# Evaluation of Vascular endothelial growth factor (VEGF) and its family member expression after peripheral nerve regeneration and denervation.

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**Key words:** VEGF, peripheral nerve regeneration, crush injury, end-to-end repair, median nerve transaction, neuropilin, VEGF receptors.

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**Abstract:**

Vascular endothelial growth factor (VEGF) represents one of the main factor involved not only in angiogenesis and vasculogenesis but also in neuritogenesis, VEGF plays its function acting via different receptors: VEGF receptor1 (VEGFR-1), VEGF receptor2 (VEGFR-2), VEGF receptor3 (VEGFR-3) and co-receptors Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2).

This study reports on the first *in vivo* analysis of the expression of VEGF and VEGF family molecules in peripheral nerve degeneration and regeneration: for this purpose, different model of nerve lesion in rat were adopted, the median nerve crush injury and the median nerve transaction followed or not by end-to end microsurgical repair.

Results obtained by Real Time polymerase chain reaction showed that VEGF and VEGF family molecules are differentially expressed under regenerating and degenerating condition, furthermore, in order to study the modulation and involvement of these factors in two different regenerative models, crush injury and end-to-end repair, protein expression analysis was evaluated. In addition, immunohistochemical analysis allowed to state a glial localization of VEGF and VEGFR-2 after peripheral nerve crush injury.

Finally *in vitro* assay on Primary Schwann cells culture show that VEGF165 stimulation increases Schwann cells migration, a major process in the promotion of neurite outgrowth.

## Introduction

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that increases blood vessels permeability and promotes angiogenesis. For this reason VEGF is mainly expressed by endothelial cells but also by activated macrophages and during cancer pathogenesis (Berse, Brown et al. 1992).

VEGF belongs to a family of homodimeric glycoproteins structurally related to the platelet-derived growth factors (PDGF); in mammals, VEGF family consists of five members, VEGF-A, B, C, D and placenta growth factor (PLGF).

Through alternative RNA splicing, different VEGF-A isoforms are generated: VEGF121,145, 165, 183, 189 and 206. VEGF121 is a freely diffusible molecule that lacks the basic amino acid residues and does not bind the extracellular matrix (ECM), VEGF165 contains some basic residues and it is partly diffusible while VEGF189 contains even more basic residues, showing a spatially restricted localization to the matrix around the VEGF-producing cell (Cohen, Gitay-Goren et al. 1995; Ruiz de Almodovar, Lambrechts et al. 2009; Grunewald, Prota et al. 2010).

VEGF binds three tyrosine kinase receptor: VEGF receptor1 (VEGFR-1), VEGF receptor2 (VEGFR-2), VEGF receptor3 (VEGFR-3) and also receptors of the neuropilin family, Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2) considered co-receptor for VEGF (Neufeld, Cohen et al. 1999; Ferrara, Gerber et al. 2003; Takahashi and Shibuya 2005; Ruiz de Almodovar, Lambrechts et al. 2009; Rosenstein, Krum et al. 2010; Carmeliet and Ruiz de Almodovar 2013).

Since the vascular and the nervous system show similar anatomical features and despite the main role of VEGF as a pro-angiogenetic factor, an increasing number of studies focus the attention on VEGF activity on different neural cell types and recent evidence shows a role for VEGF as a neurotrophic and neuroprotective factor for neurons and glial cells. In fact, VEGF stimulates the proliferation of neuronal precursors, increasing the BrdU labeling, in *in vitro* and *in vivo* models of neurogenesis (Jin, Zhu et al. 2002). Yet, it supports the survival of mesencephalic neurons in explants cultures (Silverman, Krum et al. 1999).

Furthermore it has been reported that VEGF administration enhances axonal outgrowth from dorsal root ganglia adult mice explants promoting the survival of neurons and satellite glial

cells (Sondell, Lundborg et al. 1999; Sondell, Lundborg et al. 1999; Hobson, Green et al. 2000; Brockington, Lewis et al. 2004; Pereira Lopes, Lisboa et al. 2011).

Evidence has also been provided that VEGF administration increases the functional recovery after peripheral nerve injury since it was shown that after end-to-end neurorraphy (ETE) and end-to-side neurorraphy (ETS) of transected muscolocutaneous rats nerves, plasmid VEGF transfection in the distal stumps resulted in a better axon regeneration in terms of fibers density, axons diameter and myelin sheath thickness of regenerated axons (Haninec, Kaiser et al. 2012).

The aim of our work is to investigate the expression of VEGF, VEGF receptors and VEGF co-receptors after nerve injury and regeneration. In the present study we carried out a biomolecular and immunohistochemical analysis on rat median nerve experimental models. In particular three different nerve injuries models will be used (crush injury, end-to-end repair and degenerating nerve) in order to analyze mRNA expression and protein expression and localization.

## Materials and methods

Animals

All experiments were carried out on adult female Wistar rats (Charles River Laboratories, Milan, Italy) weighing approximately 190-220g.

Surgery

All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC).

Animal well-being assessment was carried out using careful animal surveillance to check for passive and active movement, auto-mutilation, skin ulcers, and joint contracture, especially during early post-operative times.

Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress.

For mRNA expression a total of 48 rats were used and three different surgeries were applied: crush injury, end to-end repair and degenerating nerve.

For the crush injury group ($n$=3), the median nerve of both forelimb were crushed with a non-serrated clamp at mid-humerus level according to the procedure described (Ronchi, Raimondo et al. 2010). For the end-to-end repair ($n$=3), median nerve was bilaterally transected at the same position described for the crush injury, proximal and distal stumps were immediately sutured using 9/0 epineurial sutures. In the degenerating group, median nerve was bilaterally transected and unrepaired.

For the animals belonging to the control groups (CTRL) nerve was exposed and the skin was closed immediately after ($n$=3).

For protein analysis, only rats resulting from crush injury and end-to-end repair were used, whereas uninjured rats were used as control (see Total Protein Extraction and Western Blot Analysis section).

For morphological evaluation (see Immunofluorescence section) a total of 6 animals were used, $n$=3 for crush injury and $n$=3 for control groups.

All surgical procedures were carried out under deep anaesthesia obtained with Tiletamine + Zolazepam (Zoletil) i.m. (3 mg/kg).

For mRNA and protein analysys, median nerves were harvested 1d, 3d, 7d, 15d and 30d after the different surgeries, for morphological analysis rats were sacrified 7 days after crush injury.

RNA isolation, cDNA preparation.

Samples were frozen at -80° C and processed for RNA extraction. Total RNA was extract with the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. 0.5 µg total RNA were subjected to a reverse transcriptase (RT) reaction in 25 µL reaction volume containing: 1X RT-Buffer (Fermentas); 0.1 µg/µL bovine serum albumin (BSA, Sigma); 0.05% Triton X-100; 1 mM dNTPs; 7.5 µM random exanucleotide primers (Fermentas); 1 U/µl RIBOlock (Fermentas) and 200 U RevertAid™ M-MuLV reverse transcriptase (Fermentas). The reaction was performed for 10 min at 25°C, 90 min at 42°C, 10 min at 90°C. Control reaction "$RT^-$" (without the enzyme RT) and "$H_2O$", without RNA, was also carried out. RNA concentration was quantified using the Nanodrop® ND-1000 spectrophotometer (Celbio, Milano, Italy). cDNA was stocked at -20°C until used as a template for the real-time RT-PCR analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR).

cDNA was diluted 5 times before analyses and 1 µl was analyzed in a total volume of 10 µl using 1X SYBR Green Supermix (Applied Biosystems) and 300 nM forward and reverse primers. Quantitative real-time PCR analysis was performed using chemistry with the StepOne Sequence Detection System (Applied Biosystems), dissociation curves were routinely performed to check the presence of a single peak in agreement to the required amplicon. Reactions were performed in technical and biological triplicate.

Data were analyzed by ΔΔCt relative quantification method normalizing to the housekeeping gene ANKRD (Ankyrin repeat domain 27) and RICTOR (RPTOR Indipendent Companion of MTOR) (Gambarotta, Ronchi et al. 2014).

Primers were designed using ANNHYB software (http://www.bioinformatics.org/annhyb/) and synthesized by Invitrogen (Life Technologies Europe BV, Monza, Italy).

Primers sequences are reported in Table1. Relative expression levels were calculated by (ΔΔCt) method. The normalized relative quantity (NRQ) was determined using the formula: $NRQ = 2^{-(\Delta\Delta Ct)}$. Results were expressed as mean + S.D.

Total Protein Extraction and Western Blot Analysis.

Total proteins were extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Protein pellets were resuspended in boiling Laemli buffer (2.5% SDS, 0.125 M Tris-HCl pH6.8) and followed by 3 min at 100°C. Protein concentration was determined using the Bicinchoninic Acid assay kit (BCA, Sigma), and equal amounts of proteins (40 µg, denaturated at 100°C in 240 mM 2-mercaptoethanol and 18% glycerol) were loaded onto each lane, separated by SDS-PAGE, transferred to iBlot® Transfer Stacks nitrocellulose membrane using the iBlot® Dry blotting Transfer Device (Invitrogen).

Primary antibody used were: mouse monoclonal anti-VEGF-A (1:500, ab171828 Abcam), rabbit polyclonal anti-VEGFR-1 (1:1000, #2893 Cell-Signalling Technology), rabbit polyclonal anti-VEGFR-2 (1:1000, #2479 Cell-Signalling Technology), rabbit polyclonal anti-NRP1 (1:1000, #3725 Cell-Signalling Technology), rabbit polyclonal anti-NRP2 (1:1000, #3366 Cell-Signalling Technology), mouse monoclonal anti-β-actin (1:4000 #A5316 Sigma).

Secondary used antibodies were horse-radish peroxidase-linked anti-rabbit (#NA934), and anti-mouse (#NA931) both used 1:20000 (GE Healthcare Life Science, Europe).

Immunoflurescence and confocal laser microscopy.

Nerve samples were fixed by immediate immersion in 4% paraformaldehyde for 2 h, washed in a solution of 0.2% glycine in 0.1 M phosphate buffer (pH 7.2), and embedded in OCT. Specimens were cut 10µm thick by a Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were permeabilized, blocked [0.1% triton X-100, 10% normal goat serum (NGS)/0.1% $NaN_3$, 1h] and processed for an immunohistochemical protocol. See Table 2 for the list of primary antibodies used. Samples were incubated overnight in primary antibody or in case of double-immunofluorescence experiments, in a mixture of primary antibody and visualized using a solution containing the appropriate secondary antibodies: goat anti-mouse IgG Alexa-Fluor-488-conjugated (1:200, Molecular Probes, Eugene, Oregon), CY3-conjugated anti-rabbit IgG (dilution 1:400, Dako, Milano, Italy). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma) diluted 1:1000 in PBS.

The samples were finally mounted with a Dako fluorescent mounting medium (DAKO) and analyzed by a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany).

Primary Schwann Cells (SC) Cultures.

Primary Schwann cells (SC) cultures were obtained from fresh adult rat sciatic nerves.
The sciatic nerves were collected and immediately kept in cold DMEM plus glutamax (Invitrogen, UK) containing 100 U/mL penicillin and 100 g/mL streptomycin. Nerves were dissected and the epineurium was stripped off. Nerve fragments were plated in a Petri dish in SC growth medium (DMEM plus glutamax containing 100U/mL penicillin, 100 g/mL streptomycin, 14 M forskolin, and 100 ng/mLNRG11, R&D Systems, UK) and incubated for 2 weeks at 37˚C with fresh medium added approximately every 72h.

Nerve fragments were incubated with 0.125% (w/v) collagenase type IV and 117 u/mg dispase for 24 hours and mechanically dissociated using a sterile Pasteur glass pipette in order to obtain a cell suspension. Cell suspension was filtered using a 70µm cell strainer (Falcon; BD Biosciences Discovery Labware, Bedford, MA) and centrifuged at 100×g for 5min to obtain the cell pellet. Finally, the cell pellet was resuspended in SC growth medium, seeded into a Petri dish pre-coated with poly-D-lysine (Sigma, St Louis, MO, USA), and incubated in the same conditions. SC were purified by an antibody complement method to roll out the remaining fibroblasts (Tohill, Mann et al. 2004; Kaewkhaw, Scutt et al. 2012; Pascal, Giovannelli et al. 2014)

Proliferation Assay.

Primary Schwann cells were seeded at a concentration of 1000 cells/cm$^2$ on poly-D-lysine coverslips in complete DMEM containing 2%FBS as the control condition, or medium added with VEGF (50 ng/ml) (human VEGF-A165, R&D). After 1, 3, and 7 days, cells were fixed, stained, photographed and counted.
Culture medium was removed, substrates with attached cells were rinsed with PBS with $Ca^{2+}$ and $Mg^{2+}$ and fixed by the addition of 4% paraformaldehyde solution. After 20 min, samples were rinsed with PBS with $Ca^{2+}$ and $Mg^{2+}$ and then stained with 4, 6-diamidino-2-phenylindole (DAPI) diluted 1:1000 in PBS. Cells were photographed using an optical video-confocal microscope (Nikon Eclipse 80i) and the supporting software Image ProPlus (Media Cybernetics USA). For each sample, four images were taken with at low magnification. The number of proliferated cells were counted by using Image J Software. All conditions were

performed in triplicate. The counts obtained from assays were analyzed, averaged, and expressed as the number of proliferated cell number/$mm^2$ ± standard deviation.

Three-dimensional migration: transwell assay.

The Transwell migration assay was used to measure three-dimensional movement. Primary Schwann Cells ($10^5$) resuspended in 200 µl of DMEM containing 2% FBS were seeded in the upper chamber of a Transwell (cell culture insert, no. 353097, BD Biosciences) on a porous transparent polyethylene terephthalate membrane (8.0-µm pore size, $1 \times 10^5$ pores/$cm^2$). The lower chamber (a 24-well plate well) was filled with DMEM containing 2% FBS with or without VEGF165 (50 ng/ml, R&D). The 24-well plates containing cell culture inserts were incubated at 33°C in a 5% $CO_2$ atmosphere saturated with $H_2O$. After 18 h of incubation, cells attached to the upper side of the membrane were mechanically removed using a cotton-tipped applicator. Cells that migrated to the lower side of the membrane were rinsed with PBS, fixed with 2% glutaraldehyde in PBS for 15 min at room temperature, washed five times with water, stained with 0.1% crystal violet and 20% methanol for 20 min at room temperature, washed five times with water, air-dried, and photographed using a Nikon ECLIPSE TS100 inverted microscope equipped with a Nikon Digital Sight DSL1 camera; the images were analysed with ImageJ software. The experiments were repeated three times independently (biological triplicate). Each set included three control condition transwells and three transwells stimulated with VEGF165. Cell counts were expressed as percentage of migrated cells/total number of cells ± standard deviation. All conditions were performed three times independently (technical triplicate). Five images were analyzed for each transwell using the ImageJ software. Cell counts were expressed as percentage of migrated cells/total number of cells ± standard deviation.

Statistical analysis.

For *in vivo* experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post hoc* LSD. Two-way analysis of variance (ANOVA) and *post-hoc* Bonferroni was performed using the Prism Software Package (GraphPad, La Jolla, CA, USA) and SPSS. For *in vitro* proliferation experiments, statistical analysis was performed using one-way analysis of variance (ANOVA) and *post-hoc* Bonferroni using the Prism Software. For *in vitro* migration assay, statistical analysis was performed using Two-Sample t-Test. The

level of significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Values are expressed as mean ± standard deviation (SD).

**Results**

The expression of VEGF, VEGFR-1, VEGFR-2, VEGFR-3, NRP1 and NRP2 mRNAs were assessed in crush injury, end-to-end repair and degenerating conditions.
The relative quantification (RQ) was determined using the control sample that better represents the mean of the control samples ΔCt; therefore, the relative (and not absolute) gene expression shown in the graphs cannot be compared among different genes.

VEGF mRNA expression is differentially regulated in regenerating and degenerating condition.
VEGF mRNA expression significantly increases in crush group at day 1 ($p \leq 0.01$ (**)), day 3 ($p \leq 0.05$ (*)) and day 7 ($p \leq 0.01$ (**)) after injury. Whereas VEGF mRNA expression is significantly down regulated in degenerating condition at day 3($p \leq 0.05$ (*)), day 7 ($p \leq 0.01$ (**)) and day 30 ($p \leq 0.05$ (*)). In end-to-end repair group no significant differences are observed (Figure1).

VEGFR-1 is strongly upregulated after crush injury.
In crush injury group the mRNA level of VEGFR-1 is significantly upregulated peaking at day 1 after injury ($p \leq 0.001$ (***)). The expression significantly decreases starting from day 3 ($p \leq 0.05$ (*)) and a significant downregulation is also detectable at day 30. In the end-to-end repair and degenerating condition, mRNA expression of VEGFR-1 does not show significant variations (Figure 2).

VEGFR-2 mRNA expression is strongly down regulated in regenerating and degenerating condition.
Results on mRNA expression of VEGFR-2 in crush injury group showed that expression is significantly decreased 30 days after crush injury ($p \leq 0.05$ (*)). In the end-to-end repair group a significant down regulation of VEGFR-2 is detectable at day 1 ($p \leq 0.05$ (*)) and 30 ($p \leq 0.05$ (*)) after surgery.
VEGFR-2 mRNA expression goes back to control condition level from day 3 up to day 7. Under degenerating condition mRNA VEGFR-2 expression levels did not change compared to control condition (Figure 3).

VEGFR-3 mRNA expression decreases in regenerating and degenerating condition.

mRNA expression of VEGFR-3 significantly decreases starting from day 1 ($p \leq 0.01$ (**)) in crush injury group. Decrement is also detectable at day 3 ($p \leq 0.01$ (**)), day 7 ($p \leq 0.01$ (**)) day 15 ($p \leq 0.001$ (***)) lasting until day 30 ($p \leq 0.001$ (***)) after injury. In degenerating nerve and end-to-end repair groups no regulation occurs (Figure 4).

VEGF co-receptors NRP1 and NRP2 are significantly upregulated in degenerating condition.

The mRNA expression of VEGF co-receptors NRP1 and NRP2 are both upregulated under degenerating condition. NRP1 is significantly upregulated ($p \leq 0.05$ (*)) at day 3 and 15 ($p \leq 0.05$ (*)) and a higher upregulation is detectable at day 30 ($p \leq 0.01$ (**)) in degenerating nerve. The mRNA expression of NRP2 in degenerating condition is strongly upregulated 30 days after injury ($p \leq 0.05$ (*)) (Figure 5).

Comparison between surgery: two-way ANOVA analysis

Through two-way ANOVA analysis several relevant mRNA expression changes have been detected. The most relevant data to us is the VEGFR-3 expression since it is involved in inflammatory response. Its mRNA expression in degenerating nerve is higher compared to degenerating control condition, end-to-end repair and crush injury *p*-value ranging from $p \leq 0.05$ (*) to $p \leq 0.01$ (***). (See Figure 6 for details).

VEGF, VEGFR-2, NRP1 and NRP2 proteins are differentially expressed after crush injury.

In order to focus the attention on nerve regeneration process, western blot analysis was performed only on crush injury and end-to-end repair group to confirm protein expression of some of genes analyzed by qRT-PCR.

Western blot analysis showed that VEGF expression is detectable in both crush and end-to-end nerves. The 43-kDa band is strongly detectable in control condition, 1 day, 3 days and day 7 after crush injury while a weak band is detectable at day 15 and 30 after injury. In end-to-end nerves a high VEGF protein expression is observed in all time points post repair.

Interestingly VEGFR-2 protein appears in end-to-end nerves starting from day 3 until 30 days. In crushed nerves, high protein expression is found between day 1 and 15 after injury. No band appears in healthy nerves.

NRP1 antibody detects endogenous levels of total NRP1 protein through a 120-kDa band. This antibody also recognizes an 80-kDa protein of unknown origin.

Protein expression of NRP1 results with a very small band detectable in healthy control nerves although protein expression level increases in crushed nerves starting from day 1 until day 7 and then decreases until day 30. However the expression is higher compared to control condition. In end-to-end-nerves NRP1 protein expression is higher between day 3 and day 30.

Finally, NRP2 antibody detects endogenous levels of total NRP2 protein recognizing a 130-kDa band. The protein expression level of NRP2 is barely detectable especially in crushed nerves in which a weak band appears starting from day 7 up to day 30 after injury. In end-to-end repair group protein expression of NRP2 appears between day 1 and day 15 with a stronger band detectable at day 3 after surgery (Figure 7).

VEGF, VEGFR-2 have a glial localization.

Since Real Time PCR and western blot analysis do not allow to obtain a morphological evaluation, different immunohistochemical reactions were carried out using VEGF, VEGFR-2, NRP1 and NRP2 as markers. In order to study the localization of the different markers, double immunostaining with glial marker (S-100B) and neuronal marker (β-tubulin) were performed on crushed nerves. The employment of the crush injury, which interrupts nerve fibers without severing the connective tissue of the nerve trunk represents a suitable model for the study of the regenerative process providing an optimal regeneration pathway. Furthermore the crush lesion was applied using a standardized and reproducible method, in terms of force, pressure and duration of the compression represented by the use of the not-serrate clump (Ronchi, Nicolino et al. 2009). For this reason, transversal sections of 10 μm of thickness were used to perform immunofluorescence staining only on crushed nerves harvested 7days after injury.

According to Western blot analysis an immunoreactivity for VEGF was found around axons of healthy control nerves (Figure 8A).

Interesting, the same VEGF immunoreactivity was found in nerves 7 days after crush injury, suggesting a glial expression of this marker (Figure 8A-B).

Immunofluorescence analysis was performed with other markers, VEGFR-2, NRP1, NRP2. As shown in Figure 8C, double immunostaining for VEGFR-2 and β-tubulin that represent a

typical neuronal marker showed absence of co-localization of these markers suggesting a glial expression of VEGFR-2.

Finally, a double labeling for NRP1/β-tubulin and NRP2/β-tubulin was performed in order to identify co-receptors localization. A co-localization of NRP1 and β-tubulin was detected at nerve axons level (Figure 8D); absence of co-localization was found in case of double labeling NRP1/S-100b (Figure 8E). Co-localization of NRP2 and β-tubulin suggest the same expression pattern found for NRP1 (Figure 8F) with an axonal expression of NPR2.

Administration of VEGF165 do not increase Primary Schwann cells proliferation.

Proliferation assay was performed on Primary Schwann cells (SC) cultures seeded in complete DMEM containing 2%FBS as control condition, or in complete DMEM containing 2%FBS added with VEGF165 (50 ng/ml) (human VEGF-A165, R&D). The number of proliferating cells was then counted quantifying the number of fluorescence labeled nuclei at different time points 1, 2 and 3 DIV after seeding. Schwann cells seeded with complete DMEM containing 2%FBS added with VEGF165 showed a proliferation rate comparable to control conditions, no significant differences have been observed between the two different conditions(Figure 9). Therefore, primary Schwann cell stimulation with VEGF165 did not affect cell proliferation.

Three-dimensional migration assay on Primary Schwann cells culture showed a higher migration rate after VEGF165 stimulation.

To determine whether VEGF165 stimulation on Primary Schwann cell cultures increases the migration of these cells a three-dimensional migration assay was performed.

Primary Schwann cells ($10^5$) were seeded in the upper chamber of a transwell. In the lower chamber DMEM containing 2% FBS was supplemented with 50 ng/ml of VEGF165 as treatment (see three-dimensional migration: transwell assay section for details).

After 18 h of incubation Primary Schwann cells added with 50 ng/ml of VEGF165 showed a significant higher migration rate ($p \leq 0.05$ (*)) compared to Primary Schwann cells cultured in DMEM-2% FBS (Figure 10).

## Discussion

Peripheral nerve injury represents a very complex process that involves different morphological and molecular changes occurring to both proximal and distal stumps (Geuna, Raimondo et al. 2009; Allodi, Udina et al. 2012; Muratori, Ronchi et al. 2012).
After injury, axons distal to regeneration site are interrupted, myelin sheath is degradated and Wallerian degeneration occurs leading to a series of phenotypic changes that promote axonal regeneration. It is well accepted that during the regenerative process various molecular factors are involved in order to form a favorable microenvironment for axonal outgrowth (Navarro, Vivo et al. 2007).
For this reason, various molecules have been investigated in experimental models of neural repair in order to study promising strategies to improve very important aspects of the regenerative process such as axonal regrowth and target reinnervation (Raimondo, Fornaro et al. 2011; Kang, Kim et al. 2014; Chang, Quan et al. 2016).

Vascular and nervous systems share common molecular pathways during development and regeneration, furthermore the anatomical parallelism between vessel and nerve patterning is well documented (Ruiz de Almodovar, Lambrechts et al. 2009).
Anatomically, both systems are composed of afferent and efferent networks, arteries and veins, motor and sensory nerves and share similar patterning, with vessels running in parallel alongside nerve fibers as a mutual guidance alignment.
Furthermore evidences show that axon guidance and vessel navigation are regulated by similar classes of molecules (Slits, Semaphorins, Netrins,and Ephrins) (Carmeliet and Tessier-Lavigne 2005; Carmeliet and Ruiz de Almodovar 2013).

Even if it was demonstrated that VEGF plays a role during the development of the Central Nervous System (CNS), little is known about its presence and role in Peripheral Nervous System (PNS) (Rosenstein, Krum et al. 2010). This study focuses the attention on VEGF and its receptors and co-receptors expression in three different surgical models used to study peripheral nerve regeneration: the crush injury and the end-to-end repair represent regenerating conditions, while complete nerve transaction reflects a condition in which no signs of axonal regrowth are found (Ronchi, Haastert-Talini et al. 2016).

mRNA expression level for VEGFR-3 after crush injury showed a very low expression in all time points examined compared to control condition. Concerning end-to-end and degenerating nerve, no mRNA expression difference is detectable in all time points. This is probably due, as well reported, to restricted expression of the VEGFR-3 for lymphatic epithelium (Kaipainen, Korhonen et al. 1995; Jussila and Alitalo 2002; Le Bras, Chatzopoulou et al. 2005). However if we consider the two-way ANOVA comparison, we observed higher VEGFR-3 expression from day 1 until day 30 in degenerating condition compared to crush and end-to-end repair. Crush and end-to-end represents regenerative models, so the immune response may be slow down from day 1 up to 30. In degenerating condition there is no regeneration thus the inflammatory condition may persists over time. This can explain why the VEGFR-3 expression is maintained at high level.

NRP1 and NRP2 are a single pass transmembrane glycoproteins, originally identified as semaphorin receptors mediating axon growth cone collapse. Although, many study reported that NRP1 is involved in neuronal migration, dendritic guidance and repair of the adult nervous system (He, Wang et al. 2002).

NRP2, can bind VEGF165 thus, in addition to neuronal guidance, plays a role in angiogenesis and cardiovascular functions (Favier, Alam et al. 2006).

Our study showed that mRNA expression levels for NRP1 and 2 display a significant increase in degenerating condition. Since NRP1 and NRP2 are involved in several regenerative mechanisms as described above we suppose that in our degenerative model regeneration will not occur, explaining why their expression is manteined.

mRNA and protein expression levels were evaluated by Real Time and western blot analysis respectively. NRP1 and NRP2 display similar mRNA and protein expression levels in both regenerative models (crush injury and end-to-end repair). Finally, double labeling for β-tubulin/NRP1 and β-tubulin/NRP2 performed only on crush injury nerve, the surgical technique that provides clearest regeneration process, shows a co-localization for β-tubulin/NRP1 and β-tubulin/ NRP2 allowing to state an axonal localization of these co-receptors.

VEGF mRNA expression is significantly upregulated during the early phases after peripheral nerve crush injury whereas a strong down-regulation occurs in degenerating nerve suggesting a possible role during the regenerative process.

Furthermore, mRNA expression levels of VEGFR-2, the most implicated in migration and survival of neural and glial cell types of both CNS and PNS (Sondell, Lundborg et al. 1999a; Sondell, Lundborg et al. 1999b; Jin, Mao et al. 2000; Jin, Zhu et al. 2002; Ogunshola, Antic et al. 2002), is highly expressed over time in crush injury; a significant decrease is detectable at 30 days after injury allowing to suppose that the involvement of VEGFR-2 is restricted to the early phases of the regenerative process.

In order to better characterize the expression of VEGF and VEGFR-2 under regenerating condition, protein expression levels were investigated after crush injury and end-to-end repair. Data obtained from western blot analysis showed a strong VEGF protein expression in control condition and following time points (1 day, 3 days and 7 days after crush injury). VEGF protein expression is also observed in end-to-end repair suggesting a similar expression pattern in the early phase of the regeneration process. Although further *in vitro* experiments need to be done in order to better characterize the molecular pathway involved in these mechanisms.

Concerning VEGFR-2 protein results show that is expressed starting from day 3 until day 30 in end-to-repair. To better characterize protein expression and localization, morphological analysis on crushed nerves were performed showing an intense immunoreactivity for VEGF around axons suggesting a glial expression of this marker.

Furthermore, a double labelling for VEGFR-2 and β-tubulin show absence of co-localization between these two markers suggesting a glial expression also for VEGFR-2.

Interestingly, data are supported by *in vitro* analysis on Primary Schwann cells cultures that significantly increases their migration after VEG165 stimulation compared to control condition suggesting a positive effect of VEGF on glial cells migration which represents a very important process during the peripheral nerve regeneration.

**Conclusion**

Findings of the present study showed a modulation for VEGF and its family members including VEGFR-1, VEGFR-2, VEGFR-3, NRP1 and NRP2 under degenerating and regenerating conditions. Furthermore, morphological analysis allowed to understand the localization of the VEGF and the VEGFR-2 to Schwann cells after crush injury, used in this study as a suitable model for the study of the regenerative process (Ronchi, Nicolino et al. 2009; Ronchi, Raimondo et al. 2010).

Immunohistochemical results on VEGFR-2 showed also in this case a glial localization of this marker suggesting a potential autocrine VEGF/ VEGFR-2 pathway on Schwann cells.

Morphological evaluation performed by immunofluorescence allowed to identify the glial localization for both factors. Furthermore *in vitro* experiments on primary Schwann cells culture let evaluate the effect of VEGF on the migratory property of the Schwann cells suggesting that VEGF could influence the migration of Schwann cells that represents an important step during the regeneration process.

**Acknowledgement**

This study was supported by Compagnia di San Paolo (InTheCure project).

**Conflicts of Interest**

The authors declare no conflict of interest.

**Figure Legends**

**Figure 1:** VEGF mRNA expression significantly increases in crush group starting from day 1 ($p \leq 0.01$ (**)), day 3 ($p \leq 0.05$ (*)) and day 7 ($p \leq 0.01$ (**)) after injury. A downregulation is detectable in degenerating condition at day 3($p \leq 0.05$ (*)), day 7 ($p \leq 0.01$ (**)) and day 30 ($p \leq 0.05$ (*)). In end-to-end repair group a weak increase in VEGF mRNA expression level is detectable at day 30. N=3 in each time point condition.

**Figure 2:** mRNA level of VEGFR1 is significantly upregulated in crush injury group the peaking at day 1 after injury ($p \leq 0.001$ (***)). The expression significantly decreases starting from day 3 ($p \leq 0.05$ (*)) and a significant downregulation is also detectable at day 30. In the end-to-end repair group mRNA expression of VEGFR-1 decrease at day 1, at day 3 expression level increase while a strong reduction is detectable at day 30. VEGFR-1 mRNA expression level increase at day 1 and 3 decreasing gradually in degenerating nerve from day 7. N=3 in each time point condition.

**Figure 3:** mRNA expression of VEGFR-2 significantly decreased 30 days after crush injury ($p \leq 0.05$ (*)). In the End-to-end repair group a significant down regulation of VEGFR-2 is detectable at day 1 after surgery. mRNA expression of VEGFR-2 remains low from day 1 after injury until day 30 with a very weak increase at day 30 in degenerating nerve. N=3 in each time point condition.

**Figure 4:** mRNA expression of VEGFR-3 significantly decreases starting from day 1($p \leq 0.01$ (**)) until day 30 ($p \leq 0.001$ (***)) in crush injury group. In degenerating condition a strong down regulation occurs starting from day 1 until day 30. In end-to-end repair group mRNA expression of VEGFR-3 increases at day 1 and decreases from day 3 to day 30 after surgery. N=3 in each time point condition.

**Figure 5:** Two-way analysis of variance (ANOVA) and *post-hoc* Bonferroni showed mRNA expression in degenerating nerve is higher compared to degenerating control condition, end-to-end repair and crush injury *p*-value ranging from $p \leq 0.05$ (*) to $p \leq 0.01$ (***).

**Figure 6:** NRP1 and NRP2 are significantly upregulated in degenerating condition. NRP1 is significantly upregulated ($p \leq 0.05$ (*)) at day 3 and 15 ($p \leq 0.05$ (*)) and a higher

upregulation is detectable at day 30 ($p \leq 0.01$ (**)) in degenerating nerve. The mRNA expression of NRP2 is strongly upregulated 30 days in degenerating condition ($p \leq 0.05$ (*)).N=3 in each time point condition.

**Figure 7:** VEGF expression is detectable in both crush and end-to-end nerves. The band is strongly detectable in control condition, 1 day, 3 days, 7 days and 30 days after crush injury. In end-to-end nerves a high VEGF protein expression is observed in all day points post repair. VEGFR-2 band appears in end-to-end nerves starting from day 3 until 30 days. In crushed nerves high protein expression is found between day 1 and day 30 after injury. Protein expression of NRP1 results with a very small band detectable in control nerves although protein expression level increases in crushed nerves starting from day 1 until day 30. In end-to-end-nerves NRP1 protein expression is higher between day 3 and day 30. Protein expression of NRP2 is barely detectable in crushed nerves; in end-to-end repair NRP2 appears between day 3 and day 30 after surgery.

**Figure 8:** immunofluorescence staining for VEGF (red) in healthy control nerve (A) and crushed nerve 7 days after injury (B); double immunostaining for VEGFR-2 (red) and β-tubulin (green) showed absence of co-localization of these markers (C). Co-localization of NRP1 (red) and β-tubulin (green) is detected at nerve axons level (D); absence of co-localization is found in case of double labeling NRP1 (red) S-100b (green) (E). Co-localization of NRP2 and β-tubulin suggest the same expression pattern of NRP1 (F). Scale bar =100 μm. Nuclei are stained with DAPI (blue). Panel A 63X, panel B-F 40X.

**Figure 9:** Proliferation assay on Primary Schwann cell culture in control condition and after VEGF165 (50 ng/ml) stimulation.

**Figure 10:** Three-dimensional migration assay on Primary Schwann cells culture in control condition (DMEM/ 2% FBS) and after stimulation with (50 ng/ml) of VEGF165. Data show a higher migration rate after VEGF165 stimulation ($p \leq 0.05$ (*)).

**Table1:** Primers used for qRT-PCR.

**Table2:** Primary antibodies used for immunofluorescence.

## References


Allodi, I., E. Udina, et al. (2012). "Specificity of peripheral nerve regeneration: interactions at the axon level." Prog Neurobiol **98**(1): 16-37.

Berse, B., L. F. Brown, et al. (1992). "Vascular-Permeability Factor (Vascular Endothelial Growth-Factor) Gene Is Expressed Differentially in Normal-Tissues, Macrophages, and Tumors." Molecular Biology of the Cell **3**(2): 211-220.

Brockington, A., C. Lewis, et al. (2004). "Vascular endothelial growth factor and the nervous system." Neuropathol Appl Neurobiol **30**(5): 427-446.

Carmeliet, P. and C. Ruiz de Almodovar (2013). "VEGF ligands and receptors: implications in neurodevelopment and neurodegeneration." Cell Mol Life Sci **70**(10): 1763-1778.

Carmeliet, P. and M. Tessier-Lavigne (2005). "Common mechanisms of nerve and blood vessel wiring." Nature **436**(7048): 193-200.

Chang, B., Q. Quan, et al. (2016). "Molecular mechanisms in the initiation phase of Wallerian degeneration." European Journal of Neuroscience **44**(4): 2040-2048.

Cohen, T., H. Gitay-Goren, et al. (1995). "VEGF121, a vascular endothelial growth factor (VEGF) isoform lacking heparin binding ability, requires cell-surface heparan sulfates for efficient binding to the VEGF receptors of human melanoma cells." J Biol Chem **270**(19): 11322-11326.

Favier, B., A. Alam, et al. (2006). "Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration." Blood **108**(4): 1243-1250.

Ferrara, N., H. P. Gerber, et al. (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-676.

Gambarotta, G., G. Ronchi, et al. (2014). "Identification and validation of suitable housekeeping genes for normalizing quantitative real-time PCR assays in injured peripheral nerves." PLoS One **9**(8): e105601.

Geuna, S., S. Raimondo, et al. (2009). "Histology of the Peripheral Nerve and Changes Occurring during Nerve Regeneration." Essays on Peripheral Nerve Repair and Regeneration **87**: 27-46.

Grunewald, F. S., A. E. Prota, et al. (2010). "Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling." Biochim Biophys Acta **1804**(3): 567-580.

Haninec, P., R. Kaiser, et al. (2012). "Enhancement of musculocutaneous nerve reinnervation after vascular endothelial growth factor (VEGF) gene therapy." BMC Neurosci **13**: 57.

He, Z., K. C. Wang, et al. (2002). "Knowing how to navigate: mechanisms of semaphorin signaling in the nervous system." Sci STKE **2002**(119): re1.

Hobson, M. I., C. J. Green, et al. (2000). "VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy." J Anat **197 Pt 4**: 591-605.

Jin, K., Y. Zhu, et al. (2002). "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo." Proc Natl Acad Sci U S A **99**(18): 11946-11950.

Jin, K. L., X. O. Mao, et al. (2000). "Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia." Proc Natl Acad Sci U S A **97**(18): 10242-10247.

Jussila, L. and K. Alitalo (2002). "Vascular growth factors and lymphangiogenesis." Physiol Rev **82**(3): 673-700.

Kaewkhaw, R., A. M. Scutt, et al. (2012). "Integrated culture and purification of rat Schwann cells from freshly isolated adult tissue." Nat Protoc **7**(11): 1996-2004.

Kaipainen, A., J. Korhonen, et al. (1995). "Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development." Proc Natl Acad Sci U S A **92**(8): 3566-3570.

Kang, J. G., T. H. Kim, et al. (2014). "Peripheral nerve regeneration through nerve guide conduit with nerve growth factor gradient." Journal of Tissue Engineering and Regenerative Medicine **8**: 402-402.

Le Bras, B., E. Chatzopoulou, et al. (2005). "Oligodendrocyte development in the embryonic brain: the contribution of the plp lineage." Int J Dev Biol **49**(2-3): 209-220.

Muratori, L., G. Ronchi, et al. (2012). "Can regenerated nerve fibers return to normal size? A long-term post-traumatic study of the rat median nerve crush injury model." Microsurgery **32**(5): 383-387.

Navarro, X., M. Vivo, et al. (2007). "Neural plasticity after peripheral nerve injury and regeneration." Prog Neurobiol **82**(4): 163-201.

Neufeld, G., T. Cohen, et al. (1999). "Vascular endothelial growth factor (VEGF) and its receptors." FASEB J **13**(1): 9-22.

Ogunshola, O. O., A. Antic, et al. (2002). "Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system." J Biol Chem **277**(13): 11410-11415.

Pascal, D., A. Giovannelli, et al. (2014). "Characterization of glial cell models and in vitro manipulation of the neuregulin1/ErbB system." Biomed Res Int **2014**: 310215.

Pereira Lopes, F. R., B. C. Lisboa, et al. (2011). "Enhancement of sciatic nerve regeneration after vascular endothelial growth factor (VEGF) gene therapy." Neuropathol Appl Neurobiol **37**(6): 600-612.

Raimondo, S., M. Fornaro, et al. (2011). "Perspectives in regeneration and tissue engineering of peripheral nerves." Ann Anat **193**(4): 334-340.

Ronchi, G., K. Haastert-Talini, et al. (2016). "The Neuregulin1/ErbB system is selectively regulated during peripheral nerve degeneration and regeneration." Eur J Neurosci **43**(3): 351-364.

Ronchi, G., S. Nicolino, et al. (2009). "Functional and morphological assessment of a standardized crush injury of the rat median nerve." J Neurosci Methods **179**(1): 51-57.

Ronchi, G., S. Raimondo, et al. (2010). "Standardized crush injury of the mouse median nerve." J Neurosci Methods **188**(1): 71-75.

Rosenstein, J. M., J. M. Krum, et al. (2010). "VEGF in the nervous system." Organogenesis **6**(2): 107-114.

Ruiz de Almodovar, C., D. Lambrechts, et al. (2009). "Role and therapeutic potential of VEGF in the nervous system." Physiol Rev **89**(2): 607-648.

Silverman, W. F., J. M. Krum, et al. (1999). "Vascular, glial and neuronal effects of vascular endothelial growth factor in mesencephalic explant cultures." Neuroscience **90**(4): 1529-1541.

Sondell, M., G. Lundborg, et al. (1999). "Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system." J Neurosci **19**(14): 5731-5740.

Sondell, M., G. Lundborg, et al. (1999). "Vascular endothelial growth factor stimulates Schwann cell invasion and neovascularization of acellular nerve grafts." Brain Res **846**(2): 219-228.

Takahashi, H. and M. Shibuya (2005). "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions." Clin Sci (Lond) **109**(3): 227-241.

Tohill, M. P., D. J. Mann, et al. (2004). "Green fluorescent protein is a stable morphological marker for schwann cell transplants in bioengineered nerve conduits." Tissue Eng **10**(9-10): 1359-1367.