Diploma thesis

Cultivation of human Schwann Cells and subsequent Colonisation of the Polymer OPTIMAIX 2D

Kultivierung humaner Schwann`scher Zellen in Zellkultur und auf dem Polymer Optimaix 2D zur Erstellung eines tissue engineered bioartifiziellen Implantats für die Therapie von peripheren Nervenverletzungen mit Defektstrecken

submitted by

Elisabeth Katharina Trapp

Date of birth : 03-26-1988

attaining the academic degree

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

Medical University of Graz

conducted at the

Division of Plastic, Aesthetic and Reconstructive Surgery

under supervision of

Ass. Dr. Gerlinde Weigel,

Univ. Prof. Dr. Stephan Spendel

University Hospital Graz,

Division of Plastic, Aesthetic and Reconstructive Surgery

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Abstract

Background: Peripheral nerves are frequently severely injured as a result of trauma. This causes deficiency in sensory and motor qualities within the affected innervation area. First line therapy in treating peripheral nerve injury accompanied by nervous tissue defect is the autologous nerve graft. This method however is fundamentally flawed for two major reasons. The first being that harvesting nerves results in innervations deficiency at the donor site, and secondly, there is limited availability of appropriate grafting nerves that can be utilised for reconstruction. A tissue engineered nerve construct could represent a solution for these problems. In animal models it was shown that scaffolds containing Schwann cells have highly positive effects on regenerating nerves. To harness these results for treating peripheral nerve injury in humans it is necessary to harvest and culture Schwann cells from human peripheral nerves and finally apply them to a scaffold.

Methods: Within this pilot study human Schwann cells were obtained by explant-reexplant technique and cultured from human peripheral nerves. For harvesting human Schwann cells, peripheral nerves were explanted from amputates of patients at the Department of Plastic, Aesthetic and Reconstructive Surgery at the Medical University Graz. The isolated nerves were dissected from the epineurium and either cut into 1 mm sized pieces or the fascicles itself were pulled out of the nervous explant and cultured under standard conditions. After forming a confluent monolayer of cells, the tissue and respectively the cells of the monolayer were harvested, applied to OPTIMAIX 2D and cultured for another four weeks. Afterwards each polymer was frozen to obtain 7 µm slices at the cryomicrotome. The sections were stained with H&E for detecting cells on the polymer OPTIMAIX 2D. Additionally Schwann cell specific S100 staining was performed to ensure that the H&E positive cells were Schwann cells. Furthermore MIB staining was conducted on all H&E and S100 positive sections, to depict mitosis for indicating culture growth on the polymer OPTIMAIX 2D.

Results: In H&E stained sections a sprouting in all nervous tissue colonised polymers was observed, while the polymers colonised with cells harvested from the monolayer did not show any H&E stained cell at all. S100 staining of the nervous tissue colonised polymers showed a sprouting of Schwann cells in all sections. Furthermore, MIB staining was performed for detecting cell mitosis within the S100 positive cells, which depicted proliferating Schwann cells within the sprouting, indicating culture growth on the polymer OPTIMAIX 2D.

Zusammenfassung

Hintergrund: Im Rahmen traumatischer Ereignisse kommt es häufig zur Verletzung peripherer Nerven. Dies geht oft mit Einbußen der Motorik und Sensorik des betroffenen Innervationsgebietes einher. Goldstandard zur Versorgung von Nervenverletzungen mit Defektstrecken ist das autologe Nerventransplantat. Problematik dieser Methode ist zum einen der Sensibilitätsverlust an der Entnahmestelle, zum anderen die begrenzte Verfügbarkeit an Spendernerven. Ein artifizielles Implantat könnte diese Probleme lösen. Im Tierexperiment konnte ein Implantat besiedelt mit Schwann`schen Zellen sehr gute Ergebnisse in Bezug auf die Regeneration der peripheren Nerven zeigen. Um diese Ergebnisse für den Menschen nutzbar zu machen, ist es nötig humane Schwann`sche Zellen zu gewinnen, sie zu kultivieren und auf einem Polymer anzusiedeln.

Methoden: Im Rahmen dieser Pilotstudie wurden aus peripheren Nerven humane Schwann`sche Zellen mittels explant-reexplant Technik gewonnen. Hierfür wurden periphere Nerven aus Amputaten von PatientInnen der klinischen Abteilung für Plastische, Ästhetische und Rekonstruktive Chirurgie des Universitätsklinikums Graz herangezogen. Das Explantat wurde vom Epineurium befreit und in 1 mm große Stücke zerteilt bzw. einzelne Faszikel aus dem Nervenbündel extrahiert und unter Standardbedingungen inkubiert. Nach dem Erscheinen eines konfluenten Monolayers an Zellen wurden das neurale Gewebe bzw. die Zellen der Monolayer entnommen, um sie auf das Polymer OPTIMAIX 2D aufzutragen und für weitere vier Wochen zu inkubieren. Anschließend wurden die Polymere tiefgefroren, um am Gefriermikrotom 7 µm-Schnitte anzufertigen. Diese Schnitte wurden mittels H&E Färbung ausgewertet. Zeigte sich eine Aussprossung an Zellen auf dem Polymer, wurde mittels Schwann-Zell spezifischer S100 Färbung nachgewiesen, dass es sich hierbei um Schwann`sche Zellen handelte und mittels MIB Färbung, dass Mitosen auf dem Polymer stattfanden.

Ergebnisse: Auf den H&E gefärbten Schnitten konnte eine Aussprossung der mit nervalem Gewebe besiedelten Polymere festgestellt werden, während auf den nur mit Zellen besiedelten Polymeren keine H&E gefärbte Zelle nachgewiesen werden konnte. Die H&E positiven Schnitte wurden daraufhin mittels S100 und MIB Färbung weiterevaluiert. Auf den S100 gefärbten und mit nervalem Gewebe besiedelten Polymeren zeigte sich eine Aussprossung an Schwann`schen Zellen. Des Weiteren wurde eine MIB Färbung der H&E und S100 positiven Schnitte durchgeführt, welche Mitosen innerhalb der Zellaussprossung darstellen konnte, was ein Wachstum der Schwann`schen Zellen auf dem Polymer OPTIMAIX 2D anzeigt.

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Dornseifer U, Matiasek K, Fichter MA, Rupp A, Henke J, Weidner N, et al. Surgical therapy of peripheral nerve lesions: current status and new perspectives. Zentralblatt Für Neurochir. 2007 Aug;68(3):101–10.

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http://www.matricel.net/en/products/optimaix/product-description.html

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Single fascicles are pulled out of the explant (left side); nerve cut into 1 mm sized segments embedded in a pre-coated 6-well culture plate (right side)

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Schwann cells in culture showing the typical parallel orientation by lining up end to end (left side); one single Schwann cell displaying the bipolar spindle shape with an oval cell body (right side)

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Frozen pieces of OPTIMAIX 2D are cut into 7 μm sections and applied to super frost slides

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In the future treatment of peripheral nerve injury with defects is including nerve biopsy, cell expanding Schwann cell culture for finally constructing an autologous tissue engineered prosthesis. Hood B, Levene HB, Levi AD. Transplantation of autologous Schwann cells for the repair of segmental peripheral nerve defects. Neurosurg Focus. Februar 2009;26(2):E4.

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1 Introduction

1.1 Back to the roots

1.1.1 Back to the roots - Historic milestones in treating peripheral nerve injury

The concept of treating peripheral nerve injury is a very old one and has existed through many eras. Due to the fact that history has to be understood before marching on to the present, the major milestones in treating peripheral nerve injury will be conciselyoutlined in this chapter.

It is well known that even until today peripheral nerve injury often occurs due to accident or as a result of battlefield injuries. It is imaginable that in the ancient time, where the sword was the most common weapon on the battlefield, cutting injuries of the upper or lower limb were a prevalent problem.

The first famous name that occurs in connection with peripheral nerve injuries caused by a sword attack is the one of Hippocrates. According to a text passage in the Alâim-i Cerrâhîn, which was written by the surgeon Ibrahim in 1505, Hippocrates allegedly performed a nerve suture. Surgeon Ibrahim was a military physician in the war at Peloponnese from 1498 to 1502. During his early military service the citadel of Methone was conquered, where he discovered a medical book, which was written in Syrian language. He subsequently recognized its inestimable value immediately and as he was able to translate it, he began with his work as soon as he was back in his hometown, Istanbul. The book contained medical issues covered by Hippocrates, Galen and Avicenna, but besides that also this decent anecdote within the introductory part of the book(1).

“When the caravan I travelled together with overnight on the bank of Tigris River, a thief attempted to steal a horse. However, the owner of the horse stabbed him in the leg and caused splitting a nerve over the ankle region. Upon the accident, I cleaned off the

wound and applied bandage at proximal and distal sides. I pointed the 2 cut ends of the nerve and united them by using a woman's hair as suture material and then I closed the wound. However, although the man did not limp while walking, a lump persisted in the wound.“(1)

Although it is a very compelling notion to attribute the first achievements in treating peripheral nerve injury to Hippocrates, the founder of today’s medicine, there still is the contradictory fact that this is the only text passage that depicts Hippocrates treating peripheral nerve injury.

Indeed Hippocrates and his contemporaries were confused by the bewildering anatomical structure of the human body, which meant that distinguishing between nerve and tendon still was an occasional problem back then. This even illustrates the origin of the word “neuron” which basically means “sinew”.

First steps in clarifying these anatomical obstacles made Herophilus (335-280 BC). He was anatomist at the famous school of Alexandria and performed as one of the first scientific autopsies on the human body. Besides his great work on the human vessels, he also traced down peripheral nerves to its origin, the spinal cord and the brain respectively. Based on these new insights Galen of Pergamon (129-200 BC) could further distinguish between motor and sensory nerves for the first time(1).

Despite of these new anatomical approaches, the cut-off injury of a peripheral nerve still was seen as untreatable condition *per se*. All through history of peripheral nerve repair Paulus Aeginatus (626-696 AD) was the first, who supposed that restitution of divided nerves could be possible. The idea of suturing nerves was resumed by Avicenna (980-1037 AD) who postulated “epidural end-to-end” adaptation as a possible solution and treatment.

However, the modern concept of peripheral nerve injury`s treatment goes back to one name, Gabriele Ferrara. Ferrara (1543-1627) dedicated his life to surgery and subsequently wrote one of the most famous surgical books at that time, the “Nuova Selva di Cirugia”(2).

The “Nuova Selva di Cirugia” is divided into three parts: the surgical part, covering performing surgical procedures and its pitfalls, the medication part and finally the distillation part. Ferrara is well known for writing one of the most fundamental works of

surgery, but besides that he is especially famous for giving the first instruction for treating peripheral nerve injury in history.

In part one*, more* precisely in “Observationum Chirurgicarum: Observatio XVII: De Modo Consuendi Nervos Magnos Incisos it is written:

Figure 1
Gabriel Ferrara (1543-1627)

„Ad consuendos nervos incisos, oportet eos prius ab omni carne et aliis tegumentis denudare, ut capita utrinque retracta possint inveniri, quibus inventis, sit in promptu acus cum excavato secundum longitudinem, ut filum abscondi possit, foramine, per illud foramen trahatur chorda testudinis ex minutissimis, eaque, una cum acu in vino rubro, cui rosmarinus et rosae sint incoctae, aliquandiu emolliatur: hoc facto forcipe unum ex duobus nervi incisi capitibus apprehende, alteri, quantum potes, adiunge, invicemque; cum acu et chorda consue, ita tamen, né nimium nervi acu comprehendas, nec etiam minus, ne iterum propter teneritatem et mollitiem dislaceret. Et postquam nervus ita consutus fuerit, applicetur oleum nostrum hypericonis magistrale cum oleo abietino mistum, calidumque, ut et nostrum digestivuum, usque; dum tota curatio absolvatur, qua durante sauciatus in

lecto et quiete permaneat, nec membrum hinc inde cum impetu movear, ne suturae dissolvantur et cura impediatur.“(2)

„The first impression conveyed by this passage is the need for precise identification of the nerve stumps, separating them from the surrounding tissues. Suture of the nerve, preceded by a type of “alcoholic disinfection” of the needle and suture thread in a decoction of red wine, rosemary, and roses, must be performed gently, taking care to sew the retracted stumps without damaging them. A hot mixture of hypericum (a herbaceous plant) oil and spruce oil was then applied and the patient confined to bed to immobilize the limb and avoid damaging the suture.“(2)

Ferrera`s instructions for treating the peripheral nerve injury were trend-setting. Even 400 years later we use the same modus operandi implemented by this renowned surgeon(2).

1.1.2 Back to the roots - What tissue are we talking about, anatomy of the peripheral nervous system

The nervous tissue is the system that enables the human body to keep in touch with internal structures and its external environment. Through this system the central organizer, the brain, is able to receive information from the periphery of its body, to process it and to react to any stimulus accordingly.

The nervous system of the human body consists of three major parts, the central nervous system, the peripheral nervous system and the vegetative nervous system. The main control station, as already mentioned, is the CNS, which consists of the brain and its caudal prolongation, the spinal cord. The peripheral nervous system is built up by all of the peripheral nerves, connecting the central nervous system with its target organs. However, the vegetative nervous system again consists of two major, physiological contrary acting parts, the sympathicus and the parasympathicus. These two are regulating the activity of our metabolism according to the “flight or fight” precept (3).

The peripheral nervous system includes the twelve pairs of cranial nerves, the 32 pairs of spinal nerves and the peripheral components of the sympathetic nervous system. By definition, the peripheral nerves consist of motor- and sensory fibres. The neurons forming the anterior funiculus are located in the anterior cornu of the spinal cord and are named the “alpha motor neurons”. The cell bodies of the dorsal root are situated in the dorsal root ganglion, which is located near the intervertebral foramen. The dorsal and the anterior root join each other to form the peripheral nerve, which divides itself into the anterior and posterior rami directly after passing the intervertebral foramen. The posterior ramus supplies for the dorsal profound muscles and the overlying dorsal skin(3).

However, the anterior primary rami either innervate their target organs immediately, or form branches and unite to plexus, called the cervical, brachial and lumbosacral plexus. The cervical plexus innervates the skin and the superficial muscle layer of the neck, while the brachial and lumbosacral plexus innervate the extremities(4).

The cervical plexus includes the spinal nerves from C2 to C4 and is located at the posterior rim of the sternocleidomastoid muscle. It forms the sensible nerves called the

smaller *occipital nerve*, great *auricular nerve* and *supraclavicular nerves*, as well as the mixed *transverse cervical nerve* for the innervation of the Platysma and the deep lying motor portion called the phrenic nerve and the lower part of the profound ansa cervicalis(3).

The brachial plexus follows directly the cervical plexus, which consists of the anterior rami of C5,C6,C7,C8 and TH1(5).

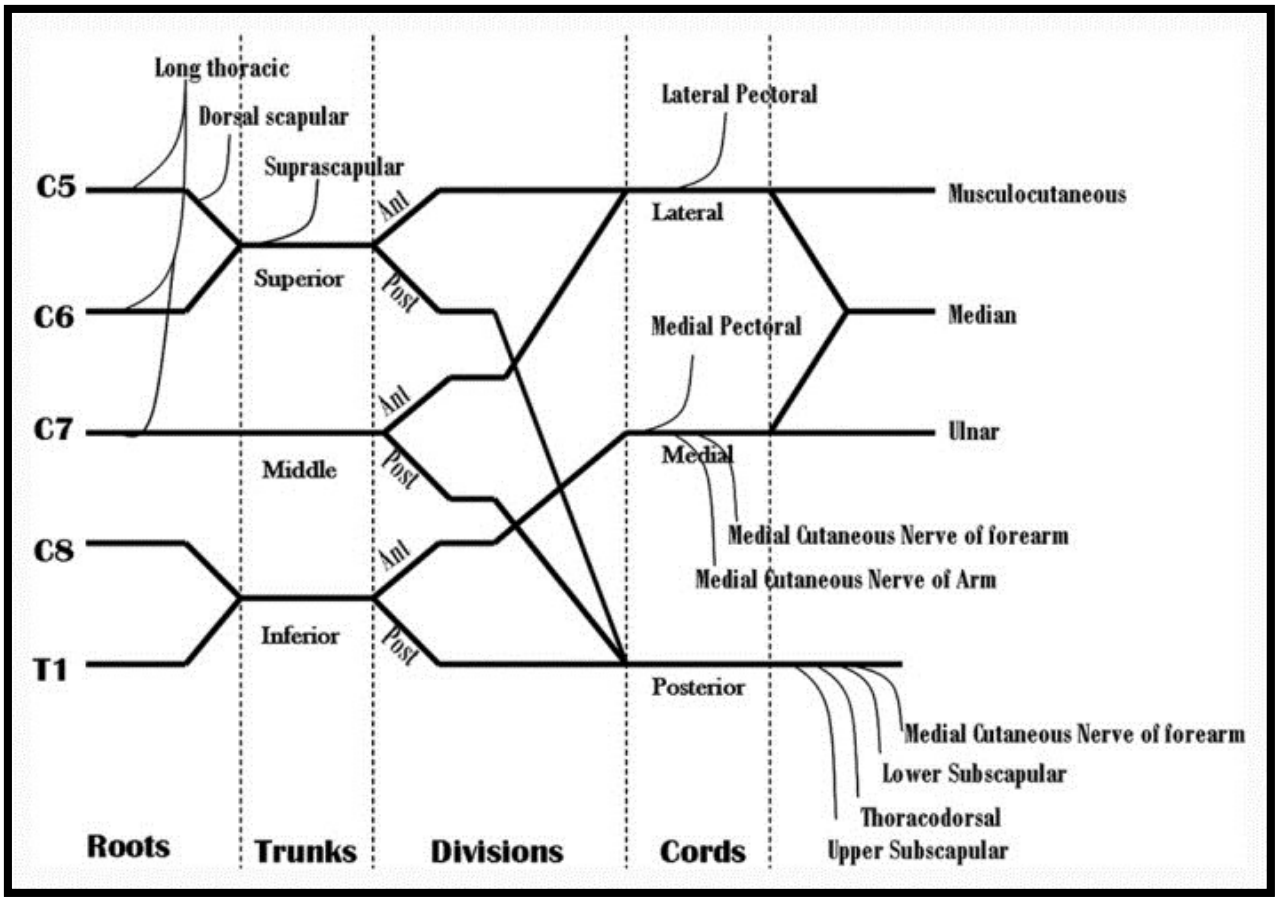

Figure 2
Brachial plexus and its branches

These segmental nerve roots merge together to form three trunks, the superior (C5-C6), middle (C7) and inferior (C8,TH1) trunk. Again each of these trunks divides into a dorsal and ventral part. All dorsal parts unite to the posterior cord, whilst ventral parts of the superior and middle trunk to the lateral cord and the remaining ventral part of the inferior trunk forms the medial cord by itself. These three cords branch into the main terminal nerves of the upper limb(4). The lateral cord forms the musculocutaneus nerve and the

lateral root of the median nerve, while middle cord forms the medial root of the median nerve and the ulnar nerve(6).

Both cords innervate the flexor muscles and the overlying skin. From the posterior cord branch the radial nerve and the axillar nerve, which both innervate the extensor muscles and overlying skin of the upper limb(7).

Thoracic nerves do not form plexus. These nerves supply the thoracic muscles and its attached skin by a lateral and anterior skin branch.

The anterior rami of the lumbar, sacral and coccygeal nerves form the lumbosacral plexus. Therefore the segments of Th12 to L4 compose the lumbar plexus, while the segments of L5 to S4 form the sacral plexus(5).

The segmental nerves reach in form of the so called “cauda equine” their intervertebral foramen and merge together to form the both plexus, right between the superficial and profound parts of the iliac muscle. The lumbar plexus branches into six major terminal nerves. The segments of Th12 to L1 form the iliohypogastric nerve, ilioinguinal nerve and genitofemoral nerve, which innervate the abdominal muscles and the attached skin, as well as the skin of the loin, the groin and major parts the external genitals. The segments of L2 to L4 form the three other major terminal nerves, the femoral nerve, the obturator nerve and the lateral femoral cutaneous nerve. The most cranial one of these is the lateral femoral cutaneous nerve, which pierces the iliac muscle, runs next to its lateral rim and becomes subcutaneous in the area of the anteriosuperior iliac spine in order to supply the sensation of the lateroventral upper thigh(3).

However, the obturator nerve is the only one of these nerves, which crosses the pelvis to reach its bony canal, the obturator canal, for supplying its muscle group, the adductor muscles and a tiny skin area directly overlying these muscles. The femoral nerve runs within the psoas major muscle and emerges from between the psoas major muscle and the iliac muscle, approximately 4 cm away from the inguinal ligament. From below the ligament the nerve enters the upper thigh and innervates the quadriceps femoris muscle as well as the sartorius muscle. The sensible portion of the nerve runs renamed as saphenous nerve right next to the great saphenous vein to reach and innervate the inner medial side of the foot(4).

The sacral plexus branches into five terminal nerves. The anterior ramus of the segments of L4 to S4 form the superior gluteal nerve, which supplies for the gluteus medius and minimus muscle, as well as for the tensor fasciae latae muscle. It runs through the suprapiriform foramen, while the inferior gluteal nerve supplies the gluteus maximus muscle by passing the infrapiriform foramen. However, the skin of the upper dorsal thigh to the area of the popliteal fossa is innervated by the posterior femoral cutaneous nerve. The most important terminal nerve of this plexus is the sciatic nerve. It enters the thigh through the infrapiriform foramen and divides right before entering the popliteal fossa into the tibial nerve and the common fibular nerve(5). The tibial nerve innervates the flexor muscles of the calf and foot, as well as the skin of the calf and the sole, while the common fibular nerve branches into a superficial and deep part. It supplies the two extensor compartments of the calf, as well as the extensor muscles on the foot and overlaying skin of the dorsal shape of the foot. The segments of S2 to S4 merge together and unite to the pudendal nerve. This nerve is responsible for the function of the anal and urethral muscles, as well as the skin innervation of the genital area(3).

1.1.3 Back to the roots- Taking a closer look, Histology of the peripheral nervous system

Nervous tissue is composed of about one trillion neurons a multitude of interconnections to form a complex system of neuronal communication in the human body. Neurons transduce different types of stimuli received by their receptor organs into nerve impulses. They conduct these stimuli to their adjacent neurons, in order to process and transmit it to higher centres for descrying sensation and starting motor action.

Functionally this system is divided into a sensory or afferent part, which receives stimuli from the periphery of the body and conducts nerve impulses to the CNS, as well as a motor or efferent part, which emerges from the CNS and conducts nervous impulses to its effector organs lying within the periphery of the body. Additionally to nerve cells, nervous tissue also contains the neuroglial cells.(8) These cells do not conduct any nerve stimulus, but support the neurons within their function. It is thought that there may be ten times more neuroglial cells than neurons within the whole nervous system.(9)

The peripheral nervous tissue is build up by these two different cell components. On the one hand there are the neurons, including alpha motor neurons and pseudounipolar neurons and on the other hand the neuroglial cells, in the peripheral nervous system called Schwann cells(3).

The major cell of the efferent compartment within the peripheral nervous system is the alpha motor neuron. This neuron consists of three different parts. The cell body, also called perikaryon, the dendrites for interconnecting with other neurons, and the axon, for conducting nerve impulses to its target organ, the muscle fibre. The perikaryon contains the large usually ovoid nucleus full of finely scattered chromatin and a cytoplasm enriched with endoplasmatic reticulum. Its endoplasmatic reticulum shows a large number of cisternae structured in parallel arrays and dispersed polyribosomes, called the Nissl bodies. Prominent Golgi complexes are mostly found very close to the central nucleus thought to produce neurotransmitter, as well as constantly moving neuronal mitochondria, appearing more slender than those in other tissues. Additionally liposomes, melanin granules, lipid droplets and cytoskeletal components can be seen all over the cytoplasm(9).

Projecting from the perikaryon arise the dendrites for receiving nerve impulses from sensory cells, axons or adjacent neurons. Dendrites are the afferent ends of the nerve cell and most neurons possess more than one dendrite. Dendrites typically arise directly from the perikaryon itself and contain the same cytoplasm and cell organelles like its perikaryon. They appear as short trunks that branch several times and interconnect with adjacent cells(9).

However the longest branch is called the axon. It also originates from the perikaryon at the so-called axon hillock. Axons of alpha motor neurons can grow to a length of approximately 1 m. The thickness of an axon is proportional to conduction velocity, which means that velocity increases with the diameter of its axon. Axons are filled with axolemm containing endoplasmatic reticulum, neuronal mitochondria and cytoskeletal components, called microtubules. These are gathered in small strands at the axon hillock and supply for the axonal transport, which is essential for trophic relationship between the alpha motor neuron and its target organ(9).

Alpha motor neurons usually are myelinated axons. This means they are wrapped in neuroglial cells, called Schwann cells. This special glia can be found only in the peripheral nervous system. The end of the axon may branch into several axon terminals and each of them ends in a small distension, the end bulb. These end bulbs are packed with a structure that allows the neuron to transduce its electric stimulus into a chemical message to its effector organ, the synapse(10).

However, the second type of neurons that can be found within the peripheral nervous system is the pseudounipolar neuron. Pseudounipolar neurones belong to the afferent component of the nervous tissue and differ in many ways from its efferent component, the alpha motor neurone. These neurons possess only one axon.

Its axon directly arises from the perikaryon and divides into a peripheral and central branch. The central branch enters the central nervous system by invading the dorsal root of the spinal cord and forming the dorsal white column-medial lemniscus system, whereas the peripheral branch runs into the periphery of the body supplying the sensation for its target organ. Each of the branches conducts nerve impulses and therefore is identified morphologically as axonal.

The pseudounipolar neuron does not possess dendrites, which referrers to its origin. These neurons develop from the neuronal crest cells and thus are localized outside the spinal cord within the dorsal root ganglion. These neurons develop from ordinary embryonic bipolar neurons, whose processes do not develop into dendrites and one single axon in the first place. Instead the processes merge together and fuse before developing, which results in one big, branched axon.

It is thought that the peripheral branch used to be of dendritic origin, due to the fact that it branches in its end. This chance in development results in transmitting the nerve impulse passing from peripheral branch, the original “dendritic” end to the central branch, the original “axonal” end, without involving the perikaryon at all(9).

Besides these two neuronal types of cells the peripheral nervous tissue also comprises neuroglial cells, as it has already been mentioned before. These cells are called Schwann cells and cover the axons of the previous described neurons.

They envelope the axons in two different ways - myelinated and non-myelinated. Myelinated axons are wrapped by the Schwann cells, which means that the covering of the axon includes the whole body of the Schwann cell. In absence of this type of myelin sheaths the axons are called non-myelinated. Although plasmalemma processes of adjacent Schwann cells wrap their neighboured axons partly, too.

Deducing from this behaviour the main difference between non-myelinated and myelinated axons is the velocity of conducting nerve impulses(10).

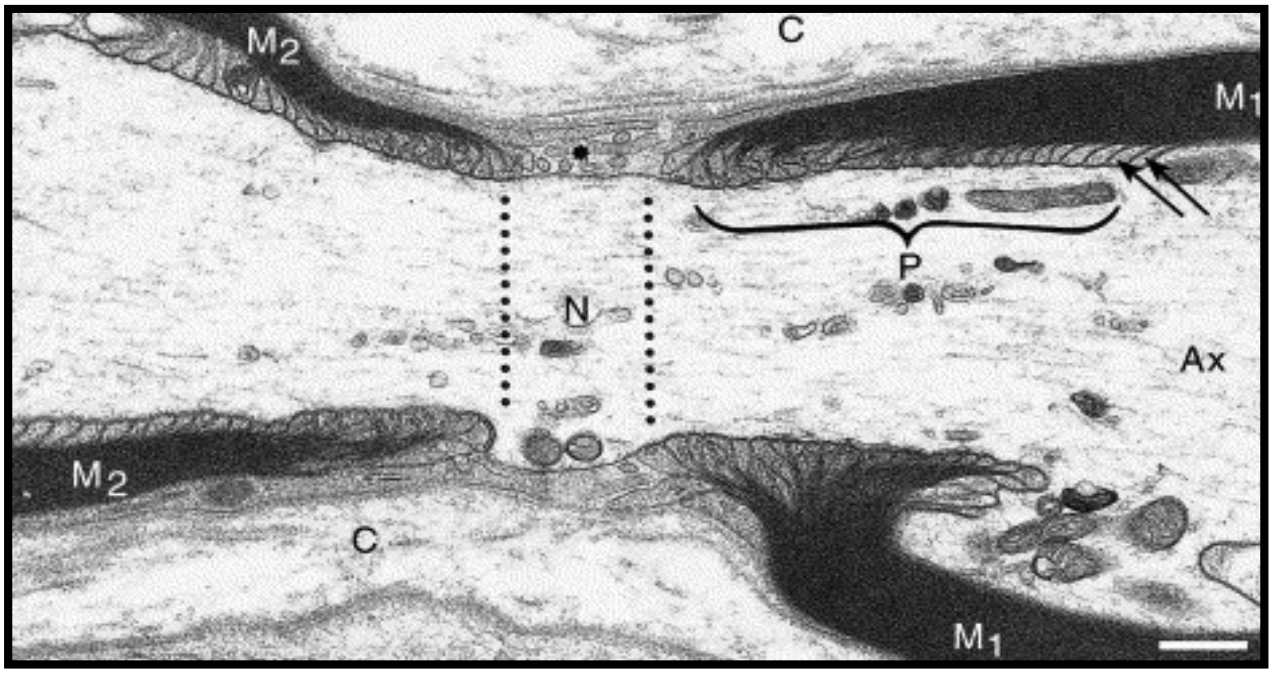

Figure 3

Adjacent Schwann cells (M1 and M2), wrapped around their axon (Ax), building the node of Ranvier (N)

Schwann cells of myelinated axons show to be flattened containing narrowed cell organelles like nucleus, small Golgi complex and a few scattered mitochondria. These Schwann cells form myelin sheaths with the cytoplasm of their body by wrapping about 50 times around the axon of the peripheral nervous neuron. Gaps occur in regular distance within the myelin sheath through forming some space between the adjacent Schwann cells (M1 and M2). They can be seen all along the length of the axon and are called the nodes of Ranvier (N). In these particular gaps the plasma membrane of the axon (Ax) is exposed to its extracellular environment (C), while the rims of the node of Ranvier are covered in basal membrane coming from the two adjacent Schwann cells.(9)

This is of great importance when it comes to peripheral nerve injury, due to the fact that this envelope of basal membrane becomes the guiding structure for the sprouting axons. However, the cell filled spaces between the nodes of Ranvier are called the intermodal segments, which range from 200 µm to 1000 µm in width and include approximately 50 turns of wrapping. Within these intermodal segments occur cone like, oblique incisures, called the clefts of Schmidt-Lantermann(11). These incisures can be described as cytoplasm trapped within the lamellae of the sheath. Furthermore there is a major dense

line and an intermediate line described. The major dense line depicts the merged intercellular cytoplasmic membrane of the wrapped Schwann cell, while the intermediate line represents the extracellular oriented cytoplasmic membrane(10).

Although sensory and motor Schwann cells look similar in light microscopy, new studies have shown, that there exists different genotypic specialization into motor and sensory Schwann cells. Due to differences in gene expression there were particular gene patterns detected indicating sensory or motor Schwann cells. It was found that VEGF and PTN are up regulated in motor Schwann cells, while up regulation of GDNF and BDNF indicate sensory Schwann cells.(12)

Thus these genes can be analysed and used to identify the genotype in Schwann cells, for instance in Schwann cell cultures. Especially in the cultures dedicated to be used in tissue engineering it should be considered, that motor Schwann cells might create better conditions and support for regenerating alpha motor axons than sensory Schwann cells(13).

In addition to all the cells already mentioned, nervous tissue is also build up by connective tissue cells, like fibroblasts and fat cells. These cells envelope the nerve fibres and build its extracellular environment by producing extracellular components, like collagen(10).

All together these cells build the peripheral nerve by forming nerve fibres that are arranged in bundles and wrapped in several layers of connective tissue. These bundles are also called fascicles and are visible to the unaided eye. Each fascicle contains both sensory and motor neuronal cells wrapped in their Schwann cells. All fascicles are bundled again by three different layers of connective tissue. The outermost layer of these is called the epineurium, which is the continuation of the Dura mater(9). Therefore it is composed of dense and erratic fibrouid tissue, as well as thick elastic fibres. Also special collagen fibres oriented in straight direction to prevent the nerve from overstretching can be described in large numbers. There are blood vessels running in the epineurium to supply oxygen to the nerve. Furthermore the epineurium integrates the nerve into its surrounding environment(10).

One layer beneath the epineurium the fascicles are located within their own surrounding connective tissue, the perineurium. The perineurium consists of 20 lamellae of dense connective tissue and perineural epitheloid cells. The outer part of the perineurium is called the pars fibrosa, whereas the inner part is called the pars epitheloidea. The pars epitheloidea is formed of several layers of epithelial cells connected by zonulae occludentes and surrounded by basement membrane. Between these layers also collagen fibres are located, which are longitudinally aliened with interweaved elastic fibres. Reaching the periphery the perineurium thins out until the pars fibrosa vanishes completely, whereas the pars epitheloidea remains covering the terminal branches of its peripheral nerves. Due to the fact that the pars epitheloidea forms a sort of “perineural barrier” for preventing diffusion due to tight junctions, it can be observed in nerves of every calibre(10).

The innermost layer of the connective tissue is the so-called endoneurium. It surrounds every Schwann cell covered axon and consists of loose connective tissue.

Within the extracellular fluid thin layers of reticular fibres, produced by the axon covering Schwann cells, macrophages, as well as scattered fibroblasts can be detected. The endoneurium is attached to the basement membrane of the Schwann cells, thus these neuroglial cells directly affect the innermost layer of the connective tissue surrounding the peripheral nerve fibres(9).

This specialized structure and the highly specified cells in the peripheral nervous tissue make it finally possible to descry sensation and initiate motor action in higher centres. The mechanism that allows transferring nerve impulses and within this process the information for action and sensation, is called the salutatory conduction(14).

Nerve impulses are generally electric signals produced by membrane depolarization originating from the already mentioned spike trigger zone at the hillock of its neuron. They are conducted all along the axon and transferred by synapses to other neurons, muscle cells or glands. For the event of depolarisation, the neuron has to have a resting potential. This resting potential is about -70 mV, which means on the inner side of the axonal membrane there is less positive potential comparing to its outside. This effect is caused by ion distribution differences. In detail it is achieved by a much higher concentration of K^+ ions within the cell, while outside especially Na^+ ions can be

detected. Additionally the outer side of the cell is also enriched with CL- ions(14). The K+ ions are connected to the outside through K+ leak channels located in the plasma membrane, maintaining a way for escaping to the outside according to a K+ concentration gradient.

However, there also exist a few open Na+ and Cl- channels. At this point especially the Na+/K+ pumps have to be mentioned, transporting actively Na+ to the outside, while K+ is brought into the plasmalemma. For every three Na+ ions transported out, two K+ ions enter the cell in order to maintain the resting potential(9). Hence, there is a relative positive charge on the outer side of the axonal membrane resulting from different concentrations at the inner and outer side of the neurone. All together Na+ ions count 143 mEq/l on the outer side, while only counting 14 mEq/l on the inner side of the neurone. In comparison K+ ions show a concentration of only 4.5 mEq/l in the extracellular liquid, while 120 mEq/L in the cytoplasm.(15)

This changes at once when the nerve becomes excited. By emission of excitatory transmitters into the presynaptic cleft excitatory receptors at the neuronal postsynaptic membrane are stimulated causing an increase in permeability for Na+ ions. Suddenly it comes to an influx of Na+ ions causing a more positive change compared to the resting potential meaning from -70 mV to -45 mV. This is now called the excitatory postsynaptic potential. If this spike rises high enough it will reach the point of initiating an action potential(15).

But first the potential has to reach this threshold level before it can aggravate all voltage-dependent Na+ channels, especially those in the axon hillock where twenty times more Na+ channels can be found than in the perikaryon itself. This elicits an action potential, which then travels along the axon by salutatory conduction(11).

Depolarisation results in opening the voltage-dependent Na+ channels for about for 2 milliseconds causing a Na+ influx. Due to the fact that the internodal Schwann cells myelin sheath isolate the axonal part between two adjacent nodes of Ranvier, the action potential has to skip these internodes. This results in a very high velocity of signal transmission making a speed of up to 350 km/h(14).

Now these phenomena arise the question how it comes, that the action potential only travels one direction. Indeed every node of Ranvier has two adjacent nodes, a peripheral and proximal one. So the depolarisation must also affect both of its neighbours(9). Retrograde depolarisation is prevented by the fact that action potential rises up to a level of 30 mV, which is called the overshoot. Overshoot and following refractory period make the proximal node non-responded to the electric stimulation of its distal neighbour. This ensures, that action potential can only be transmitted one way, resulting in efferent conduction in motor neurons and afferent conduction in sensory neurons(14).

1.1.4 Back to the roots- tracing back the origin, Embryology of the peripheral nervous tissue

The development of the peripheral nervous tissue goes back to the very beginning of human being. Starting as one cell zygote, containing the just combined pronuclei of its mother and father, the mitotic division begins. After three days the zygote reaches the 16 cell stage. Now it is called the morula or mulberry and is built up by an inner and outer cell mass. The inner cell mass is the portion that will give rise to the embryo, while the outer cell mass develops further into the trophoblast and placenta respectively.

By the time the morula reaches the uterine cavity the inner cell mass fills with fluid, forming the blastocyst cavity, causing a polarity within the cell mass. The outer and cavity forming part is from this stage on called the embryonal trophoblast, while the inner part is now named the embryoblast.

Within the second week the embryoblast has just differentiated into the epiblast and hypoblast, forming a sort of bilaminar disc, which within two important steps, the gastrulation and neurulation, create the grassroots for the development of our nervous system.

Gastrulation starts at the end of the second week. In the first step the primitive streak forms on the surface of the epiblast. At its upper end the primitive streak forms the primitive node surrounding the primitive pit. This stage marks the start of invagination, meaning the epiblast cells migrate towards the primitive streak and slip beneath it. This inward movement of epiblast cells causes displacement of the underlying hypoblast cells. The epiblast cells, having now displaced the hypoblast cells, are creating the embryonic endoderm, while the cell layer between the epiblast and embryonic endoderm is called the embryonic mesoderm. However, the cells remaining within the epiblast, but overlying the embryonic mesoderm, are known as embryonic epiblast.

By the end of day 16 a trilaminar germ disc has developed and will give rise to all sorts tissues(16).

The development of the nervous system is called the neurulation and follows directly after the step of gastrulation. It begins with the central mesodermal cells transforming into a specialized tissue, the prenotochordal cells. These prenotochordal cells invaginate into the primitive pit and migrate cranially to reach the chordal plate. This causes the chordal plate to be detached from the endoderm.

Furthermore, both of them form a solid chord, the notochord, which can be seen as a midline and supporting structure. It has certain importance when it comes to the development of the axial skeleton and the induction of the nervous system.

The notochord expresses factors, like chordin and noggin that cause an inhibition of BMP-2 and BMP-4. BMP-2 and BMP-4 are responsible for ectodermal skin development, whereas their blockage neuralizes ectoderm. Their presence finally support the paraxial mesoderm lying next to the notochord developing into somites. By the end of week five there will be 42 segmental somites and although not all of them develop further these entities later on give rise to myotome, sclerotome and dermatome, depending on the interaction with their adjacent tissues(16).

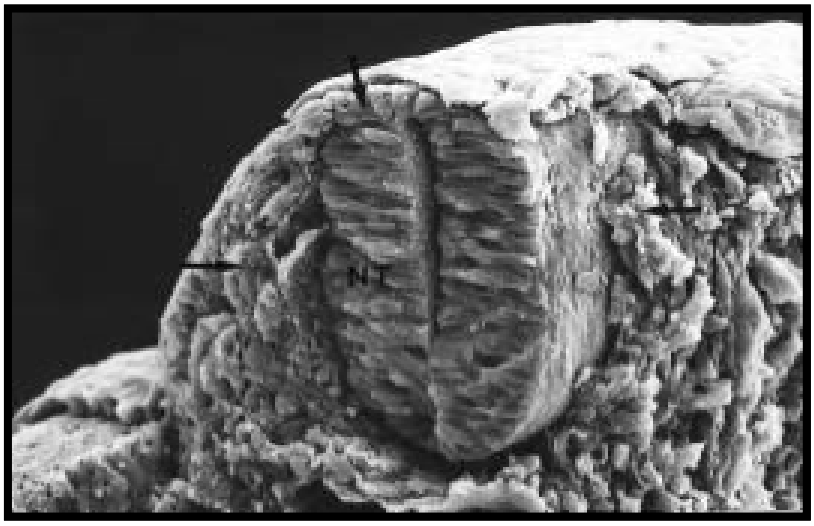

Figure 4 The neural tube (with arrows demonstrating the neural crest cells)

However, for induction of neurulation these differentiation factors have to be blocked. This initiates the development of neuroectoderm, which then evolves into brain, whereas in presence of FGF the same tissue will develop further into spinal cord tissue. Elevation of the lateral parts of the neutralized ectoderm forms the neural plate and central groove. Gradually the neural groove deepens so that the edges become folds, called the neural folds. These neural folds start to fuse from the cranial area, forming a pipe-shaped entity, the neural tube(16).

Before fusion has completed a special group of cells organizes due to the overlying ectoderm interacting with its adjacent ectodermal fold tissue within the lips of the neural tubes.

These cells detach from the folds and are called the neural crest cells(16).

After separating from the neural tube they migrate towards their later position in the developing body. This population of cells gives rise to a variety of structures, like melanocyte, C cells of the thyroid, sympatric and enteric nerves and especially to point out- also to Schwann Cells. However, some of the neural crest cells settle down very close to the neural tube and develop into the dorsal root ganglia, the afferent part of the peripheral nervous tissue(17).

Closure of the neural tube continues and by the beginning of the fourth week only its two ends remain open, called the cranial and caudal neuropores.

However, the neural tube gives final shape to itself by closing the cranial neuropore on the 24^{th} day and the caudal neuropore on the 26^{th} day(17).

At this stage the ectoderm within the neural tube is called neuroepithelial cells. These cells build up a pseudostratified epithelium, with high mitotic activity. Gradually the outer located portion of these cells develope into neuroblasts. They are allocated within the mantle layer, which furthermore gives rise to the grey matter of the spinal cord. This especially includes the alpha motor neurons, the afferent part of the peripheral nervous system(16).

As development proceeds the axons of the precursor motor neurons are the first of all, who spread. They grow towards their target, always guided by their growth cone. The growth cone is an apical structure of filopodiae that help the sprouting nerve to find and innervate its end organ. It is estimated, that the activity and function of the growth cone is influenced by tropic and trophic substances, for example like BDNF, IGF and Netrin.

While growing towards their target, their apposite segmental myotome, these axons induce also the dorsal root ganglia to develop. So these offshoots of neuronal crest cells develop two branches. One is penetrating the dorsal root of the developing spinal cord, whereas the other branch joins the sprouting of its segmental ventral root ganglion. This mixed trunk of dorsal and ventral root axons is now called the spinal nerve(16).

The axons applying to the ventral root seek out their specific muscular bundles and form synapses for finally innervating them. However, although the dorsal root axons have

joined the ventral root fibres, they grow slower than the ventral root. Hence, for most of their way they follow the path of the efferent portion for finally reaching their target tissue, the sensory end organs, like temperature receptors or touch receptors.

Like the ventral root also the dorsal root innervates their end organs within the dermis in a segmental way. These segments are called dermatomes and according to location and distribution it can be deduced to its innervating nerve and its segment respectively(17).

On the ventral and dorsal axon`s growth towards their target the closely allocated neural crest descendants, who by now transform into neural glia, are induced to accompany the axons(17).

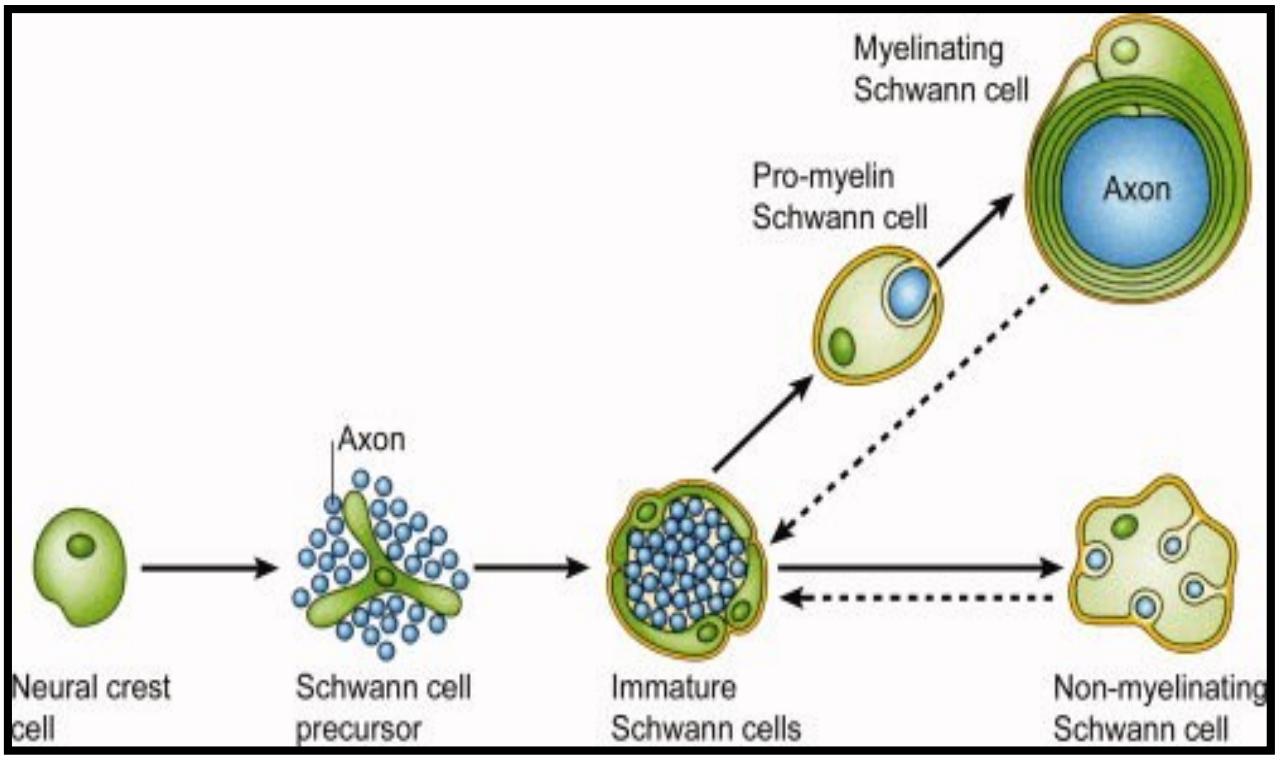

Figure 5

Phases in gliogenesis

At this early stage of embryonic development these immature cells have to transform over two transitional stages: These are known as gliogenesis, whereby these specific portion of crest cells reaches the Schwann cell precursor state coincidental with the axons reaching their target organ and as maturation, which describes the process of

developing further into immature Schwann cells. These immature Schwann cells then give rise to either myelinating or non-myelinating mature Schwann cells(18).

Schwann cell precursors depend on their neuron, due to the fact that it secrets neuregulines for keeping the immature Schwann cells alive.

The most important neureguline for Schwann cell precursors is NRG-1(18).

The newly transformed cells attend apoptosis, if they are separated from their spreading and NRG-1 producing source, their axon. Schwann cell precursor can be found on the edge and within the merging nerve bundles, where their many little cell processes form adherent junctions. This ends in surrounding large groups of axons with their connected processes for influencing the perineural and endoneurial development and by that the future microenvironment of these peripheral nerves(19).

Three different main transcription factors are important for developing from neural crest cells into precursor Schwann cells and further into immature Schwann cells: Sox-10, belonging to the SRY mobility group TF, PUO domain factor Oct-6, and Krox 20, a zinc-finger protein(20).

Sox-10 is present in the earliest state of glial development in at least a portion migrating crest cells and initiates the transformation into glial precursor cells, but also affects pigmentation and enteric innervation. In conclusion Sox-10 deficiency causes agenesis of Schwann cells and therefore has a key function in development of the whole peripheral glial lineage(19).

Oct-6 is present in their further state, the Schwann cell precursor cell and its suppression is responsible for triggering as well as progression in myelination. But the most important contributor for reaching the step of maturation is the transcription factor Krox-20. Krox-20 leads to an up-regulation in the key genes involved in myelination, for example P0, which is the major peripheral myelin protein and MBP(19).

For example, by activating P0, Krox-20 induces a striking large range of phenotypic change. Hence Krox-20 is responsible for the withdrawal from proliferation state and entering the differentiation state. Through this mechanisms the cells change into Schwann cell precursors.(19)

This processes are marked by a down regulation of cell adhesion molecules, the CAM and N-cadherine, and up regulation of myelin production proteins, like P0 and MBP and secretion of connective nerve sheath inducing Desert Hedgehog molecule(20).

The down regulation of adhesion molecules is in keeping with a transformation in nerve architecture from compact glial structure towards endoneurial extracellular space. This newly arisen endoneurial extracellular space gives room for the development of mesenchymal tissue ,which finally will surround the immature nerve(21).

In the end this will result in building up the endoneurium, perineurium and epineurium with its blood vessels(19).

With this step Schwann cell precursor cells have entered the myelination process and by reaching this stage, the maturation respectively. From now on they are called immature Schwann cells. In the next step, called the radial sorting, they undergo profound phenotypic changes. This process will result in two different sorts of mature Schwann cells, the myelinating or (Pro-)myelin Schwann cells and non-myelinating Schwann cells. Still during the intrauterine period of development the immature Schwann cells send their cytoplasmatic processes to defaciculate the axons they have surrounded in the stage before. Axons with a diameter >1 µm establish a 1:1 relationship with their surrounding immature Schwann cells, which induces final myelination. Axons less then 1 µm in diameter remain unmyelinated and therefore are accommodated in clefts within the cell`s surface(18).

However, the (Pro)myelin Schwann cells start myelination very similar to the non-myelinating Schwann cells. They also start forming a patch into which the axon is allocated. The two lips of the patch begin to merge around the surface of their axon and finally build a mesaxon. Finally they encounter each other by coating the whole axon radially, which induces the next step. Thereby the future inner lip slides beneath the future outer lip and begins to wrap around the axon for several times.

By that time the wrapping cell`s endoplasmatic reticulum is producing myelin proteins at full blast to induce the compaction of myelin. This means the amount of transmembrane proteins, like MBP and PMP 22, are up-regulated for sticking together the adjacent myelin lamellae. These lamellae still can be observed under the microscope in mature myelinating Schwann cells and are called, as already has been mentioned in the histological part, the intermediate line(10).

In comparison to the ordinary cytoplasmic membrane the myelin consists of an increased portion of lipids, like phospholipids, glycolipids and sterols and a decreased portion of proteins. In dry matter this means, that 70% are lipids(10).

2 Main Part

2.1 Background

2.1.1 Epidemiology of the peripheral nerve injury- who is affected

The peripheral nerve injury can cause problems that directly impacts patient`s everyday life. Loss in function, disability and neuropathy rank among the list of impairments and they all basically lead to loss of quality in life(22). The peripheral nerve injury is a worldwide problem and occurs in times of war caused by blast or penetrating injury, as well as in times of peace for example within an motor vehicle accident(23).

In our western society peripheral nerve injury mostly is caused by traumatic events and therefore stretch-related trauma is the most common type in our western population(23).

The typical patient is rather male and in average about thirty years old(24)(22).

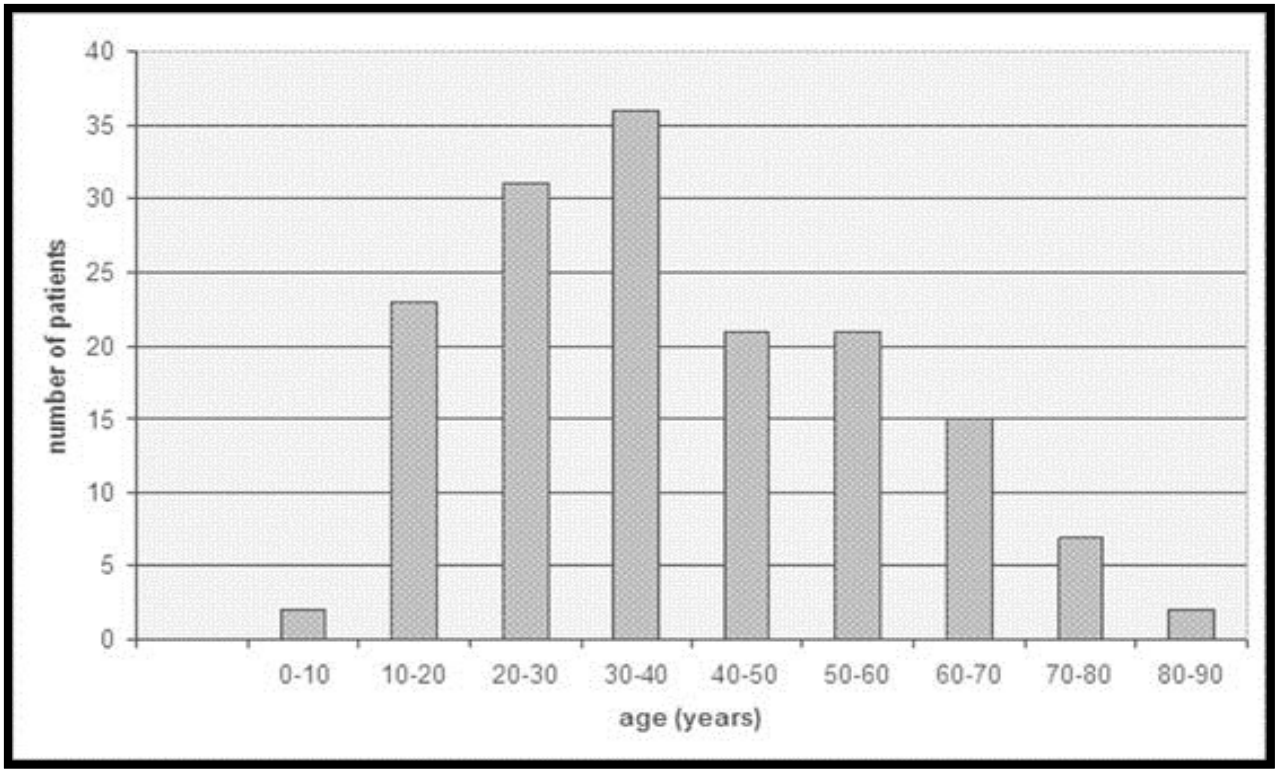

Figure 6

Epidemiology of Peripheral nerve injury concentrating on the age of affected patients

46% of the patients, who suffer from peripheral nerve injury, are involved in a motor vehicle crash contracting lacerations, penetrating or stretching injuries. Also domestic, work and leisure time accidents are very common events causing peripheral nerve injury(25). According to Eser et al.,iatrogenic factors represent about 11.2% of all events and mostly are caused by intramuscular injections(26).

Often peripheral nerve injury is associated with disruption of major vessels and bone fractures.

In the study conducted by Eser et al., in 77% the upper limb and in 23% the lower limb was injured(26).

However, it is a worldwide observation, that the upper limb is more often affected than the lower limb(25)(23). The ulnar and radial nerve are the most common nerves affected in the upper limb, sciatic and peroneal nerve are the predominantly affected ones in the lower limb(27).

Although offering the best microsurgical treatment, full recovery, especially in sensation and proprioception, is very rare(28).

In a study conducted on peripheral nerve injury causing neuropathic, pain Ciaramitaro et al. were able to show that 50 % of all included patients presented with neuropathic pain after being treated. Furthermore, they were able to show, that the severity of pain strongly correlates with decreased quality in life(22).

2.1.2 Classification of peripheral nerve injuries

Peripheral nerve injuries can be classified based on clinical or morphological alterations. Over many decades different groups have tried to create optimal classification systems for clinical use. In general peripheral nerve injury could lead either to loss of function or to persistent or transient perversion of function and pain. Both clinical manifestations could include efferent and afferent qualities of nerves.

Based on the type of injury, they can be further divided into lacerations, compressions, stretch related injuries and deformations(29).

Lacerations could be caused by exposure of nerves to sharps (e.g. blades), leading to loss of contact between proximal and distal part of the injured nerve. It is one of the most commonly and widely used models for peripheral nerve injuries in experimental studies, as it can be easily reproduced.

Compressions such as the “Saturday night palsy” are caused by chronically increased pressure on the nerve itself. There are two mechanisms suspected to be involved in this injury type. First of all compression itself may cause some mechanical damage to the cells of the peripheral nerve. On the other hand there is evidence of transient ischemia being involved in the mechanism of nerve compression as well as impaired axonal transport(30).

Case reports from patients, where compressed nerves had to be resected, revealed that chronic compression causes histological alteration, including wall thickening of vessels in the peri- and epineurium, as well as thickening, edema and fibrosis of the peri- and epineurium at the injury site(31).

Dependent on duration of compression there will be no, an incomplete or complete loss of function.

Stretch related injuries seem to be the most common types of peripheral nerve injuries. One important example is radial nerve stretching after fracture of the humerus. In such cases traction forces exceed elastic capacity of the endoneurium and cause structural damage to nerves. In cases of strong longitudinal stress, complete avulsion could occur(32).

Deformation may be seen in severe forms of compression. Experiments performed by Ochoa et al. in 1971 showed that severe compression with inflated pneumonic cuffs up to 1000 mmHg in upper limbs of baboons caused degenerative processes in peripheral nerves. Damage predominately occurred under the edges of the cuffs, including demyelination(33).

Clinical and morphological classifications alone are insufficient for clinical use. Therefore it has been tried to integrate those, the morphological classification mentioned above, into clinical classifications. The most famous classification for peripheral nerve injury were created by Seddon and Sunderland(34)(35). Both of them include changes in structure of the nerve, as well as clinical symptomatology.

Cohen and Seddon divided peripheral nerve injuries into three different types, also stating that there may be many more forms of mixed character. Those three injuries are called Neurotmesis, Axonotmesis and Neurapraxia(34).

Neurotmesis is defined as a disconnection of the nerve. At the proximal side of injury there occurs a neuroma consisting of scar tissue and nerve fibres. At the peripheral stump of the injured nerve a glioma is formed, which consists of Schwann cells and again scar tissue. Scar formation and loss of mesenchymal tubes that usually guide nerve outgrowth make axonal growth unlikely. This causes consecutively degeneration of end-organs. Therefore, Neurotmesis most often is associated with severe loss of function, including total motor and sensory paralysis. Regarding clinical outcome, Seddon stated that in this kind of injury, recovery is unlikely, unless surgical intervention is performed(34).

Axonotmesis is characterized by discontinuation of nerve fibres but preservation of its neural sheath. Before Seddon’s classification the term “division in contiuity” has often been used, describing this kind of injury. After injury endoneurium and perineurium form pathway structures allowing spontaneous regeneration. Although scar tissue is formed at site of damage the preserved mesenchymal structures make it easier for growing nerve fibers to overcome this barrier. Hence excellent recovery after Axonometesis can be seen. However immediately after injury there are no differences in clinical manifestation between Axonometesis and Neurotmesis.

Neurpraxia is defined as transient block of neural transmission and is typically caused by compression and deformation. Examples again are tourniquet paralysis, Saturday-night paralysis and crutch paralysis. It is the mildest form of nerve injury leading to transient loss of function(34).

Seddon further defined it as a clinical syndrome, characterized by typical clinical findings such as a predominately motor paralysis, unchanged electrical reactions of the muscle, and subjective sensory disturbances, often accompanied by loss of postural sensibility and vibration sense. Although recovery appears in all of the cases, its progress is irregular, lasting from hours to weeks.

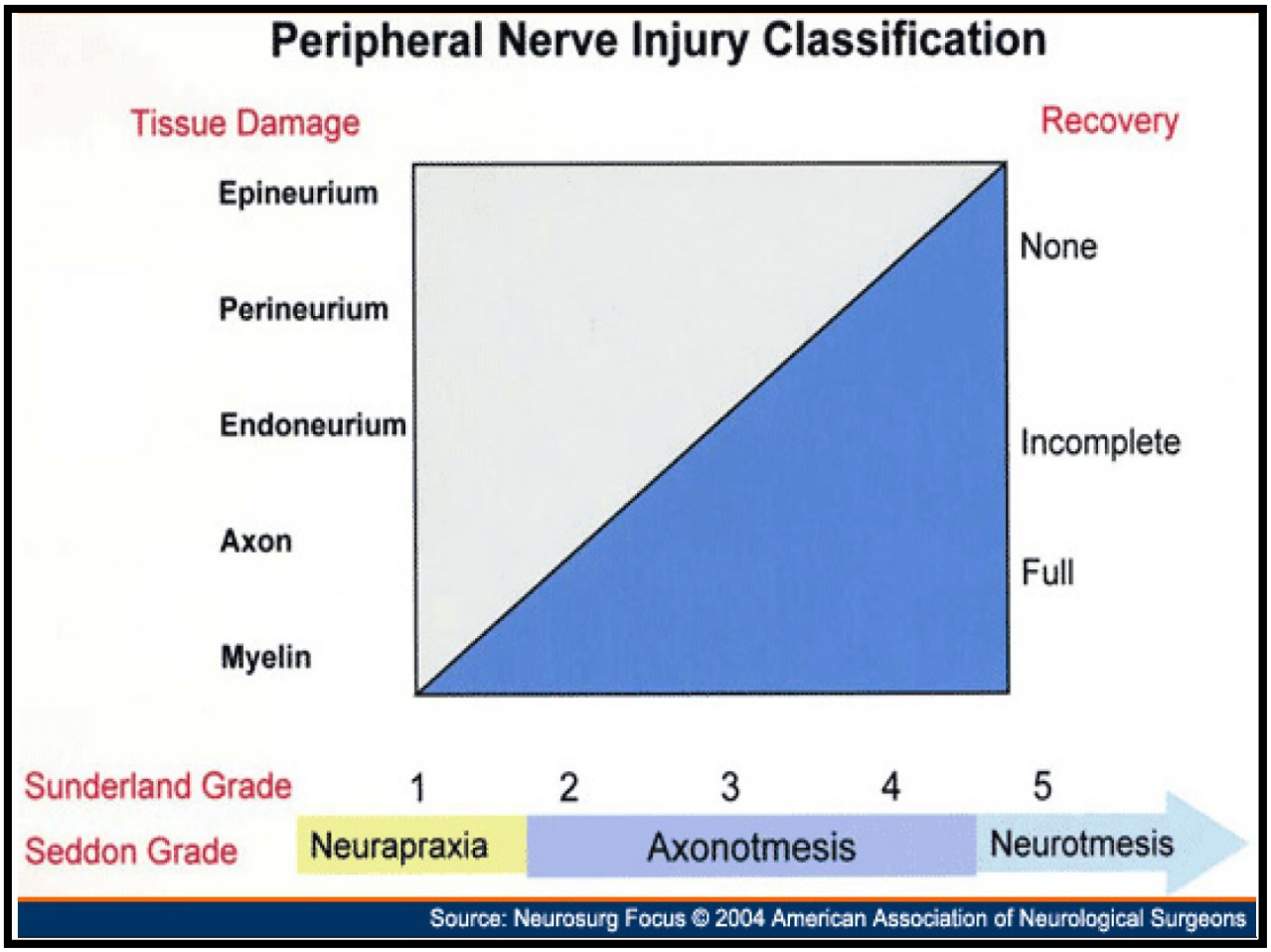

Figure 7

Peripheral nerve injury classified by Sunderland and Seddon

Sunderland performed further sub-classification of Seddon's types of peripheral nerve injuries. According to Sunderland there are five degrees of nerve injury(32). First and

second grade injuries are equal to Seddon's Neurapraxia and Axonotmesis. Neurotmesis was divided into further subgroups including third grade injury (disruption of the nerve plus only partial injury of the endoneurial sheath), fourth grade injury (disruption of all parts except the epineurium) and fifth grade injury, defined as total discontinuation(35).

2.1.3 Pathophysiology

In the mildest form of peripheral nerve injury, the neurapraxia, all wrapping mesenchymal structures, as well as the axon itself, are intact. Despite of some minor changes observed in the myelin sheath, there exists no severe pathological change, which is consistent with the reversible character of neurapraxia. It is estimated that a block in conduction due to insufficient ion distribution causes this transient phenomena(29).

In axonotmesis prospects of cure are excellent, but due to denervation caused by complete interruption of the axon, it takes a prolonged period of regeneration. Nevertheless, only the injured axon has to recover, while the surrounding structures, the peri- and epineurium, are untouched and continuous. This means that the regeneration processes only include Wallerian degeneration, chromatolysis and axonal regrowth along the surrounding mesenchymal latticework(29).

Whereas an injury classified as neurotmesis has much poorer prospects of recovery than axonotmesis due to the fact that not only the axon, but also the surrounding tissue are disrupted. In further consequences this means additionally to the three processes mentioned above, an inflammatory cascade in the tissue around the nerve is started, including total breakdown of the blood-nerve barrier and subsequent haemorrhage resulting in axonal regrowth hampering formation of scar tissue at the injury site(29).

To which extend regeneration processes take place is depending on the grade and severity of peripheral nerve injury, whereas the processes, which contribute to axonal regeneration, are almost always made up by certain pathophysiological steps.

The loss in axonal continuity causes denervation of the effector organ, which means a block in saltatory conduction and axonal retrograde transport of growth factors, like NGF secreted from its supplied organ. Especially the interruption of NGF inflow from the periphery caused by axotomy is estimated to trigger the regeneration processes following peripheral nerve injury(29).

However, under the mentioned conditions several degenerative processes at the neuronal and distal segment are induced and seen as direct preludes to regeneration.

According to its anatomical zone at the site of injury these processes include the Wallerian degeneration, chromatolysis and axonal regrowth.

The distal segment- Wallerian degeneration

Although axonal degeneration does not begin immediately in the distal segment and can take up to a period of days in humans, a persistent denervation will result in an influx of extrinsic and intrinsic (from intracellular stores) Calcium at the injury site. An increased concentration of Calcium in the plasmalemma activates caplain, which is a protease, and the ubiquitin-proteasome system initiating granular disintegration, cytoskeletal degeneration and disarray of myelin(36)(37).

This step is necessary to induce further degenerative processes, like breakdown of myelin sheath and blood-nerve barrier(38). In the beginning only the site of injury is affected, but because Wallerian degeneration travels along the axon also the injury distant segment up to the end organ loses its blood-nerve barrier. This leads to a double increased permeability facilitating acute inflammatory response and invasion of immune cells, like macrophages(38). Gradually axonal contours become fuzzy, which goes hand in hand with loss in axonal continuity and ability of conduction.

Simultaneously to axonal degeneration the axon`s coverage, the Schwann cells are also affected by denervation. Moreover it has been shown that Schwann cells seem to act like PNS-sentinel cells “specialized” on sensing peripheral nerve injury for enhancing regeneration. They use TLRs and possess TLR3, TLR4 and TLR7 constantly, while TLR1 only after axotomy. Binding endogenous ligands, which are only present during injury, like mRNA or degenerated ECM, activates TRL- receptors. This leads to TRLs initiating an intracellular signalling cascade that induces an activation of transcription factors. These induce expression of those cytokines, which are vital for recruiting immune response and establishing a regeneration enhancing microenvironment.

Boivin et al. observed a lack of cytokine expression leading to reduced macrophage recruitment and an overall delayed Wallerian degeneration causing poor recovery in TRL signalling deficient rodents(39).

However, in the first step of Wallerian degeneration Schwann cells change their gene expression from “myelination” to “non-myelination-precursor-like” and stop producing P0,

PMP22 and MBP, whereas the production of p75, the receptor for NGF, extracellular matrix, adhesion molecules and growth factors is up-regulated. Consistent with changes in gene expression they show an increased mitotic activity accompanied by phenotypic dedifferentiation. The newly dedifferentiated daughter cells begin to phagocytise the myelin and axonal debris accumulated in the endoneural tubes, which is in a later state passed on to recruited macrophages(29). While phagocytosis still is going on the endoneural tubes become swollen, but after a period of five to eight weeks the tubal diameter decreases due to being only filled with dedifferentiated Schwann cells forming the bands of Büngner.

Extend in shrinkage of tubal diameter depends on the time of denervation and explains, why the chances of total functional recovery decrease with time. Due to the fact, that sprouting axons have to fit through their specific endoneural tube to finally reconnect with its effector organ, they have to pass the site of injury within a certain time, which in turn correlates with degree of damage and the grade of peripheral nerve injury respectively.

Besides phagocytising the debris, the invaded macrophages induce enhanced NGF production through releasing interleukin-1β. The increased concentration of NGF contributes to keeping the injured neuron alive, but also serves bound to the p75 receptors at the surface of the dedifferentiated Schwann cells as guiding track for the axonal growth cone(28).

In the next step the highly mitotic active Schwann cells express adhesion molecules, especially N-Cadherin, for arranging themselves in the bands of Büngner(37). Furthermore they produce special chemotactic ECM molecules, like laminin and collagen, which accumulate along the outer surface of the basal lamina(29).

Its growth cone leads the sprouting axon. This growth cone advances along the adhesive molecules, the NGF bound to p75 and the trail of regeneration supporting ECM, which in conclusion means along the bands of Büngner.

The neural segment and its soma-Chromatolysis

The preconditions for survival of the neuron require older, fully differentiated axons, distant site of division and low grade of injury(37).

Like in the distant segment the regeneration of the proximal segment starts with axonal degeneration similar to Wallerian degeneration. Degeneration and subsequent phagocytosis is beginning at the site of injury, traveling along the axon in proximal direction. In contrast to the distal segment degeneration processes only affect the peripheral section of the injured axon ranging from minimal, stopping at the next node of Ranvier, to major extent, which means that degeneration goes all the way down to the neuron. In case it hits the neuron, for example due to sever nerve damage, this could lead to cell body death and therefore no axonal regrowth will occur(29).

Meanwhile chromatolysis is induced in the cell soma. Within this process the nucleus migrates from its central position to the periphery. Simultaneously endoplasmatic reticulum and the Nissl bodies scatter and disperse(29). The neuron up-regulates gene expression for axonal outgrowth supporting proteins, like tubulin and actin as well as growth factors and lipids, whereas the protein synthesis for neurotransmitter is switched off.(37) The adjacent proliferating Schwann cells isolate the in diameter shrunken and injured rest of the distal axon from synaptic connections. It is estimated that this habit should give the neuron time to recover and for preparation of axonal regrowth(29).

Axonal regrowth

Depending on the severity of injury, the regeneration processes can start relatively early in minor and later in severe damage. In humans the neuron can initiate axonal regrowth for a period of up to one year postinjury. The period of axonal regrowth can take up to months with an average rate of 1 mm per day. Functional recovery depends on the time of denervation, which is causing end organ impairment, like muscular atrophy occurring already after only two month(27). In lesions without gaps the sprouting axons are guided by their surroundings, easily invade the site of injury and innervate their specific end organ guided by the endoneural tubes. Whereas in lesions with gaps there are very poor prospects of full recovery, because of formation of scar tissue within the gap impeding axonal advancing at the site of injury. Commonly this leads to misdirection in finding the

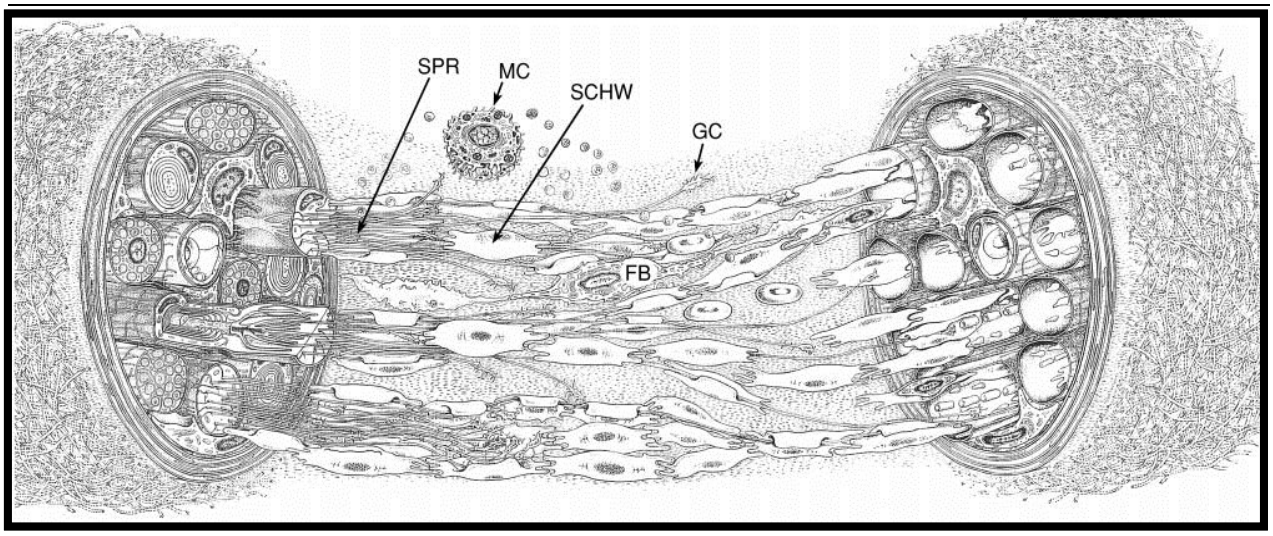

Figure 8

Wallerian degeneration and axonal outgrowth (SPR) guided by Schwann Cells (SCHW)

right endoneural tube with subsequently innervating the wrong end organ. This is consistent with a prolonged period of denervation leading to fibrosis and death of the effector organ(27).

After a period of 24-48 hours regeneration is induced at the proximal segment. By complex interaction between soma and axonal tip the regeneration is started. The parent axon starts to swell and finally gives rise to a large number of sprouts (SPR). This process is provided by anterograde axoplasmatic transport of regrowth supporting materials from the neuronal soma. At the axonal tip of each sprout develops the growth cone (GC), which is a bulb with multiple filopodia. The axonal sprouts need their growth cones to adhere to the basement membrane of Schwann cells (SCHW) and for following and exploring the chemotropism directing them to the end organ(28). Guided by NGF bound to the surface of Schwann cells and the growth enhancing ECM provided by the glia the growth cone traces down a trail directing across the site of injury and to the endoneural tube respectively. Due to the fact that their diameter has decreased by the time the regrowing axon reaches its distal segment entering its tube is considered with a terminal slowing down in regeneration(29). Guided by the bands of Büngner the axons finally reach the denervated end organ, for example motor endplates and form new synapses for reinnervation(29). Contact to the regrown axon effects in Schwann cells switching back into myelinating gene expression and maturation respectively(37).

2.2 Treatment

Transection and basically impairment in nerve impulse transduction affects the whole physiologic system and therefore accurate and successful treatment is very important.

It has serious consequence for the central nervous system and requires immediate functional reorganization in the cortex. The therapeutic result after treating nerve injury depends on several factors, like the quality of axonal sprouting in terms of orientation and growth, and the survival of its target organs(28).

Conventional treatment of peripheral nerve injury includes the coaptation of the proximal and distal ends, nerve transplantation, implants, and the rarely used nerval neurotisation, where a healthy nerve is transferred to the distal end of a proximally irreversible injured nerve.

First the injury has to be evaluated using Hofmann Tinel sign, clinical presentation and Electromyogram.

According to severity of presentation the injury can basically divided into lesions with gaps, also described as Sunderland IV and lesions without gaps, according to Sunderland classification grade I-III. Furthermore, the lesions without gaps have to be subdivided according to Millesi`s classification regarding their degree of fibrosis after trauma in particular(40).

There are two major types, the pseudoneuromas, where scaring takes place interfascicular or perineurial according to Millesi A/B, and axional and transduction impairing neuromas, where scaring affects the endoneurial space according to Millesi C(40).

In lesions with interruption of continuity a direct suture can be attempted by end-to-end or end-to-side coaptation. Especially bland cut injuries have good chances to be treated with this method, due to achieving a tension-free coaptation.

Direct suture is achieved by microsurgical epineurial or epiperineurial, as well as perineural (interfascicular) coaptation of the proximal to its distal stump and its fascicles, respectively.

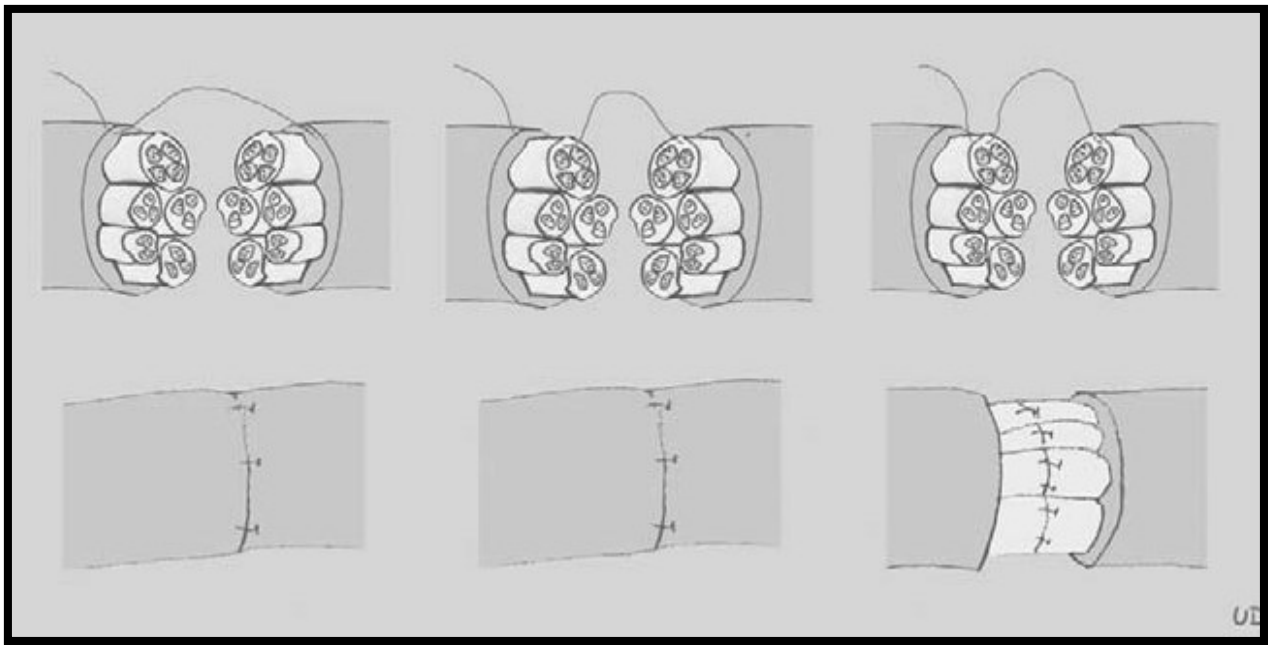

Figure 9

Suture technique used in peripheral nerve injury with loss in continuity demonstrating epineural,epiperineural and perineural coaptation

Regarding the right time for the procedure, there are two different approaches, the primary and secondary intervention. In secondary intervention treatment takes place three weeks up to six month after injury. In primary intervention the nerve is treated straight after injury has taken place or at least within the first two weeks(41).

A huge advantage of primary care is avoiding the formation of scar tissue at the proximal and distal end of the nerve, whereas the big disadvantages appoint the lack of proper assessment of its full extent at this early stage and the absence of the optimal metabolic milieu at the site of injury. However, Lundborg points out, that there lays no disadvantage in primary care concerning the emergence of an optimal metabolic environment(28).

This means that both secondary and primary care are possible approaches in treating peripheral nerve injury as long as treatment is decided according to the circumstances, as well as dimension of injury. Primary treatment is seen as today`s standard and is performed in smoothly separated nerves, clean wound bed and if no other associated side-injuries are present. Therefore mono- to oligofascicular nerves are treated by epineural suture, while in case of clear group structure, interfascicular coaptaption is performed(42).

In terms of recovery and regaining lost function of the effector organ, it is estimated that the sooner the divided nerve ends can be reunited, the better it is for its outcome. In case injury does not meet the motioned criteria and immediate assessment is not accurately manageable secondary or delayed care is recommended. The chances for recovery in complicated lesions or even continuous peripheral nerve injuries can be predicted by assessing action potentials on electromyogram after eight to twelve weeks delay(43). Advantage of delayed intervention also includes that the damaged parts within the nerve are demarcated by scaring tissue, which makes it easier to recognize the damage to its full extent and secondarily conduct sufficient debridement more accurately(44).

In both, primary and secondary intervention it has to be considered that direct coaptation is only a possibility if it is carried out without tension on the reconstructed nerve. Tension on direct sutured nerves has shown to cause enhanced and disorientated axonal sprouting resulting in painful neuroma at the suture site(44).

As already mentioned, in lesions where its dimension can not be assessed immediately delayed procedure is recommended(44).

In case of absent signs for healing progress and persistent neurological deficits pseudoneuroma or neuroma may be present. In pseudoneuroma with fibrosis arising from the epineurium, the fibroid tissue is removed by microsurgical decompressing intervention, called an epineurectomy. However, Interfascicular neurolysis is performed, if scaring originated from the perineurium and occurs between the strands. In neuromas, where the scaring tissue arises from the endoneurium, impairing axonal electrophysiological transduction, radical resection of the affected fascicles is required with subsequent interfascicular reconstruction(40).

In case tension-free direct reconstruction can not be achieved, may it be either due to substantial loss of tissue in the first or due to resection of massive fibrosis or neuroma in the second place, using an interponate for bridging the defect is recommended(44).

Millesi et al. were able to show that autologous nerve grafting performed a better outcome than direct nerve suture under tension(45).

Hence, nerval heterografts, homografts and autografts were tried as bridging material to achieve tension-free reconstruction(41).

Due to immunologic reaction heterografts are not favourable. Homografts are still studied extensively, but the superiority of autografts has been shown due to its immune compatibility and ability of providing a natural conduit to the injured nerve(28).

By undergoing Wallerian degeneration within the graft itself, the graft Schwann cells form the bands of Büngner and serve as natural guiding structure for axonal outgrowth. Furthermore the graft cells provide the required environment for nerve regeneration(28).

Commonly used autografts are trunk grafts, where an autologous nerve is harvested and the whole segment is interposed. Unfortunately this type of autograft tends to turn fibrotic in its centre, which hinders the sprouting nerve in passing the implanted tissue to regain access to its distal end(41).

There is also the possibility to use cable grafts described by Seddon in 1947, which is a multistrand free nerve graft, interposed into the nerve gap as one unit for bridging the defect between the stumps of the injured nerve.

Interfascicular nerve grafting, a technique developed by Millesi et al., is used especially when electrophysiological transduction impairing fibrous tissue is present within or between the fascicles(40). Interfascicular nerve grafting is performed by implanting the strands of a grafted nerve between the corresponding and microsurgically isolated major fascicles or in groups mobilized minor fascicles(45).

Sources for autologous nerve grafting have to meet certain criteria: the graft must be expandable for tension-free suture, the ratio between the nerve fibres and its connective tissue must be known and the diameter of the graft should be large enough to maintain closure, but also as thin as it is needed to stay viable during free grafting(41).

The most popular nerve or otherwise said the “gold standard” grafting nerve is the sural nerve. A length up to 30 cm can be excised, while only causing “minimal” functional deficit at the grafting site. Another nerve at the lower limb that is possible to consider for autologous grafting, is the lateral femoral cutaneous nerve. At the upper limb also the medial antebrachial cutaneous nerve, the lateral antebrachial cutaneous nerve and the dorsal antebrachial nerve hit the mentioned criteria(41).

Alternative biological nerve grafts include arteries, veins, muscle and combinations of these. A more experimental approach also suggests human amnion matrix(46).

Büngner was able to bridge tiny gaps of injured peripheral nerves using small segments of arteries even in 1891. The advantages of this technique are roughly the same like in autologous nerve grafting: no immunologic reaction to the graft and serving as a natural conduit containing collagen and fibroid tissue. Additional to these factors the lumen conserves the regeneration enhancing metabolic milieu and does not turn fibrotic, like it may occur in cable grafts. The big disadvantage of autologous artery grafting for treating peripheral nerve injury is the fact that they cannot be harvested without serious consequences regarding perfusion on the donor site(44).

Therefore it was switched towards vein grafting. They can be harvested in large numbers without causing any serious consequences at the donor site, like loss of perfusion or sensation. But the big problem with autologous vein grafting turned out to be the stability of the graft. Because of the physiological fact that veins belong to the low-pressure system they have not the same muscular layer within the vascular wall like arteries leading to collapse, when used on bridging of bigger deficits(41). Nevertheless, Chiu and Stauch were able to show that venous grafting works very well in defects not bigger than 30 mm(47).

To archive reconstruction of bigger defects it was tried to fill the vein grafts in order to prevent them from collapsing. Therefore the lumen was filled up with muscular tissue. Muscular tissue showed to be a good matrix for the sprouting nerves, due to its longitudinal orientated muscular fibres, and especially due to its laminin and collagen containing basal lamina.

The longitudinal fibres serve as an alternative to the main guiding structure in peripheral nerves, the bands of Büngner. These physiological supporting structure are only found within the autologous nerve graft of course, but because of the fact that longitudinal orientated muscular fibres have a similar effect on the regenerating nerves, they can be seen as an alternative guiding scaffold, serving sprouting axons in growing towards their distal stump(44)(45).

For finding new approaches in treating peripheral nerve injury a huge amount of experimental research has been conducted on pathophysiology of the healing process in divided nerves. Autologous nerve grafting showed the big advantage to be closest to its physiological environment and nature.

Hence, this method is, as already mentioned, still “gold standard” for treating peripheral nerve injuries with defects.

Whereas all other grafts are preserving the regenerator milieu between the nerve stumps, the autologous nerve graft is the only graft providing Schwann cells, which are the major component in contributing to create this regenerator milieu. Additionally the graft provides the longitudinally orientated connective tissue, like collagen and laminin. The specific nerve architecture and tissue composition of this type of autologous grafts naturally supplies the healing nerve with the perfect environment required for axonal sprouting(28).

However, there are also some critical factors to consider in autologous grafting and especially autologous nerve grafting. All grafts have to be harvested from the patient and this comprises the procedure of explantation. It requires a second incision and scar at the donor site. Explanting autologous tissue may also cause infection, bleeding and fibrosis, which accounts for every surgical procedure and in most cases are unlikely to happen, but it is the loss of function at the donor site, which will occur for sure.

In veins and especially arteries this may impair perfusion, while nerve graft eplantation causes loss of sensation at the donor site and in the worst case the forming of painful amputation neuromas(48). Additionally there is a lack of availability, risk of disease spread and the problem of tissue incompatibility when thinking of reconstructing motor nerve injuries with sensory nerve grafts(49).

Considering that in most adult patients even autologous nerve grafting results in impaired recovery, it may raise the question for alternatives(28).

Non-autologous conduits could solve these problems. They do not have to be harvested from the patient and are unlimited available like a kind of “off the shelf prosthesis”.

Non-autologous conduits are tubular interponates conserving diffusion of growth factors secreted by the nerve stumps while preventing infiltration of scar tissue from the

surroundings. Each, the proximal and distal stump of the injured nerve, are placed into the open ends of the tube and are hold in place by two single stitches at each side. This simplifies the procedure and also reduces extensive scaring at the suture site(44).

The different devices can be subdivided into biological and artificial interponates depending on the material they are made off, which can then be further specified according to their absorbability.

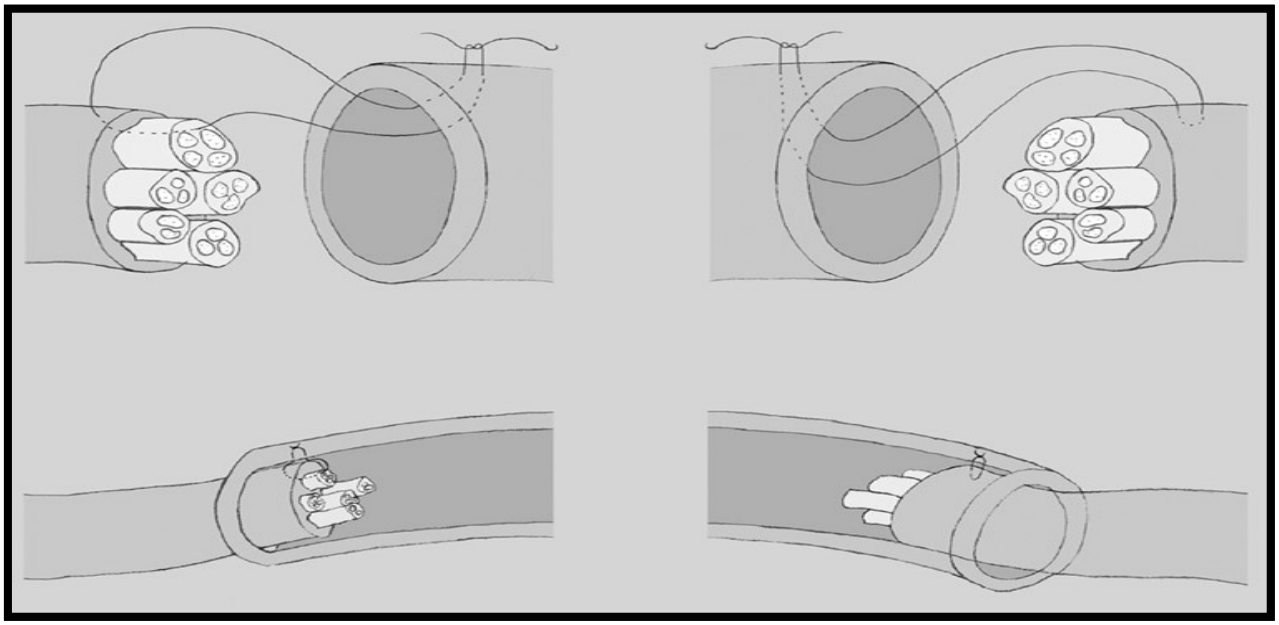

Figure 10

Applying a tubular interponate for bridging peripheral nerve injury with gap

The biological conduits are made of extracellular matrix components, like Collagen. Longitudinally orientated collagen is also found in large numbers in extracellular matrix of nerves and is responsible for the orthograde guidance of sprouting axons.

Archibald et al. were able to show, that collagen tubes work as good as direct suture in treating iatrogenic median nerve dissection in monkeys. Collagen shows to be completely absorbable and therefore causes only minimal scaring at the suture site, which means it has not to be removed by second surgery(50).

The same results also were observed in humans, who underwent primary reconstruction of peripheral nerve injury based on collagen tubes after laceration of mixed nerves.

After 24 month there were no differences between the direct sutured and the collagen tube treated group of patients regarding sensory and motor conduction(51).

Therefore collagen tubes were approved by FDA and CE and are registered under the name NeuraGen®, or Integra LifeSciences, which is made of bovine collagen I. Both are reserved for treating defects with several millimetres(52).

To extend the idea of using biological conduits without foreign body reaction, much research has been made on decelluarisation of allogeneic bridging tissue.

It suggests providing extracellular matrix in its natural three-dimensional architecture, which is so crucial for sprouting axon`s guidance, without triggering immune reaction. But its biggest advantage comprises the fact that there is no need for harvesting tissue form the same individual(53).

Avance® is a commercial product, which consist of cadaveric acellular nerve and is already in use in clinical case studies at the moment(54).

Besides these already mentioned techniques there also have to be mentioned the non-biologic artificial conduits.

Non biologic artificial conduits are sub-dividable into non-absorbable tubes, like silicone or polytetrafluoroethylene conduits and the absorbable tubes, made of polyglycolic acid or poly(DL-lactide-ε-caprolactone), for only to mentioning the FDA and CE approved ones(52).

Non-absorbable silicone tubes were one of the first synthetic bridging devices used in clinical trials and are nowadays in clinical use for treating small defects in peripheral nerve injuries. Due to their non-permeability they have the big advantage to conserve the metabolic milieu consisting of neurotropic and neurotrophic factors produced by the two nerve ends stuck into the tube.

Lundborg et al. were able to show that long-term outcome of silicone tube treated nerves is equal to neurorrhaphy treated ones.(55) However, the disadvantages of silicone are its lack in flexibility with an increased risk of damage. Furthermore, it is non-absorbable in keeping with occurrence of nerve compression-syndrome and foreign-body-reaction(54).

Neurotube® is an empty tubular and bioabsorbable PGA conduit, used in peripheral nerve injury reconstruction defect with minor gaps. Compared to standard repair the Neurotube® reconstruction did not show any different outcome regarding regaining nerve function and showed to be superior in terms of sensational regain compared to end-to-end anastomosis in gaps under 4 mm(56).

Under the name Neurolec® the poly(DL-lactide-ε-caprolactone) conduit is approved and listed. It is an absorbable tubular device allowing optimal interchange with its environment. Chiriac et al. treated 23 patients presenting with defects averaged 11.03 mm with Neurolec® and did not favour it, because of severe complications, like fistulization, difficulty in handling and expensiveness(57).

In small defects these devices show to be equal to neurorrhaphy and therefore are put in clinical use and are certificated for administration in humans. However, they did not show the same success in large lesions with defects over 3 cm in humans(52).

The lack of supporting cells, who are responsible for creating a microenvironment enriched in neurotropic and neurotrophic factors, seems to be the main reason for the failure of these conduits in large defects(44).

This finding and its understanding induced the idea of manipulating the artificial conduits in the way to create the perfect environment for regeneration.

The working hypothesis of creating something like an “artificial nerve” was borne and with it the idea of using tissue engineering as a tool in finding an alternative way of treating peripheral nerve injury.

2.3 Tissue Engineering

2.3.1 A new approach- designing a tissue engineered nerve

Tissue engineering was defined as a discrete field only since the mid 1980s(58). Its roots go back to several well-established disciplines, like molecular biology, cell biology, biochemistry and found its implementation within “in vitro” methods and cell physiology based on molecular genetics and cell signalling. With the idea of creating a bioengineered surrogate nerve also skills in clinical science, like microsurgical skills in terms of tissue transfer and surgical transplantation techniques were required. All these skills are essential in facing the challenges which arise when planning realisation of this idea, like cell sourcing, design of a tissue like scaffold and manipulation of cell function(58).

Furthermore the result and finally its product should be of predictable and reproducible quality for finally elaborating a commercial tissue engineered device that perfectly meets the criteria of success in treatment, easy handling, capability of sterilization and(59).

The main question is how it can be achieved to finally hit the requirements mentioned above?

For success in treatment it is suggested that the tissue engineered construct should mimic peripheral nerve tissue grafts. Therefore four major components come to mind:

- The scaffold, the structure giving component.
- Fillers, like extracellular matrix.
- Growth factors, promoting regeneration and axonal outgrowth.
- Supporting cells, like the Schwann cells.

Furthermore, these components should be easily available, biodegradable and flexible for not causing compression symptoms(49).

The tissue engineered construct must support direct axonal sprouting, provide the mechanical support during the sprouting process and allow diffusion for neurotrophic and neurotropic factors, as well as nutrition from outside, while obviating infiltration of surrounding scaring tissue(54).

2.3.2 The scaffold

It should support appropriate behaviour in sprouting by promoting molecular and mechanical signals, without causing any undesirable local and systemic side effects.
This includes the scaffold to be blood compatible, histocompatible and mechanical compatible(54).
By blood compatibility it is meant that the scaffold must not induce haemolysis, coagulation and thrombus formation. In terms of histocompatibility the scaffold should be non-toxic including toxicity to the surroundings, as well as to the systemic body and must not cause gene mutation in body cells. Mechanical compatibility appoints the fact of compliance to the nerve tissue for allowing processes, like moving and swelling without kinking, tearing or braking of the device(59).
These criteria can be met by careful selection or modification of material and fabrication.
In terms of material natural and synthetic scaffolds are available and are mostly the same like the tubal grafts discussed before. Hence, natural scaffold material includes veins, muscles, tendons, amnionic membranes or extra cellular matrix. Especially extracellular matrix like laminin and collagen are known to support axonal outgrowth.(38)
But also acellular allogeneic or xenogeneic tissue fall into this category and provide extracellular matrix conserved in its natural three dimensional architecture(54).
Furthermore, chitin and chitosan have been introduced into peripheral nerve tissue engineering(60). It can be isolated from the outer shell of crustaceans, insects and fungal cell walls(60).
Non-biological or synthetic alternatives include the already mentioned degradable polyglycolic acid and poly(DL-lactide-ε-caprolactone) respectively, but also polyphosphoester, poly(l-lactic-co-glycolic acid), poly(2-hydroxyethyl methacrylate-co-methyl methacrylate, polysulfone and polyaniline are used in research.
Non-degradable synthetic neural scaffolds can be made of silicon, like mentioned above and polytetrafluoroethylene (Groe-Tex®) or polyethylene(54).
In terms of fabrication and structure the single hollow tube is the most frequently used neural scaffold structure. It is a tubal cylinder that may either be fabricated of natural or artificial biomaterials. It is achieved by melt extrusion or using a spinning-mandrel followed by air-drying(61).

Moreover, they can be formed by injection moulding, electro spinning but also fabrication techniques like crosslinking and physical film rolling can be used(54).

Single hollow neural tubes represent the basic structure and prototypes of conduits and are tried to be modified to come closer to the real and natural architecture of peripheral nerves. By incorporating an internal framework like filaments, sponges or multichannel nerve tubes it was tried to enhance stability and ability of axonal guiding.

Multichannel conduits are elaborated by injection moulding. As a positive side-effect the multiple channels increases the surface of the scaffold, which implies the possibility of incorporating a greater amount of growth factors or support cells in the next step on the scale of improving a tissue engineered conduit for treating peripheral nerve injury(59). Considering that more cells also means more cell metabolisms it has to be thought about the limitation of diffusion in this type of scaffold. Hence its superiority to single hollow tubes still has to be clarified(54).

2.3.3 Fillers

The other possibility suggests filling the lumen of the basic structure, the tubular cylinder, with fibres, foams, gels or filaments. By using these filler techniques the internal is designed to mimic the endoneurial environment.

It already has been shown what happens to the nerve after applying a tubular device. A fibrin clot will form across the gap already in the first hours post implantation followed by invasion of supportive cells, including Schwann cells forming the Bands of Büngner, creating a physiological way of filling(62).

A first attempt to mimic this process was undertaken filling silicone tubes with polyamide filaments in a rat model. The polyamide filaments worked better compared to the group, which was not treated with any bridging method(63).

This indicated to conduct further research on tubular fillers, which included synthetic and biological materials.

It was to find a filling material, which induced axonal regeneration and grafts filled with extracellular matrix showed to promote axonal outgrowth and even were comparable to autograft treated nerves(64).

Putting all these findings together, the fact that collagen has superior features facilitating axonal elongation and laminin (65), which has been shown to elicit neuronal growth, as well as filling the tube itself gives more supportive architecture to the regeneration process, a tube was designed containing all these components(66).

Matsumoto et al. were able to show successful bridging of an 80 mm sciatic nerve gap in a beagle dog model by applying PGA tubes incorporated with laminin coated collagen fibres. After 12 month the implanted dogs had recovered to almost normal in non-loaded walking, whereas the control group, which consisted of 4 dogs with unimplanted 80 mm sciatic nerve gaps, still were limping(66).

Besides using extracellular matrix fibres and filaments, also gels and three-dimensional sponges were used. Additionally to these also alginate foams, hyaluronic acid and extracted proteins, like keratin were tried as possible tubal fillers(67). Gels of laminin and collagen showed the same effect like laminin or collagen filaments, while in terms of distribution more homogeneity was observed along the tube (59), imitating the natural processes already mentioned in tubular devices without prefilling(63).

The scaffold and its filling are two components in elaborating a tissue engineered conduit and choice of material and fabrication techniques are not the only possibility to modify them. Imprinting the scaffold and its filling with growth factors and supporting cells are the third and fourth components for creating a tissue engineered nerve conduit(62).

2.3.4 Growth factors

Growth factors basically are cell-produced products with the ability to stimulate and regulate cell proliferation and maturation. From the processes in pathophysiology of the peripheral nerve injury it is well known that growth factors are very important in modulating regeneration progresses.

These neurotrophic and neurotropic factors play a key role in maintaining survival of neurons, inducing proliferation processes, differentiation and migration of supporting cells to the site of injury.

After acute axotomy the injured neuron up-regulates neurotrophic factors, like BDGF or NGF within the first week.

But also glial sources for growth factors have to be mentioned. The Schwann cells at the distal stump of the injury promote axonal regeneration by providing a variety of growth factors, which then are absorbed by the regenerating axons and transported retrogradely to its neuronal soma.

By activating intracellular pathways they modulate key processes in cellular differentiation and productivity. This leads to an up regulation of growth and axonal regeneration by affecting for example genes like cytoskeletal related genes, resulting in an increased production of tubulin and actin, which are vital for axonal outgrowth.(68)

However, in chronic axotomy injured peripheral nerves regenerate slower and over long distances because of being separated for a prolonged period of time from their distal stump, their distal glia and target organ respectively. Gordon et al. were able to demonstrate that prolonged axotomy, implying chronic denervation, is accompanied by a decline in cellular production of neurotrophic factors in injured neurons and denervated Schwann cells, which is leading to an decreased regenerative capacity of less than 10%(69).

A solution for the problematic lack of growth factors would be creating an exogenously delivered microenvironment that continuously supplies the required type and amount of growth factors to the regenerating nerve.

But which type of growth factor seems to promote axonal sprouting and can be delivered exogenously?

Neurotrophins represent an important class of growth factors and work as stimulators and controllers in the various steps of neurogenesis and peripheral nerve regeneration.
Much research has been conducted on these four structurally related classes of growth factors implying NGF, BDNF, neurotrophin-3 and neurotrophin 4/5.
They initiate a variety of intracellular pathways by binding to two classes of cell surface receptors, the p75 and TrK.

The most important of these four is NGF, which is a small protein that promotes axonal survival and neuronal differentiation. In small amounts NGF is also present in healthy axons, but when it comes to peripheral nerve injury it has been shown, that NGF production is up-regulated in denervated Schwann cells and the surrounding macrophages enhancing nerve regeneration(28).
To use this effect for treating peripheral nerve injury Rich et al. conducted an experiment on rodents comparing NGF- solution and saline-only filled silicone tubes in bridging sciatic nerve defects. Although they found no difference in the number of regenerating axons, they were able to show, that the NGF treated sciatic nerves presented with more myelinated axons, as well as thicker myelin sheaths than the saline solution only treated group(70).

BDNF works very similar to NGF and also supports axonal survival and promotes axonal outgrowth(68). It is expressed by the injured neuron and therefore has to be delivered in high concentration at the site of injury(54).
According to these findings Utley et al. compared a collagen tube with BDNF covalently cross-linked to the collagen matrix to epineural coaptation and plain collagen tube treatment in a rat model. They were able to show, that animals treated with collagen tubes containing BDNF covalently cross-linked to the collagen matrix demonstrated superior functional recovery after a period of 90 days compared to the standardly treated groups. Histologic evaluation of the sciatic nerve segment harvested from the former site of injury showed the largest mean axon diameter in the collagen tube with BDNF covalently cross-linked to the collagen matrix. This indicates that BDNF affects the sprouting axons in a very positive way.

NT-3 and NT4/5 also support axonal survival, outgrowth, formation of neuronal synapses and myelin sheaths. In several rat models the supportive effects of these neurotrophic factors were observed, showing improvement in number, axonal diameter and myelin thickness compared to the groups of standardly treated rats(71)(72).

Additionally to the already mentioned neurotrophins there also exist some other classes of growth factors, cytokines and insulin derivatives that demonstrated positive effects on regeneration axons.

Glial cell-line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor (IGF-1, IGF2), platelet-derived growth factor (PDGF), Fibroblast growth factor (FGF) showed to support axonal regeneration as well as proliferation of supporting cells, like Schwann cells(54).

One big problem when working with growth factors is the maintenance of concentration over the period of regeneration.

This means that usage of exogenously applied neurotriophic and neurotropic factors brings up concerns about the right way of delivery. Incorporating growth factors without losing the ability of maintaining the right dosage over the period of regeneration is still a big challenge.

Like in the experiments described above, the first attempts were delivering the growth factor directly incorporated into the tubular device. This was realized by using growth factor containing coatings and covalently linking or reservoir-based delivery strategies. However, there still exists the problem of declining concentration of neurotrophic factors over time due to degradation and absorption(54).

New approaches tackle this problem by using subcutaneously implanted pumps, repeated injection or models of controlled gene expression by utilizing virus vectors or plasmids(62).

2.3.5 Supporting cells

Another promising approach in modifying the plain scaffold is incorporating supportive cells. These cells then themselves create the regenerative milieu containing all the mentioned components for peripheral nerve regeneration. The big advantage of applying supportive cells lies in the fact that they produce the required growth factors directly at the site of injury in the right dosage and composition. Deducing from the pathophysiology of the peripheral nerve injury Schwann cells seem to be a very promising candidate, but also stem cells with the ability to differentiate into nerves or Schwann cells are studied extensively.

Schwann Cells

Schwann cells take over a major role in the pathophysiology of peripheral nerve injury. They directly contribute to the regenerative milieu crucial for survival of the proximal segment by producing growth factors like NGF, BDNF and IGF. Additionally they phagocytize the debris of Wallerian degeneration, which means they remove axonal regrowth impeding tissue. Due to the fact, that the most successful functional recovery depends on the shortest possible period of denervation, by removing debris Schwann cells indirectly contribute to degeneration as well as regeneration. Furthermore they produce a large number of adhesion molecules, like N-Cadherin and neural cell adhesion molecule N-CAM that enables the regenerating axons to find their way through the site of injury. Their basal membrane acts as a natural scaffold guiding axonal regrowth due to containing laminin and collagen(49).
Deducing from all these observations Schwann cells were the first choice for creating an artificial peripheral nerve graft when it was striking the first hour of tissue engineering.

In the 1980ies first attempts were undergone with allogeneic Schwann cells, but the results were devastating, because of immune response to the foreign cells. So it was tried to administer immunsupressive agents and the most widely used drug for this purpose was Cyclosporin A. With this approach the delivered Schwann cells were able to persist until chronic rejection occurred, which meant that endogenous Schwann cells

got the time required to gradually replace the foreign donor Schwann cells. An alternative to Cyclosporin A was Tacrolimus (FK506), which is a calcineurin inhibitor(54). FK506 was found not only to keep the allogeneic Schwann cells alive, but also showed to enhance axonal regeneration(73). Although immunosuppressive drugs controlled the problems with antigenicity very well, the administration of immunosuppressive agents in humans still is in argument against clinical use. Therefore experiments with autologous Schwann cells were started in the 1990ies. Adding exogenous mitogens, like heregulin and forskolin allowed to expand the initial number of cells obtained from a tiny nerve tissue source with in vitro culture techniques(61).

Then Schwann cells were isolated from the autologous peripheral nerve and harvested either by enzymatic dissociation or multiple passages(74). Afterwards these cells were delivered to the scaffold, which was tried by seeding them onto the conduit or introducing the cells with fillers or by direct injection into single hollow or multichannel tubes. The cells showed to be vital after implantation and HNK-1 (specific marker for human myelin) labelling in nude mice indicated that the delivered human Schwann cells were able to form myelin and enhance axonal regrowth(75). Furthermore, Keilhoff et al. were able to show, that implanted Schwann cells aligned forming columns of cells and produce neurotrophic and neurotropic factors(76)(77).

These observations suggested, that transplanted Schwann cells not only survived the procedure of being seeded onto a polymer and being transplanted into the gap, but also showed that they still took over their mediator role in the key processes of axonal regeneration.

Stem cells

Characteristics of stem cells include unlimited capacity of renewal and the virtual ability to differentiate into any kind of body cell. With these characteristics it was tried to modulate the pluripotent cells in their differentiation by using neural growth factors. First attempts were undertaken with NGF, which showed to be a strong enhancer of neuronal differentiation.

These pretreated stem cells showed to express the same growth factors like Schwann cells do, when applied to the site of peripheral nerve injury.

In a rat model Cui et al. resected a segment of the sciatic nerve and after a period of one hour they applied the undifferentiated cells to the site of injury. Resecting the healed nerve after three month showed that the stem cells had differentiated into myelinating cells providing a perfect environment for axonal regrowth(78).

On the other hand comparing stem cell and Schwann cell delivering grafts directly indicated that Schwann cell delivering grafts achieved better results(79).

2.4 Material

2.4.1 Pilot study

The concept EK-23-127 “Isolating and culturing adult Schwann cells from peripheral nerves explanted from amputates of patients at the Department of Plastic, Aesthetic and Reconstructive Surgery of the Medical University of Graz” was accepted by the ethics committee of the medical University of Graz on the 20th of January 2011.

2.4.2 Patients

Our patient collective included patients treated at the Department of Plastic, Aesthetic and Reconstructive Surgery of the Medical University Graz. The peripheral nerves were excised from amputates after infectious disease was excluded, whereas age, sex, race and anatomical quality of peripheral nerve did not cut down the selection of patients. In summary eight peripheral nerves were harvested.

2.4.3 Agents, instruments and equipment

2.4.3.1 Agents

Amphotericin B	Sigma Aldrich GmbH, Vienna
Dulbecco’s Modified Eagle’s Medium (DMEM)	Sigma Aldrich GmbH, Vienna
Fetal Calf Serum (FCS)	Sigma Aldrich GmbH, Vienna
Forskolin	Sigma Aldrich GmbH, Vienna
Heregulin-β	Sigma Aldrich GmbH, Vienna
Laminin	Sigma Aldrich GmbH, Vienna
L-Glutamine–penicillin–streptomycin solution (GPS)	Sigma Aldrich GmbH, Vienna
Phosphate Buffered Salt Solution (PBS)	Biochrom AG, Berlin
Poly-L-ornithine solution	Sigma Aldrich GmbH, Vienna

OCT Compound medium Tissue-Tek®,	Sakura Finetek Germany GmbH, Staufen

Table 1 Agents used for experiment

2.4.3.2 Instruments

Culture plate (6-well)	IWAKI Europe GmbH, Willich
Petri culture dish	BD Biosciences Austria, Schwechat
Cell scraper	BD Biosciences Austria, Schwechat
Falcon tube	BD Biosciences Austria, Schwechat
Pipette (100-1000µl)	Eppendorf Austria GmbH, Vienna
Pipette (10-100µl)	Eppendorf Austria GmbH, Vienna
Pipette (0,5-10µl)	Eppendorf Austria GmbH, Vienna
Pipette tip	Eppendorf Austria GmbH, Vienna
Microforceps	Aesculap AG, Tuttlingen
Anatomical forceps	Aesculap AG, Tuttlingen
Surgical scissors	Aesculap AG, Tuttlingen
Spring type micro scissors	Aesculap AG, Tuttlingen
Surgical scalpel	Aesculap AG, Tuttlingen
Superfrost plus microscope slides	Menzel GmbH & CO KG, Braunschweig

Table 2 Instruments used for experiment

2.4.3.3 Equipment

Centrifuge	Heraeus Holding GmbH, Hanau
Inverse microscope	Olympus Europa GmbH, Hamburg
Incubator	Eppendorf Austria GmbH, Vienna
Vortex mixer	IKA Werke GmbH & Co. KG, Staufen
Workbench	Heraeus Holding GmbH, Hanau
Water bath shaker	JP SELECTA S.A., Abrera (Spanien)

Slide stainer (H&E stain) DRS2000	TTISSUE-TEK
Fluorescence microscope,Basic BX51	OLYMPUS
Microtom-Cryostar HM560	Thremo SCIENTIFIC

Table 3 Equipment used for experiment

2.4.3.4 The Polymer-OPTIMAIX 2D

OPTIMAIX 2D is a two dimensional scaffold produced by Matricel GmbH, Herzogenrath in Germany. The scaffold is made of highly purified porcine collagen containing low levels of cysteine, tryptophan and hexosamines and shows a very high capacity of medium up take assessed with the wet/dry ratio by Botzkurt et al.(80).

Figure 11
Polymer OPTIMAIX 2D

Therefore OPTIMAIX 2D provides an optimized scaffold for in vitro and in vivo experiments. Furthermore, it has proven compatibility with a wide range of different cell types, also including Schwann cells. Applied in vivo this scaffold showed to be biodegradable without triggering significant inflammation at the site of implantation.

The scaffold has two different sides, a dense fibrous and open fibrous side. Nevertheless OPTIMAIX 2D is at present not indicated for human use(81).

Bozkurt et al. have already used OPTIMAIX 2D and 3D for creating a Schwann cell seeded microstructured nerve conduit. The Schwann cells were obtained by dissociation from sciatic nerves of Lewis rats and a Schwann cell suspension was applied to the dense fibrous side of OPTIMAIX 2D. The viability after culture on OPTIMAIX 2D was assessed by mitochondrial metabolic activity and morphologically. The seeded cells showed to be viable and had their metabolic climax on day 14 of culture. Morphological assessment revealed that the Schwann cells were aligning in cell columns mimicking the bands of Büngner, indicating preserveration of cell function(80).

2.4.3.5 The culture medium

44.43 ml DMEM	20 µl Forskolin
5.00 ml FCS	50 µl Amphotericin B
0.5 GPS	9.1µl Heregulin

Table 4 Culture medium used for experiment

Based on experimental results of Morrissey et al. in rodents we used DMEM and 10% FCS as basic ingredients for the Schwann cell culture medium. This basic culture medium was complimented by GPS and amphotericin B to prevent infection, which has already successfully been used in the culture medium of Scarpini et al(74)(82).

Additionally to these ingredients forskolin and heregolin β were added.

Forskolin is acting mitogenic on Schwann cells and therefore is used to expand the number of cells obtained from harvested peripheral nerves. Forskolin is known to elevate the intacellular cyclic AMP level, which promotes proliferation in Schwann cell cultures. Besides that it was observed, that forskolin in the same way inhibits perineural fibroblasts from proliferation, which is ideal for working according to the explant-reexplant protocol of Askanas et al(83).

Heregulin β belongs to the polypeptide growth factor family promoting Schwann cell proliferation and survival by binding to erbB2 and erbB3, which activate the MAP kinases pathway. Due to the fact that this effect is enhanced in presence of in cyclic AMP elevating conditions, cyclic AMP elevating agents, like forskolin, have to be added.

Furthermore, forskolin and heregolin β synergistically elevate the expression of proliferation-required components, like cyclin D and enhance the phosphorylation of retinoblastoma gene product.(84) By adding heregulin β and forskolin the number Schwann cells can be extended maximally in culture, while the harvested cells maintain their ability of responding to axonal signals in appropriate ways(85)(86).

Moreover, the culture dishes, as well as the polymer OPTIMAIX 2D were pre-coated with extracellular matrix components, including ornithine/laminin and collagen, which also has been observed to contribute positively to Schwann cell proliferation(87)(88).

2.5 Methods

2.5.1 Schwann cell Isolation

The peripheral nerves were harvested from amputates of patients who underwent treatment at the Department of Plastic, Aesthetic and Reconstructive Surgery of the Medical University Graz. The nerves were explanted directly in theatre and transported to the laboratory in DMEM/GPS storage medium.

Meanwhile in the laboratory a 6-well culture plate was prepared and incubated for 30 minutes under standard conditions, which means 37 °C, 5 % CO_2 and 90% humidity. Therefore each well was pre-coated with 500 µl of 1:50 diluted collagen in Schwann cell culture medium. Then the freshly explanted human nerve was prepared in a petri dish. Therefore the nerve was dissected under the microscope with microsurgical instruments. Always paying attention to working as less traumatizing to the nerve as possible the epineurium was stripped off. Afterwards the remaining nerve tissue was either cut into 1 mm sized segments or, if the explant was long enough, single fascicles were pulled out of the explant. Now the obtained nerve tissue was embedded into the previously prepared wells of the 6-well culture plate and incubated for another 30 min before adding another 500 µl of Schwann cell culture medium to each well. Schwann cell culture medium was changed every third day and the growth of the culture was assessed by light microscopy.

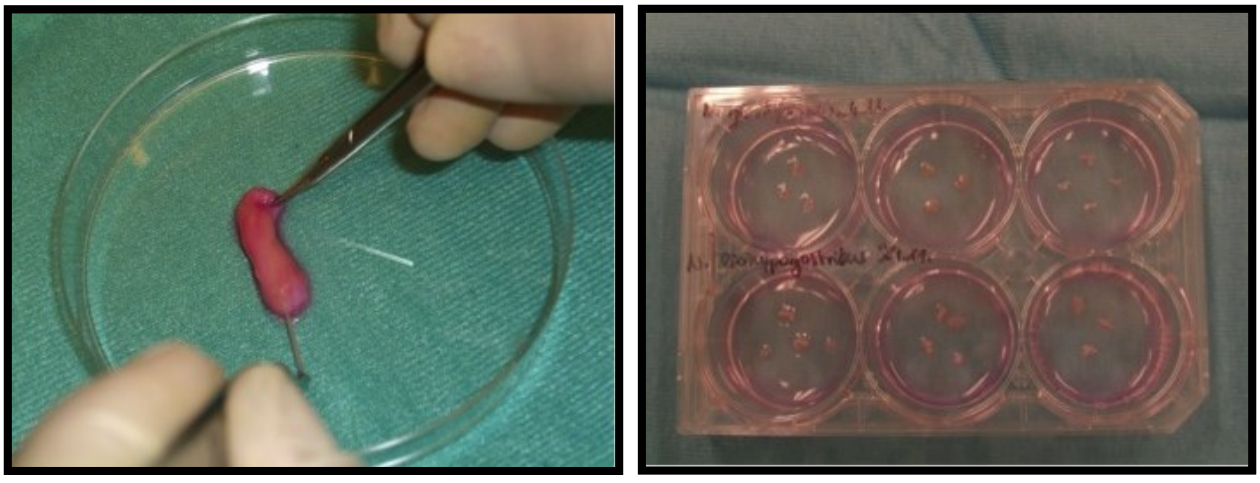

Figure 12

Single fascicles are pulled out of the explant (left side); nerve cut into 1 mm sized segments embedded in a pre-coated 6-well culture plate (right side)

2.5.2 Application of Schwann cells to the polymer OPTIMAIX 2D

The fascicles and 1 mm pieces were cultured under standard conditions with the previously described Schwann cell medium. After a confluent monolayer of morphologically identified Schwann cells had formed around the pieces or fascicles respectively, they were explanted and replanted on the laminin pre-coated polymer OPTIMAIX 2D.

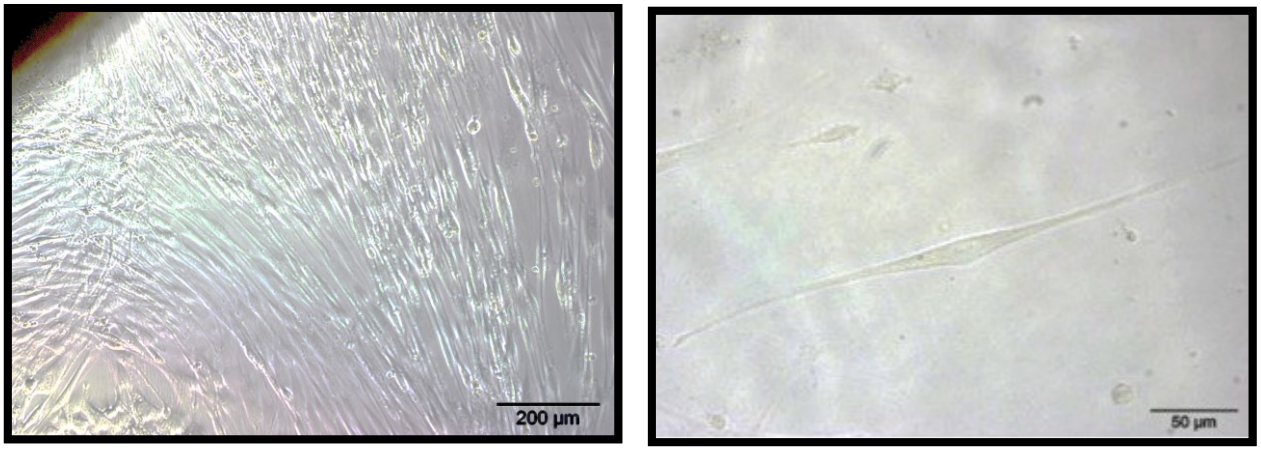

Figure 13
Schwann cells in culture showing the typical parallel orientation by lining up end to end (left side); one single Schwann cell displaying the bipolar spindle shape with an oval cell body (right side)

Therefore the Polymer OPTIMAIX 2D was cut under sterile conditions into six equally sized piece. Then each piece was applied to one well of a plain 6-well culture plate and pre-coated with 210 µl of a 5 % laminin/ornithine mixture. Now either the fascicles or the 1 mm pieces were applied via drop on technique on the open fibrous side of the polymer OPTIMAIX 2D. After 30 minutes of incubation under standard conditions each well was filled with 1000 µl of Schwann cell culture medium.

Also the cells remaining in the 6-well culture plate were harvested to apply them all on one of the pieces of Polymer OPTIMAIX 2D prepared in the same way, like it already has been described above. Therefore 1000 µl phosphate buffered saline (PBS) were filled into the explanted wells. With a cell scraper the cells were detached from the dish and the surface was washed by pipetting up and down several times. From each well of the 6-well culture plate the cell suspension was pipetted into a 50 ml falcon and centrifuged for 12 minutes with a speed of 1200 turns per minute. Afterwards the

supernatant fluid was thrown away and the remaining cell pellet mixed with 1000 µl Schwann cell culture medium by vortexing and pipetting up and down for several times. Afterwards the cell suspension was again applied to the open fibrous side of the laminin pre-coated OPTIMAIX 2D.

The Schwann cell culture medium was changed on every third day. After a period of 4 weeks the OPTIMAIX 2D pieces were explanted and frozen to – 20 °C.

2.5.3 Assessing Schwann cell culture growth on OPTIMAIX 2D

The frozen OPTIMAIX 2D pieces were embedded in OCT compound medium to obtain 7 µm sections at the cryomicrotome, which were applied to superfrost plus microscope slides for subsequent staining.

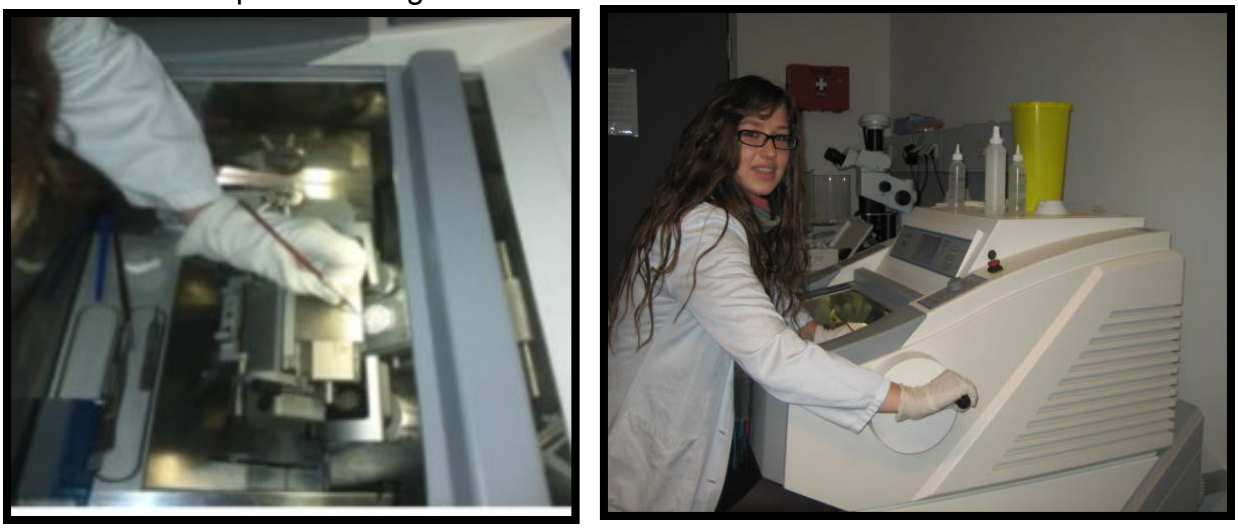

Figure 14

Frozen pieces of OPTIMAIX 2D are cut into 7 µm sections and applied to superfrost plus slides

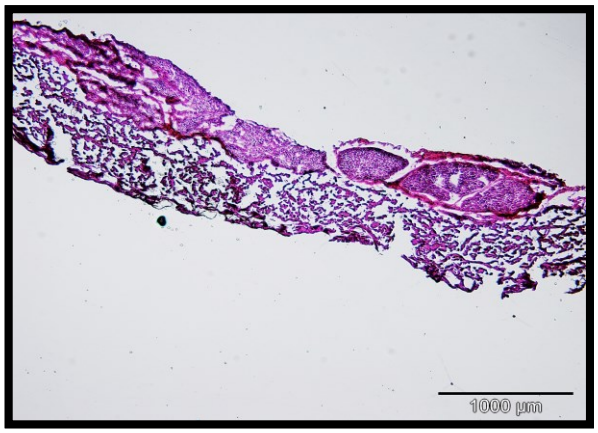

Figure 15

H&E stained slide

In the first step of assessment all plain slides were stained with haematoxylin and eosin to detect, if cells had survived on the polymer OPTIMAIX 2D.

Thereby hemalum, which means haematoxylin oxidated to haematein, the active staining component, bound in a complex with aluminium ions, stains

basophile structures, like DNA, blue, whereas the counterstaining eosin colours the rest, like cytoplasm, in bright pink.

Every 10th slide was stained H&E in a H&E slide stainer. In the first chambers of the stainer the slides were applied to Xylol and Mayer`s haematoxylin solution. Afterwards the slides were blued in water and counterstained with eosin and finally washed. In the last step they were dehydrated by increasing alcohol concentrations(89).

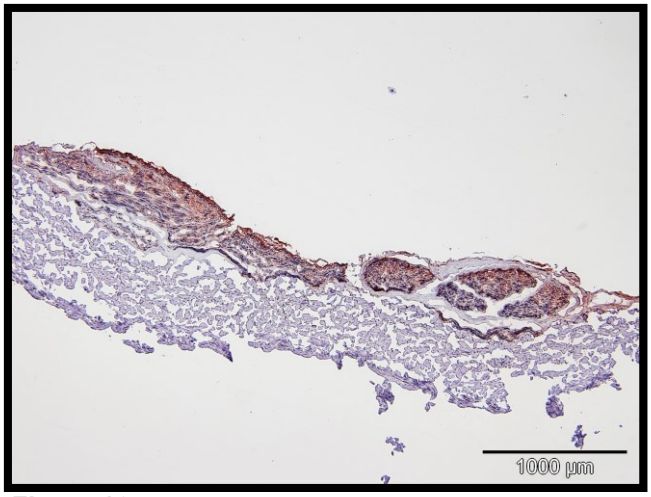

Figure 16
S100 stained slide

All slides showing cells seeded on the polymer OPTIMAIX 2D were stained for their identity. Schwann cell specific immunocytochemical S100 staining was used to confirm that the H&E stained cells were indeed Schwann cells(82). S100 is an intracellular protein bound to Calcium and involved in several intercellular processes, like cell communication, intracellular signal transduction and proliferation(90).

For S100 staining every slide following a H&E positively stained slide was fixed in acetone and blocked with ULTRA V (LAB VISOIN). For labelling cytoplasmic S100 the slides were incubated with the primary antibody rabbit anti-S100, then washed in PBS before the secondary antibody was applied in form of biotinylated goat anti-polyvalent (LAB VISION). Again the slides were rinsed in PBS for the application of streptavidin (LAB VISION). Afterwards the slides were rinsed for a last time in PBS, for finally labelling the S100–antibody complex with ACE+(DAKO), staining them in bright red, and counterstaining with Mayer`s haematoxylin (MERCK) respectively.

All S100 and H&E positively stained explants were additionally stained for MIB. MIB staining is used for detecting mitosis, thus growth on the polymer OPTIMAIX 2D within previously S100 positively stained cells, Schwann cells. It is an immunocytochemical marker for the K-67 protein, which only is present during mitosis and absent in Go phase. Ki-67 is important for ribosomal RNA transcription in body cells and is widely used as a prognostic tool for cancer diagnostics(91).

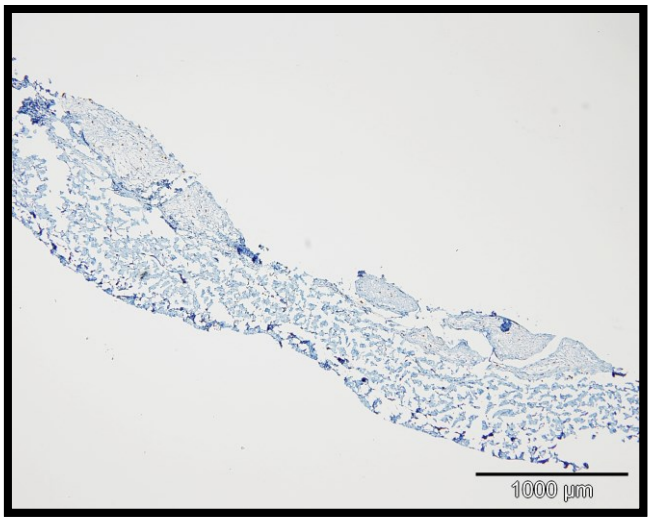

Figure 17

MIB stained slide

For the MIB staining all slides were fixed in 30 ml of 37% formaldehyde in 70 ml PBS. Afterwards the slides were given into the MIB stainer. Within the staining process the monoclonal rabbit antibody, anti Ki-67 (VENTANA), was applied and they counterstained with haematoxylin.

The MIB positive cells are coloured in dark brown, whereas the Ki-67 lacking tissue was stained in light blue.

2.6 Results

2.6.1 Cell culture

All of the 8 harvested nerves were applied to a 6-well culture dish and cultured in Schwann cell medium, described above, under standard conditions. From these eight nerves two showed an outspread forming a confluent monolayer of bipolar spindle shaped cells with an oval cell body, whereas nerve three showed an outspread of polygonal cells. In the two vital nerves, mentioned above, the first cells spread from the fascicle explant after a culture period of 19 days, whereas the first cells in the 1 mm sized explants were seen on day 14. Culture growth and phenotype of the sprouting cells was assessed by light microscopy.

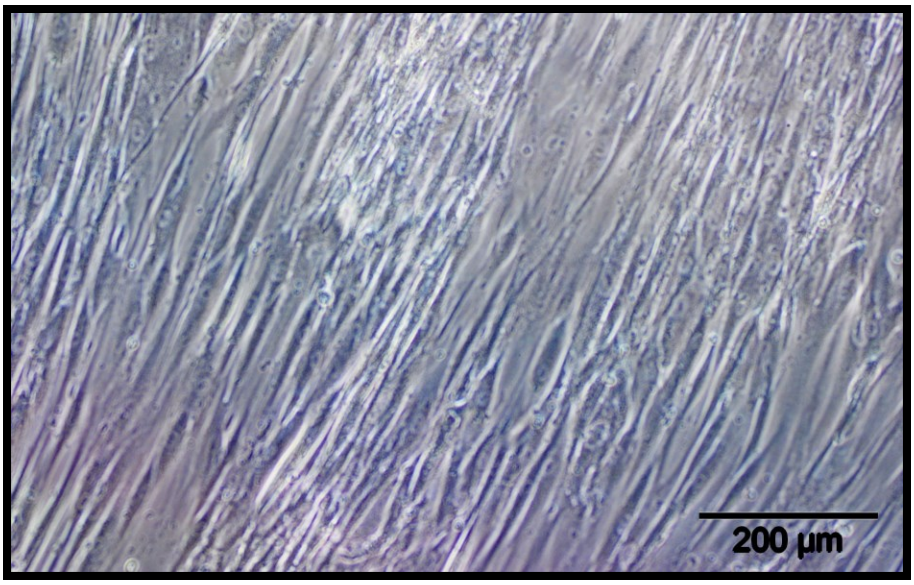

Figure 18
Schwann cells in culture

The Schwann cells showed a spindle shaped bipolar appearance and spread parallel orientation, being aligned end to end. Contrary to sprouting Schwann cells fibroblasts displayed a rather polygonal morphology growing dispersed and much faster than Schwann cell cultures. They were observed in one nerve and spread on day 7, which was much earlier than spindle-shaped Schwann cells normally occur in culture. The appearance of fibroblasts was followed by reduced outspread of Schwann cells. Therefore this nerve was not used for culturing on the polymer OPTIMAIX 2D.

Furthermore, it was observed, that pre-coating culture dishes with collagen contributes to a better attachement of the neural tissue, which resulted in optimal conditions for cell sprouting. Explants that loosely swam around in the Schwann cell culture medium did not show any outspread of cells.

2.6.2 Assessing Schwann cell culture growth on the polymer OPTIMAIX 2D

The tissue explants and the cells of the confluent monolayer were harvested and applied to the laminin pre-coated polymer OPTIMAIX 2D, which was cut into 6 pieces. Applied to each well of a 6-well culture dish these pieces were cultured for four weeks before they were harvested and frozen at -20 °C.

For assessing whether the cells remained viable following culture on the polymer OPTIMAIX 2D the colonised pieces of the polymer were cut into 7 μm sections and applied to superfrost thermo plus microscope slides for subsequent H&E staining.

The polymer pieces colonised with the fascicles showed H&E positive tissue, as well as the samples colonised with the 1 mm sized explants. Whereas the two pieces of polymer colonised with cells harvested from each monolayers of the two different nerve explants, the 1 mm sized explant and the fascicle cultured explant respectively, showed to be H&E negative. These results indicated, that no cell had survived on the polymer and therefore no further histocytochemical staining was conducted on these polymer pieces.

In the next step it had to be clarified, whether the cells displayed in the H&E staining were Schwann cells or fibroblasts. Therefore immunocytochemical S100 staining was conducted. This staining showed that the fascicle colonised polymer pieces, as well as the polymer pieces colonised with 1 mm explants showed S100 positive cells. This indicated, that the H&E stained cells were indeed Schwann cells.

To be determined was whether the Schwann cells on the polymer OPTIMAIX 2D were resident and senescent Schwann cells belonging to the neural tissue of the explanted nerves, or if these cells were proliferating and showing the same behaviour analogous to culture. Immunocytochemical assays were therefore conducted to probe MIB staining to depict mitosis, which represents culture growth. In both nerves there were MIB positive cells detected, indicating that cellular outspread and growth also occurred on the polymer OPTIMAIX 2D.

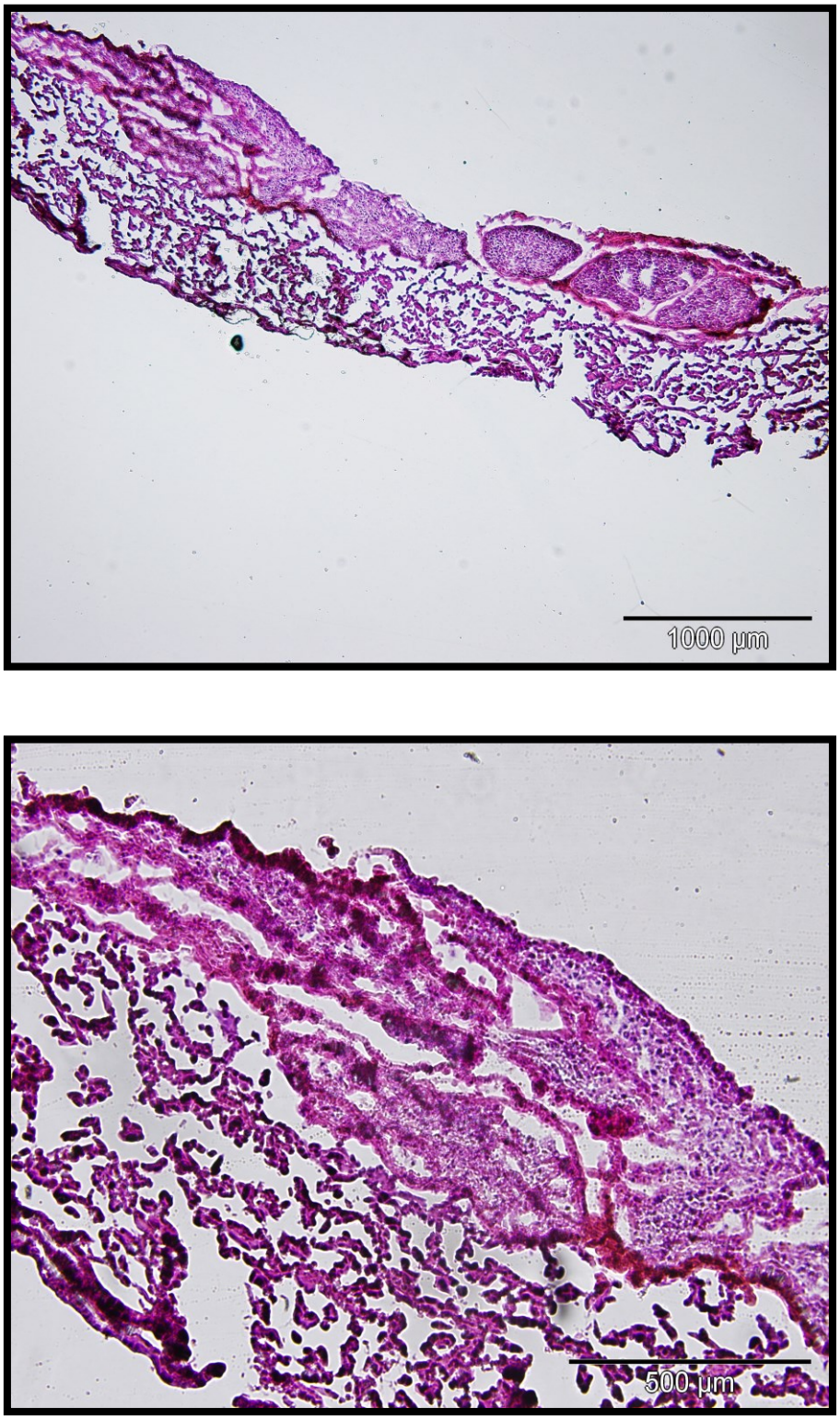

Figure 19

H&E stained slide of the polymer OPTIMAIX 2D colonised with neural tissue; the nuclei are stained in dark blue, while the cell bodies are coloured in violet. On the right side of the picture the architecture of the explant is still visible, while on the left side the loose structure indicates outspread.

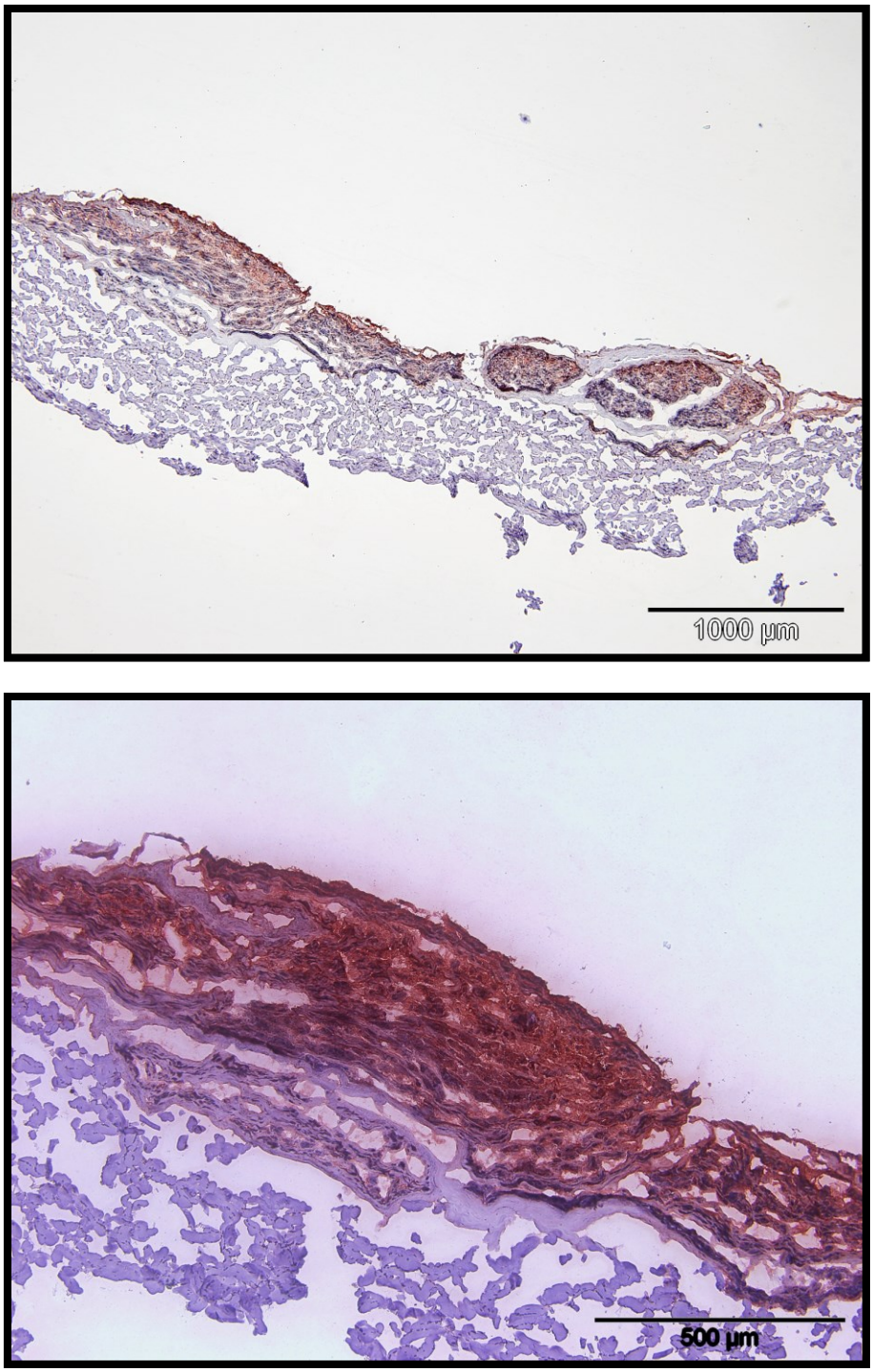

Figure 20

S100 stained slide of the Polymer OPTIMAIX 2D colonised with neural tissue;

The cell bodies of Schwann cells are stained in bright red. There are stained Schwann cells visible within the neural tissue on the right side, but also within the outspread on the left side of the picture.

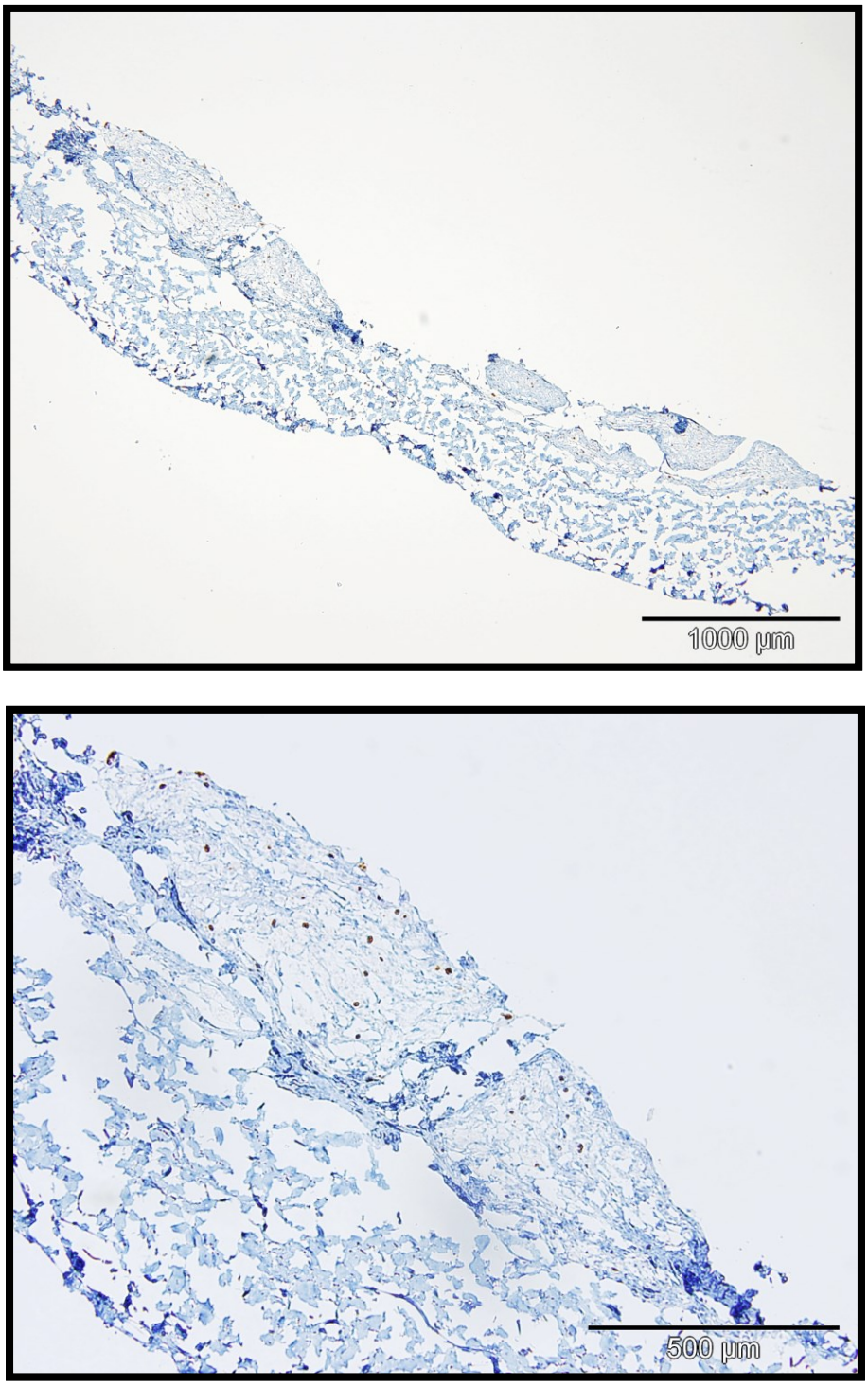

Figure 21

MIB stained slide of the polymer OPTIMAIX 2D colonised with neural tissue;

The nuclei of proliferating cells are stained in dark brown, while the cell bodies of non-mitotic cells are coloured in blue. There are mitotic cells in the explant on the right side, as well as in the loose structure on the left side of the picture depicted, which is indicating outspread.

Moreover, it was tried to grossly assess the growth of the cells cultured on the polymer OPTIMAIX 2D. For this purpose an indication of the average count MIB positive cells was obtained by assessing the polymer pieces showing the best cellular growth of the group colonised with 1 mm sized expants and the group of fascicle colonised OPTIMAIX 2D. Therefore, the brown cells within the location showing the most positively stained cells per microscopic field with magnification x 20 were counted.

Slide stained MIB/ magnification x20	Polymer OPTIMAIX 2D colonized with 1 mm sized explant	Polymer OPTIMAIX 2D colonised with fascicle
Slide 12	0/ field	0/ field
Slide 22	1/ field	0/ field
Slide 32	6/ field	6/ field
Slide 42	4/ field	9/ field
Slide 52	28/ field	7/ field
Slide 62	26/ field	6/ field
Slide 72	15/ field	13/ field
Slide 82	24/ field	12/ field
Slide 92	32/ field	6/ field
Slide 102	37/ field	6/ field

Table 5 Count of MIB positively stained cells.
Each chosen section of neural tissue-colonised polymer OPTIMAIX 2D was analysed in the region showing the highest concentration of brown cells per field/ magnification x20.

The polymer piece colonised with the 1 mm sized explants thereby showed a higher mitotic activity than the fascicle colonised one. For this reason a full count of S100 positive cells in the slides of the same polymer pieces, chosen for MIB cell assessment, was conducted in order to finally assess whether the fact that there were more cells on the polymer was creating the impression of higher mitotic activity or not.

Therefore all red stained cells were counted through utilisation of the microscope Basic BX 51 connected to a computer providing the cell count programme CELL D (OLYMPUS).

For counting purposes a grid overlays the image, allowing the user to zoom into every little quadrant. This ensures better identification of cell structures for counting only the cells stained positively. Every cell, the user selects by mouseclicks, is marked with a red cross preventing double counting. Additionally every click is counted in an Excel table (MICROSOFT) in the background of the programme.

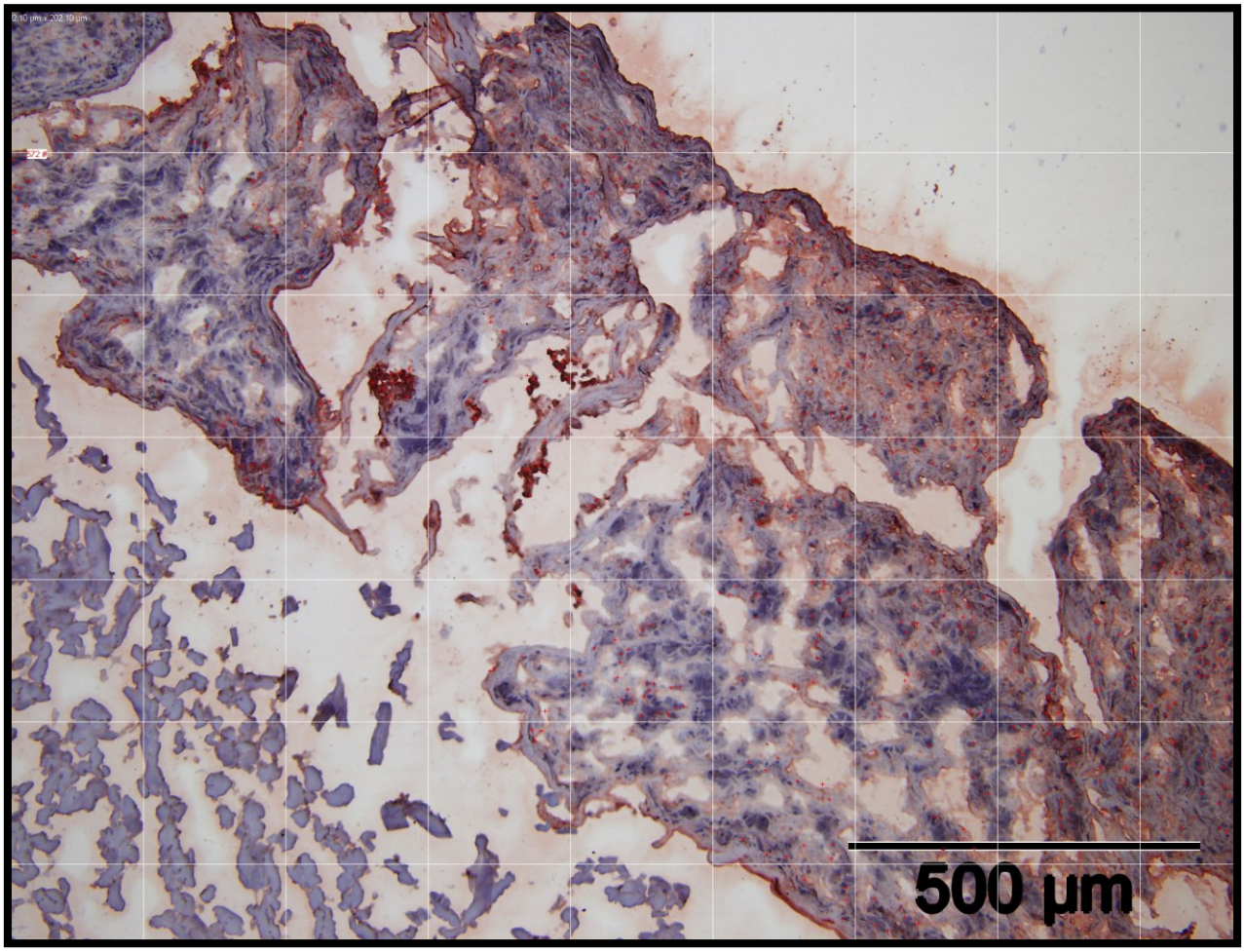

Figure 22

Total cell count of S100 positive cells by cell count programme CELL D

For counting purposes a white coloured grid is burned into the image. All S100 positive cells on the polymer OPTIMAIX 2D are marked with a red cross. The tiny number on the right upper side gives the total number of cells marked with a red cross.

Slide stained S100	Polymer OPTIMAIX 2D colonised with 1mm sized explant	Polymer OPTIMAIX 2D colonised with fascicle
Slide 11	0/ section	0/ section
Slide 21	0/ section	9/ section
Slide 31	81/ section	44/ section
Slide 41	212/ section	150/ section
Slide 51	551/ section	102/ section
Slide 61	1353/ section	575/ section
Slide 71	1487/ section	941/ section
Slide 81	1199/ section	1122/ section
Slide 91	2598/ section	1237/ section

Table 6 Full cell count of S100 positively stained cells on the tissue-colonised polymer OPTIMAIX 2D.

On average there were 1075.3 Schwann cells counted on the polymer OPTIMAIX 2D colonised with the 1 mm sized explants, while only an average of 464.4 Schwann cells was detected on the fascicle colonised piece. The stained cells counted included all red cells on the polymer, the newly grown Schwann cells as well as the originally to the neural tissue belonging cells.

Set in relation to the MIB assessment results, which indicated that the polymer piece colonised with the 1 mm sized explants showed a higher mitotic activity than the fascicle colonised one, the S100 total count results suggests, that there was more neural tissue on the with explant colonised polymer piece, than on the with fascicle colonised OPTIMAIX 2D.

In general, this pilot study has shown, that the explant–reexplant technique first described by Askanas et al, can not only be used for obtaining a primary Schwann cell culture from human peripheral nerves, but also for colonisation of a polymer, such as OPTIMAIX 2D.

2.7 Discussion

The objective of this pilot study was colonising the polymer OPTIMAIX 2D with human Schwann cells obtained by expant-rexplant technique described by Askanas.

Three main pillars characterise this experiment. These include the polymer OPTIMAIX 2D, the human Schwann cells obtained from human peripheral nerves and finally, the explant-reexplant technique of Askanas. Therefore, the critical points regarding polymer OPTIMAIX 2D, the quality of human Schwann cells and the efficiency of explant-reexplant technique have to be discussed.

The polymer OPTIMAIX 2D is produced of highly purified porcine collagen with a very high capacity of medium uptake. It already has shown great results when used for creating a Schwann cell seeded microstructured nerve conduit by Botzkurt et al(80).
Moreover, it has proven compatibility with a wide range of different cell types and applied in vivo, the biodegradable and collagen based scaffold triggered no significant inflammation at the site of implantation(81).
Nevertheless, OPTIMAIX 2D is at present not registered for use in humans and therefore a tissue engineered tubular device made of OPTIMAIX 2D cannot currently be used for treating peripheral nerve injury in patients (81).
However, it has to be considered, that other devices, such as NeuraGen® or Integra are produced from xenogeneic collagen, as well. Contrary to OPTIMAIX 2D, they already have been registered by FDA and are are being utilised in the clinic. Moreover, Boeckstyns et al. were able to show that a collagen tube made of bovine collagen performs as well as direct suture in treating peripheral nerve injury presenting with a small defect. 24 month after the procedure the collagen tube treated patient group was compared to the standardly treated patient group by electrophysiological and hand function testing. There were no statistical significant differences found regarding conduction velocity and motor function. Additionally to these phenomenal results the implantation of the collagen tube was much easier than performing neurorrhaphy, which appoints the fact that collagen devices not only comprise huge advantages for the patient, but also for the surgeon(51).

Regarding the supporting cells used in this pilot study the selection of patients and the culture medium must be outlined.

Our patient collective included patients treated at the Department of Plastic, Aesthetic and Reconstructive Surgery of the Medical University Graz. Over all, eight peripheral nerves were excised from amputates, after infectious disease was excluded. Age, sex, race and anatomical quality of peripheral nerve did not cut down the selection of patients. A closer look to the eight patients who consented for donating peripheral nerves for this study shows, that all of them were adults and in general about 60 years old partly suffering from chronic disease impairing neural health, like polyneuropathy.

Unfortunately this fact implied that nerve tissue obtained from amputates of these patients tended to die after blood supply was cut down.

Thus, this might explain, why five of the nerves cultured in Schwann cell medium containing forskolin and heregulin β did not respond with cellular outgrowth. This observation also addresses another issue. The use of in mitogenic substances cultured supporting cells for neural devices, when implantation into humans is aimed, is highly controversial. It is argued that mitogenic ingredients alter the implanted cells giving them the potential of malignant transformation. Although these cells have demonstrated to maintain their ability of responding appropriately to axonal signals regarding myelination and promoting axonal outgrowth, formation of tumours in rodent models has been observed(85)(86).

This raises the question, if this phenomena also occurs in human Schwann cells implanted into human peripheral nerves?

For clarifying whether mitogen-extended human Schwann cells are safe in vivo or not, Emery et al. have conducted a study based on this particular question. They injected heregulin β and forskolin treated human Schwann cells into sciatic nerves of severe combined immunedeficient rats. Although they found formation of connective tissue sheath, there was no emergence of malignant tissue detected. These results indicate that mitogen-extended human Schwann cells are safe and do not cause malignancy(92). In the future this will be of certain importance, especially when considering harvesting the right amount of autologous Schwann cells from patients undergoing peripheral nerve reconstruction with a tissue engineered prosthesis.

For preventing damage to the donor nerve only very small amounts of tissue can be harvested. Hood et al. even suggest obtaining autologous nervous tissue from a biopsy of an appropriate autologous donor nerve, which implicates the need for expanding culture techniques. Otherwise the amount of Schwann cells obtained from the biopsy material is too small for subsequent construction of the future patient`s tissue-engineered autologous nerve prosthesis(93).

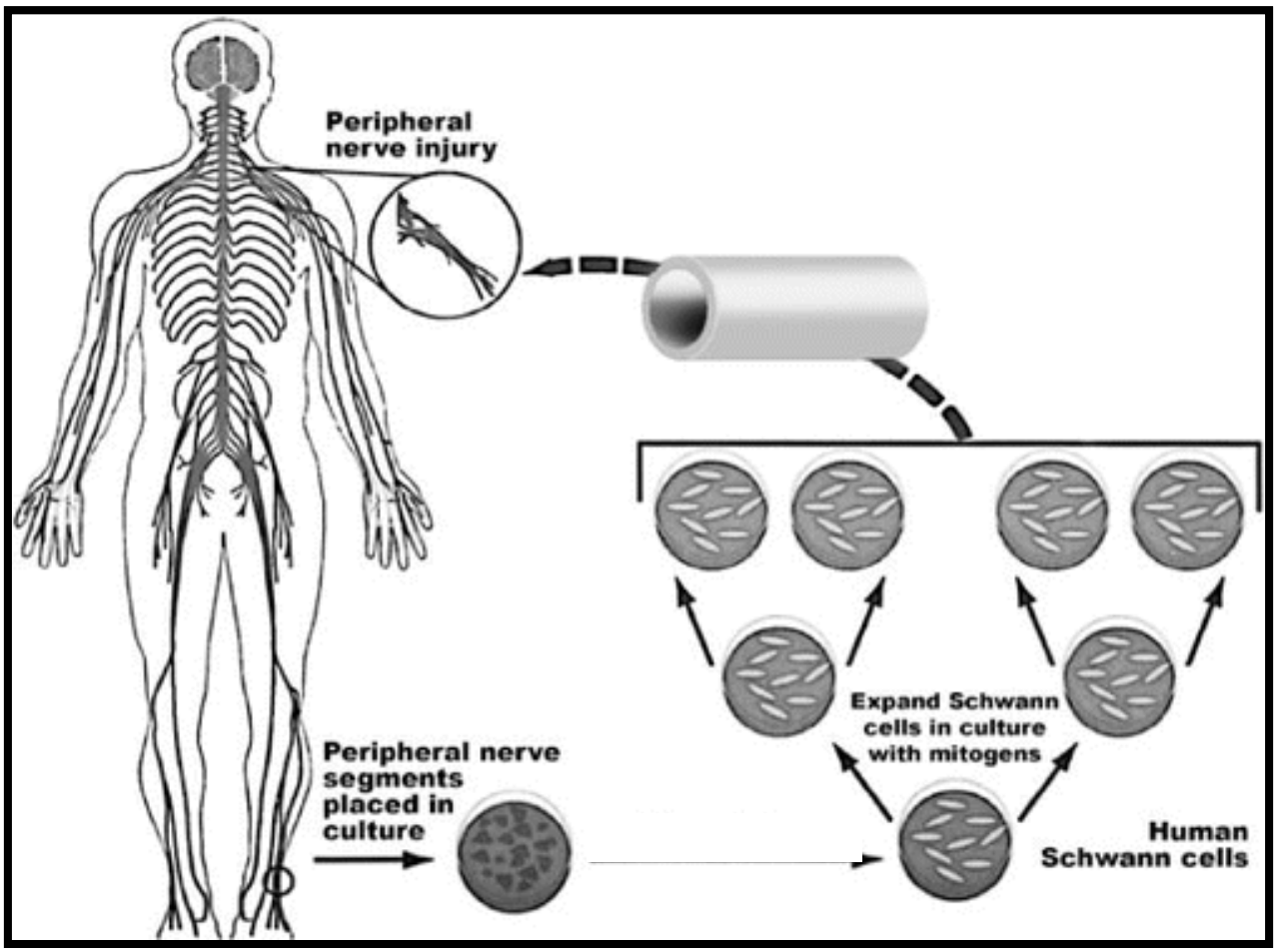

Figure 23 Future Treatment of peripheral nerve injury

In the future treatment of peripheral nerve injury with defects is including nerve biopsy, cell expanding Schwann cell culture for finally constructing an autologous tissue engineered prosthesis.

At present we unfortunately only can think about this scenario, nevertheless we have to bear in mind, that the most important advantage of using a tissue engineered prosthesis still should be the fact that no healthy nerve has to be scarified for treating peripheral

nerve injury with defects. Therefore one must focus on obtaining the maximum yield of human Schwann cells from the smallest nervous tissue explant possible. On the one hand this can be archived by adding mitogens to the culture medium, but on the other hand also by using the piece of tissue obtained from the biopsy for several times. With the explant-reexplant technique of Askanas several primary Schwann cell cultures, increasing in purity with every passage, can be created. But also direct colonisation of the prosthesis can be achieved by this technique. Contrary to the commonly used method of enzymatic dissociation, where the nerve tissue is digested, the explant-reexplant technique is conducted by multiple passages of the same explant. The big advantage of this technique represents the fact that the explant is not harmed by enzymatic dissociation and therefore it still supplies the positive effects of Wallerian degeneration. The big disadvantage appoints the questionable purity of cell yield(83).

Although we tried to create a medium exclusively enhancing Schwann cell proliferation, still outspread of fibroblasts was observed in one nerve. Thus, it has to be suspected that also the confluent monolayer of bipolar cells cultured according to the explant-reexplant technique contained a certain percentage of fibroblasts.

Unfortunately we were not able to clarify this suspicion by S100 staining, due to the fact that the loose cells harvested from these monolayers did not settle down on the polymer OPTIMAIX 2D. In this sense, it would be advisable to clarify the identity of monolayer cells by conducting a culture staining with S100(83). Additionally the harvesting technique has to be modified. Cell scraper seemed not to be the right method for detaching the adherent Schwann cells from the culture dish and may be using trypsin would represent a solution for this problem.

Although the colonisation of the polymer OPTIMAIX 2D with culture harvested Schwann cells failed, pieces of OPTIMAIX 2D colonised with nervous tissue showed positive results. In the HE stained slides cells were observed on the nervous tissue colonised OPTIMAIX 2D, which subsequently were identified as Schwann cells by S100 staining. Additionally growth assessment with MIB staining proved proliferation on OPTIMAIX 2D, which indicates outspread on this polymer.

2.8 Conclusion

This pilot study aimed to colonise the polymer OPTIMAIX 2D with human Schwann cells obtained by explant-reexplant technique first described by Askanas. It was conducted in view of developing a tubular, tissue engineered device for treating peripheral nerve injury presenting with defects.

Three main pillars characterize this experiment. The use of polymer OPTIMAIX 2D, harvesting human Schwann cells from patients treated at the Department of Plastic, Aesthetic and Reconstructive Surgery and finally the colonisation of polymer OPTIMAIX 2D according to the explant-reexplant technique.

Moreover, mitogenic ingredients were added to the basic culture medium in order to subtilize the culture technique. For increasing purity of Schwann cells forskolin was added. Furthermore, heregulin β was applied for enhancing proliferation and extending the number of Schwann cells.

Moreover, the explants were directly cultured on the polymer OPTIMAIX 2D for provoking outspread of cells straight on the polymer. This approach focuses on obtaining the maximum yield of human Schwann cells from very small portions of nervous tissue.

In conclusion we were able to show, that the explant-reexplant technique described by Askanas works very well in culture, but also for straight colonisation of the polymer OPTIMAIX 2D. Contrary to these positive results, the colonisation of the polymer OPTIMAIX 2D with cells harvested from a confluent monolayer, obtained from the first passage of the nerve tissue explant, failed.

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Abbreviations

A

AMP (Adenosine-monophosphate)

B

BDNF (Brain-Derived Neurotrophic Factor)

BMP (Bone morphogenetic proteins)

C

CAM (cell adhesion molecule)

CE (Conformité Européenne)

CNS (Central nervous system)

CNTF (ciliary neurotrophic factor)

D

DMEM (Dulbecco's Modified Eagle's Medium)

E

ECM (Extra cellular matrix)

EGF (Insulin-like growth factor)

F

FDA (Food and drug administration)

FCS (Fetal Calf Serum)

FGF (Fibroblast growth factors)

FK 506 (Tacrolimus)

G

GDNF (glial cell derived neurotrophic factor)

GPS (Glutamine Penicillin Strepto-mycin)

H

H&E (Haemotoxylin&Eosin)

I

IGF (Insulin-like growth factor)

M

MAP (Mitogen-activated protein)

MBP (Myelin basic protein)

MIB (Monoclonal immunohisto-chemical staining)

N

N-CAM (Neural adhesion molecule)

NGF (Nerve growth factor)

NRG (Neuregulin)

P

PCA (polyglycolic acid)

PDGF (platelet-derived growth factor)

PNS (Peripheral nervous system)

T

TF (tissue factor)

TLR (toll-like receptors)

TrK (Tyrosine kinase receptor)

POU (Pituitary specific/ Octomer transcription factor/ Neural Unc-86 transcriptionfactor)

PTN (Pleiotrophin)

PMP (peripheral myelin protein)

R

RNA (Ribonucleic acid)

S

SRY (sex determining region Y)

V

VEGF (Vascular endothelial growth factor)

Curriculum vitae

Elisabeth Katharina Trapp

Address: 28/1/1 Geidorfgürtel
8010 Graz
Austria / Europe

E-mail: elisabeth.trapp@stud.medunigraz.at

Date of birth: March 26th, 1988

Place of birth: Wassertrüdingen, Bavaria, Germany

Nationality: Austrian

University studies: **Medicine at the Medical University of Graz**
Dentistry at the Medical University of Graz

Professional activities:

Tutor at the Institute of Anatomy
(Medical University of Graz)
Scientific collaborator for tissue engineering
(Department of plastic, aesthetic, and reconstructive Surgery)

Extracurricular activities:

Mentor for incoming students
(Medical University of Graz)

Oral Presentations:

2012*: Colonization of two dimensional scaffolds with Schwann cells*
53rd Annual Meeting of the Austrian Society of Surgery

2011: *Tissue engineering with Schwann cells*
35th Seminar of the Austrian Society for Surgical Research

Electives:

Oxford

Department of Obstetrics and Gynaecology (November 2013)

King`s College London

Department of Endocrinology (September 2013)

The Royal Liverpool University Hospital

Department for Breast and Endocrine Surgery (July 2013)

Medical clerkships:

Department of Dermatology
University Hospital of Graz 2013 (2W)

Department of Neurology
Charite` Berlin 2012 (2W)

Department of Obstetrics and Gynaecology
Ludwig- Maximilian University Munich 2012 (4W)

Department of Psychosomatic and General Internal Medicine
University Hospital Heidelberg 2011 (4W)

Department of Internal Medicine
Hospital of Wolfsberg 2011 (2W)

Department of Trauma Surgery
University Hospital Graz 2010 (2W)

Department of General Surgery
Hospital of Wolfsberg 2010 (2W)

Department of Paediatrics and Neonatology
University Hospital Graz 2010 (2W)

Basic education:

2007-2008	Academic Studies of Pharmacy and Law at the Karl-Franzens University
June 2007:	Matura (High School Certificate)
2004-2007:	Stiftsgymnasium der Benediktiner St.Paul
1998-2004:	Laurentiusgymnasium Neuendettelsau

Languages: German as a native speaker
English fluent in speech and writing
Italian and Latin