrounded by a thin layer of connective tissue containing perineural cells [Fig. 14(f)]. Myelinated [Fig. 14(g)] and unmyelinated axons [Fig. 14(h)] showed neurofilaments and neurotubules.

At LM and TEM analyses, nerve tissue of the distal stump showed nerve fibers with a normal pattern of myelination, although the axons were smaller than in the control [Fig. 14(i) vs. (o) and Fig. 14(l) vs. (p)]. The presence of neurofilaments and neurotubules was attested [Fig. 14(m,n)].

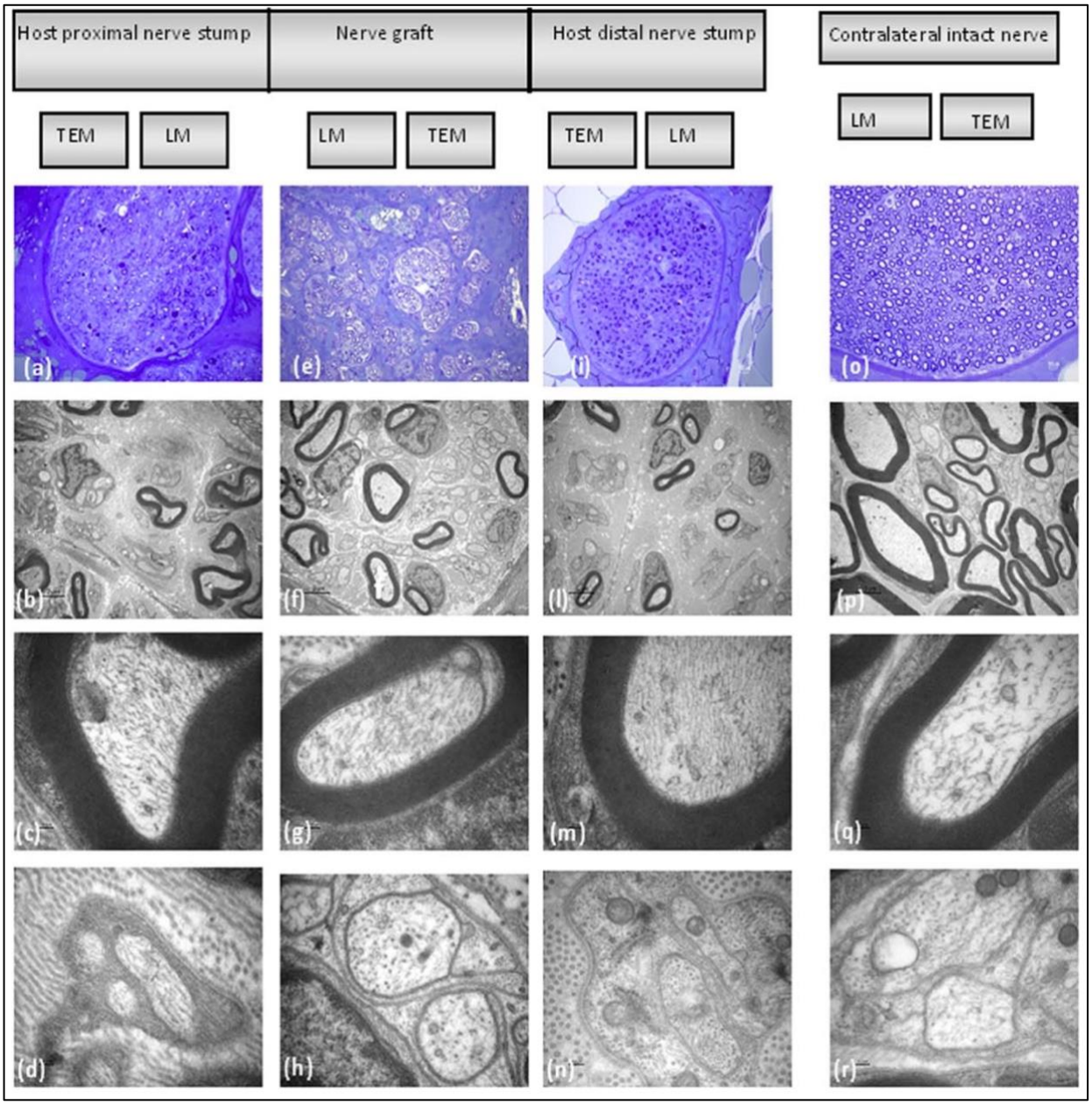

FIGURE 14
*In vivo study on rabbit tibial nerve. Regenerating nerve with **ANAs**.*
*a–d: Proximal nerve stump. **a**: LM of semithin cross-section (toluidine blue); **b**: TEM of ultrathin cross-section; **c and d**: TEM at higher magnification.*
*e–h: Nerve graft. **e**: LM of semithin cross-section: (toluidine blue); **f**: TEM of ultrathin cross-section; **g and h** TEM at higher magnification.*
*i–n: Distal nerve stump. **i**: LM of semithin cross-section (toluidine blue); **l**: TEM of ultrathin cross-section; **m and n**: TEM at higher magnification.*
*o–r: Contralateral intact nerve. **o**: LM of semithin cross-section (toluidine blue); **p**: TEM of ultrathin cross-section; **q and r**: TEM at higher magnification.*

The same aspects described above were also evident in the regenerating nerves implanted with autografts. *Figure 15* shows semithin [Fig. 15(a,e,i)] and thin cross-sections [Fig. 15(b–d), (f–h), and (l–n)] from normal nerve, graft, proximal, and distal stumps.

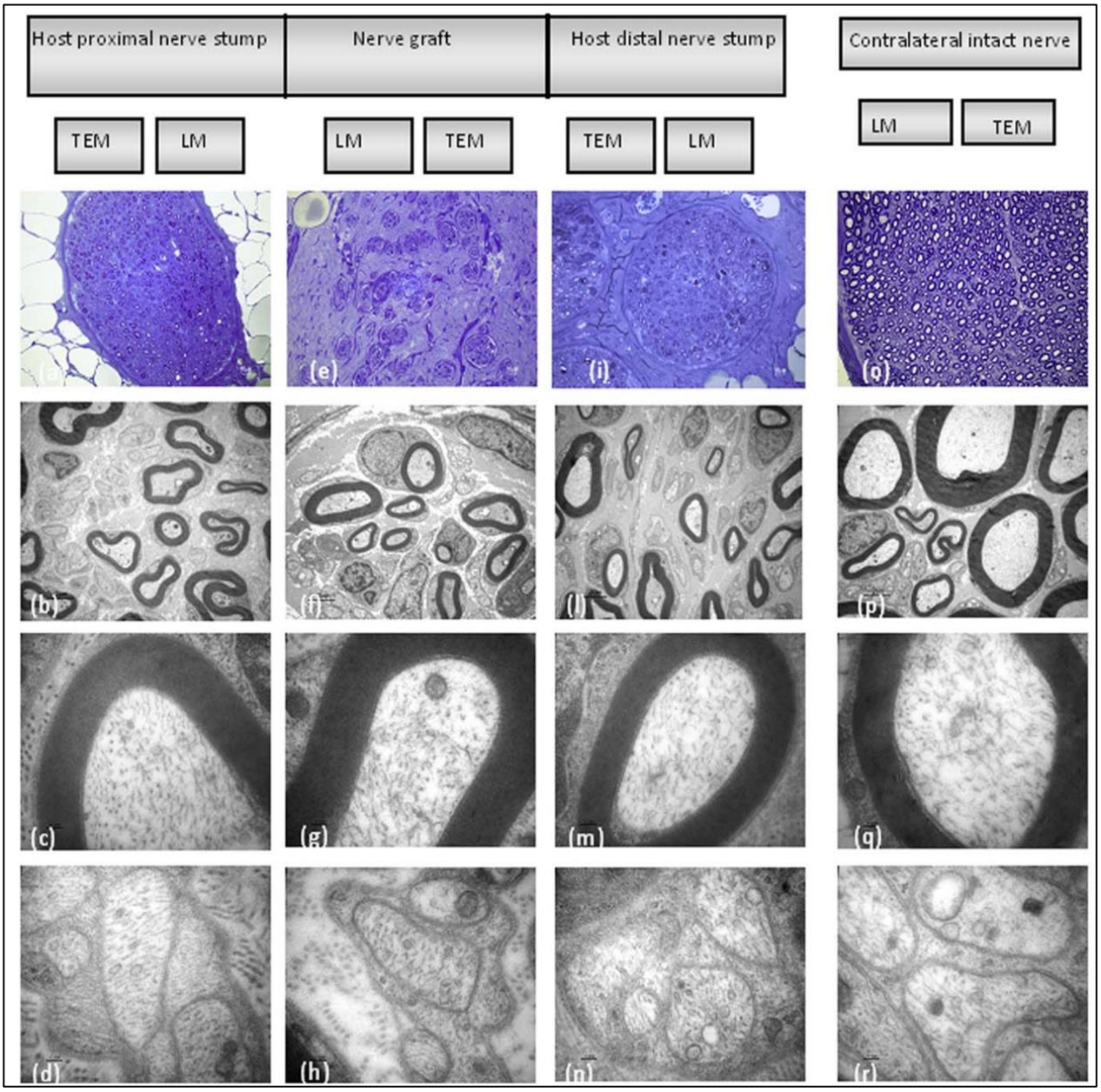

FIGURE 15
In vivo study on rabbits. Regenerating tibial nerve with ***AUTOGRAFT****.*
a–d: Proximal nerve stump. ***a****: LM of semithin cross-section (toluidine blue);* ***b****: TEM of ultrathin cross-section;* ***c and d****: TEM at higher magnification.*
e–h: Nerve graft. ***e****: LM of semithin cross-section: (toluidine blue);* ***f****: TEM of ultrathin cross-section;* ***g and h*** *TEM at higher magnification.*
i–n: Distal nerve stump. ***i****: LM of semithin cross-section (toluidine blue);* ***l****: TEM of ultrathin cross-section;* ***m and n****: TEM at higher magnification.*
o–r: Contralateral intact nerve. ***o****: LM of semithin cross-section (toluidine blue);* ***p****: TEM of ultrathin cross-section;* ***q and r****: TEM at higher magnification.*

Figure 16 (A) showed the histomorphometric data measured on the toluidine blue stained semithin sections evaluating the fiber density and diameter of the nerves, the axon diameter, the myelin thickness, and the g-ratio. *Figure 14 (B)* shows the comparison between distal segments.

The comparison between autograft and ANA regeneration, performed by applying the Mann–Whitney U test, demonstrated no significant difference in all analyzed parameters. Between autografts and ANAs, by separately comparing data in proximal, distal, and graft segments, there were no significant differences.

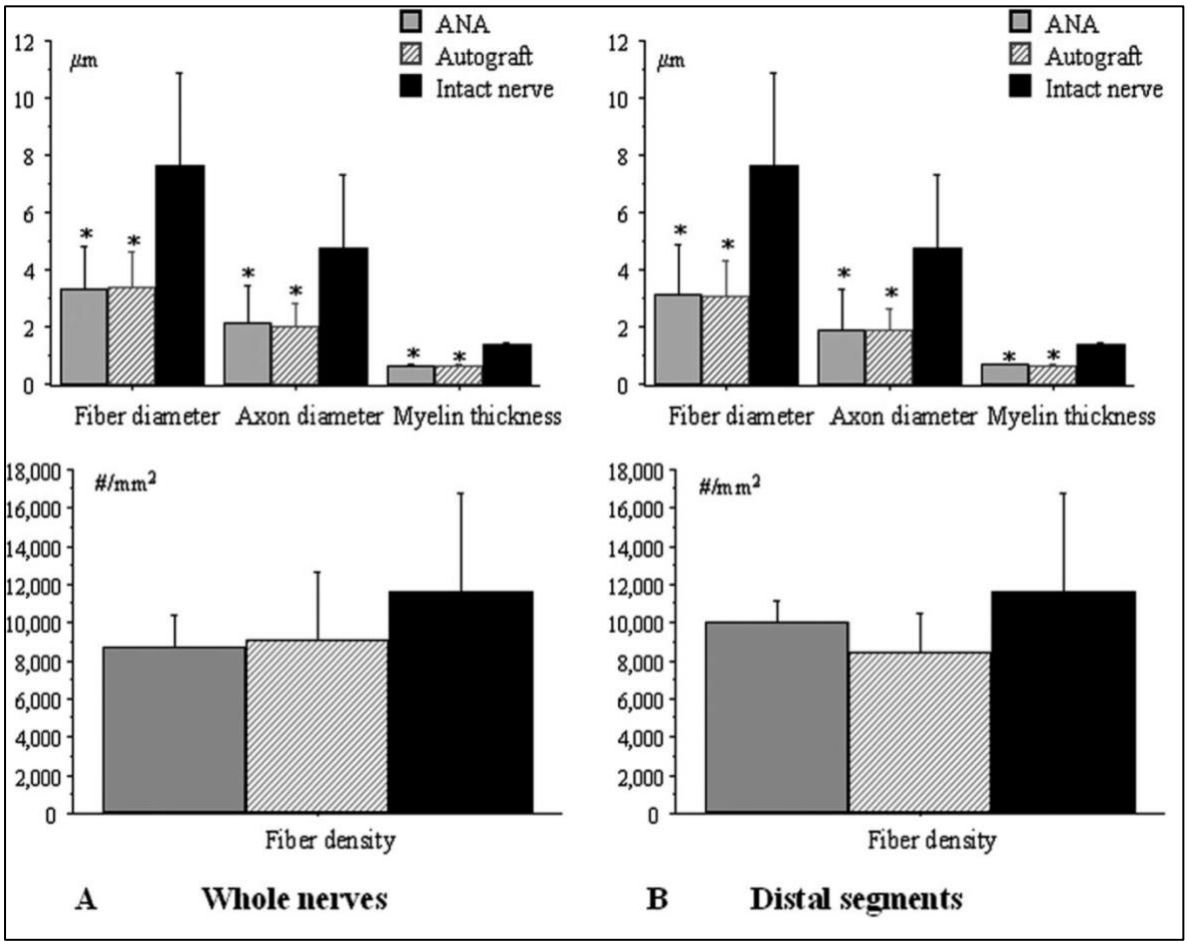

FIGURE 16: *Histomorphometric data in rabbit nerve biopsies (A) and in distal segments (B) comparing ANAs, autografts, and intact nerves. Values are reported as mean 6 SD. *p < 0.05 versus intact nerve (Mann–Whitney U test).*

The in vitro and in vivo results of Rizzoli decellularization protocol demonstrated the potential to achieve a fully decellularized and aseptic graft in a shorter treatment time than pre-existing methods. Indeed, this new method was conceived to make it more compatible with the requirements of the clean room manufacturing, by limiting the protocol duration to only one day of manipulation within the sterile environment, and by reducing the tissue handling time under the 5 h of a common aseptic working session.
According to Azhim et al., [165] such expedient allowed to reduce the whole process costs and to avoid the post-manufacturing sterilization techniques, such as the gamma irradiation, whose effects on the ANAs integrity is still controversial [162-163].

2.1.3 Parallel multifascicular combination of autologous and allogenic nerve allograft

Removal of the myelin sheath, often referred to in the experimental literature as "nerve predegeneration," [167-168] appears to accelerate and promote Schwann cell activity and their role in nerve regeneration. Danielsen have shown that predegenerated nerve grafts (PNG; taken from the distal nerve stump of a previously injured donor nerve) are superior to fresh nerve grafts (FNG) in terms of repair of peripheral nerve defects in the rat [169]. Predegenerated nerve grafts (PNG) have a reduced initial delay period, i.e. the time interval before the axons enter the graft while the rate of regeneration is unaffected and this can be related to non-neuronal cells (e.g., Schwann Cells), that are more numerous in the predegenerated grafts. These cells produce a variety of factors which stimulate axonal growth, including neurotrophic factors, like NGF, or lamina components, such as laminin [167]. They performed some studies in which the regenerative potential of the fresh allogenic grafts (FNG) was compared to the one of the predegenerated acellular nerve grafts (PANG or ANA). Both axons and SCs can grow into acellular nerve graft. Danielsen et al. proved that Predegenerated Acellular Nerve Grafts (PANG) are superior to Acellular nerve grafts, showing that PANG have regenerative capacity similar to fresh nerve grafts.
Acellular nerve allografts are unmyelinated and empty predegenerated nerve fibers that are immediately ready and prone to accept regeneration, which may be facilitated by the presence of an autograft, which presumably provides Schwann

cells and their pro-regenerative environment and guidance effect throughout the length of the grafted complex. Since this concept, advances in microsurgical techniques and graft processing enable better clinical outcomes. One example of a microsurgical technique that has improved peripheral nerve regeneration is the method of autologous interposed segments by Sugita et al. [170]. In this work, Sugita and coworkers demonstrated the migration of host Schwann cells from autologous grafts to interposed allografts in the same nerve defect.

We therefore hypothesized that the described phenomenon could be extended to the juxtaposition of allogeneic grafts and autografts, within a larger caliber nerve defect, combining the advantages of autologous and allogeneic components within a single repair, i.e., maintaining a single autograft segment but reducing donor site morbidity due to less nerve harvesting, as ANAs would serve to complete the caliber of the bridging graft. In addition, the presence of an autograft, due to the abundance and viability of Schwann cells, could ideally allow or facilitate neurorigeneration in ANAs, as demonstrated by Sugita et al.

During the validation studies of Rizzoli method, performed on a larger sample of rabbits, the possibility of a parallel combination of autologous and allogeneic grafts into a single bridging multifascicular cable was therefore tested.

In the validation study, Boriani et al [171-172], has used 3 groups of 5 rabbits:

- Grup A: it was treated with ANAs;
- Group B: treated with Autologous grafts;
- Group C: treated with an innovative model consisting of a cabled nerve graft containing a nervous autologous fascicle centrally placed and 2-3 parallel ANA fascicles, to recreate the recipient nerve calibre (*Figure 17*).

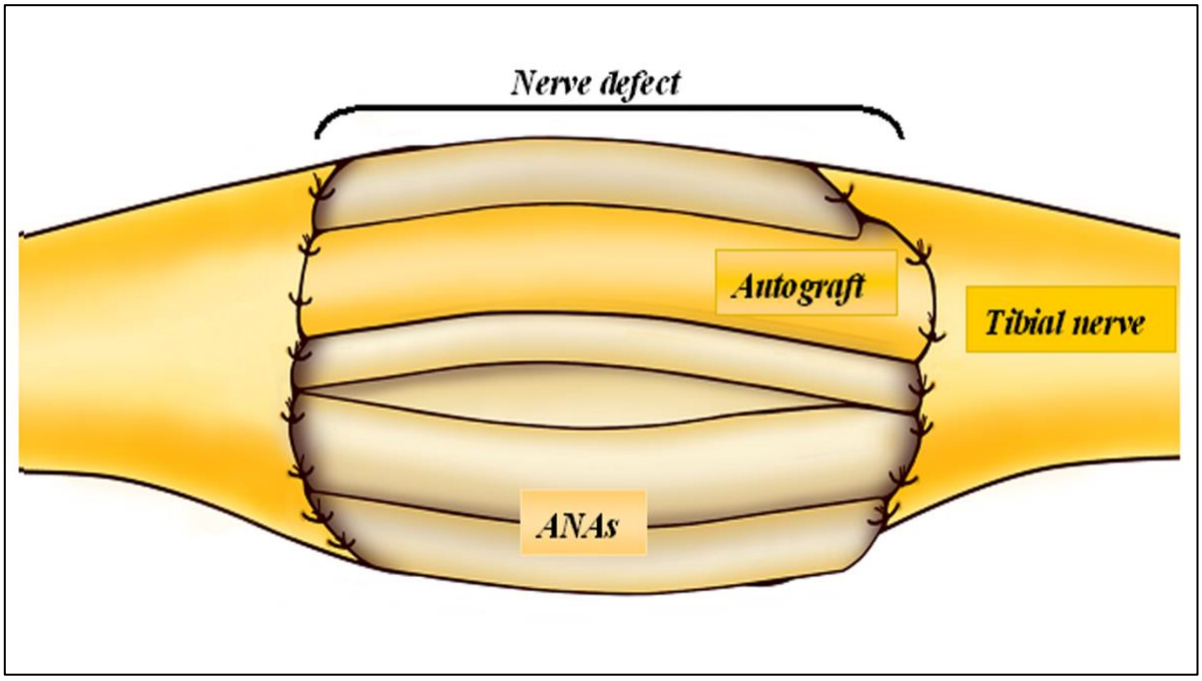

FIGURE 17: *Diagram of parallel multifascicular combination of autologous and allogenic nerve allograft [171].*

Electrophysiological studies (*Figure 18*) showed a better recovery of nerve function in Group C compared to Groups A and B, in terms of wave amplitude and latency, whereas nerve conduction velocity was highest in the autograft group, although no statistical difference was found.

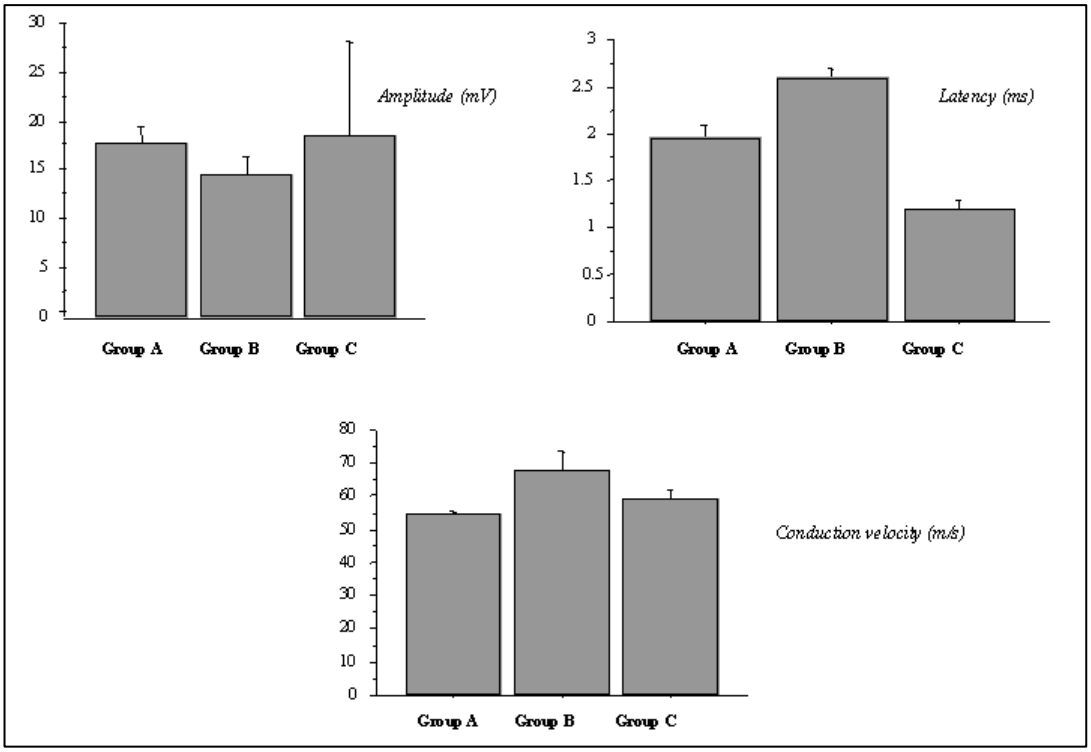

FIGURE 18: *Summary graphs of the EMG tests [172]*

Regarding histology and immunohistochemistry, Group C samples demonstrated the best regeneration pattern (*Figure 19*), shown by a fascicle structure very similar to that of the intact nerves [Fig. 19(e) vs. 19(g)] by a regular nerve arrangement and a homogeneous S-100 fluorescence [Fig. 19(f) vs. 19(h)].

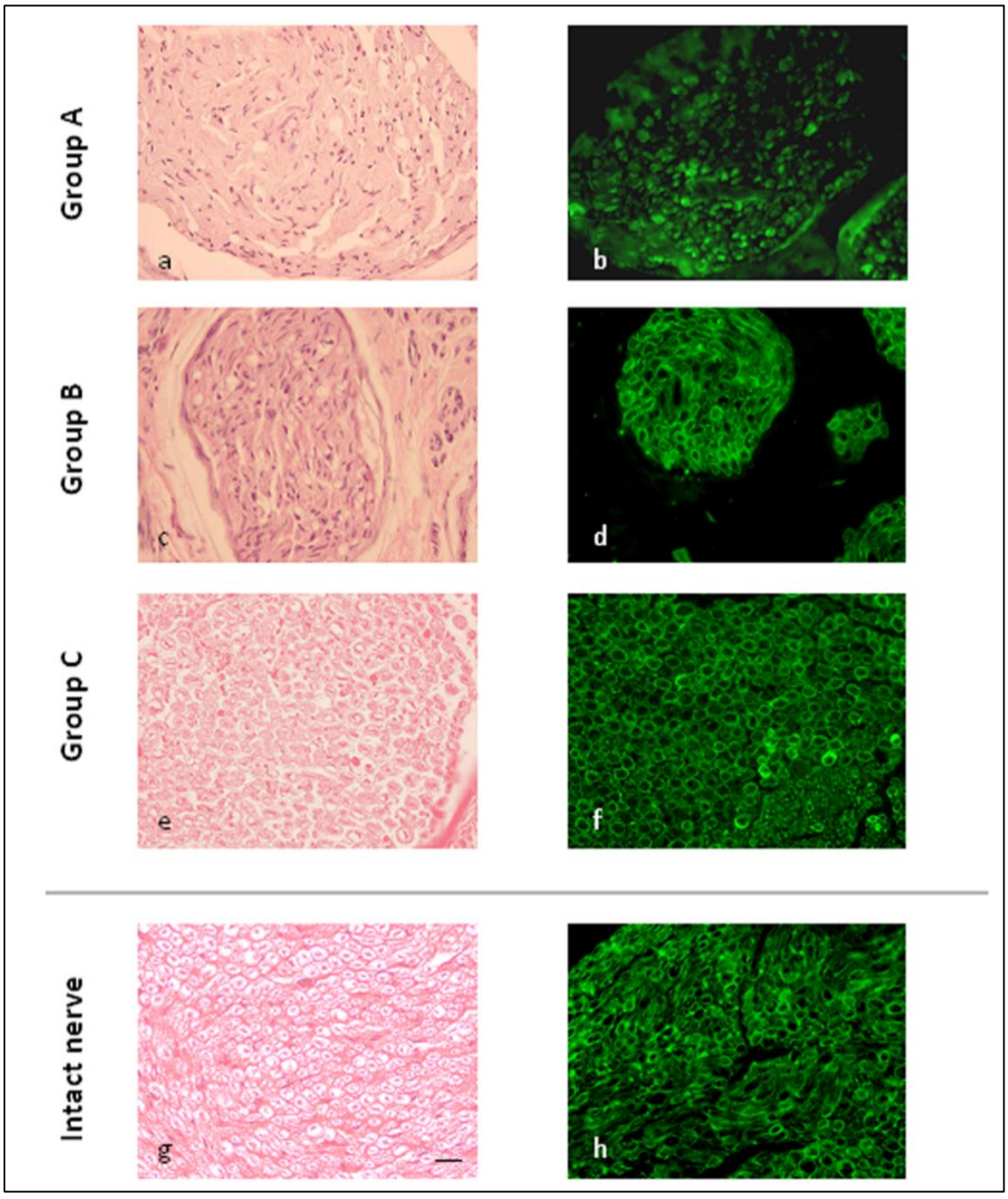

FIGURE 19: *histology and immunohistochemistry study results. Sample stained in E/E observed with LM (first column) and immunohistochemistry with anti-S100 antibody (second column) [172].*

The Group C segments (*Figure 20.1*) presented viable nerve fibers with a normal pattern of myelination [Fig. 20.1(a-c)]. Neurofilaments and neurotubules were present [Fig. 20.1(c)]. The histological appearance of neuroregeneration was very similar to the structure of the intact nerve [Fig. 20.1(d-f)].

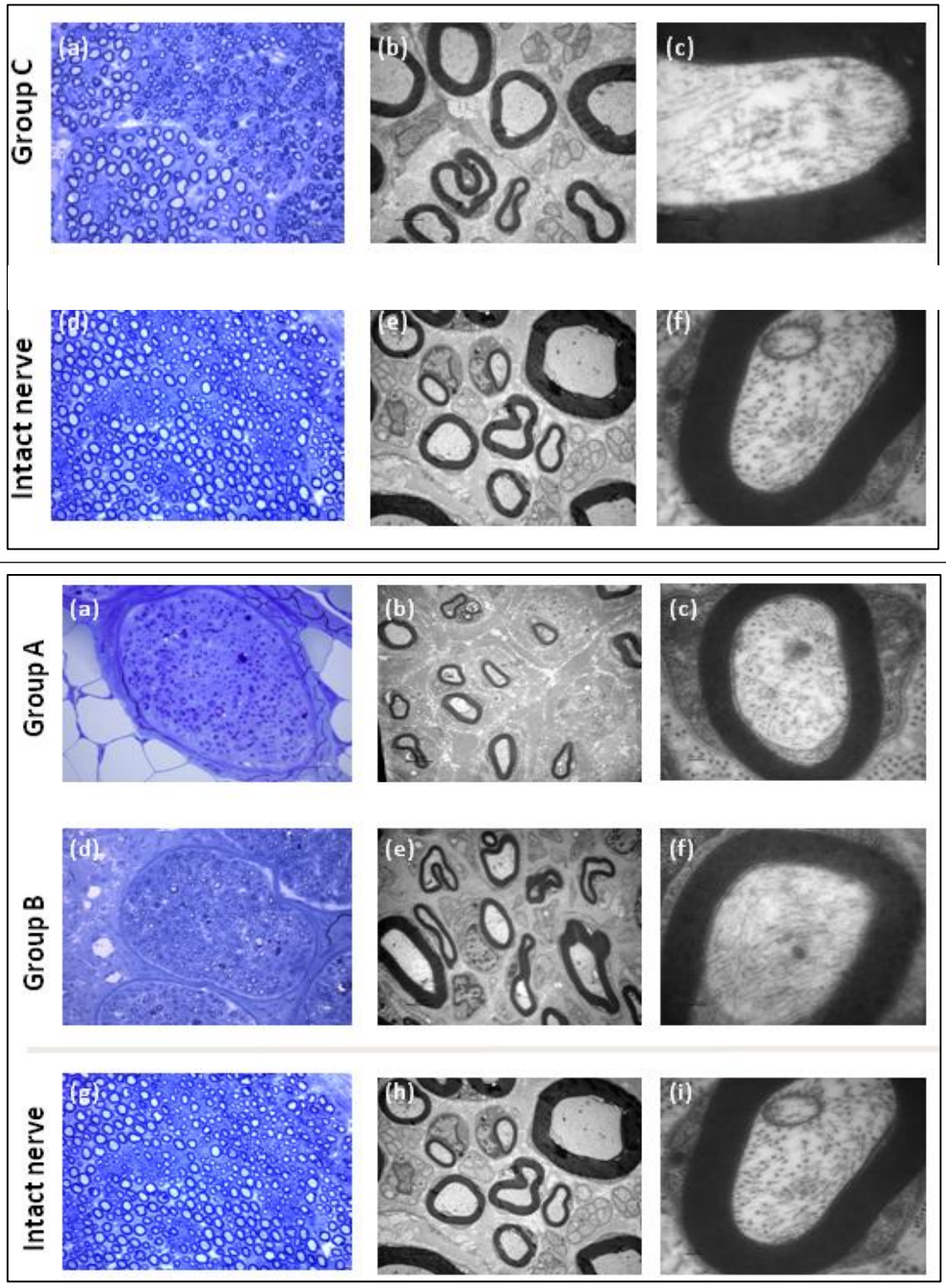

FIGURE 20: *Analysis of neuroregeneration made by LM and TEM. Part 1: Group C and Intact Nerve; Part 2: Group A, Group B and Intact Nerve [169]. First column sample stained with toluidine-blu, Second column TEM 3'000x and third column TEM 12'000x [172].*

ANAs and autografts combined demonstrated a significant difference regarding myelin fibre and axon diameter, whereas myelin thickness and fibre density did not show any significant difference. Differently, myelin fibre and axon diameter in the regenerating nerves reconstructed through the innovative surgical model (Group C) were significantly greater than in Groups A and B, as well as myelin thickness and fibre density. Especially, myelin in Group C showed the same thickness as in the control nerves.

Figure 21 illustrates how Schwann cells might migrate and repopulate the acellular fraction of the complex multifascicular graft, not only from the stumps, but also from the whole longitudinal centrally-posed autologous graft source, as allowed by the newly conceived microsurgical method. Differently, in previous simple ANA's based graft models, Schwann cells could migrate from the host stumps only.

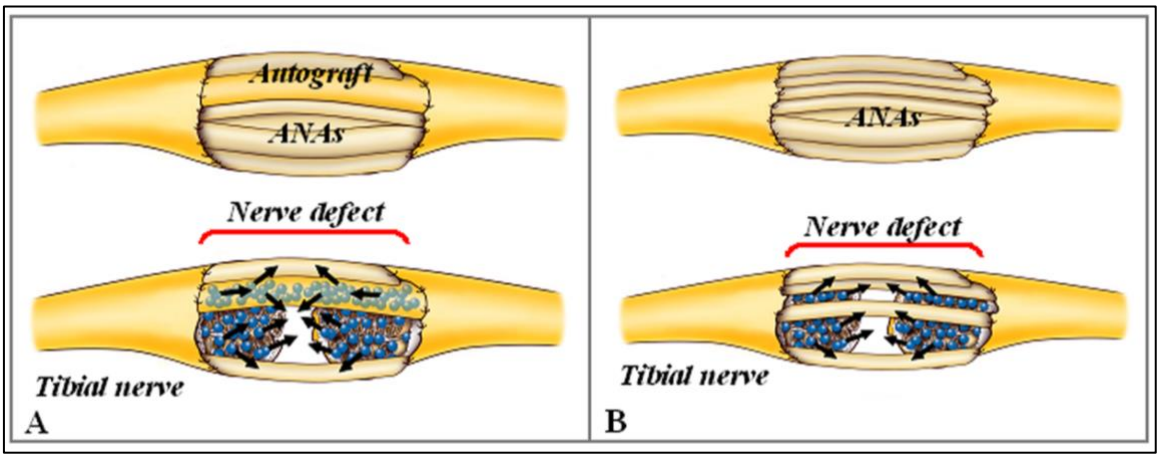

FIGURE 21: *Schwann cells migration in innovative method (A) and in ANAs (B) [171].*

The autologous-allogenic combined grafting proposed by Our Group induced a better regeneration process versus pure autografts and allografts, although more research is necessary to better understand the underlying processes [172].

2.2 Validation of Rizzoli decellularization method on HUMAN NERVES

The positive results of Rizzoli method tested on animal models (rabbit) obtained from these studies [156,171,172] encouraged the Authors to validate the proposed protocol on human nerves.

The new method demonstrated:

- *Effectiveness*: based on the results of histological examinations, performed with LM and TEM, immunohistochemistry, and EMG, the method was found to be as effective as traditional methods and major products on the market

- *Safety*: the method ensures sterility by maintaining the "sterile chain," preserving the nerve from gamma radiation.

The human validation aims to evaluate the results of the Rizzoli decellularization method on human nerves, in terms of histology, immunohistochemistry and microbiology, in order to prepare the field for its clinical translation.

2.2.1 Materials and Methods

This study was conducted following approval of local Ethical Committee (Comitato Etico Indipendente Dell'Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola-Malpighi, Authorization 1293/2017 of 24 April 2017).

1. Preparation and collection of cadaver donor nerve

The nerve were taken from a "non-beating heart" donor inside the autopsy room of the Sant'Orsola-Malpighi Hospital by surgeons. Harvesting was done with the approval of the family, after explaining the method of collection and the purposes of the study.

Inclusion criteria:

- death at the authors' institutions with the indication to undergo autoptic examination, individual age between 30 y.o. and 80 y.o.

Exclusion criteria:

- neurodegenerative pathology, HCV, HIV, diabetic neuropathy, sepsis, metastatic neoplasia with associated paraneoplastic syndrome, meningitis.

After death, sural nerve grafts from the right leg were harvested within 48 hours. A clean area was set up for nerve harvesting, similar to surgical procedures in the operating room, isolating and sterilizing the area.
Samples were taken for a maximum length of 20 cm, and perineural adipose tissue was removed during the harvesting procedure. We reserved, for nerve processing and histological analysis, the sural nerve segment (6-7 cm) centered at the midpoint of the nerve, which had a constant caliber. The caliber of the sural nerve at the midpoint varied between 2.4 and 2.9 mm. Small segments of the same nerves

were collected separately for microbiological analysis. Finally, the surgical site was sutured (*Figure 22*).

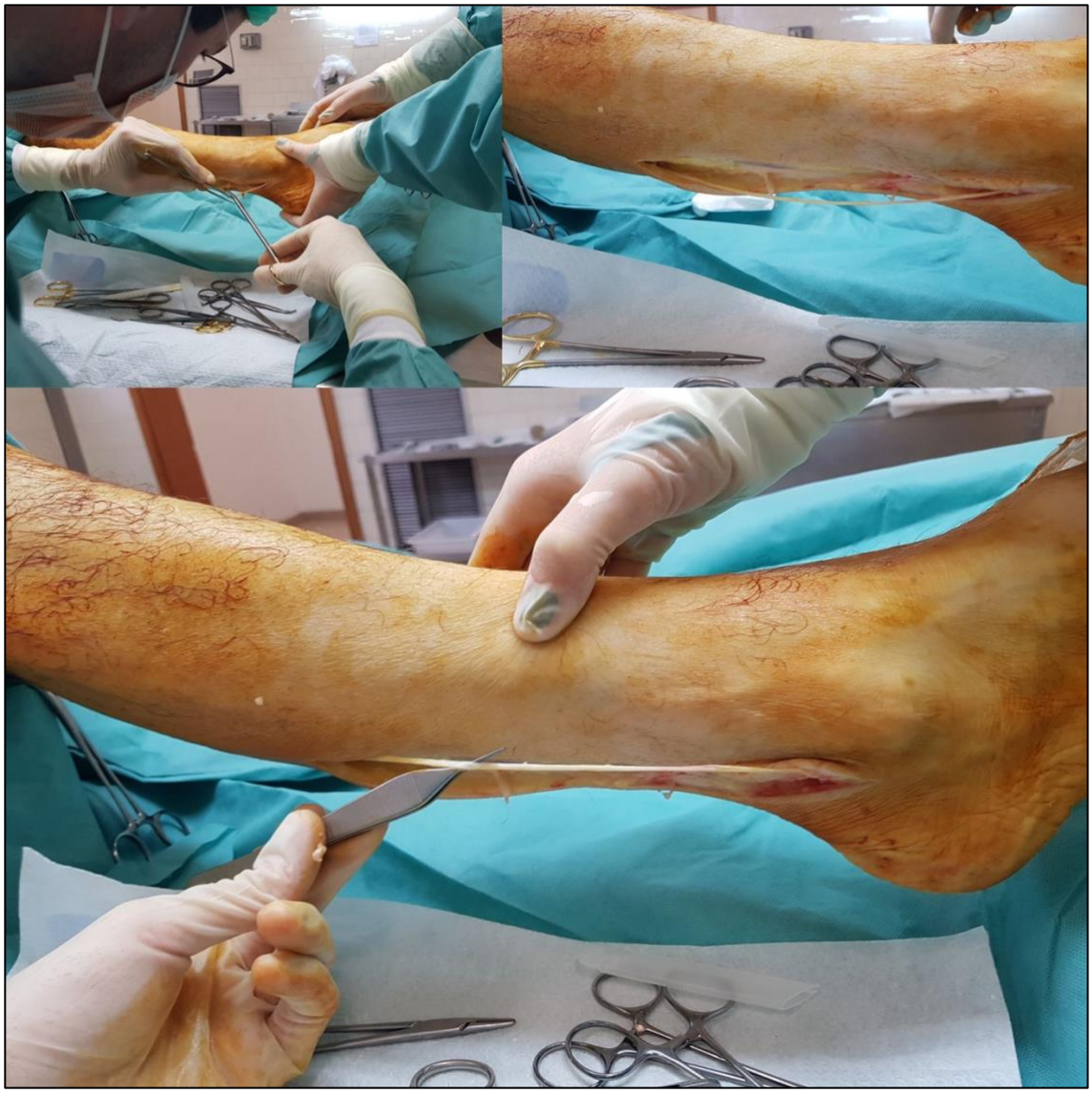

FIGURE 22: *Isolation and harvesting process of the Sural nerve.*

A total of seven different donors were available, with an average age of 67.5 years. Donor ages ranged from 51 years to 79 years.

Each nerve tissue harvested was divided into three samples: one was treated by the novel method, one was decellularized by the Hudson method (control method) and one was left as an untreated nerve.

All nerves were then observed by light microscopy (LM) and transmission electron microscopy (TEM) to assess the removal of myelin, cells and cellular debris and were subjected to immunohistochemical tests to assess the degree of decellularization.

2. Decellularization protocol

The initial decellularization protocol was the same one already tested on rabbits, but it was refined to be adapted to human nerves as follows:

- **A:** nerves were immersed in 40 mL of phosphate-buffered saline (PBS) containing Sulfobetaine-10 (SB-10) 125 mM (SB-10, Soltec Bio Science, Beverly, MA, USA), 0.2% v/v Triton X-100 (Sigma-Aldrich S.r.l., Milan, Italy) (Triton X-200 was no longer available) and 2% v/v Pen Strep (Pen Strep, Thermo Fisher Scientific, Inc., Waltham, MA USA), incubated for 120 h at room temperature in the orbital shaker, and then frozen.

- **B:** on the day of manipulation, nerves were transferred to a Class A glove box to simulate sterile conditions and thawed.

- **C:** nerves were rinsed three times with PBS (total 30 min) and then immersed in sterile PBS containing 0.25% Sodium Dodecyl Sulfate (SDS, Sigma- Aldrich S.r.l., Milan, Italy) for 180 min. During this phase, ultrasounds (40 Hz) were applied for 3 min every 30 min. Sonication cycles were performed with nerves soaked in 30 mL of decellular- ization solution sealed inside sterile 50 mL tubes, which, in turn, were immersed in a sonicator (Branson 2510 DTH Bath Sonicator; ultrasound frequency 40 Hz). During incubation, tubes were kept in agitation on a radial shaker.

- **D:** at the end, ANAs were rinsed three times with PBS for 30 min and immersed in 10% v/v Dimethyl Sulfoxide (DMSO) in isotonic saline solution, then frozen at −80 ◦C for long-term preservation.

After the first nerves, better results were obtained by changing the initial incubation period (**A**) from 48h to 120h.

The **C** phase sonication time also changed from 5 min to 3 min per cycle. This represents the method currently used for decellularization.

3. Microbiology

Microbiological investigations were performed in 3 stages: T0 time of harvesting, T1 mid-process and T2 end of process.
Microbiological analyses were performed with different bacterial broths, depending on the time of examination: at T0 and T1, tryptic soy broth (TSB) and thioglycolate broth (THIOG); at T2, BacT/ALERT® iFA Plus (Bio Merieux, Florence, Italy) (aerobic microorganisms) and BacT/ALERT® iFN Plus (anaerobic microorganisms). iFA and iFN were adopted to simulate the release criteria required by the local competent authority to anticipate future translation to the clinical field.

4. Histological and immunohistochemical investigation

Hematoxylin-eosin (HE) staining was performed to assess general morphology and the presence of cell nuclei.
Luxol Fast Blue staining was used to analyze the presence of myelin.
Immunohistochemistry was performed in an automated stainer using 7 pre-diluted antibodies purchased from Ventana (Ventana, Tucson, AZ, USA).
Ultrathin cross-sections (0.1 μm) were stained with tannic acid, uranyl acetate and lead citrate for transmission electron microscope observation with a Zeiss EM 109.

The antibodies were used according to standardized protocols: **anti-S100** (polyclonal) demonstrated Schwann cells, **anti-EMA** (epithelial membrane antigen, clone E29) was used for perineurium, **anti-NF** (neurofilaments, clone 2F11) demonstrated axons, and **anti-Collagen IV** (clone CIV22) with **anti-Laminin** (clone D18) stained the extracellular matrix (ECM) of the nerve [*Table 1*].

Antibody	Clone	Company
Anti-S100	Polyclonal	Ventana
Anti-EMA	E29	Ventana
Anti-Neurofilament	2F11	Cell Marque
Anti-Collagen IV	CIV22	Cell Marque
Anti-Laminin	D18	Thermo Scientific

TABLE 1. *Technical information about antibodies used according to standardized protocols.*

The Authors proposed the following scores to assess the different nerve histological and immunohistochemical characteristics:

- ENDONEURIUM, PERINEURIUM AND EPINEURIUM PRESERVATION (global nerve preservation): **badly preserved**, **moderately preserved**, **well-preserved** (for the evaluation, see Results section below).

- NUCLEAR DENSITY: **absent** = 0; **low** < 25/10 High Powered Field (HPF); **medium** = 25–50/HPF; **high** > 50/HPF.

- NUCLEAR STATUS: **intact** or **degenerated** (nuclear debris without distinct shape).

- SCHWANN CELLS DENSITY (distinct immunopositivity for S-100 both in the nucleus and cytoplasm): **absent** = 0; **low** < 25/10 High Powered Field (HPF); **medium** = 25–50/HPF; **high** > 50/HPF.

- PERINEURIAL CELLS DENSITY (distinct membrane immunopositivity for EMA): **absent** = 0; **low** < 2 5/10 High Powered Field (HPF); **medium** = 25–50/HPF; **high** > 50/HPF.

- AXONAL NETWORK: **well-preserved** (bright immunointensity, preserved shape, par- allel orientation for NF) or **badly preserved** (reduced immunointensity for NF and ax- onal swelling).

- ECM PROTEINS: **well-preserved** (bright, continuous and homogeneous immunoin- tensity for Collagen IV and Laminin) or **badly preserved** (weak and dishomogeneous immunoreactivity).

2.2.2 Results

Microbiological, histological and immunohistochemical analyses were performed on each sample to evaluate the effectiveness of the method on the human nerve.

1. Microbiology

The microbiological investigations were performed in 3 times: T0 at the time of sampling, T1 at half decellularization and T2 at the end of the process.

The samples were always negative at T0, T1 and T2. The only exception was sample No. 1 at T0, which was positive for streptococcus salivarius. However, the positivity disappeared at T1 after incubation with the first solution, which contains antimicrobial agents.
The microbiological analyses were carried out with different culture media based on the examination time (*Figure 23*):

- **T0 and T1**: *TSB* (Tryptic Soy Broth) and *THIOG* (Thioglycollate) were used as broths.

- **T2**: it was based on *iFA plus* (for aerobic microorganisms) and *iFN plus* (for anaerobes), to simulate the parameters required by the CRT, as these broths were approved by the official pharmacopoeia.

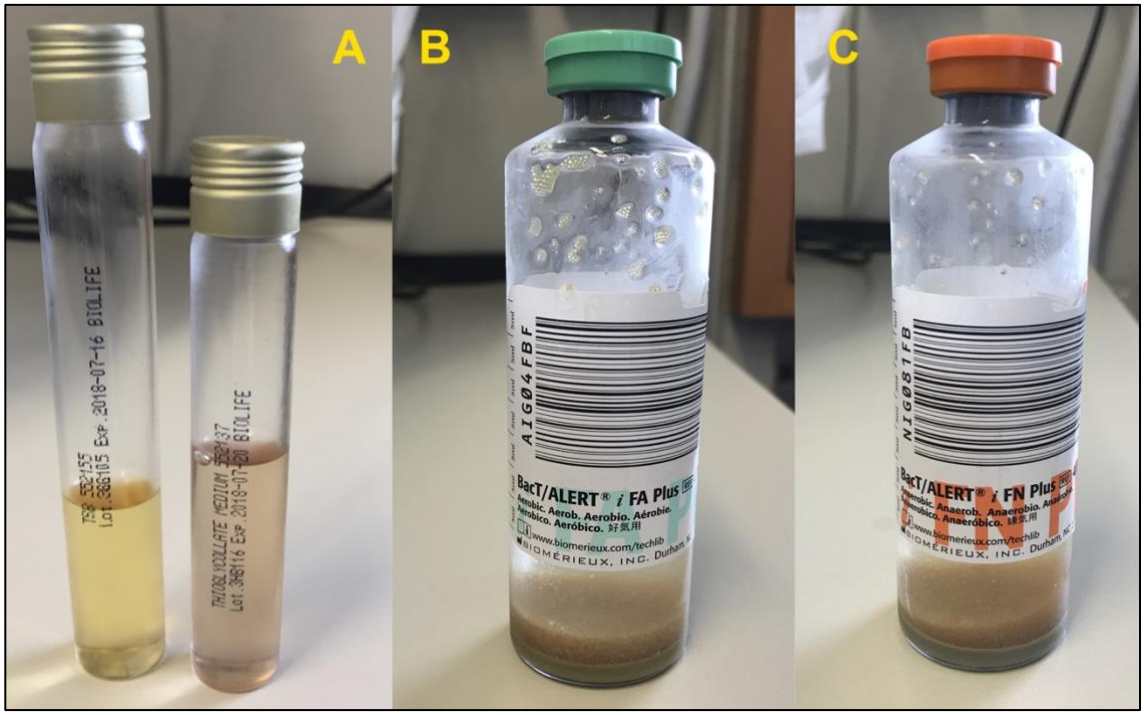

FIGURE 23:
- ***a.*** *TSB and THIOG broths*
- ***b.*** *iFA plus*
- ***c.*** *iFN plus*

The table below [*Table2*] shows that, with the exception of the first sample in T0, there was no bacterial growth. This may be due to increased caution in collection and improved method.

Sample	Time	Media	Date of receipt	Broth/instrument result	Subculture result
7982	T0	TSB	29/09/17	Murky	Streptococcus salivarius
7982	T0	THIOG	29/09/17	Murky	No growth
7982	T1	TSB	02/10/17	No growth	
7982	T1	THIOG	02/10/17	No growth	
7982	T2	iFA plus	13/10/17	No growth	
7982	T2	iFN plus	13/10/17	No growth	
7988	T0	TSB	06/10/17	No growth	
7988	T0	THIOG	06/10/17	No growth	
7988	T1	TSB	09/10/17	No growth	
7988	T1	THIOG	09/10/17	No growth	
7988	T2	iFA plus	13/10/17	No growth	
7988	T2	iFN plus	13/10/17	No growth	
7997	T0	TSB	09/11/17	No growth	
7997	T0	THIOG	09/11/17	No growth	
7997	T1	TSB	13/12/17	No growth	
7997	T1	THIOG	13/12/17	No growth	
7997	T2	iFA plus	13/12/17	No growth	
7997	T2	iFN plus	13/12/17	No growth	
8043	T0	TSB	14/02/18	No growth	
8043	T0	THIOG	14/02/18	No growth	
8043	T1	TSB	13/03/18	No growth	
8043	T1	THIOG	13/03/18	No growth	
8043	T2	iFA plus	13/03/18	No growth	
8043	T2	iFN plus	13/03/18	No growth	

TABLE 2: *Summary chart of the microbiological analyses made by the microbiology laboratory of the Sant'Orsola-Malpighi hospital*

2. Transmission Electronic Microscopy

TEM analysis showed that cell debris was detectable within the axons and the ECM was severely damaged, in the first two nerves decellularized by the novel method (*Figures 24 and 25*).

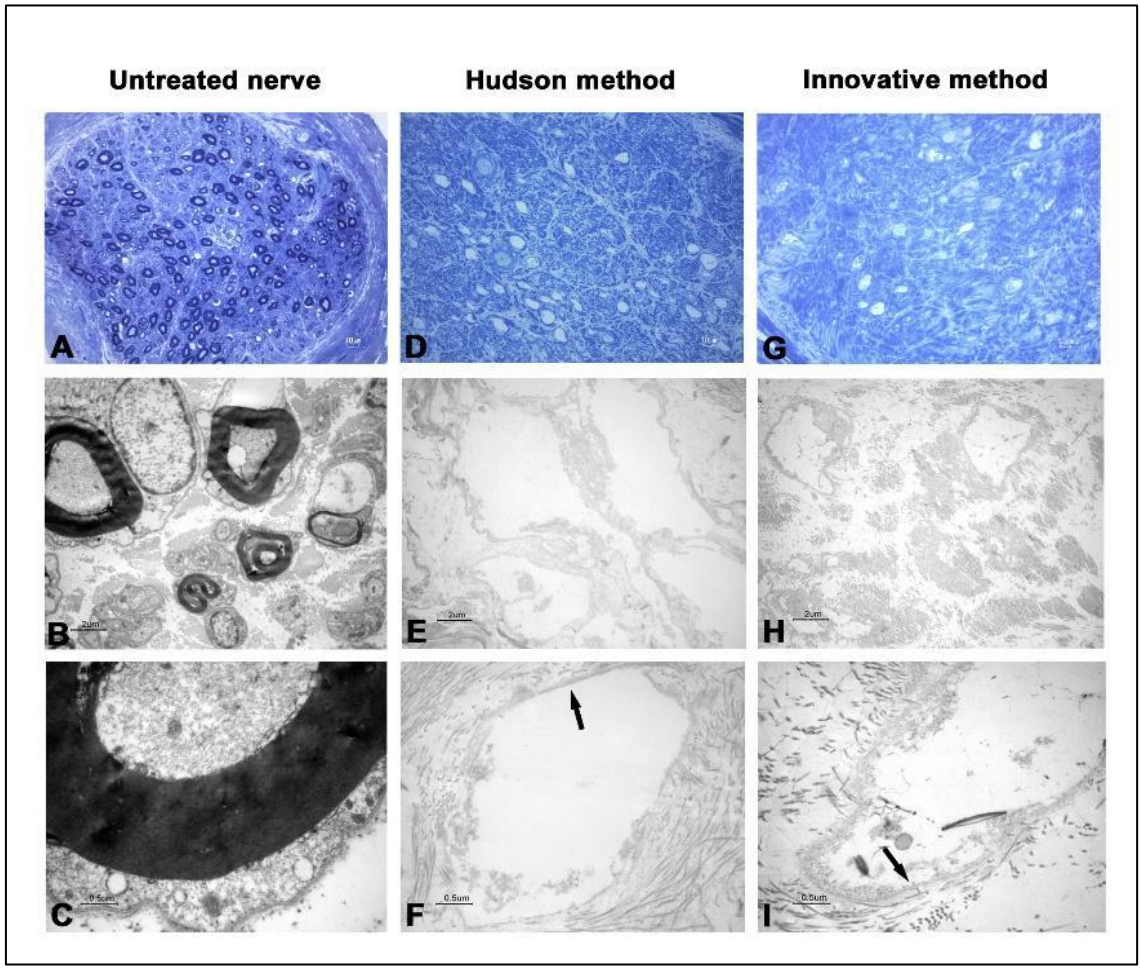

FIGURE 24. *Light and electron microscopy of human nerve sample n.1. (**A**–**C**) Untreated nerve; (**D**–**F**) control decellularization method (Hudson method); (**G**–**I**) innovative decellularization method. (**A**, **D**, **G**) LM 40× of semithin cross-sections (toluidine blue, bar: 10 μm); (**B**, **E**, **H**) TEM 3000× of ultrathin cross-sections (bar: 2 μm); (**C**, **F**, **I**) TEM 12,000× at higher magnification (bar: 0.5 μm). Arrows indicate the preserved basal lamina.*

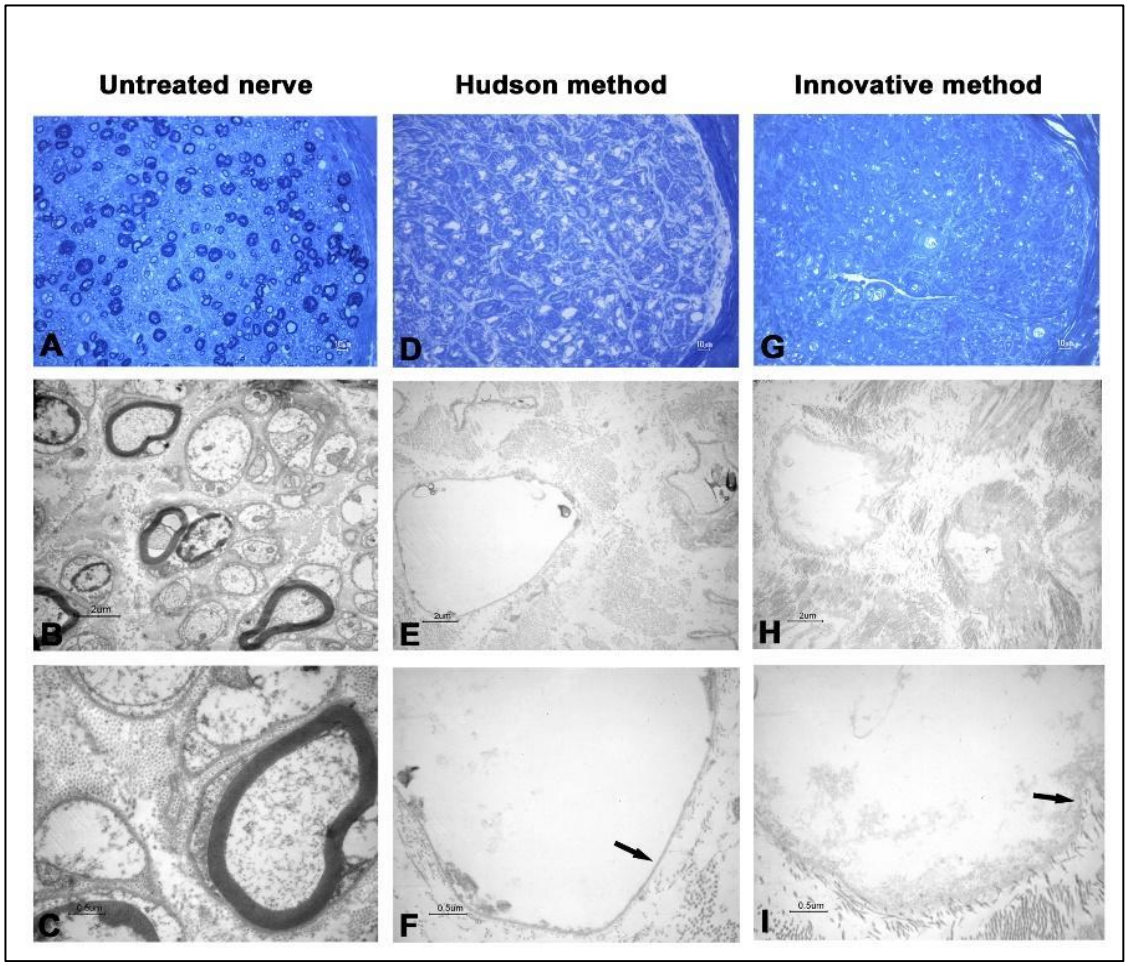

FIGURE 25. *Light and electron microscopy of human nerve sample n.2. (A–C) Untreated nerve; (D–F) control decellularization method (Hudson method); (G–I) innovative decellularization method. (A, D, G) LM 40× of semithin cross-sections (toluidine blue, bar: 10 μm); (B, E, H) TEM 3000× of ul-trathin cross-sections (bar: 2 μm); (C, F, I) TEM 12,000× at higher magnification (bar: 0.5 μm). Arrows indicate the preserved basal lamina.*

These structures were less detectable in samples prepared by the Hudson method. No evidence of residual myelin was present in either preparation. Initial fluorescent immunohistochemistry results showed that almost no residual Schwann cells remained in the samples after the treatments (*Figures 26 and 27*), indicating that the new method is effective in terms of decellularization on human nerves.

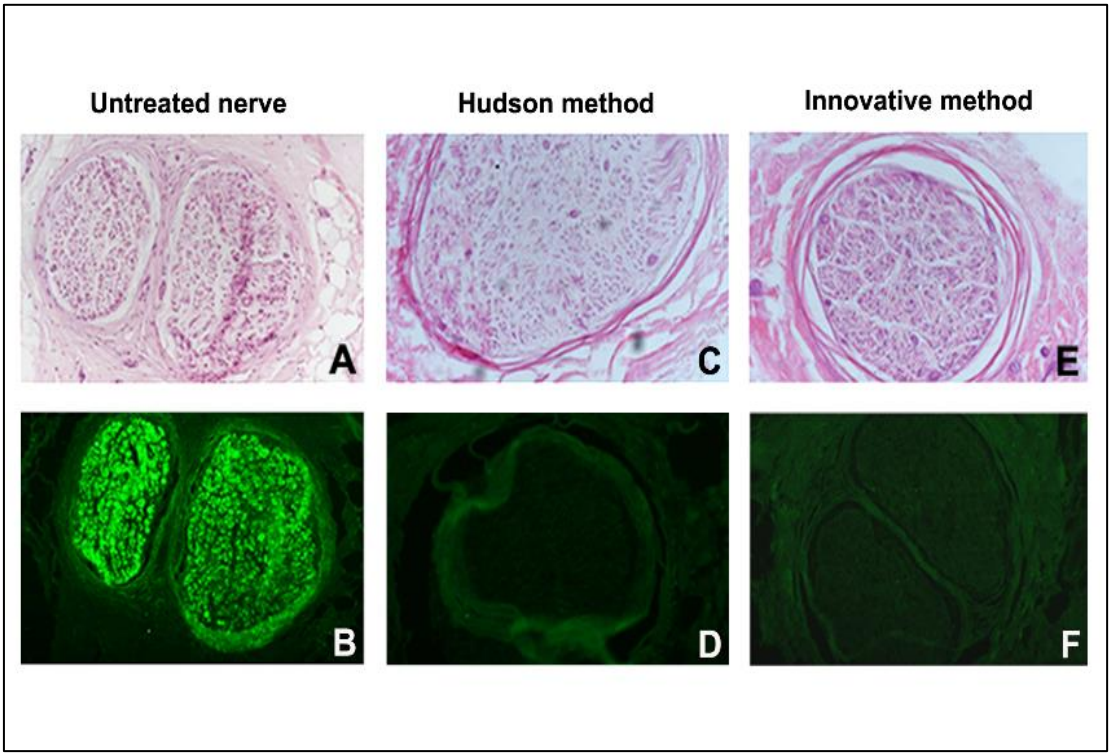

FIGURE 26. *Fascicle morphology (**A**, **C**, **E**) (hematoxylin–eosin) and S-100 immunohistochemical analysis (**B**, **D**, **F**) of human nerve sample n.1. (**A**, **B**) Untreated nerve; (**C**, **D**) control decellularization method (Hudson method); (**E**, **F**) innovative decellularization method. Original magnification 20× (**A**–**F**).*

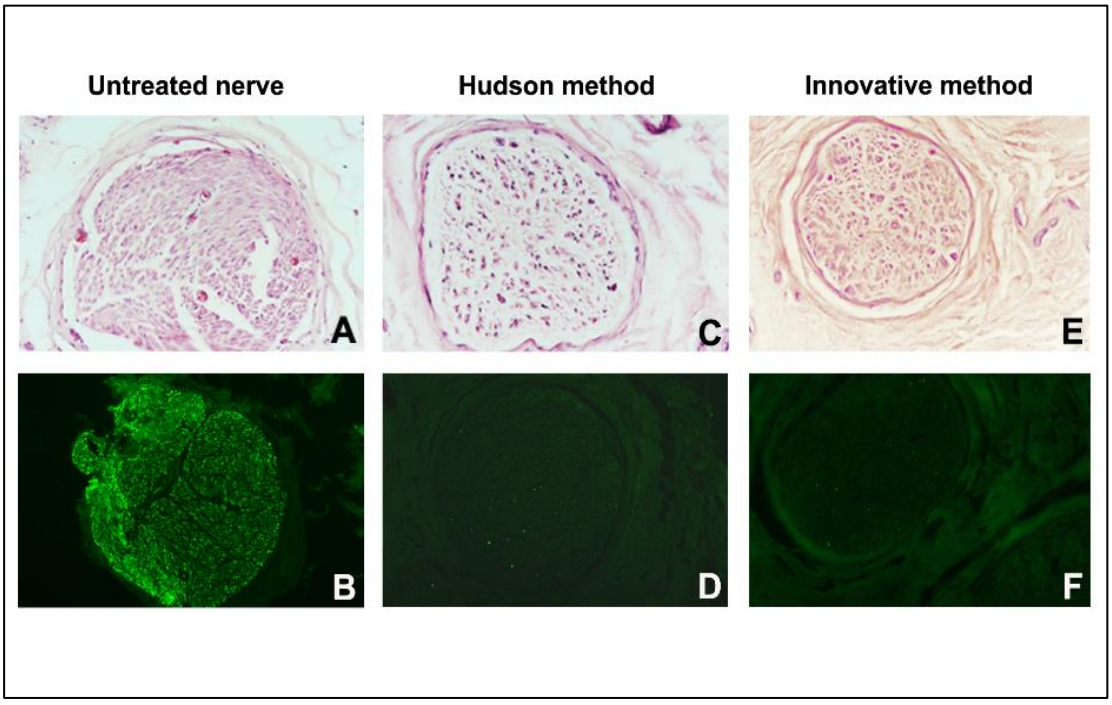

FIGURE 27. *Fascicle morphology (**A**, **C**, **E**) (hematoxylin-eosin) and S-100 immunohistochemical analysis (**B**, **D**, **F**) of human nerve sample n.2. (**A**, **B**) Untreated nerve; (**C**, **D**) control decellularization method (Hudson method); (**E**, **F**) innovative decellularization method. Original magnification 20× (**A**–**F**).*

Since the hypothesis that the ultrasound cycles were too aggressive and damaged the integrity of the treated nerves, we applied two modifications to the initial protocol. More specifically, we increased the initial incubation period from 48 h to 120 h and changed the sonication time from 5 min to 3 min per cycle. These modifications was first applied to sample No. 3 (*Figure 28*), improving both the clearance of cell fragments and the histomorphological integrity of the ECM. No major deterioration of the ECM was observed at TEM, axons showed no traces of residual cells and myelin, and the basal lamina was preserved. Images obtained from other samples were comparable (*Figures 29 and 30*).

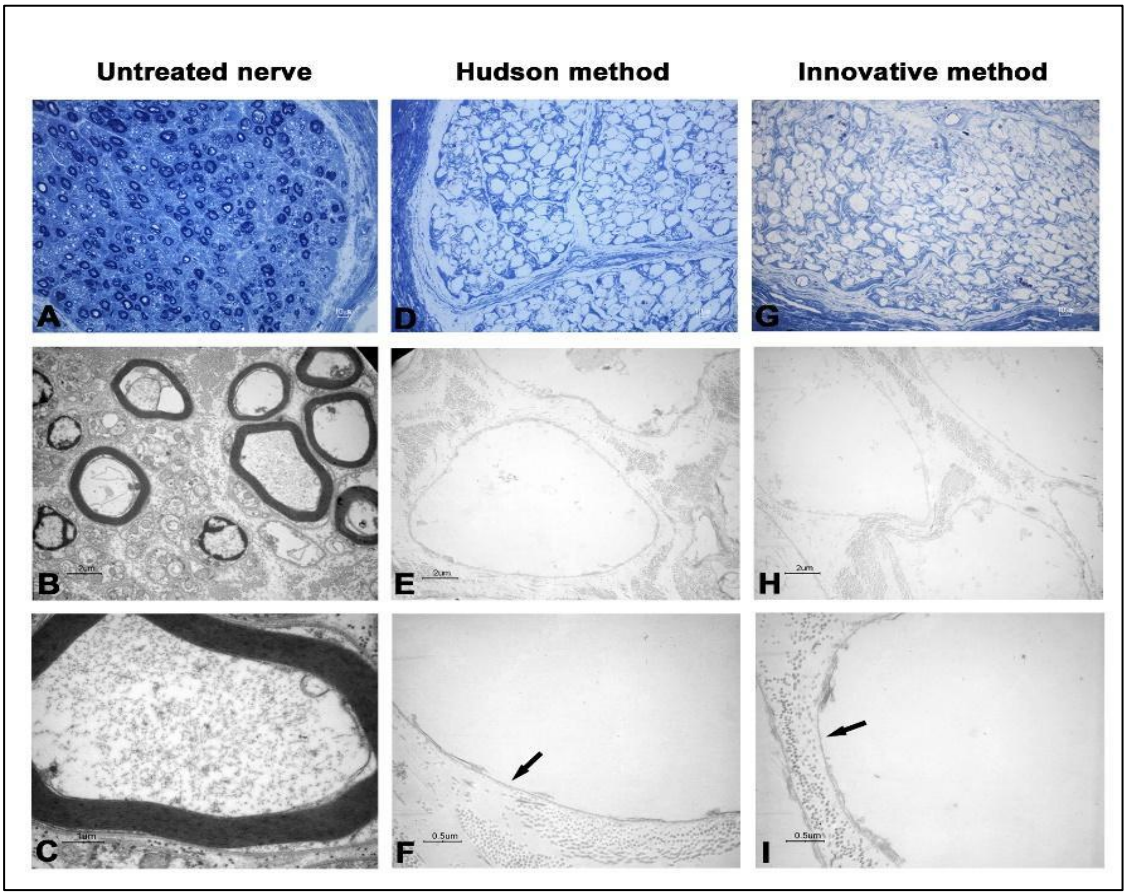

FIGURE 28. *Light and electron microscopy of human nerve sample n.3.* ***(A–C)*** *Untreated nerve;* ***(D–F)*** *control decellularization method (Hudson method);* ***(G–I)*** *innovative decellularization method.* ***(A, D, G)*** *LM 40× of semithin cross-sections (toluidine blue, bar: 10 µm);* ***(B, E, H)*** *TEM 3000× of ultrathin cross-sections (bar: 2 µm);* ***(C, F, I)*** *TEM 12,000× at higher magnification (bar: 0.5 µm. Arrows indicate the preserved basal lamina.*

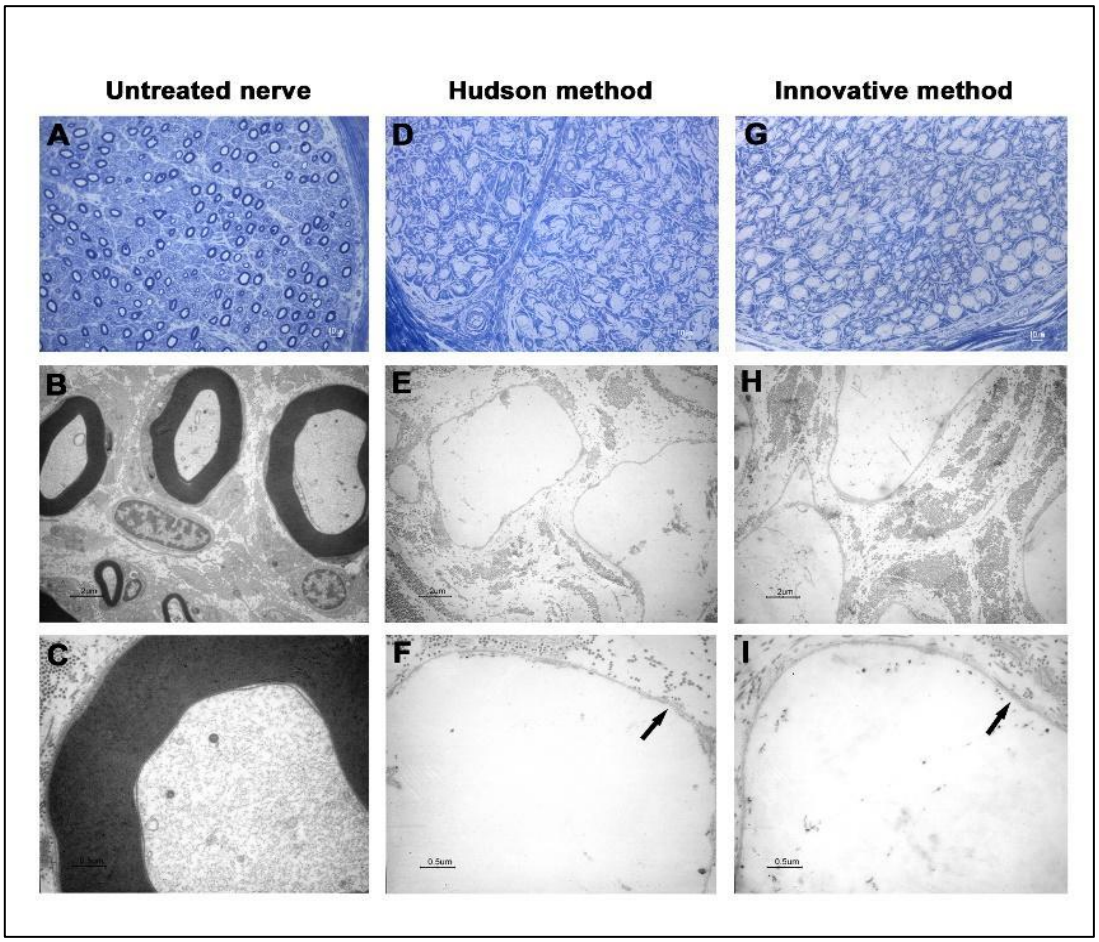

FIGURE 29. *Light and electron microscopy of human nerve sample n.6. (A–C) Untreated nerve; (D–F) control decellularization method (Hudson method); (G-I) innovative decellularization method. (A, D, G) LM 40× of semithin cross-sections (toluidine blue, bar: 10 μm); (B, E, H) TEM 3000× of ultrathin cross-sections (bar: 2 μm); (C, F, I) TEM 12,000× at higher magnification (bar: 0.5 μm). Arrows indicate the preserved basal lamina.*

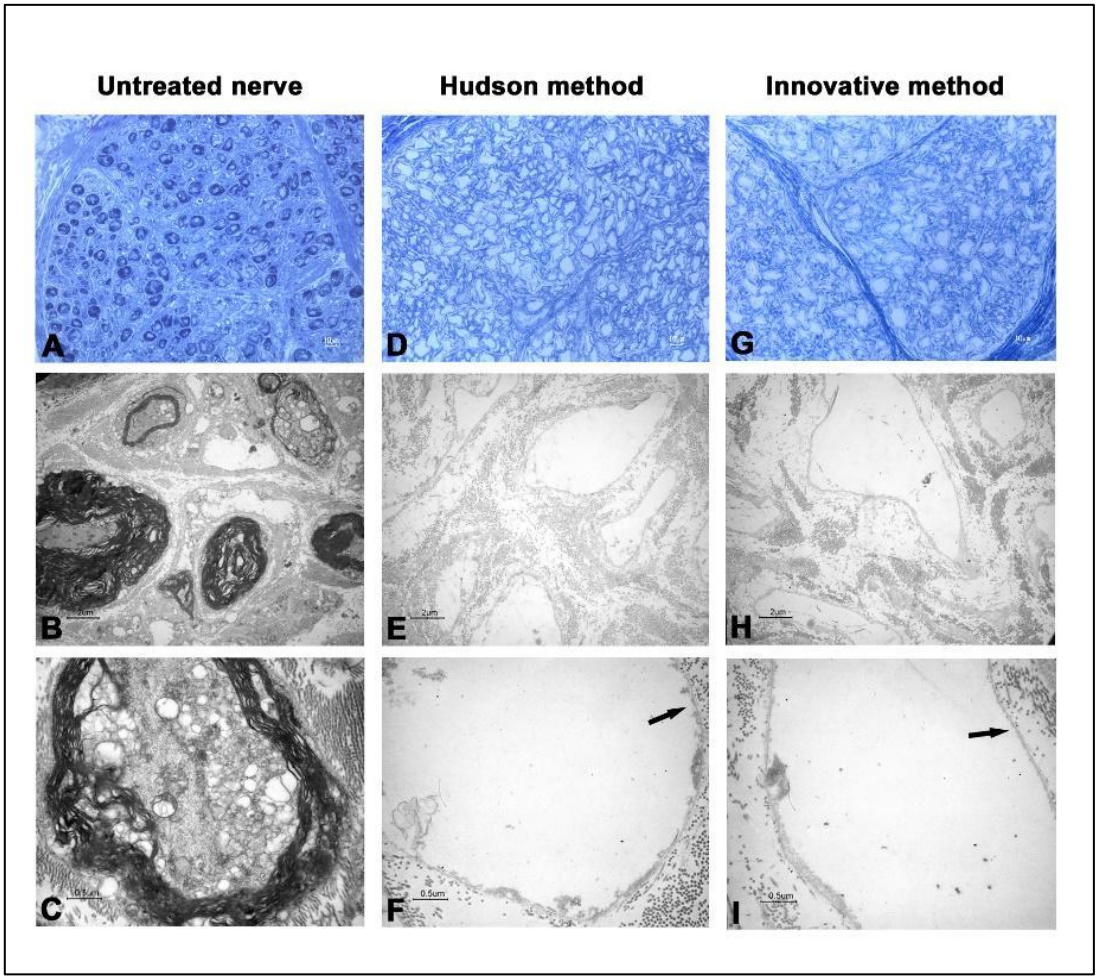

FIGURE 30. *Light and electron microscopy of human nerve sample n.7. (A–C) Untreated nerve; (D–F) control decellularization method (Hudson method); (G–I) innovative decellularization method. (A, D, G) LM 40× of semithin cross-sections (toluidine blue, bar: 10 µm); (B, E, H) TEM 3000× of ultrathin cross-sections (bar: 2 µm); (C, F, I) TEM 12,000× at higher magnification (bar: 0.5 µm). Arrows indicate the preserved basal lamina.*

3. Histology and Immunohistochemistry

General morphology was assessed by HE staining. Native nerves consisted of three distinct and well-preserved compartments: endoneurium composed of axons and Schwann cells; perineurium composed of flattened, polygonal cells arranged concentrically and separated by a thin layer of collagen; and epineurium composed of fibrous and adipose tissue with small arteries, veins and lymphatics [*Figure 31 A-C*].

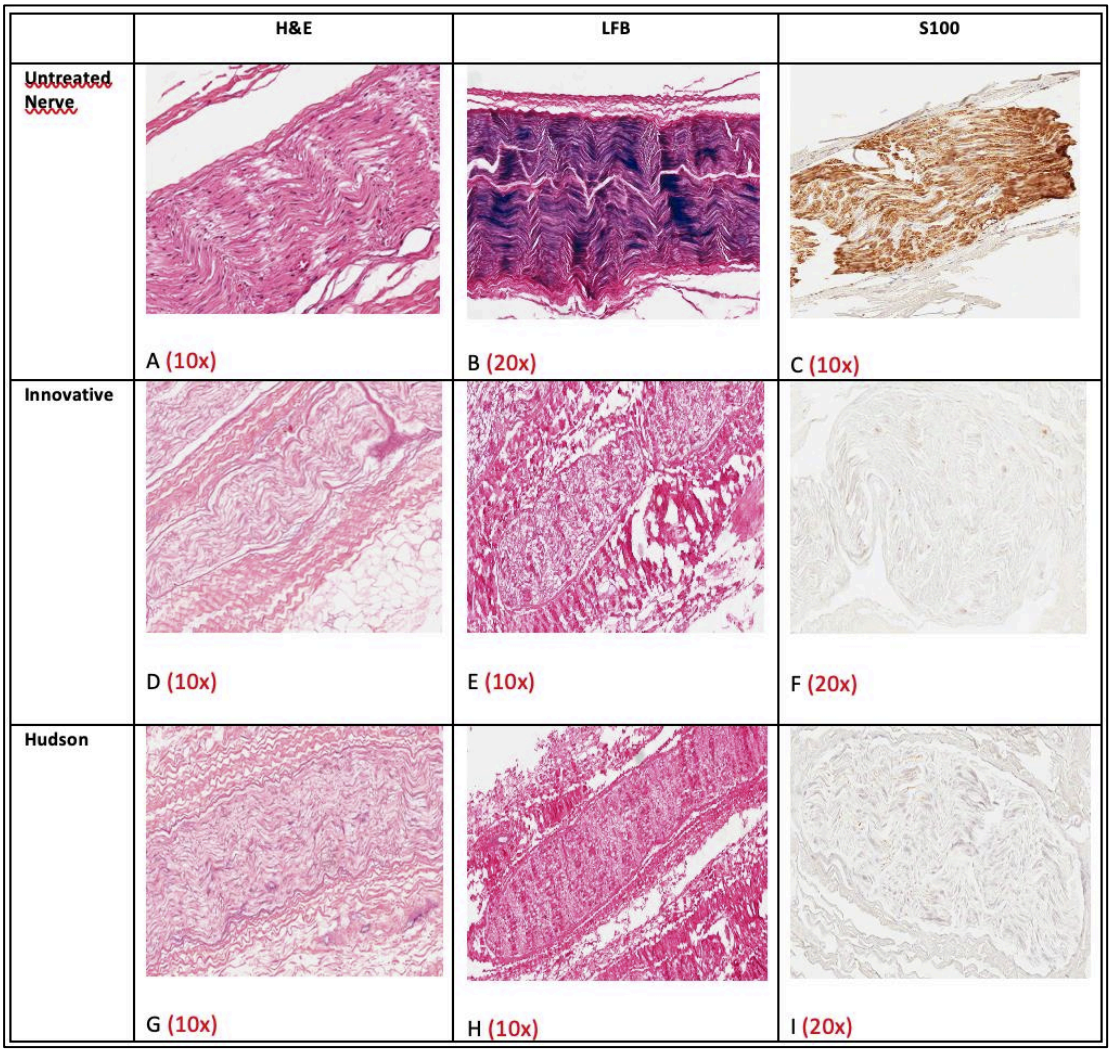

FIGURE 31. *Example of structural and cellular comparison between the nerves before (**A**, **B**, **C**) and after decellularization: innovative method (**D**, **E**, **F**) and Hudson method (**G**, **H**, **I**). Note the absence of well-preserved nuclei in (**D**) and the presence of nuclear debris in (**G**).*

After decellularization, the epineurium and perineurium fibrous tissue were well preserved in both groups, but intact cell nuclei were no longer detectable [*Figure 31 D-I*]. The structures of the endoneurium were generally preserved, although a small number of degenerate nuclei and chromatin debris were still visible in the endoneurium [*Figure 31G*].

Luxol Fast Blue showed myelin removal in both decellularization groups [*Figure 31E,H*].

Immunohistochemical staining of S100 and EMA supported these observations: in the native nerves, Schwann and perineurial cells were highly evident [*Figure 31C*], whereas after decellularization no distinct positivity could be seen [*Figure 31F,I*]. Native nerves contained moderately retained axons [*Figure 32A*] and preserved ECM [*Figure 32B,C*], whereas decellularized groups retained only traces of axonal

proteins and very well preserved ECM components, as supported by immunohistochemical staining for laminin and type IV collagen [*Figure 32D-I*]. The latter two stains were often more clear in decellularized samples; presumably, after decellularization, the proteins were more exposed to antibody binding.

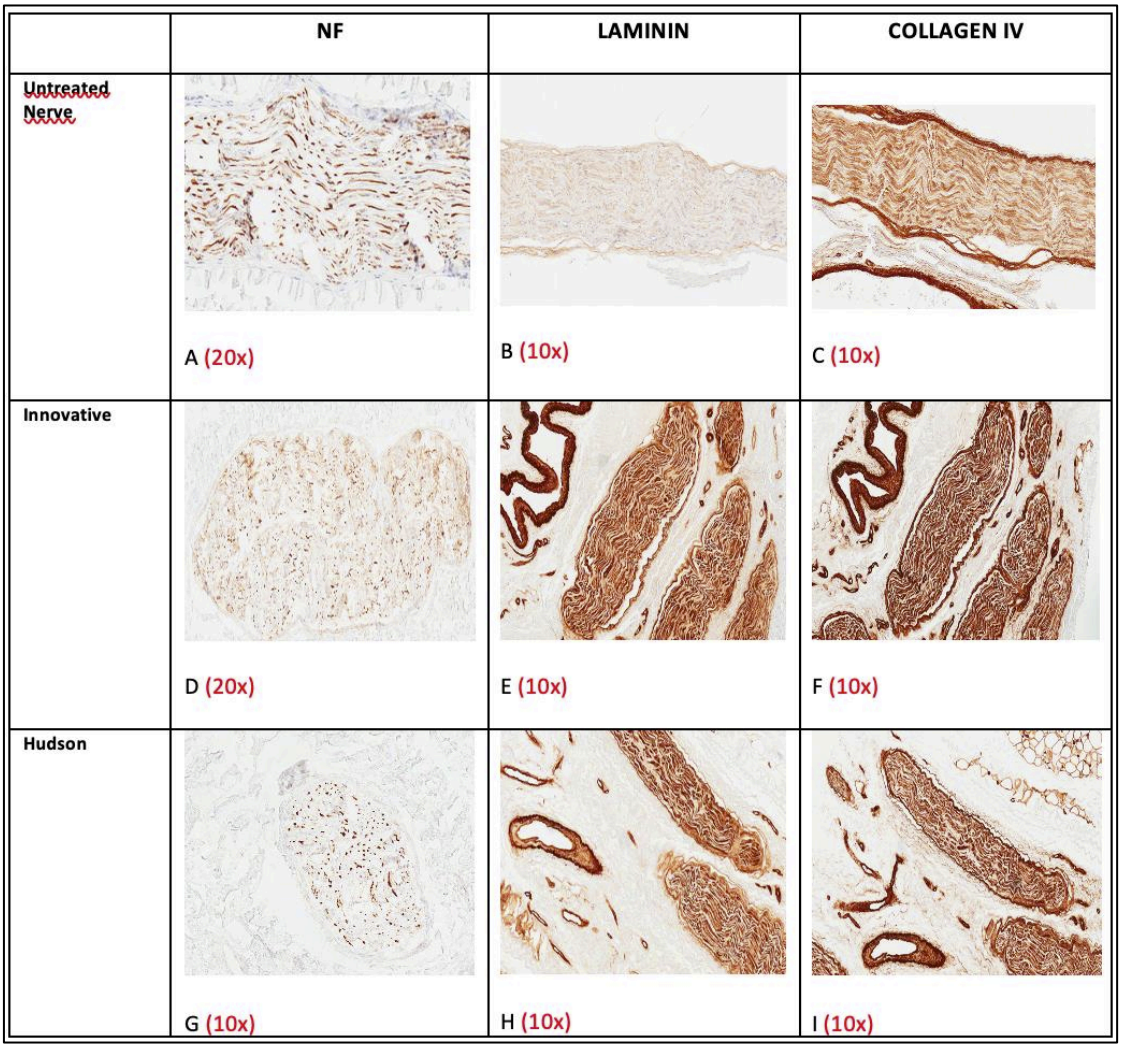

FIGURE 32. *Example of axonal and ECM preservation in nerves before (**A**, **B**, **C**) and after decellularization: innovative method (**D**, **E**, **F**) and Hudson method (**G**, **H**, **I**). Note the increasing density and the different distribution of the ECM after decellularization.*

The novel method showed a higher degree of decellularization than the Hudson one in five samples [cases 1, 2, 3, 6, 7], with well-preserved nerve structure [*Figure 32D,G*]. In two samples (cases 4 and 5), the Hudson process yielded better results [*Table 3*].

Cases	Decellularization Techniques	Parameters for the Evaluation of Decellularization Process					Evaluation of Neural Support Structure	Results
		Global Nerve Preservation	Nuclear Density and Status	Schwann Cells (S100)	Perineural Cells (EMA)	Axons Preservation (NF)	ECM Preservation (Laminin and Collagen IV)	
1	Native Innovative Hudson	well well well	High, intact Low, degenerated Medium, degenerated	High Absent Absent	High Absent Absent	Well Badly Badly	Well Well Well	Innovative better than Hudson
2	Native Innovative Hudson	well well well	High, intact Low, degenerated Medium, degenerated	High Absent Absent	High Absent Absent	Badly Badly Badly	Well Well Well	Innovative better than Hudson
3	Native Innovative Hudson	well moderately moderately	High, intact Absent Low, degenerated	High Absent Absent	High Absent Absent	Well Badly Badly	Well Well Well	Innovative better than Hudson (Innovative with complete decellularization)
4	Native Innovative Hudson	well well moderately	High, intact Medium, degenerated Low, degenerated	High Absent Absent	High Absent Absent	Badly Badly Badly	Well Well Bad	Hudson better than Innovative but without ECM preservation
5	Native Innovative Hudson	well well well	High, intact Medium, degenerated Low, degenerated	High Absent Absent	High Absent Absent	Badly Badly Badly	Well Well Well	Hudson better than Innovative
6	Native Innovative Hudson	well well well	High, intact Low, degenerated Medium, degenerated	Medium Absent Absent	Medium Absent Absent	Well Badly Bad	Well Well Well	Innovative better than Hudson
7	Native Innovative Hudson	moderately moderately moderately	High, intact Low, degenerated Medium, degenerated	High Absent Absent	High Absent Absent	Well Badly Badly	Well Well Badly	Innovative better than Hudson

TABLE 3. *Different decellularization techniques based on morphological and immunohistochemical results (abbreviations: EMA, epithelial membrane antigen; NF, neurofilament; ECM, extracellular matrix). Scores for evaluation are described in the section "Materials and methods: Histology and immunohistochemistry".*

2.2.3 Discussion

With these studies Authors propose and histologically validate e new method for decellularization of nerves, which appears to be microbiologically safe and effective but also sufficiently delicate to preserve ECM, whose role to condition nerve regeneration is essential. The use of ANAs for peripheral nerve reconstruction has recently increased and widely spread as shown by the recent medical literature. Isaacs and coworkers [173] have implanted ANAs in combination with nerve connectors in rats; Li and collaborators [174] have successfully utilized ANAs in a clinical context for high-level, greater, and mixed nerves, specifically the brachial plexus. Similarly, the use of acellular nerve allograft allows for a minimally invasive approach to successful corneal neurotization, as shown in humans by Leyngold and colleagues [175] and is demonstrated to be of benefit for human trigeminal nerve reconstruction [176] and upper limbs [177].

Even for a challenging problem as painful neuroma, ANAs have been successfully tested in an animal setting by Hong and coworkers [178]. Supercharging ANAs through a nerve transfer has allowed overcoming the limits in neuroregeneration related to longer defects, as verified by Isaacs and his group on rats [179]. Biological enhancement of ANAs has also been tested: Yang and coworkers have shown that adipose stem cells-based seeding of ANAs promote neuroregeneration [180]. Clinical application of ANAs is promising based on Zhu and colleagues' clinical trial [181]. The present study shows that the method, previously optimized in vitro and in vivo presented by our research group [156, 182], can be applied on human nerve samples, obtaining similar, and sometimes even better results compared with the Hudson technique.

The first step was to use several ancillary tests to confirm the success of the decellularization method.

Observation at LM of HE-stained sections revealed degeneration of cells, absence of myelin, and preservation of morphological features and basal lamina with preservation of interstitial endoneurium.

Immunohistochemistry and TEM showed that the cellular debris observed at LM corresponded neither to intact nuclei nor to cytoplasmic structures; they also confirmed the preservation of ECM components.

The second step was to compare the results obtained by adopting the novel protocol with those obtained with the Hudson technique. Interestingly, the innovative method revealed a better degree of decellularization than the Hudson process with a well-preserved structure of the nerve in 5 out of 7 cases (cases 1, 2, 3, 6, 7 = 70%), whereas in the remaining 2 out of 7 cases (cases 4, 5 = 30%) the Hudson process had slightly better results [*Table 3*].

Besides, the innovative technique revealed histologically complete nerve decellularization in case 3 [see also Fig. 31D]. Although case 1, 2, 6, 7 revealed few nuclei with highly degenerated features in the routine staining with HE, it can be noticed that the density and distribution of the debris were higher in Hudson treated nerve than in innovatively treated specimens. Moreover, the immunohistochemistry and the TEM did not confirm any intact nuclear and cytoplasm structures. The axonal network was reduced after both decellularization treatments in all specimens. TEM did not confirm any intact axonal structures. As shown by other groups [183], also in this experimental protocol ECM components were well-preserved after both decellularization techniques: again, the innovative method revealed a higher degree of ECM preservation than the Hudson process in 2 out of 7 cases (cases 4, 7 = 30%).

Reducing the duration of the protocol to a single day of manipulation in a sterile environment and limiting the tissue handling time to the 5 hours of a common aseptic work session make this technique as compatible as possible with the requirements of cleanroom production. As suggested by Azhim et al. [165], reducing tissue exposure reduces the cost of the entire process and avoids post-production sterilization, particularly γ-irradiation, a procedure considered controversial and presumably detrimental for ANA stromal integrity [162-163]. As already showed [156], freeze-thaw cycles did not cause an increased loss of ECM proteins, even in human nerves. This protocol employs low concentrations of chemical detergents, which renders the graft in a more suitable environment for cellular proliferation and colonization. Using multiple detergents increases ECM

deprivation [184] but also allows a more extensive detergent clearance after decellularization due to lower detergent doses[185-187]. After the mentioned variation of exposure to sonication, from 5 minutes to 3 minutes' cycles, nerve ECM ultrastructure was nearly entirely preserved from damage, which affected other protocols in previously published studies [48, 163]. This sonication time was different from that used in the experimental in vivo studies [156] where 5 minutes' cycles achieved the best morphological results. As shown in Figures 23 and 24, human nerves cycle longer than 3 minutes appears to have detrimental effects on nerve histomorphological integrity. The described mild effect of sonication treatment on the biomechanical properties of decellularized nerves is in line with the medical literature. Azhim et al. [165, 188] demonstrated that sonication-based decellularization treatment preserved tissue biomechanical strength in several tissues, such as aorta and meniscus.

Sonication appears to be a proper coupling and complement to freeze-thawing according to the results of Szynkaruk et al. [55], who compared different decellularization protocols and showed that freeze-thawing eliminates cells only in the case where a long procedure, i.e., the Hudson method, is used; our experiments showed in human nerves that coupling sonication to freeze-thawing achieved an aseptic and cell-free allograft within a reasonable time. Furthermore, based on the negative microbiological results obtained in the various stages of nerve processing, the final product does not require post-production γ-ray sterilization, which is suspected to compromise tissue integrity and function [155, 158]. For transposition to clinical practice, the production of the decellularized nerve allografts proposed by our group will be feasible in a Class A or ISO 1 clean room, as defined by ISO 14644 classification.

3. CONCLUSION AND FUTURE PERSPECTIVES

The peripheral nerve injuries are a very frequent condition in the world population with discomfort and worsening of the patients' quality of life.

They are also an important cost for the national healthcare systems, both in terms of therapeutic expenses and of days of absence from work.

The current therapeutic options have known limits that we tried to overcome with this innovative method. Although the method was effective in terms of decellularization, it is still necessary to further investigate the study method before moving on to the clinical arena.

Our decellularization protocol, previously studied *in vivo* [172, 182] to functionally compare with the Hudson method and now optimized *in vitro* on human nerves, has the potential to achieve an aseptic, fully decellularized nerve tissue graft in shorter processing times than pre-existing protocols and without the need for post-production γ-ray sterilization. In addition, the entire method has the great advantage of being easily performed in certified aseptic environments, such as clean rooms. In Europe, there is a principle that "*the human body and its parts should not, as such, give rise to financial gain*" and only licensed Tissue Banks can distribute donated tissue; however, to date, none of these facilities produce and distribute cadaveric donor acellular nerve allografts for surgical reconstructions. This work represents the first step in providing a novel, safe, and inexpensive tool for use by European tissue banks to democratize the use of nerve tissue transplantation for nerve injury reconstruction.

The main future prospect deals with the possibility of replacing the cadaver collection with a nerve taken from a beating-heart donor. This would avoid any kind of degenerative process. In addition, it would allow the nerve to be taken in the operating room, guaranteeing an optimal sterility standard of the sample and the maintenance of the “sterile chain”.

Finally, the collection in the operating room would lead to: a larger availability of the nerves; the possibility of setting up a tissue bank, going to ameliorate the huge limits of the products present on the market, which are mainly logistic:

- Price: being reduced to the cost of the nerve production only;
- Shipping: shorter collection times and lower shipping costs;
- Selection of donors with European criteria.

A limitation of the study is the absence of clinical translation, even in the form of a pilot study, to evaluate the safety of ANA implantation. Therefore, after conducting two literature reviews [71, 144] and the two preclinical studies mentioned above [172, 182], our research group is now planning a preliminary clinical validation of human nerve grafts obtained by the described method.

4. REFERENCES

1. Huebner EA, Strittmatter SM. *Axon Regeneration in the Peripheral and Central Nervous Systems.* Results and problems in cell differentiation. 2009; 48:339-351.

2. Siemionow M, Brzezicki G. Chapter 8: *current techniques and concepts in peripheral nerve repair.* International Review of Neurobiology, vol. 87, no. C, pp. 141–172, 2009

3. Campbell WW. (2008). *Evaluation and management of peripheral nerve injury*. Clinical Neurophysiology. 119 (9): 1951–1965.

4. Arslantunali D, Dursun T, Yucel D, Hasirci N, Hasirci V. *Peripheral nerve conduits: technology update*. Med Devices (Auckl). 2014 Dec 1;7:405-24.

5. Beirowski B, Adalbert R, Wagner D, Grumme DS, Addicks K, Ribchester RR, Coleman MP. *The progressive nature of Wallerian degeneration in wild-type and slow Wallerian degeneration (WldS) nerves*. BMC Neurosci. 2005 Feb 1;6:6.

6. Pfister BJ, Gordon T, Loverde JR, Kochar AS, Mackinnon SE, Kacy Cullen D. *Biomedical engineering strategies for peripheral nerve repair: surgical applications, state of the art, and future challenges*. Critical Reviews in Biomedical Engineering. vol. 39, no. 2, pp. 81–124, 2011.

7. Lee SK, Wolfe SW. *Peripheral nerve injury and repair.* Journal of the American Academy of Orthopaedic Surgeons. vol. 8, no. 4, pp. 243–252, 2000

8. Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld, T. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nature Medicine. 2005. 11 (5): 572–577.

9. Vargas ME, Barres BA. *Why Is Wallerian Degeneration in the CNS So Slow?*. Annual Review of Neuroscience. 2007. 30 (1): 153–179.

10. Zimmerman UP, Schlaepfer WW. *Multiple forms of Ca-activated protease from rat brain and muscle*. The Journal of Biological Chemistry. 1984 259 (5): 3210–8.

11. Guertin AD. *Microanatomy of Axon/Glial Signaling during Wallerian Degeneration*. Journal of Neuroscience. 2005. 25 (13): 3478–3487.

12. Dailey AT, Avellino AM, Benthem L, Silver, J, Kliot M. *Complement depletion reduces macrophage infiltration and activation during Wallerian degeneration and axonal regeneration*. The Journal of Neuroscience. 1998. 18 (17): 6713–22.

13. Lundy-Ekman L. *Neuroscience: Fundamentals for Rehabilitation*. 3rd ed. Saunders, 2007.

14. Heumann R, Korsching S, Bandtlow C, Thoenen H. *Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection*. The Journal of Cell Biology. 1987. 104 (6): 1623–1631.

15. Lindholm D, Heumann R, Hengerer B, Thoenen H. *Interleukin 1 increases stability and transcription of mRNA encoding nerve growth factor in cultured rat fibroblasts*. The Journal of Biological Chemistry. 1988. 263 (31): 16348–51.

16. Thomas PK, King RH. *The degeneration of unmyelinated axons following nerve section: an ultrastructural study*. Journal of neurocytology. 1974. 3 (4): 497–512.

17. Araki T, Milbrandt J. *Ninjurin, a Novel Adhesion Molecule, Is Induced by Nerve Injury and Promotes Axonal Growth*. Neuron. 1996. 17 (2): 353–361.

18. Noble J, Munro CA, Prasad VS, Midha R. *Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries*. J Trauma. 1998. 45, 116.

19. Belkas JS, Shoichet MS, Midha R. *Peripheral nerve regeneration through guidance tubes*. Neurol Res. 2004. 26, 151.

20. Ashley WW Jr, Baty JD, Hollander T, Noetzel MJ, Park TS. *Long-term motor outcome analysis using a motor score composite following surgical brachial plexus repair.* J Neurosurg. 2007. 106(4 Suppl), 276.

21. Kelsey JL, Praemer A, Nelson L, Felberg A, Rice LM. *Upper Extremity Disorders: Frequency, Impact, and Cost*. New York: Churchill Livingstone, Inc., 1997

22. Menorca RMG, Fussel TS, Elfar JC. *Peripheral Nerve Trauma: Mechanisms of injury and recovery*. Hand Clin. 2013 August; 29(3): 317-330

23. Upton AR, McComas AJ. *The double crush in nerve entrapment syndromes*. Lancet. 1973. 2(7825):359-62.

24. Zochodne DW, Levy D. *Nitric Oxide in damage, disease and repair of the peripheralnervous system*. Cell Mol Bio (Nosi-le-grand). 2005; 51:255-67

25. Dellon AL, Mackinnon SE, Seiler WA. *Susceptibility of the diabetic nerve to chronic compression*. Ann Plast Surg. 1988; 20:117-119.

26. Seddon HJ. *A classification of nerve injuries*. Brit Med J. 1942; ii:237-239.

27. Payton OD, Di Fabio RP. *Manual of physical therapy*. Churchill Livingstone Inc. Page 24

28. Burnett MG, Zager EL. *Pathophysiology of peripheral nerve injury: A brief review*. Neurosurgical focus. 2004. 16 (5): E1.

29. Sunderland S. *A classification of peripheral nerve injuries producing loss of function*. Brain. 1951;4: 491-516.

30. Hems T. *Nerve injury: Classification, clinical assessment, investigation, and management.* In: Handchirurgie Weltweit e.V., editor. Living Textbook of Hand Surgery. Version. 2016. Cologne: gms; 2014.

31. Dong-Hee L. *Clinical Efficacy of Electroneurography in Acute Facial Paralysis.* Journal of Audiology & Otology. 2016. 20. 8. 10.7874.

32. Medical Research Council. *Peripheral nerve injuries: Medical Research Council special report series no. 282*. London Her Majesty's Stationary Office, 1954.

33. Lundborg G, Rosen B. *The two-point discrimination test--time for a re-appraisal?*. J Hand Surg Br. 2004;29(5): 418-22

34. Slutsky DJ, Hentz VR, Jabaley M. *Primary nerve repair Peripheral nerve surgery: Practical applications in the upper extremity*. Churchill Livingstone Elsevier.; 2006. pp. 23–38.

35. Kline DG. *Physiological and clinical factors contributing to the timing of nerve repair*. Clin Neurosurg. 1977;24:425–55.

36. Brown PW. *Factors influencing the success of the surgical repair of peripheral nerves*. Surg Clin North Am. 1972;52:1137–55

37. Seddon HJ. *Surgical Disorders of the Peripheral Nerves*. Churchill Livingstone; 1972.

38. Ray WZ, Mackinnon SE. *Management of nerve gaps: Autografts, allografts, nerve transfers, and end-to-side neurorrhaphy*. Exp Neurol. 2010; 223(1): 77–85.

39. Weber RV, Mackinnon SE. *Bridging the neural gap*. Clin Plast Surg 2005; 32(04):605–616, viii.

40. Zhao Q, Dahlin LB, Kanje M, Lundborg G. *Specificity of muscle reinnervation following repair of the transected sciatic nerve.* J Hand Surg Br. 1992. 17(3):257-61.

41. Driscoll PJ, Glasby MA, Lawson GM. *An in vivo study of peripheral nerves in continuity: biomechanical and physiological responses to elongation*. J Orthop Res 2002; 20(02):370–375.

42. Millesi H. *Factors affecting the outcome of peripheral nerve surgery.* Microsurgery 2006;26(04):295–302.

43. Rasuli L. *Current Concept in Adult Peripheral Nerve and Brachial Plexus Surgery*. Journal of Brachial Plexus and Peripheral Nerve Injury Vol. 12 No. 2017 pp e7-e14.

44. Norkus T, Norkus M, Ramanauskas T. *Donor, recipient and nerve grafts in brachial plexus reconstruction: anatomical and technical features for facilitating the exposure*. Surg Radiol Anat 2005. 27:524–530.

45. Terzis J, Faibisoff B, Williams B. *The nerve gap: suture under tension vs. graft.* Plast Reconstr Surg. 1975. 56(2):166-70.

46. Dorsi MJ, Chen L, Murinson BB, Pogatzki-Zahn EM, Meyer RA, Belzberg AJ. *The tibial neuroma transposition (TNT) model of neuroma pain and hyperalgesia*. Pain 2008. 134:320–334.

47. Kehoe S, Zhang XF, Boyd D. *FDA approved guidance conduits and wraps for peripheral nerve injury: a review of materials and efficacy*. Injury. 2012. 43:553–572.

48. Karabekmez FE, Duymaz A, Moran SL. *Early clinical outcomes with the use of decellularized nerve allograft for repair of sensory defects within the hand.* Hand. 2009. vol. 4, no. 3, pp. 245–249.

49. Cho MS, Rinker BD, Weber RV, Chao JD, Ingari JV, Brooks D, Buncke GM. *Functional outcome following nerve repair in the upper extremity using processed nerve allograft*. The Journal of Hand Surgery. 2012. vol. 37, no. 11, pp. 2340–2349, 2012.

50. Fox IK, Mackinnon SE. *Experience with nerve allograft transplantation*. Semin Plast Surg. 2007. 21, 242.

51. Moore AM, Macewan M, Santosa KB, Chenard KE, Ray WZ, Hunter DA, Mackinnon SE, Johnson PJ. *Acellular nerve allografts in peripheral nerve regeneration: a comparative study*. Muscle Nerve. 2011. 44, 221.

52. Evans PJ, Mackinnon SE, Midha R, Wade JA, Hunter DA, Nakao, Y, Gregory MT, Hare MD. *Regeneration across cold preserved peripheral nerve allografts*. Microsurgery. 199. 19, 115.

53. Strasberg SR, Hertl MC, Mackinnon SE, Lee CK, Watanabe O, Tarasidis G. *Peripheral nerve allograft preservation improves regeneration and decreases systemic cyclosporin A requirements*. Exp Neurol. 1996. 139, 306.

54. Grand AG, Myckatyn TM, Mackinnon SE, Hunter DA. *Axonal regeneration after cold preservation of nerve allografts and immunosuppression with tacrolimus in mice.* J Neurosurg. 2002. 96, 924.

55. Szynkaruk M, Kemp SWP, Wood MD, Gordon T, Borschel GH. *Experimental and Clinical Evidence for Use of Decellularized Nerve Allografts in Peripheral Nerve Gap Reconstruction*. Tissue Engineering: Part B Volume 19, Number 1, 2013.

56. Hudson TW, Zawko S, Deister C, Lundy S, Hu CY, Lee K. *Optimized acellular nerve graft is immunologically tolerated and supports regeneration*. Tissue Engineering. 2004. 10, 1641.

57. Johnson PC, Duhamel RC, Meezan E, Brendel K. *Preparation of cell-free extracellular matrix from human peripheral nerve*. Muscle Nerve. 1982. 5, 335.

58. Sondell M, Lundborg G, Kanje M. *Regeneration of the rat sciatic nerve into allografts made acellular through chemical extraction*. Brain Res. 1998 795, 44.

59. Hudson TW, Liu SY, Schmidt CE. *Engineering an improved acellular nerve graft via optimized chemical processing*. Tissue Engineering. 2004. 10, 1346.

60. Axogen site, *www.AxogenInc.com/for-patients/ways-to-diagnose-peripheral-nerve-injuries-pni* .

61. Moore A, Kasukurthi R, Magill CK, Farhadi FH, Borschel GH, Mackinnon SE. *Limitations of conduits in peripheral nerve repairs*. Hand. 2009. 4(2):180-6.

62. Saheb-Al-Zamani M, Yan Y, Farber SJ, et al. *Limited regeneration in long acellular nerve allografts is associated with increased Schwann cell senescence*. Exp Neurol. 2013;247(Sep):165-177.

63. Wood PM. *Separation* of functional Schwann cells and neurons from normal peripheral nerve tissue. Brain Res. 1976; 115(3):361–75.

64. Johnson PJ, Wood MD, Moore AM, Mackinnon SE. *Tissue engineered constructs for peripheral nerve surgery*. Eur Surg. 2013. 45(3).

65. Evans MJ, Kaufman MH. *Establishment in culture of pluripotential cells from mouse embryos*. Nature. 1981; 292(5819):154–6.

66. Martin GR. *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells*. Proc Natl Acad Sci U S A. 1981; 78(12):7634–8.

67. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. *Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response*. Faseb J. 2007; 21(7):1345–57.

68. Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechav G. *Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient*. PLoS Med. 2009; 6(2):e1000029.

69. Jiang L, Jones S, Jia X. *Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities*. Int. J. Mol. Sci. 2017, 18, 94.

70. Tse C, Whiteley R, Yu T, Stringer J, MacNeil S, Haycock JW, Smith PJ. *Inkjet printing Schwann cells and neuronal analogue NG108-15 cells*. Biofabrication. 2016 Mar 1;8(1):015017.

71. Pedrini FA, Boriani F, Bolognesi F, Fazio N, Marchetti C, Baldini N. *Cell-Enhanced Acellular Nerve Allografts for Peripheral Nerve Reconstruction: A Systematic Review and a Meta-Analysis of the Literature*. Neurosurgery. 2019 Nov 1;85(5):575-604.

72. Aloe L. *Rita Levi Montalcini: The discovery of nerve growth factor and modern neurobiology.* Trends Cell Biol 2004;14:395–399.

73. Gravvanis AI, Tsoutsos DA, Tagaris GA, Papalois AE, Patralexis CG, Iconomou TG, Panayotou PN, Ioannovich JD. *Beneficial effect of nerve growth factor-7S on peripheral nerve regeneration through inside-out vein grafts: An experimental study.* Microsurgery 2004; 24:408–415.

74. Skaper SD. *Nerve growth factor: A neurokine orchestrating neuroimmune-endocrine functions*. Mol Neurobiol 2001;24:183–199.

75. Yang Y, De Laporte L, Rives CB, Jang JH, Lin WC, Shull KR, Shea LD. *Neurotrophin releasing single and multiple lumen nerve conduits.* J Control Release 2005;104:433–446.

76. Piotrowicz A, Shoichet MS. *Nerve guidance channels as drug delivery vehicles.* Biomaterials 2006;27:2018–2027.

77. Lamm XM, Duenas ET, Cleland JL. *Encapsulation and stabilization of NGF into PLGA microspheres.* J Pharm Sci 2001;90:1356–1364.

78. Menei P, Daniel V, Montero-Menei C, Brouillard M, Barthelaix BP, Benoit JP. *Biodegradation and brain tissue reaction to poly (D,L-lactide-co-glycolide) microspheres.* Biomaterials 1993;14:470–478.

79. Pean JM, Venier-Julienne MC, Boury F, Menei P, Denizot B, Benoit JP. *NGF release from poly (D,L-lactide-co-glycolide) microspheres: Effect of some formulation parameters on encapsulated NGF stability*. J Control Release 1998;56:175–187.

80. Xu XY, Yu H, Mao HQ, Gao SJ, Leong KW, Wang S. *Polyphosphoester microspheres for sustained release of biologically active nerve growth factor.* Biomaterials 2002;23:3765–3772.

81. Boyer RB, Sexton KW, Rodriguez-Feo CL, Nookala R, Pollins AC, Cardwell NL, et al. *Adjuvant neurotrophic factors in peripheral nerve repair with chondroitin sulfate proteoglycan-reduced acellular nerve allografts*. J Surg Res. 2015;193:969-977.

82. Yu H, Peng J,Guo Q, Zhang L, Li Z, Zhao B, et al. *Improvement of peripheral nerve regeneration in acellular nerve grafts with local release of nerve growth factor.* Microsurgery. 2009;29:330-33.

83. Ovalle FJ, Patel A, Pollins A, de la Torre J, Vasconez L, Hunt TR III, et al. *A simple technique for augmentation of axonal ingrowth into chondroitinase-treated acellular nerve grafts using nerve growth factor*. Ann Plast Surg. 2012;68: 518-524.

84. J. Boyd, T. Gordon. *Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury*. Mol. Neurobiol. 27 (3) (2003) 277–324.

85. M. Jubran, J. Widenfalk. *Repair of peripheral nerve transections with fibrin sealant containing neurotrophic factors*. Exp. Neurol. 181 (2) (2003) 204–212.

86. J. Boyd, T. Gordon. *GDNF and BDNF sustain the axonal regeneration of chronically axotomized motoneurons in vivo*. Exp. Neurol. 183 (2003) 610–619.

87. R. Eggers, W. Hendriks, M. Tannemaat, J. van Heerikhuize, C. Pool, T. Carlstedt. *Neuroregenerative effects of lentiviral vector-mediated GDNF expression in reimplanted ventral roots*. Mol. Cell. Neurosci. 39 (1) (2008) 105–117.

88. Y. Wu-Fienberg, A.M. Moore, L.M. Marquardt, P. Newton, P.J. Johnson, S.E. Mackinnon, et al. *Viral transduction of primary Schwann cells using a Cre-lox system to regulate GDNF expression*. Biotechnol. Bioeng. 111 (9) (2014) 1886–1894.

89. I. Guilhem, B. Balkau, F. Lecordier, J. Malecot, S. Elbadii, A. Leguerrier. *Insulin pump failures are still frequent: a prospective study over 6 years from 2001 to 2007*. Diabetologia 52 (12) (2009) 2662–2664

90. Tajdaran K, Gordon T, Wood MD, Shoichet MS, Borschel GH. *A glial cell line-derived neuro- trophic factor delivery system enhances nerve regeneration across acellular nerve allografts*. Acta Biomater. 2016;29:62-70.

91. Hsiong SX, Mooney DJ. *Regeneration of vascularized bone*. Periodontol 2000 2006;41:109–122.

92. van Weel V, van Tongeren RB, van Hinsbergh VW, van Bockel JH, Quax PH. *Vascular growth in ischemic limbs: A review of mechanisms and possible therapeutic stimulation*. Ann Vasc Surg 2008;22: 582–597.

93. Katsube K, Bishop AT, Simari RD, Yla-Herttuala S, Friedrich PF. *Vascular endothelial growth factor (VEGF) gene transfer enhances surgical revascularization of necrotic bone*. J Orthop Res 2005;23: 469–474.

94. Larsen M, Willems WF, Pelzer M, Friedrich PF, Dadsetan M, Bishop AT. *Fibroblast growth factor-2 and vascular endothelial growth factor mediated augmentation of angiogenesis and bone formation in vascularized bone allotransplants*. Microsurgery 2014;34: 301–307.

95. Wongtrakul S, Bishop AT, Friedrich PF. *Vascular endothelial growth factor promotion of neoangiogenesis in conventional nerve grafts*. J Hand Surg Am 2002;27:277–285.

96. Hattori Y, Doi K. *Vascularized ulnar nerve graft*. Tech Hand up Extrem Surg 2006;10:103–106.

97. Weber RV, Mackinnon SE. *Bridging the neural gap*. Clin Plast Surg 2005;32:605–616, viii.

98. Sondell M, Lundborg G, Kanje M. *Vascular endothelial growth factor stimulates Schwann cell invasion and neovascularization of acellular nerve grafts*. Brain Res 1999;846:219–228.

99. Sondell M, Sundler F, Kanje M. *Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor*. Eur J Neurosci 2000;12:4243–4254.

100. Hobson MI, Green CJ, Terenghi G. *VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy*. J Anat 2000;197(Pt 4):591–605.

101. Giusti G, Lee J-Y, Kremer T, Friedrich P, Bishop AT, Shin AY. *The influence of vascularization of transplanted processed allograft nerve on return of motor function in rats*. Microsurgery. 2016; 36:134-143.

102. Nakamura, T., Nawa, K., Ichihara, A., 1984. *Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats*. Biochem. Biophys. Res. Commun. 122, 1450–1459.

103. Gohda, E., Tsubouchi, H., Hirono, S., 1988. *Purification and partial characterization of Hepatocyte growth factor from plasma of a patient with hepatic failure*. J. Clin. Invest. 81, 414–419.

104. Nakamura, T., Teramoto, H., Ichihara, A., 1986. *Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary culture*. Proc. Natl. Acad. Sci. U.S.A. 83, 6489–6493.

105. Matsumoto, K., Nakamura, T., 1996. *Emerging multipotent aspects of hepatocyte growth factor*. J. Biochem. 119 (4), 591–600.

106. Maina, F., Klein, R., 1999. *Hepatocyte growth factor, a versatile signal for developing neurons*. Nature Neurosci. 2 (3), 213–217.

107. Yang, X.-M., Toma, J.G., Bamji, S.X., et al. 1998. *Autocrine hepatocyte growth factor provides a local mechanism for promoting axonal growth*. 18(20), 8369–81.

108. Davey, F., Hilton, M., Davies, A.M., 2000. *Cooperation between HGF and CNTF in promoting the survival and growth of sensory and parasympathetic neurons*. Mol. Cell Neurosci. 15 (1), 79–87.

109. Oppenheim, R.W., 1996. *Neurotrophic survival molecules for motoneurons: an embarrassment of riches*. Neuron. 17, 195–197.

110. Ebens, A., Brose, K., Leonardo, E.D., Hanson Jr., M.G., Bladt, F., Birchmeier, C., Barres, B.A., Tessier-Lavigne, M., 1996. *Hepatocyte growth factor/scatter factor is an axonal chemoattractant and neurotrophic factor for spinal motor neurons*. Neuron. 17, 1157–1172.

111. Jung, W., Castren, E., Odenthal, M., et al., 1994. *Expression and functional interaction of hepatocyte growth factor-scatter factor and its receptor c-met in mammalian brain*. J. Cell Biol. 126, 485–494.

112. Krasnoselsky, A., Massay, M.J., DeFrances, M.C., Michalopoulos, G., Zarnegar, R., 1994. *Hepatocyte growth factor is a mitogen for Schwann cells and is present in neurofibromas*. J. Neurosci. 14, 7284–7290.

113. Hashimoto, N., Yamanaka, H., Fukuoka, T., Dai, Y., Obata, K., Mashimo, T., Noguchi, K., 2001. *Expression of HGF and cMet in the peripheral nervous system of adult rats following sciatic nerve injury*. Neuroreport. 12, 1403–1407.

114. Okura, Y., Arimoto, H., Tanuma, N., Matsumoto, K., Nakamura, T., Yamashima, T., Miyazawa, T., Matsumoto, Y., 1999. *Analysis of neurotrophic effect of hepatocyte growth factor in the adult hypoglossal nerve axotomy model*. Eur. J. Neurosci. 11, 4139–4144.

115. Li Z, Peng J, Wang G, Yang Q, Yu H, Guo Q, et al. *Effects of local release of hepatocyte growth factor on peripheral nerve regeneration in acellular nerve grafts*. Exp Neurol. 2008;214:47-54.

116. Kotsovilis, S., Markou, N., Pepelassi, E., and Nikolidakis, D. *The adjunctive use of platelet-rich plasma in the therapy of periodontal intraosseous defects: a systematic review*. J Periodontal Res 45, 428, 2010.

117. Andia, I., Sanchez, M., and Maffulli, N. *Joint pathology and platelet-rich plasma therapies*. Expert Opin Biol Ther 12, 7, 2012.

118. Arora, N.S., Ramanayake, T., Ren, Y.F., and Romanos, G.E. *Platelet-rich plasma: a literature review*. Implant Dent 18, 303, 2009.

119. Zheng, C., Zhu, Q., Liu, X., Huang, X., He, C., Jiang, L., Quan, D., Zhou, X., and Zhu, Z. *Effect of platelet-rich plasma (PRP) concentration on proliferation, neurotrophic function and migration of Schwann cells in vitro*. J Tissue Eng Regen Med 2013.

120. Zheng C, Zhu Q, Liu X, Huang X, He C, Jiang L, et al. *Improved peripheral nerve regeneration using acellular nerve allografts loaded with platelet-rich plasma*. Tissue Eng Part A. 2014;20: 3228-3240.

121. Rao, C.N.R., Biswas, K., Subrahmanyam, K.S., et al., 2009. *Graphene, the new nanocarbon*. Mater. Chem. 19, 2457–2469.

122. Muhammad, G., Rashid, I., Firyal, S., Saqib, M., 2017. *Successful treatment of idiopathic generalized subcutaneous emphysema in kajli a ram by large bore injection needle*. Matrix Sci. Medica 1 (1), 01–02.

123. Rahman, A.S., Kahar, A.A., Mansor, A., Murni, D.L., Hussin, A., Sharifudin, A.S., Hun, T. G., Rashid, A.N.Y., Othaman, M.A., Long, K., 2017. *Identification of potential indigenous microbe from local fermented vegetables with antimicrobial activity*. Galeri Warisan Sains 1 (1), 01–03.

124. Barnard, A.S., Snook, I.K., 2008. *Thermal stability of grapheme edge structure and grapheme nanoflakes*. Chem. Phys. 128, 09470–09477.

125. Wang, Y., Li, Y.M., Tang, L.H., et al., 2009. *Application of graphene-modified electrode for selective detection of dopamine*. Electrochem. Commun. 11, 889–892.

126. Wang, C., Feng, C., Gao, Y.J., et al., 2011. *Preparation of a grapheme-based magnetic nanocomposite for the removal of an organic dye from aqueous solution*. Chem. Eng. J. 173, 92–97.

127. Luo, Y.B., Shi, Z.G., Gao, Q., et al., 2011. *Magnetic retrieval of graphene: extraction of sulfonamide antibiotics from environmental water samples*. J. Chromatogr. A 1218, 1353–1358.

128. Barroso-Bujans, F., Cerveny, S., Verdejo, R., et al., 2010. *Permanent adsorption of organic solvents in graphite oxide and d its effect on the thermal exfoliation*. Carbon 48 (4), 1079–1087.

129. Sun, X., Liu, Z., Weisher, K., et al., 2008. *Nano-graphene oxide for cellular imaging and drug delivery*. Nano Res. 1 (3), 203–212.

130. Jeong, H.K., Lee, Y.P., Lahaye, R.J., 2008. *Evidence of graphitic AB stacking order of graphite oxides*. J. Am. Chem. Soc. 130 (4), 1362–1366.

131. Wang Q, Chen J, Niu Q, Fu X, Sun X, Tong X. *The application of graphene oxidized combining with decellularized scaffold to repair of sciatic nerve injury in rats*. Saudi Pharm J. 2017;25:469-476.

132. AI-Majed AA, Tam SL, Gordon T (2004). *Electrical stimulation accelerates and enhances expression of regeneration-associated genes in regenerating rat femoral motoneurons*. Cell Mol Neurobiol 24(3):379–402.

133. Bervar M (2005). *Effect of weak, interrupted sinusoidal low frequency magnetic field on neural regeneration in rats: functional evaluation*. Bioelectromagnetics 26(5):351–356.

134. Nicolau RA, Martinez MS, Rigau J, et al (2004). *Effect of low power 655 nm diode laser irradiation on the neuromuscular junctions of the mouse diaphragm*. Lasers Surg Med 34(3):277–284.

135. Raso VV, Barbieri CH, Mazzer N, et al (2005). *Can therapeutic ultrasound influence the regeneration of peripheral nerves?* J Neurosci Methods 142(2):185–192.

136. Liao WJ, Zeng G (1995). *Experimental study of effect of muscle weight and muscular tissue lipid peroxidation on injured peripheral nerve*. J China Rehabil Med l0(5):209.

137. Zhang LX, Tong XJ, Sun XH, Tong L, Gao J, Jia H, et al. *Experimental study of low dose ultra- shortwave promoting nerve regeneration after acellular nerve allografts repairing the sciatic nerve gap of rats*. Cell Mol Neurobiol. 2008;28: 501-509.

138. Al-Majed AA, Neumann CM, Brushart TM, Gordon T. *Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration*. J Neurosci. 2000b; 20:2602–2608.

139. Geremia NM, Gordon T, Al-Majed AA, Brushart TM, Verge VMK. *Brief electrical stimulation promotes sensory neuron regeneration and intrinsic growth-associated gene expression*. Society for Neuroscience. 2005 Program No. 28.26.

140. Al-Majed AA, Brushart TM, Gordon T. *Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons*. Eur J Neurosci. 2000a; 12:4381– 4390.

141. Ide C, Tohyama K, Tajima K, Endoh K, Sano K, Tamura M, Mizoguchi A, Kitada M, Morihara T, Shirasu M. *Long acellular nerve transplants for allogeneic grafting and the effects of basic fibroblast growth factor on the growth of regenerating axons in dogs: a preliminary report*. Exp Neurol. 1998; 154:99–112.

142. Krekoski CA, Neubauer D, Zuo J, Muir D. *Axonal regeneration into acellular nerve grafts is enhanced by degradation of chondroitin sulfate proteoglycan*. J Neurosci. 2001; 21:6206–6213.

143. English AW, Schwartz G, Meador W, Sabatier MJ, Mulligan A. *Electrical stimulation promotes peripheral axon regeneration by enhanced neuronal neurotrophin signaling*. Dev Neurobiol. 2007;67:158-172.

144. Boriani F, Fazio N, Bolognesi F, Pedrini FA, Marchetti C, Baldini N. *Noncellular Modification of Acellular Nerve Allografts for Peripheral Nerve Reconstruction: A Systematic Critical Review of the Animal Literature*. World Neurosurg. 2019 Feb;122:692-703.e2.

145. Rasulic, L. *Current concept in adult peripheral nerve and brachial plexus surgery*. J. Brachial Plex. Peripher. Nerve Inj. 2017, 12, e7–e14.

146. Grinsell, D.; Keating, C.P. *Peripheral nerve reconstruction after injury: A review of clinical and experimental therapies*. Biomed. Res. Int. 2014, 2014, 698256.

147. Santos D, Gonzalez-Perez F, Giudetti G, Micera S, Udina E, Del Valle J, Navarro X. *Preferential enhancement of sensory and motor axon regeneration by combining extracellular matrix com- ponents with neurotrophic factors*. Int J Mol Sci 2016;18:65.

148. Jia H, Wang Y, Tong XJ, Liu GB, Li Q, Zhang LX, Sun XH. *Sciatic nerve repair by acellular nerve xenografts implanted with BMSCs in rats xenograft combined with BMSCs*. Synapse 2012;66:256–269.

149. Faroni A, Mobasseri SA, Kingham PJ, Reid AJ. *Peripheral nerve regeneration: Experimental strategies and future perspectives*. Adv Drug Deliv Rev 2015;82–83:160–167.

150. Nichols CM, Brenner MJ, Fox IK, Tung TH, Hunter DA, Rickman SR, Mackinnon SE. *Effects of motor versus sensory nerve grafts on peripheral nerve regeneration*. Exp Neurol 2004;190:347–355.

151. Bellamkonda RV. *Peripheral nerve regeneration: an opinion on channels, scaffolds and anisotropy*. Biomaterials 2006;27:3515–3518.

152. Tang P, Chauhan A. *Decellular Nerve Allografts*. J Am Acad Orthop Surg 2015;23:641–647.

153. Moore AM, MacEwan M, Santosa KB, Chenard KE, Ray WZ, Hunter DA, Mackinnon SE, Johnson PJ. *Acellular nerve allografts in peripheral nerve regeneration: A comparative study*. Muscle Nerve 2011;44:221–234.

154. Harrell, C.R.; Djonov, V.; Fellabaum, C.; Volarevic, V. *Risks of using sterilization by gamma radiation: The other side of the coin*. Int. J. Med. Sci. 2018, 15, 274.

155. Gut, G.; Marowska, J.; Jastrzebska, A.; Olender, E.; Kaminski, A. *Structural mechanical properties of radiation-sterilized human bone-tendon-bone grafts preserved by different methods*. Cell Tissue Bank. 2016, 17, 277–287.

156. Boriani F, Fazio N, Fotia C, Savarino L, Nicoli Aldini N, Martini L, Zini N, Bernardini M, Baldini N. *A novel technique for decellularization of allogenic nerves and in vivo study of their use for peripheral nerve reconstruction*. J Biomed Mater Res A. 2017. 105(8):2228-2240.

157. Harrell CR, Djonov V, Fellabaum C, Volarevic V. *Risks of Using Sterilization by Gamma Radiation: The Other Side of the Coin*. International Journal of Medical Sciences. 2018; 15(3): 274-279.

158. Nguyen H, Morgan DAF, Forwood MR. *Sterilization of allograft bone: effects of gamma irradiation on allograft biology and biomechanics*. Cell Tissue Banking. 2007. 8:93–105.

159. Hudson, T.W.; Liu, S.Y.; Schmidt, C.E. *Engineering an improved acellular nerve graft via optimized chemical processing*. Tissue Eng. 2004, 10, 1346–1358.

160. Sandle T. *People in cleanrooms: Understanding and monitoring the personnel factor. Institute of Validation Technology*.
http:// www.ivtnetwork.com/article/people-cleanrooms-understanding- and-monitoring-personnel-factor.

161. Feigal EG, Tsokas K, Viswanathan S, Zhang J, Priest C, Pearce J, Mount N. *Proceedings: international regulatory considerations on development pathways for cell therapies*. Stem Cells Transl Med. 2014. 3:879–887.

162. Yang B, Zhang Y, Zhou L, Sun Z, Zheng J, Chen Y, Dai Y. *Development of a porcine bladder acellular matrix with well-preserved extracellular bioactive factors for tissue engineering*. Tissue Eng C Methods 2010;16:1201–1211.

163. Reing JE, Brown BN, Daly KA, Freund JM, Gilbert TW, Hsiong SX, Huber A, Kullas KE, Tottey S, Wolf MT, Badylak SF. *The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds*. Biomaterials 2010;31:8626–8633.

164. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, Kotton D, Vacanti JP. *Regeneration and orthotopic transplantation of a bioartificial lung*. Nat Med 2010;16:927e933.

165. Azhim A, Syazwani N, Morimoto Y, Furukawa K, Ushida T. *The use of sonication treatment to decellularize aortic tissues for preparation of bioscaffolds*. J Biomater. Appl 2014. 29:130–141.

166. Haase SC, Rovak JM, Dennis RG, Kuzon WM, Jr., Cederna PS. *Recovery of muscle contractile function following nerve gap repair with chemically acellularized peripheral nerve grafts*. Reconstr Microsurg 2003;19:241–248.

167. Danielsen N, Kerns JM, Holmquist B, Zhao Q, Lundborg G, Kanje M. *Predegeneration enhances regeneration into acellular nerve grafts*. Brain Research. 1995. 681 105-108 Elsevier.

168. Tomita K, Hata Y, Kubo T, et al. *Effects of the in vivo predegenerated nerve graft on early Schwann cell migration: quantitative analysis using S100-GFP mice*. Neurosci Lett. 2009;461:36–40.

169. Danielsen, N., Kerns, J.M., Holmquist, B., Zhao, Q., Lundborg, G. and Kanje M., *Predegenerated nerve grafts enhance regeneration by shortening the initial delay period*. Brain Res., 666 (1994) 250-254.

170. Sugita N, Ishida O, Ikuta Y, et al. *Interposed autologous nerve segment stimulates nerve regeneration in peripheral nerve allografts in a rat model*. J Reconstr Microsurg. 2004;20:167–174.

171. Boriani F, Savarino L, Fazio N, Pedrini FA, Fini M, Nicoli Aldini N, Martini L, Zini N, Bernardini M, Baldini N. *Neuroregenerative enhancement of a novel acellular nerve allograft through a new-concept microsurgical technique*. *Submitted* Plastic and Reconstructive Surgery.

172. Boriani F, Savarino L, Fazio N, Pedrini FA, Fini M, Nicoli Aldini N, Martini L, Zini N, Bernardini M, Bolognesi F, Marchetti C, Baldini N. *Auto-Allo Graft Parallel Juxtaposition for Improved Neuroregeneration in Peripheral Nerve Reconstruction Based on Acellular Nerve Allografts*. Ann Plast Surg. 2019 Sep;83(3):318-325.

173. Isaacs, J.; Mallu, S.; Patel, G.; Kite, A.; Shah, S.; Graham, G.P. *Implantation of acellular nerve allograft using nerve connectors.* HAND 2019, 15, 625–630.

174. Li, L.; Yang, J.; Qin, B.; Wang, H.; Yang, Y.; Fang, J.; Chen, G.; Liu, X.; Tu, Z.; Gu, L. *Analysis of human acellular nerve allograft combined with contralateral C7 nerve root transfer for restoration of shoulder abduction and elbow flexion in brachial plexus injury: A mean 4-year follow-up*. J. Neurosurg. 2020, 132, 1914–1924.

175. Leyngold AM, Yen MT, Tian J et al. *Minimally invasive corneal neurotization with acellular nerve allograft: surgical technique and clinical outcomes*. Ophthalmic Plast Reconstr Surg 2019;35:133-40.

176. Yampolsky, A.; Ziccardi, V.; Chuang, S.K. *Efficacy of acellular nerve allografts in trigeminal nerve reconstruction*. J. Oral Maxillofac. Surg. 2017, 75, 2230–2234.

177. Zhu, S.; Liu, J.; Zheng, C.; Gu, L.; Zhu, Q.; Xiang, J.; He, B.; Zhou, X.; Liu, X. *Analysis of human acellular nerve allograft reconstruction of 64 injured nerves in the hand and upper extremity: A 3 year follow-up study*. J. Tissue Eng. Regen. Med. 2017, 11, 2314–2322.

178. Hong, T.; Wood, I.; Hunter, D.A.; Yan, Y.; Mackinnon, S.E.; Wood, M.; Moore, A.M. *Neuroma management: Capping nerve in-juries with an acellular nerve allograft can limit axon regeneration*. HAND 2019, 16, 157–163.

179. Isaacs J, Patel G, Mallu S, et al. Effect of reverse end-to-side (supercharging) neurotization in long processed acellular nerve allograft in a rat model. J Hand Surg Am 2019; 44(5):419.e1-419.e10.

180. Yang JT, Fang JT, Li L, et al. *Contralateral C7 transfer combined with acellular nerve allografts with differentiated adipose stem cells for repairing upper brachial plexus injuries in rats*. Neural Regen Res 2019;14:1932-40.

181. Zhu S, Liu J, Zheng C. *Analysis of human acellular nerve allograft reconstruction of 64 injured nerves in the hand and upper extremity: a 3-year follow-up study*. J Tissue Eng Regen med 2017;11:2314-22.

182. Boriani F. Fazio N, Fotia C. et al. *Acellular nerve allografts for peripheral nerve defects bridging: an innovative decellularization method*. Proceedings of the 3rd Annual EURAPS Research Council Meeting May 28-29, 2014 - Isle of Ischia Napoli (Italy). Medimond, Bologna 2014.

183. Philips C, Campos F, Roosens A, Sánchez-Quevedo MDC, Declercq H, Carriel V. *Qualitative and Quantitative Evaluation of a Novel Detergent-Based Method for Decellularization of Peripheral Nerves*. Ann Biomed Eng. 2018; 46:1921-1937.

184. Alhamdani MS, Schröder C, Werner J, Giese N, Bauer A, Hoheisel JD. *Single-step procedure for the isolation of proteins at near-native conditions from mammalian tissue for proteomic analysis on antibody microarrays*. J Proteome Res 2010; 9:963e971.

185. Crapo PM, Gilbert TW, Badylak SF. *An overview of tissue and whole organ decellularization processes*. Biomaterials 2011; 32:3233-3243.

186. Cebotari S, Tudorache I, Jaekel T, Hilfiker A, Dorfman S, Ternes W, Haverich A, Lichtenberg A. *Detergent decellularization of heart valves for tissue engineering: toxicological effects of residual detergents on human endothelial cells*. Artif Organs.

187. Rieder E, Kasimir MT, Silberhumer G, Seebacher G, Wolner E, Simon P, Weigel G. *Decellularization protocols of porcine heart valves differ importantly in the efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells*. J Thorac Cardiovasc Surg 2004;127:399-405.

188. Azhim A, Ono T, Fukui Y, Morimoto Y, Furukawa K, Ushida T. *Preparation of decellularized meniscal scaffolds using sonication treatment for tissue engineering*. Conf Proc IEEE Eng Med Biol Soc 2013; 2013:6953-6956.