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Functional regeneration of supraspinal connections in a patient with transected spinal cord following transplantation of bulbar olfactory ensheathing cells with peripheral nerve bridging

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Running title: Spinal cord repair: OECs and nerve grafts

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Abstract:

Treatment of patients sustaining a complete spinal cord injury remains an unsolved clinical problem because of the lack of spontaneous regeneration of injured central axons. A 38-year-old man sustained traumatic transection of the thoracic spinal cord at upper vertebral level Th9. At 21 months after injury the patient presented symptoms of a clinically complete spinal cord injury (American Spinal Injury Association class A-ASIA A). One of the patient's olfactory bulbs was removed and used to derive a culture containing olfactory ensheathing cells and olfactory nerve fibroblasts. Following resection of the glial scar the cultured cells were transplanted into the spinal cord stumps above and below the injury, and the 8 mm gap bridged by 4 strips of autologous sural nerve. The patient underwent an intense pre- and post-operative neurorehabilitation program. No adverse effects were seen at 19 months postoperatively, and unexpectedly, the removal of the olfactory bulb did not lead to persistent unilateral anosmia. The patient improved from ASIA A to ASIA C. There was improved trunk stability, partial recovery of the voluntary movements of the lower extremities, and an increase of the muscle mass in the left thigh, as well as partial recovery of superficial and deep sensation. There was also some indication of improved visceral sensation and improved vascular autoregulation in the left lower limb. The pattern of recovery suggests functional regeneration of both efferent and afferent long-distance fibers. Imaging confirmed that the grafts had bridged the left side of the spinal cord, where the majority of the nerve grafts were implanted, and neurophysiological examinations confirmed the restitution of the integrity of the corticospinal tracts and the voluntary character of recorded muscle contractions. To our knowledge, this is the first clinical indication of beneficial effects of transplanted autologous bulbar cells.

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Key words: paraplegia, cell transplantation, repair, regeneration

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Introduction

Olfactory ensheathing cells (OECs) are a population of glial cells residing both in the peripheral and central nervous system. Together with their accompanying envelope of olfactory nerve fibroblasts (ONFs) they enfold the bundles of olfactory nerve fibers in their course from the nasal mucosa to make synaptic connections in the olfactory bulb (9,28,34,36). Animal experiments have shown that transplantation of OECs and ONFs cultured from the olfactory bulb mediate regeneration and functional reconnection of severed axons in spinal cord and brachial plexus injuries (20,22,23,35,37), whereas cells cultured from the olfactory mucosa have only minor benefits in corticospinal tract injuries (46), and do not appear to mediate regeneration of severed axons (18,46).

Clinical trials of transplantation of autologous OECs into spinal cord injuries have up to now been based on the more accessible cells obtained from the olfactory mucosa (6,14,26,27,30,43). While these studies serve to establish the safety of the procedure, there was, as in the animal studies, little (26,27,43) or no neurological improvement (6,30). In an experimental study of dorsal root repair in rats we showed that bulbar, but not mucosal OEC/ONFs can make a bridge for severed axons to cross from a peripheral nerve into the spinal cord (18). Based on this we now describe the results from the treatment of an ASIA A patient (American Spinal Injury Association class A) with an almost total transection of the thoracic spinal cord, who underwent the operation of scar resection, transplantation of cultured autologous bulbar OEC/ONFs, and reconnection of the spinal cord stumps with autologous sural nerve grafts, followed by a 19-month-course of postoperative rehabilitation.

Materials and Methods

Patient

A 38-year old male with a complete chronic thoracic spinal cord injury was qualified for the study. Thirteen months previously he had sustained a penetrating spinal cord injury at upper vertebral level Th9, caused by a knife assault. Serial magnetic resonance imaging (MRI) studies of the thoracic spine showed an 8-mm-long gap between the spinal cord stumps. The stumps remained connected only by a 2 mm rim of spared tissue in the area of the right spinal cord lateral column (Fig.1 and 2). Initial neurological examination of the patient showed a complete loss of sensory and motor function below the injury (ASIA Category A), with a zone of partial sensory preservation at level Th9. Transcranial magnetic motor-evoked potentials (MEPs) and electromyography (EMG) excluded any response of the lower extremity (LE) muscles to motor cortex activation and any voluntary muscle activity.

The patient met all the general and neurological criteria to be qualified for the OEC-transplantation protocol as described in our recently completed phase I clinical trial (43). However a diagnosis of chronic allergic sinusitis and nasal polyps (Fig.2A) was a contraindication for using the olfactory mucosa for obtaining OECs. Initially the patient was bilaterally anosmic but after performance of an endoscopic bilateral anterofrontoethmosphenoidectomy, he regained his smell perception due to the improved air flow in the nasal cavity (Fig.2B). In this situation the only OEC containing tissue not directly involved in the nasal pathology was the olfactory bulb. The patient was offered a new two-stage therapeutic approach consisting of the performance of craniotomy for obtaining one of his olfactory bulbs for OEC-isolation and subsequent transplantation of cultured OEC/ONFs into the lesioned spinal cord. The

patient provided a written informed consent according to the Declaration of Helsinki and understood the risk of the trial and the potential for no benefit. The study was approved by the Bioethics Committee of Wroclaw Medical University, according to the guidelines of the National Health Council of Poland.

Rehabilitation protocol

Before being qualified for the study it was established that the patient had shown no neurological improvement during a number of different rehabilitation programs over the first 13 months after injury. These programs were incomplete and were interrupted for longer periods because of the necessity for treatment of infections of the respiratory tract, pressure ulcers and inflammation of the deep venous system of the lower limbs.

For this reason the patient was subjected to an additional intense neurorehabilitation program for 8 months before the planned experimental treatment to confirm that there would be no spontaneous recovery. This program was previously applied in our paraplegic patients qualified for the phase I clinical trial concerning transplantation of autologous olfactory mucosal OECs (43). The patient was trained for 5 hours/day, 5 days/week. The training agenda consisted of: 1 h range of motion and stretching exercises, 3 h locomotor training and 1 h sensory training. The main emphasis was set on locomotor training that included training of individual muscle groups of the lower limbs, training for posture and balance and overground walking exercises. For each exercise the patient's legs were positioned in a way to have maximal support and a specific load (using weights attached to ropes) was used to enable the movement in the joints of paralyzed limbs. Each exercise had a fixed number of repetitions. If the patient showed any improvement in the performed exercise, the load was changed in a way to

increase the difficulty of the task as well as increase the number of repetitions. The minimal increase of the load was 100 g. The walking exercise was performed with emphasis to the assessment of the Walking Index (8), starting from the attempts to stand and walk in parallel bars with braces and the assistance of two persons. Postoperative rehabilitation was planned for at least 24 months.

Obtaining the olfactory bulb

Before operation, standard laryngological tests for evaluation of nostril patency and for assessment of respiratory epithelium function (the saccharine test) were performed.

Then patient's smell perception was tested using a scale for evaluation of smell perception we described previously (43). For obtaining the OB a left-sided fronto-lateral craniotomy was performed under general anesthesia. As he had large frontal sinuses, they were opened during craniotomy, cleared from the sinus mucosa and cranialized at the end of the procedure with autologous muscle and periosteum. Brain relaxation was achieved by a lumbar drainage and by opening of some skull base arachnoid cisterns.

Drilling of the skull base ethmoid eminences was necessary because the OB was localized deep inside the olfactory groove. The OB was obtained in two pieces with microsurgical instruments using the subfrontal microscopic endoscopy-assisted approach (Fig. 2C). The choice of the left OB was based on the better smell perception on the left and the lesser atrophy of the left OB in MRI. The tissue samples were maintained at 4°C and transported to the culture facility in a complete culture medium consisting of 1:1, v:v of Dulbecco's modified Eagle medium and Ham's F12 (CTS™ KnockOut™ DMEM/F12; Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS ATMP-Ready, gamma irradiated, PAA, Pasching, Austria), 1

mM CTS™ KnockOut™ GlutaMAX-I Supplement (complete culture medium, Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Polfa Tarchomin Warszawa, Poland).

Cell culture

Cultures of the patient's bulbar OECs were performed according to Miedzybrodzki et al (31), with slight modifications. The two fragments of olfactory bulb tissue were transferred to a 100mm polystyrene Petri dish (Becton Dickinson, Franklin Lakes, NJ, USA) and washed in 15mL of CTS™ DPBS containing calcium and magnesium, without phenol red (Gibco). The blood vessels and meninges were carefully peeled off under a dissecting microscope, and the tissue cut into pieces of 2 mm using a razor blade and incubated at 37°C in 0.25% trypsin solution (Sigma, St. Louis, MO, USA) for 15 min. The enzyme activity was stopped by adding complete culture medium and the tissue was repeatedly triturated by passing through the tip of 1ml pipette (Costar, Corning, Amsterdam, The Netherlands). The suspension was twice spun down at 300g for 5 min and the pellet again gently triturated in 2 ml of complete culture medium. The dissociated cells were seeded on two polystyrene dishes (9.6cm², Nunc, Roskilde, Denmark) and one 4-well polystyrene plate (1.9cm², Nunc) coated with 0.1 mg/ml poly-L-lysine hydrobromide (PLL, 30–70 kDa, Sigma). The culture dishes were maintained in a humidified incubator at 37°C in 5% CO₂. On the 4th day in vitro (DIV) the supernatant containing the non-adherent cells was transferred into new PLL-coated dishes. The cells were fed every second day by replacing half of the complete culture medium volume. On the 12th DIV the cells became nearly confluent, and the cultures were harvested using CTS™ TrypLE (Gibco). The enzymatic digestion was stopped by

adding complete culture medium, spun down at 300g for 5 min, and re-suspended. The tubes were placed on ice in a fridge and transported immediately to the operating theatre. After five subsequent rounds of washing using CTS™ DPBS, the cells were re-suspended in appropriate volume of CTS™ DPBS and transferred to a glass syringe (World Precision Instruments, Sarasota, FL, USA) for microinjection.

At the 10 DIV, a 5µl aliquot of the cells was fixed with 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS, IITD, Wroclaw, Poland) for 30 min, washed three times in PBS, permeabilized and blocked with 2% skim milk (Merck, Darmstadt, Germany) in PBS containing 0.1% Triton X-100 (Serva, Heidelberg, Germany).

Primary antibodies in PBS containing 2% milk and 0.1% Triton X-100 were applied overnight at 4°C. The cells were washed three times in PBS and incubated with fluorescent secondary antibodies in PBS containing 2% milk and 0.1% Triton X-100 for 90 min at room temperature in the dark. After washing four times in PBS, cells were counter-stained with Hoechst 33342 (1µg/ml, Sigma), washed twice with PBS, and mounted using ProLong Gold Antifade (Life Technologies, Rochester, NY, USA).

Primary antibodies were 1:100 monoclonal mouse anti-low affinity nerve growth factor receptor (anti-p75; clone NGFR5, Invitrogen) and 1:500 polyclonal rabbit anti-human fibronectin Ig (Dako, Glostrup, Denmark). Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG (H+L) and Alex Fluor 546 goat anti-rabbit IgG (H+L) (all 1:500; Molecular Probes, Invitrogen, Carlsbad, CA, USA). In all assays, controls were performed by incubating cells with secondary antibodies. Images of fluorescent labeled cells were captured using a Flouid fluorescence microscope (Life Technologies) equipped with Flouid Imagine Station Software. Images were exported for further analysis to the ImageJ 1.46r software (NIH, Bethesda, MD, USA).

Sterility Tests

A 100 µl aliquot of supernatant from the culture medium was taken on 5, 9 and 12 DIV for the assessment of development of bacterial or fungal infection. The samples were transferred to transport swabs (Hagmed, Rawa Mazowiecka, Poland) and delivered to the Microbiology Department of the Wroclaw Medical University (Wroclaw, Poland).

Transplantation Procedure

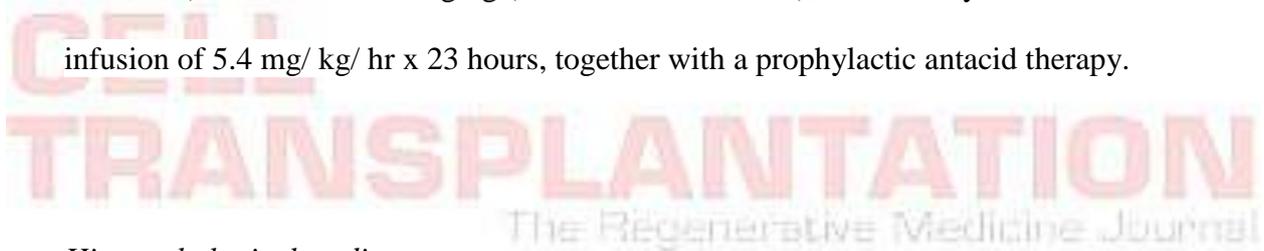
Preoperative preparation

The patient was re-admitted to the Department of Neurosurgery (Wroclaw Medical University) for the operation of cell transplantation 12 days after OB retrieval. Based on data from the axial T2-weighted MRI scans, a virtual three dimensional model of the spinal cord lesion was built. Then a schematic grid for cell microinjection was elaborated with specific topographic reference points on the surface of the spinal cord for intraoperative navigation of the stereotactic injection device, according to our previous experimental protocol (43) (Fig.3A). Briefly, the cell microinjections were planned to be done in a matrix pattern into the lateral columns of the spinal cord stumps, above and below the lesion epicenter. This matrix consisted of 4 rows, 2 mm apart. The posterolateral sulcus was chosen as the entry point for cell microinjection. The cell suspension was planned to be delivered at five depths at each injection site, 0.5 mm apart. Another target for cell microinjection was the rim of spared tissue connecting both spinal cord stumps. We also planned to reconstruct the 8-mm-gap between the spinal cord stumps with the patient's own peripheral nerve grafts.

Surgical Technique

The patient was placed under general anesthesia in a prone position. After identification of the level of operation with fluoroscopy, a midline skin incision was made, followed by dissection of the paravertebral muscles and laminectomy of the thoracic vertebrae Th7-8-9. Under an operating microscope (OPMI Pentero, Zeiss Company, Jena, Germany) an adhesion between the injured dura and the spinous process Th8 was removed. A midline durotomy was performed, followed by sharp dissection of the posttraumatic adhesions between the spinal cord surface and the dura. The area of spinal cord injury consisted of two separated spinal cord stumps, covered with yellowish scar tissue (Fig.3B) This tissue was removed and specimens taken for histology. This maneuver increased the gap between the stumps from 8 mm to 10 mm (Fig. 3C). A 2mm thin rim of spared tissue connected the margin of the cord stumps on the right. After completion of the myelo-adhesiolysis (spinal cord untethering), the system for stereotactic cell microinjection was mounted on the operating table, as described in our previous study (43). Briefly, it was composed of an automatic micropump (UltraMicro Pump III, World Precision Instruments) and a three dimensional micromanipulator (SM- 15, Narishige, Tokyo, Japan). The injector device was fitted with a 25 µl glass syringe (World Precision Instruments). The autologous OEC/ONF mixture was suspended in serum-free culture medium, centrifuged and added to an Eppendorf vial and a 25 µl glass syringe with 26 ga beveled needle was filled with the cells. The cell mixture was injected through the posterolateral sulcus between the dorsal nerve rootlets into the lateral columns of the spinal cord, proximally and distally for a distance of 8 mm on either side of the lesion epicenter. The remaining cell suspension was injected into rim of tissue connecting both spinal cord stumps. The parameters of cell

microinjection are summarized in Table 1. At the end of the procedure of microinjection, a small aliquot of cell suspension remaining in the Hamilton syringe, was taken for sterility tests and monitored in continued cell culture. In the last stage of the operation, a 6-cm-long fragment of the patient's left sural nerve was harvested. Four 12-mm strips of nerve grafts were used for reconnection of the spinal cord stumps (Fig. 3D). The nerves were positioned along the long axis of the spinal cord tracts and were fixed to the spinal cord stumps with fibrin sealant (Tisseel Lyo, Baxter AG, Vienna, Austria). The dura was closed with absorbable sutures. No duraplasty was performed. A wound drain was placed under the muscle layer and the wound was closed in layers. During the operation the patient received i/v. methylprednisolone (Solu-Medrol, Pfizer, Kent, UK) in a bolus of 30 mg/kg (over 15 to 30 minutes), followed by a constant infusion of 5.4 mg/ kg/ hr x 23 hours, together with a prophylactic antacid therapy.



Histopathological studies

Immunohistochemical examination was performed on tissue samples taken for routine diagnostic purpose from scar tissue in the spinal cord. Formalin-fixed, paraffin embedded tissue was freshly cut (4 µm). Immunohistochemistry was performed as previously described (17) using the following antibodies diluted in Antibody Diluent, Background Reducing (Dako): epithelial membrane antigen (EMA; clone E29; monoclonal mouse, dilution 1:100, Dako), S100 (rabbit polyclonal, dilution 1:400, Dako), neurofilament (NF; clone 2F11; monoclonal mouse, dilution 1:100, Dako), Vimentin (clone V9; monoclonal mouse, dilution 1:100, Dako) and glial fibrillary acidic

protein (GFAP; rabbit polyclonal, dilution 1:500, Dako). Hematoxylin-eosin (Sigma) counterstaining was performed.

Initial and Repeated Assessments

The patient's medical condition was evaluated regularly pre-and postoperatively. This included general medical assessment, otorhinolaryngological, neurological, physiotherapeutic and psychological tests as well as radiological and neurophysiological studies. All studies were performed by the same assessors. They were not blinded.

A detailed general medical assessment was conducted pre-operatively and in the first 5 weeks post-surgery and included hematology, blood chemistry and urine analysis. Tests for the presence of anti-HIV antibodies were performed preoperatively and Hepatitis B and C status was assessed. Standard electrocardiographic studies and chest X-rays (Philips, Amsterdam, Netherlands) were also performed. Microbiological studies of blood, urine or cerebrospinal fluid (CSF) were planned to be conducted only in case of suspicion of infection.

Laryngological examination included smell perception tests as well as evaluation of computed tomography (CT) and MRI scans of the head, nasal cavity, and paranasal sinuses.

Neurological examination was performed monthly and included ASIA examination, examination of deep sensation, evaluation of spasticity using the Ashworth scale, and assessment of reflex activity and the Medical Research Council muscle scale (MRC).

A spinal injuries physiotherapist undertook regularly a Functional Independence Measure (FIM), evaluated the Walking Index and recorded the achievements in each physical exercise during the locomotor training.

Psychological evaluation was performed preoperatively and at 1, 12 and 20 months after surgery. A clinical interview as well as the following psychological tests were conducted: the Minnesota Multiphasic Personality Inventory-2 test (MMPI-2) (2), The Generalized Self-Efficacy Scale (GSES) (19), The State-Trait Anxiety Inventory test (STAI) (42) and The Eysenck Personality Questionnaire- Revised (EPQR) (10). A cultural adaptation of these tests was routinely applied.

MRI images were undertaken on a 1.5 Tesla MR unit (GE Signa HDx, Milwaukee, WI, USA) and included T2-weighted, T2-weighted fat saturation (FAT-SAT) images and T1-weighted images before and after administration of contrast medium (gadolinium; Multihance, Bracco Diagnostics, Singen, Germany), in sagittal, coronal and axial planes. Diffusion tensor imaging (DTI) included an assessment of the topography of the water diffusion tracts in the spinal cord on tractography and the estimation of the values of fractional anisotropy (FA) of the spinal cord on the FA maps, at 0.5 and 2 cm above and below the lesion epicenter. MRI and DTI studies were performed preoperatively and at 1, 5, 8, 12, and 17 months post-surgery according to the previously described protocol (43).

Neurophysiological examinations included transcranial magnetic motor evoked potentials (MEPs), electroneurography (ENG) and electromyography (EMG) using the KeyPoint Diagnostic System (Medtronic, Copenhagen, Denmark), as previously described (43). Neurophysiological testing was performed twice before surgery and at 1, 5, 8, 11, 14, and 17 months postoperatively. During the MEP study, three positive

recordings with similar amplitudes and latencies of potentials were recorded from rectus abdominis, rectus femoris and extensor digiti muscles on both sides to show the integrity of long efferent neural transmission. Beside well-known parameters of amplitudes and latencies in MEPs recordings, the duration of potentials measured from the onset to the end with the reference to isoelectric line were also recorded. Bilateral EMG recordings from the rectus abdominis, rectus femoris, gastrocnemius, anterior tibial, and extensor digiti muscles were assessed using surface electrodes and needle electrodes were used to increase the measurement precision when the patient was asked to perform the voluntary contractions lasting 5 seconds.

The urodynamic study was conducted according to the protocol of the International Continence Society (44) using the Duet Logic G2 system (Medtronic). It consisted of uroflowmetry, pressure flow studies and electromyography of anal sphincter. It was performed before surgery and at 1, 5 and 12 months postoperatively.

Statistical Analysis

Statistical analyses were performed using Statistica 10 (StatSoft, Inc., Tulsa, OK, USA). A p value of 0.05 was considered significant. The U Mann-Whitney test was applied for assessment of statistical differences in measured variables. The Spearman Rank test was used to test correlations between specific measurements and features. Statistically significant correlation coefficients greater than 0.50, were considered important.

Results

Recovery after the operation of olfactory bulb retrieval

The operation of obtaining OB via craniotomy was safe. No neurological or general complications were noted postoperatively. The head CT scan performed 2 days after the surgery did not show any abnormalities. Shortly after the operation the patient lost his smell perception on the left, where the bulbectomy was performed, but unexpectedly, follow up showed a later, partial recovery of olfaction on the bulbectomised side. This persisted for the period of 20 months of observation (Fig. 4).

Cell culture

The cultures of cells isolated from the OB were not purified and contained mainly ONFs and OECs, and comprised more than 95% of the Hoechst stained cell population. OECs were identified as bi- or multipolar p75-NGFR-positive cells, with small cytoplasm and thin, long processes. They formed a network on a monolayer of flattened FN-positive ONFs (Fig. 2D). The percentage of p75-NGFR-positive cells was 16%. After 12 days, when OECs reached a confluent monolayer in culture, they were detached from the culture flasks and prepared for transplantation. The sterility tests of the cell culture, performed every 5 days, showed no evidence of bacterial or fungal contamination within the whole period of cell culture. The residual volume of cells remaining in the Hamilton syringe at the end of the operation of cell microinjection was seeded on to culture flasks and cultured for about 10 days for identification of cell populations and for assessment of culture sterility. In all cultures used for transplantation, p75NGFR-positive cells could be identified in culture and there was no evidence of microbial contamination.

Assessment of the safety of the operation of cell transplantation and spinal cord reconstruction

The operation of myeloadhesiolysis and glial scar resection, followed by cell microinjection and bridging of the spinal cord with autologous peripheral nerve grafts was safe over the period of 19 months of observation. We did not observe neurological deterioration, neuropathic pain, clinical or laboratory evidence of neuroinfection, nor any general medical complications attributed to the surgical intervention. The patient spent the first 2 days after the surgery in the Intensive Care Unit to have optimal control of his medical condition. In the first 3 days after the surgery he was suffering mainly from pain from the operative wound, requiring treatment for a period of several days with morphine (Morphini Sulfas, Polfa, Warszawa, Poland) administered

subcutaneously at a dose of 5 mg every 6 hrs. There was no spine instability or stenosis, as well as no evidence of myelomalacia, edema, inflammation or tumors of the spinal cord at the injection site, as documented in the 5 postoperative MRI studies (Fig. 5).

MRI showed a good integration of the implanted nerve strips with the host spinal cord tissue. The nerve implants were well vascularized (Fig. 5B) and retained their size (Fig. 5G). T2-weighted scans showed some mild degenerative changes in the spinal cord stumps, that were not progressive and were not associated with any negative influence on the clinical state of the patient or and the results of his electrophysiological studies (Fig. 5).

The preoperative DTI study revealed that a gap in continuity of diffusion tracts in the spinal cord was at the level of the spinal cord injury (Fig. 6). An early postoperative DTI study, performed at 5 weeks, showed restitution of the tracts of water diffusion across the area of implanted nerve grafts and also across the rim of spared tissue

connecting the spinal cord stumps. Yet tractography studies at 17 months after operation again showed a gap in the continuity of diffusion tracts which did not correlate with the continued neurological and neurophysiological recovery of the patient (Fig.6).

Assessment of the values of fractional anisotropy (FA) did not show significant changes between pre- and post-operative studies (Table 2).

Histopathological findings

Histopathological examination of the specimen obtained from the resected intraparenchymal scar tissue showed no astroglial reactivity but fibrous connective tissue intermingled with bundles of peripheral nerve fibers and Schwann cells (Fig.7).

Neurological assessment

Preoperatively the patient presented, in serial examinations, symptoms of a complete spinal cord injury (ASIA A), with a zone of partial preservation (ZPP) at dermatomal level Th9 and complete loss of any type of sensation below this dermatome, including the S4-S5 dermatomes. The patient was assigned 31 points for each side in the ASIA Light Touch and Pin Prick Score. There was a paralysis of the leg muscles (0 points according to the MRC scale) and weakness of the lower trunk muscles causing trunk instability (positive Beavor's sign) during the attempts of the patient to stand. Increased spasticity was present in the LE's between 4 and 5 on the Ashworth scale, with a bilaterally positive Babinski sign and LE hyperreflexia, except in the ankles, where there was no Achilles tendon reflex. Additionally the legs were constantly cold due to an

impaired vascular autoregulation. A small pressure ulcer was present on the lateral surface of the right hip, which was not painful because of the sensory impairment. After the operation the patient's neurological state did not change significantly during the first 4 months. Five months after surgery the first signs of recovery of sensation in dermatomes S4-S5 were present, becoming more evident at 6 months. A gradual recovery of superficial sensation, particularly in the right LE, was observed during the whole post-surgical period of 19 months, increasing in pace after the 10th month. The ASIA Light Touch score reached 35 points for the right leg, while it was 32 in the left leg. The increase of the light touch score for the period after 10 months post-surgery was statistically significant for both LE's ($p < 0.05$; Fig. 8). The patient also achieved a higher score in the ASIA Pin Prick Test (42 for the right leg and 34 for the left), but was statistically significant only for the right leg ($p < 0.05$; Fig. 8). For the first time the patient reported pain evoked by irritation of the small pressure ulcer present on the right hip. We also noticed a recovery of the deep sensation in the legs. At 6 months post-surgery the patient began to feel the tension applied to his leg muscles during training and the movement of his joints. Between the 10th and 19th month after surgery, the recovery of deep sensation was confirmed with specific tests such as the vibration test or tests for evaluation of limb position. On about 30 trials, the patient could determine with 75-85% accuracy, the direction of movement of his feet with his eyes closed, and even could discriminate the movement of his toes from the movement of the whole foot. Simultaneously with the improvement of superficial and deep sensation, we observed an evident recovery of voluntary motor function in the previously paralyzed muscle groups. This recovery appeared in a segmental pattern, starting from the lower part of abdominal and other trunk muscles. This was first evident 5 months after surgery. The

recovery of voluntary function of selected LE muscles was preceded by a marked increase in muscle mass of the left thigh 4 months postoperatively, causing visible leg asymmetry. The circumference of the left thigh, measured in the middle of the line connecting the anterior superior iliac spine and the patella, was 50 cm versus 42 cm in the right thigh and increased gradually from 50 to 54 cm within a period of 18 months. The increase of muscle mass in the right thigh did not exceed 2 cm in the same period. There was no change in the mass of the calf muscles. As the patient had sustained inflammation and thrombosis of the LE-deep-venous system in the preoperative period, Doppler-ultrasonography (Vivid 7, GE Healthcare, Horten, Norway) of the veins and arteries of LE was performed. The independent angiologic study excluded venostatic edema or post-thrombotic syndrome as a cause of the increased circumference of the left leg.

The motor recovery was more prominent in the left LE. The first voluntary adduction of the left leg was observed 5 months after surgery and increased with time reaching level 3 according to the MRC scale at 11 months. Ten months postoperatively a voluntary adduction of the right leg (MRC 2) was noted and slight hip flexion on the left (MRC 1). The increase of the strength of the left and right adductor muscles was statistically significant for the period after 7 months post-surgery ($p < 0.05$; Fig. 9). The first voluntary knee extension was recorded on the left (MRC 2) at one year, and was confirmed in later studies. A slight knee extension in the right leg was also noted at 14 months post-surgery (MRC 1). As a result, the patient's neurological state turned 6 months after surgery from ASIA A to ASIA B, and at 11 months to ASIA C (Fig. 10). The observed increase of ASIA motor scores for the left and right LE were statistically

significant for the period after 10 months when compared with the preoperative state and the first 10 months after surgery ($p < 0.05$; Fig. 9).

A decrease of spasticity in both LE was noted postoperatively, but was significant statistically only for the right ($p < 0.05$). The mean Ashworth score decreased from 5 to 3.8 for the left LE and from 4 to 3 for the right. At 5 months postoperatively the Babinski sign disappeared bilaterally and the Achilles tendon reflex re-appeared in the left LE. The left LE seemed also to have better vascular autoregulation when compared to the right.

Physiotherapeutic assessment

During the period of 8 months of intense pre-operative rehabilitation the patient did not show any improvement in performed physical exercises. There was asymmetrically increased spasticity of the trunk and LE, paralysis and loss of sensation of LE and the equinovarus positioning of left foot. This hindered any attempts of patient tilting and made walking in braces in parallel bars impossible. Walking index (WI) was 0.

Increased strength of the trunk muscles, the first voluntary movements of adduction and abduction of the left leg, decrease of muscle spasticity, and the evident recovery of proprioception in the LE, that started to be visible from the 6th month after surgery, increased the quality of performed exercises and enabled the introduction of more difficult tasks. The increase of the muscle strength and coordination enabled better trunk, pelvis and hip stabilization and could prepare the patient for the first exercises of walking re-education. In the period from 9 to 11 months after surgery, there was an evident improvement in the technique of exercise performance and an increase in the values of the loads in exercises requiring high degree of voluntary function of

abdominal and back muscles, gluteal muscles, adductors and abductors, hip flexors and knee extensors. The left leg was dominant in muscle mass, number of voluntary controlled muscles and time of appearance on first symptoms of motor recovery. This enabled the introduction of separate exercises for the left and right leg. We did not observe any notable improvement in the exercises testing muscles of the posterior part of the thigh and the calf. Table 3 summarizes the results from physiotherapy.

The observed improvement in different static physical exercises led gradually to the first attempts to walk. Six months post-surgery the patient was able to ambulate 10 m. in parallel bars with long leg braces and physical assistance of one person (WI=3), at 13 months WI increased to 5 (parallel bars, no physical assistance) and starting from 14 months the patient was able for the first time to ambulate with walker, long braces and the assistance of one person (WI =6). Additionally, in the last months of observation the patient started to walk both in parallel bars and with a walker with short braces, locked only at the ankles. The observed increases in the WI values after surgery were statistically significant ($p<0.05$).

Measurements of functional activity using the FIM scale showed a significant increase of the FIM values from 102-104 pts. before surgery to 116-120 pts. in the period between the 6th and 11th month, to reach 123-124 pts between 12th and 19th months.

Correlations between the results from neurological and physiotherapeutic studies (Table 4)

A statistically significant strong positive correlation ($p<0.05$) was found between the ASIA motor scores for both LE, the scores obtained for leg adduction, the light touch and pin prick scores in the right and left LE, between the left and right ASIA motor

scores and the WI, the right light touch and pin prick scores and WI, and between the WI and the FIM score. A negative correlation was noted between the leg adduction tests and the leg spasticity measured with the Ashworth score.

Neurophysiological evaluation (Fig. 11, Table 5)

During the first and second preoperative examinations MEP and EMG studies detected efferent transmission from motor cortex centers to spinal cord motoneurons only to the level of innervation of the rectus abdominis muscles. Postoperatively, MEPs recorded from abdominal muscles on both sides were close to the normal recorded in healthy volunteers (more than 1500 μ V). At 5 months after operation excitation reached L1 to L4 spinal motoneurons followed by clear signs of recovery of contraction of rectus femoris muscles. An increase of the amplitudes in MEPs potentials recorded in subsequent trials was prominent for the left rectus femoris muscle, and reached 300 μ V at 14 months. MEPs from the right rectus femoris muscle were comparable to the one taken from the left rectus femoris at 5 months, but decreased at 8 and 11 months, and could not be recorded at 14 and 17 months. Activation of the corticospinal connections to L5-S1 motoneurons following the magnetic field excitation was only recorded rudimentary from distal muscles of left LE. The first observed low-amplitude MEPs recordings were associated with an increased latency and duration of potentials, but a tendency for shortening was detected during recordings at 11, 14, and 17 months (Fig. 12). Postoperative EMG recordings from LE showed voluntary muscle contractions recorded as action potentials from the rectus femoris muscles and the left digital extensor during the attempt of maximal muscle contraction, that were not present preoperatively. EMG recordings (both surface and needle performed) confirmed the

voluntary contraction of muscles when recorded mainly on the left side. The frequency of positive EMG recordings in the proximal LE-muscles was higher on the left side. Low frequency of the positive EMG recordings from distal muscles of the left LE as well as absent or very weak responses were found for the distal muscles of the right LE. Electroneurographic studies suggested this was related to degenerative changes in the peripheral motor fibers in the peroneal nerves. ENG studies showed their axonal type basing on recorded low amplitudes of M-wave potentials. Needle EMG recordings performed from active muscles during voluntary contraction, showed at rest also positive sharp potentials in almost all of the recordings (20/24 trials), indicating a degenerative process at the neuro-muscular junctions. The above observations were confirmed by three independent neurophysiologists involved in the neurophysiological tests.

Urodynamic studies did not show any difference in control examinations. A hyperreflexia of the bladder detrusor and dyssynergy between the bladder detrusor and urethral sphincter activity was noted both pre-and postoperatively. There was no electromyographic evidence of voluntary functional activation of the anal sphincter. However, an improvement of bladder sensation helped the patient determine the timing for voiding. After surgery the patient reported that he had regained the ability to obtain and maintain erection without the need for pharmacological support.

Psychological evaluation

Clinical observation performed for 20 months after the operative treatment revealed positive changes in the psychological and social profile of the patient. This included a

change of the style of interpersonal relations from passive (before treatment) to a dominant (increase of MMPI-2 Leary index from 4 to 7), a better resolution of anger and aggression (increase of Leary index from 4 to 7), a decrease of anxiety level (EPQR: a decrease from 8 sten to 5 sten), an increase of the sense of self-control and efficacy in life (GSES: increase from 5 sten to 7 sten), and an increased satisfaction in interpersonal relations (including sexual). Negative observations concerned the patient's lower threshold of tolerance of frustration and higher irritability. Conclusively, an improvement of the mental state of the patient was noted, especially with respect to satisfying the most important needs like the sense of safety, social acceptability, and quality of life. According to the patient, his improved quality of life was influenced by the both the neurological improvement, giving him higher degree of independence, and the fact of participation in the therapeutic project.

Discussion

Safety of the experimental procedure

The operation of craniotomy for unilateral bullectomy, followed by isolation and culture of OECs and ONFs, and their transplantation into the spinal cord with simultaneous bridging of the gap with autologous sural nerves was safe over the period of 19 months of post-surgical observation. There was no general and neurological deterioration, neuropathic pain, infection, spine instability, stenosis, tumors or progressive myelopathy in all observational periods. Sterility tests of cell cultures and the cerebrospinal fluid did not show any bacterial or fungal infection. The implanted nerve strips retained their structure and integrated well with the host spinal cord tissue,

making efficient contact with the sectioned cord stumps. MRI revealed both pre- and postoperatively the development of small areas of myelopathy in the spinal adjacent to the lesion area. Their remained radiologically stabile and did not influence the neurological and neurophysiological state of the patient (Fig.5). We used measurements of the fractional anisotropy (FA) to assess the degree of integrity of spinal cord white mater tracts in the spinal cord (40) (Table 2). Values of FA measured at two levels above and below the lesion focus were typical for cases of spinal cord lesion (29,41) and did not deteriorate in the subsequent studies which confirms the assumption that the observed spinal cord degeneration was not progressive. Five weeks post-surgery, a DTI study showed a realignment of the tracts of water diffusion through the area of spinal cord reconstruction, followed in the next studies by a gap of water diffusion, as in the preoperative studies (Fig.6). The early realignment of the tracts in DTI is unlikely to be indicative of fiber regeneration because this process would be expected to need longer periods of time. It rather showed a good integrity of degenerated spinal cord and nerve endoneural channels. The gap of water diffusion, occurring in the later DTI studies was not associated with any worsening of the patient's neurophysiological and neurological state, and conventional MRI images showed that the nerve implants remained well integrated with the host spinal cord tissue. We believe that either the performed study was not sensitive enough to show thin regenerating fibers crossing the implant, or the pattern of fiber regrowth might not have been longitudinal and changed the direction of water diffusion in the reconstructed area.

Unexpectedly the patient regained some smell perception on the side of bulbectomy 3 weeks after the craniotomy. Our first explanation was that following the sustained operation of anterofrontoethmosphenoidectomy new anatomical connections between

the left and right nostril for the airway passage could have been created (as in septostomy etc.), enabling the right OB to be excited by the odor stimuli entering the left nostril. Yet CT scans and laryngological fiberoscopy could not confirm this hypothesis. A possibility of an efficient bilateral flow of the air through the posterior nares was also not confirmed. For example we noted in some of our patients treated for anterior skull base fracture, in whom the lesioned olfactory bulb had to be removed, that they did not regain olfaction on the side of the bulbectomy (unpublished data). As those patients underwent cranialization of the skull base with use of a periosteal flap, that separated the fila olfactoria from the olfactory cortex, and our patient did not, we suggest that a plastic response or a regenerative response within the olfactory system may have occurred in the present case. Although the phenomenon of direct reinnervation of the olfactory cortex by olfactory axons, after bulbectomy, has been described in mammals (15), further investigation of our observation is required and is beyond the scope of this paper.

The pattern of neurological recovery - spontaneous versus induced recovery?

In this paper we show an essential neurological recovery of an ASIA A patient with chronic 21-month-paraplegia to ASIA C grade (sensory and motor incomplete) within a period of 19 months after an operative intervention consisting of spinal cord scar resection, intraparenchymal bulbar cell microinjection and reconnection of the spinal cord stumps with sural nerve grafts. The first issue that has to be discussed is whether the observed recovery could have been spontaneous and triggered by rehabilitation rather than a result of the transplantation procedure. Pre-operative MRI

images as well as intra-operative exploration showed an almost total physical disconnection of the spinal cord stumps at upper vertebral level Th9. Only a thin 2-mm-thick spur of tissue was connecting the stumps (Fig. 1 and 3). Serial preoperative MEP and EMG studies indicated that this tissue was not conducting electrical stimuli to the lumbar group of motoneurons below the area of injury. We did not remove it, because we believed that it may be able to act as a scaffold to guide new regrowing axons and also might help to mechanically stabilize our grafted nerve strips.

The pre-operative clinical, electrophysiological, radiological and intra-operative data showed that the knife entry has produced a rare type of severe spinal cord lesion seen in human, resembling an experimental situation of an isolated spinal cord transection with minimal involvement of vertebral structures. In our opinion the prognostic signs made spontaneous recovery unlikely. A panel of leading experts in the field of treatment of spinal cord injuries (SCI), who analyzed the data from 3 large double-blind placebo-controlled trials on neuroprotection in SCI – the National Acute Spinal Cord Injury Study (NASCIS), Sygen (monosialotetrahexosylganglioside; GM ganglioside) and GK-11 (gacyclidine) trials - concluded that a spontaneous recovery of motor and sensory in an ASIA A patient may be possible in 20% of the cases in the first year after injury, but mainly in the first 3 months (13). Another study showed that most spontaneous motor recovery in ASIA A patients occurred in the first 6 months after injury, and was almost absent after 12 months (45). Additional data on the late spontaneous recovery of 987 SCI patients, showed that between the 1th and 5th year after injury, only 5.6 % of the ASIA A patients may recover, from which 3.5 % recover to ASIA B grade and only 1.05 % to ASIA C, and 1.05% to ASIA D (21). The data of Kirshblum et al. (21) are strong, even though they were not supported by electrophysiological studies and

information about how the patients were rehabilitated. To be sure that our patient would not improve spontaneously, and to exclude the “nocebo” effect seen in inappropriately rehabilitated patients, he underwent an additional 8 month intense pre-operative rehabilitation program in one of the Polish reference centers for rehabilitation of SCI-patients. MEP and EMG studies performed at the beginning and end of this 8-month training, together with regular neurological assessments did not show neurological recovery. For this reason we consider that the probability of spontaneous recovery in our patient was lower than 1%.

We observed a gradual recovery of both sensory and motor function that started after the 4th month postoperatively. This recovery was non-linear, having two critical periods- the 5th-6th months, when the first evident signs of sacral sparing and voluntary muscle contractions from the first motor segments below the level of injury appeared (the patient became ASIA B), and the period after the 10th month, when the patient turned to ASIA C. The motor recovery occurred in a segmental pattern, starting at 5 months from an increase of the strength of lower abdominal muscles and other trunk muscles (lower thoracic motor segments), followed by a gradual increase of voluntary LE adduction (L1 segments), reaching 3 points according to the MRC scale in the left LE and 2 points in the right LE. The voluntary control of the musculature proceeded in time downwards, predominantly to the left LE and included hip flexion (L2 segments, 1 pt. according to MRC) and knee extension (L3 segments, 2 pts. according to MRC scale) (Fig. 9). This motor recovery was preceded by a marked increase of muscle mass in the left thigh as a sign of ongoing reinnervation. The recovery concerned not only the ZPP, but several motor segments below this zone, and has been considered to be a sign of spinal cord repair (13). The observed appearance of new voluntary muscle function, coming from

reinnervated motor segments below the level of spinal cord transection, could also be confirmed more objectively by the performance of MEP and EMG studies. Repeated electrophysiological studies were predictive for the observed motor recovery in the ASIA motor investigation and indicated again a dominant motor recovery in the left LE up to the L5-S1 segments (Fig. 11 and Table 5). Difficulties in MEP recordings from the proximal muscles of the right LE, the lack of MEP recordings and low amplitudes of the EMG response in its distal muscles and the worsening of the ENG values in the right peroneal nerve (Table 5) may be explained by an ongoing peripheral nerve degeneration of central origin (33). This phenomenon was described in patients after stroke (16) and does not arise from a direct lesion of the peripheral nerves, but from a long-term lack of activation of spinal cord motoneurons from neurons of supraspinal origin. In our case such lack of activation or weak activation may have occurred in the motoneurons innervating the right LE.

The improvement in efferent spinal cord transmission was characterized by increasing MEP-amplitudes in the abdominal muscles and mainly in the left LE-muscles, but also as gradual increase of the velocity of nerve signal conduction, registered as shortening of the latency of signal conduction and the duration of motor potential (Table 5, Fig. 12). The first latency (46.5 ms) and duration of the MEP response (74.3 ms) of the left femoris muscle, registered at 5 months postoperatively, were pathologically protracted. With time, a gradual normalization of these parameters was observed. Hence, the latency of the MEP-response at 14 and 17 months post-surgery reached values typical for the healthy population (23.4 ms and 21 ms respectively). In conclusion, the improvement in efferent spinal cord transmission gave evidence of ongoing long-distance motor fiber regeneration (amplitude increase), possibly from the corticospinal

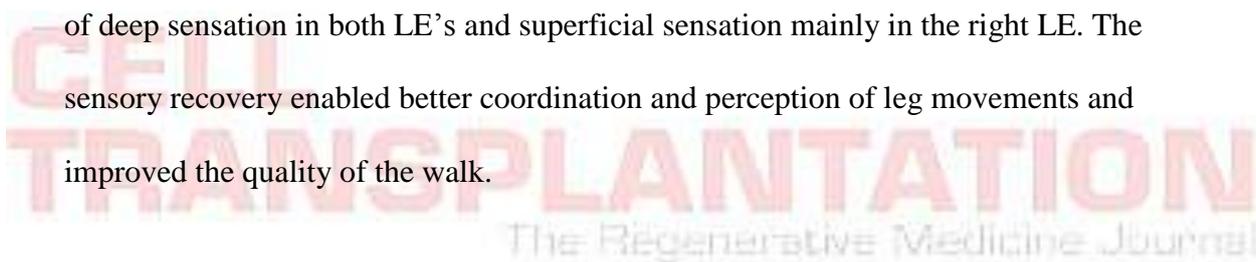
tracts, and remyelination of part of these tracts (latency and potential duration normalization), occurring mainly from the part of the spinal cord, which had been bridged with the nerve strips, and was a good prognostic factor for the observed neurological improvement.

Starting from the 5th month after surgery, as in case of the motor function tests, we observed a timely non-linear pattern of recovery of superficial sensation, with higher scores achieved in the right LE both in the ASIA Light Touch and Pin Prick scores in dermatomes from L5 to S5, depending of the type of tested modality (Fig. 8). This may be consistent with regeneration of the primary afferent fibers crossing the midline below the injury site and regenerating along the nerves implanted predominantly in the left half of the spinal cord as spinothalamic tracts. Additionally, the recovery of deep sensation on both sides, that was stronger in the feet, may be consonant with regeneration of primary afferent proprioceptive fibers from lower lumbar and sacral segments, ascending in the dorsal columns closest to the midline and crossing the bridged injury on both sides. The lesser recovery of motor function in the right LE and superficial sensation in the left LE might have been either due to plasticity of fibers that have regenerated across the nerve grafts on the left or due to minimal regrowth of fibers crossing the OEC-infiltrated scar tissue on the right side of the spinal cord.

Taken together, all observed recovery of motor and sensory function had a partial Brown-Sequard pattern and fitted exactly with the location of the repair, suggesting that the bridge of peripheral nerve grafts that preferentially reconnected the left half and the medial part of the right half of the spinal cord stumps (Fig. 13). Additional clinical observations concerned the reappearance of the left Achilles tendon reflex and the disappearance of the Babinski sign. The observed normalization of reflex activity may

also indicate an improved supraspinal control of local spinal cord neuronal circuits. We also noticed some symptoms of improved autonomic function, such an improvement of bladder sensation, confirmed in urodynamic studies, an improvement of erection control without the need for pharmacological support and improved vascular autoregulation mainly in the left LE.

The observed statistically significant improvement of sensorimotor function had a positive impact on the achievements in physical exercises during rehabilitation and significantly influenced the results from the FIM tests and the ability of the patient to walk, measured as increased Walking index (WI) (Table 4). The improved walking was not only due to the increasing strength of trunk and LE muscles but also to the recovery of deep sensation in both LE's and superficial sensation mainly in the right LE. The sensory recovery enabled better coordination and perception of leg movements and improved the quality of the walk.



Aspects of the operation contributing to the neurological recovery

Because our approach, as oriented to give the patient “best medical treatment” was complex, it is difficult to determine which aspects of the interventions contributed to the observed neurological recovery. The surgical intervention included spinal cord untethering, resection of the intraparenchymal scar tissue, injection into the spinal cord stumps and the rim of spared tissue of a mixture of bulbar OECs/ONFs, reconnection of the stumps with 4 strips of sural nerves, and was followed by a long and intense neurorehabilitation program. Each single intervention had its importance but in our opinion could not be in itself sufficient, if applied without the others.

The spinal cord untethering (myeloadhesiolysis) may have improved the vascular supply in the area of spinal cord reconstruction, and could be beneficial for the integration of the nerve grafts with the spinal cord tissue. Studies on a large group of patients with SCI undergoing late myeloadhesiolysis did not show any significant influence of this intervention on the sensorimotor recovery (11). The removal of the intraparenchymal scar tissue was beneficial because it eliminated some of the physical and chemical barriers for axonal regeneration contained in the scar (12) and turned the chronic spinal cord injury into an acute one, enabling a better interaction of the transplanted OECs and ONFs with host astrocytes. We consider it most likely that the crucial interventions during the operation were the intraparenchymal transplantation of OEC/ONFs and the reconnection of the spinal cord stumps with strips of peripheral nerves. Transplanted bulbar cells could have been responsible for realignment of the astrocytic processes and “opened the door” for regrowth of central axons (24).

The specific clinical condition of our patient, suffering from a chronic inflammatory condition of the nasal mucosa, allowed us to use for the first time OECs isolated from the human OB. There is growing evidence in the literature that bulbar OECs have stronger regeneration promoting capacity than mucosal OECs (18,32,39,46). We also observed in our previous trial on ASIA A paraplegic patients that mucosal OEC/ONFs gave minimal neurological improvement in all operated patients (43).

The only completed phase I clinical study on application of purified OECs in the treatment of human paraplegia did not show any efficacy of transplanted purified autologous mucosal OECs (30). As in our previous study, we transplanted cultures containing mixtures of OECs and ONFs. The ONFs form an intimate outer cover on the outer surface of the OECs, with the nerve fibers on the inner surface. After

transplantation into corticospinal tract or optic nerve lesions the advancing ONFs precede and establish a channel for the advance of the OECs (23,25). In all these situations OECs and ONFs seem to act together as essential and complementary components of a pro-regenerative tissue, and our experience (unpublished) is that purified OECs do not survive well after transplantation in rat spinal cord lesions. The mechanism of these complex interactions between OECs and ONFs is unknown. It certainly involves intimate surface to surface contact, implying a dependence on the interactions between membrane bound molecules and leading to a basal lamina forming on the surface of the OECs facing the ONFs.

The use of peripheral nerve grafts to bridge the sectioned spinal cord in experimental animals has been described 3 decades ago (7,38). In these studies only some populations of sensory neurons and intrinsic spinal cord neurons were seen to elongate their axons within the implants and enter for short distances the spinal cord. Newer modifications of the described methods claimed both in preclinical and clinical studies, that if the nerve grafts connect the white matter above the level of injury with the gray mater below and the white matter below the injury with the gray mater above, with local administration of acidic fibroblast growth factor, a regrowth of long-distance supraspinal axons may be possible (4,5) and may be beneficial for the patient (5). Cheng et al. (4,5) claimed that they rerouted the regenerative pathway from the nonpermissive white matter to the more permissive gray matter that is devoid of factors hindering regeneration.

In our technique multiple microinjections of a mixture of OECs and ONFs into the lateral columns of the spinal cord may have enabled the necessary interaction with host astrocytes to make possible the regeneration of both efferent and afferent axons

throughout the bridged nerves in to the caudal and rostral stumps. The nerve strips were placed along the long axis of both white and gray matter and enabled the central axons to grow within their natural anatomical compartments. Implanted peripheral nerve grafts served as guidance tubes with the appropriate extracellular environment for axon adhesion and elongation, and also as a large source of Schwann cells, known to be able to myelinate fibers of central origin and to secrete a variety of neurotrophic factors (1). The administration of methylprednisolone may have led to an increase of the number of myelinated CNS axons growing through the implants, and also may have given better integration of the transplant with the host tissue as described by Chen et al. (3). Taken together, our experimental approach of combining Schwann cell bridges and bulbar OECs was very similar to the one described by in rats by Ramon-Cueto et al. (37). In that study the authors demonstrated that supraspinal axons regenerating through the transplant re-entered the spinal cord and made synaptic connections. Thus we believe that the neurological recovery in our patient indicates regeneration of central nervous system fibers that crossed the host/peripheral nerve interfaces and grew for a considerable distance to make functional connections in the cord (Fig. 13).

Conclusion

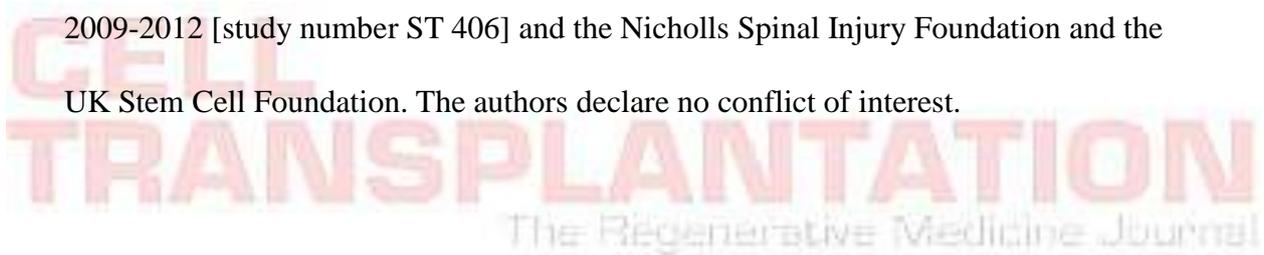
The results from the treatment of the first patient with a complete spinal cord injury receiving transplantation of bulbar OEC/ONFs and simultaneous reconstruction of the spinal cord gap with peripheral nerve implants are very encouraging, but have to be confirmed in a larger group of patients sustaining similar types of spinal cord injury. Further laboratory studies will be needed to elucidate the properties of human bulbar OEC/ONFs and their interaction with peripheral nerve bridges or artificial implants both

in vitro as well as their reparative potential in vivo. We are investigating surgical techniques for minimally invasive access to the human OB. There remains a possibility that sources of other, more readily obtainable reparative cells may be discovered.

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Figure legends

Fig. 1 T2-weighted MRI scans of the thoracic spine performed preoperatively. (A) Sagittal view of the spinal cord transection at upper vertebral level Th9. A 5-mm-gap of spinal cord continuity (orange arrow) was present 1 month after the injury. Green arrow indicates the area of the vertebral body affected by the knife injury. (B) Coronal scan of the same study showing the area of spinal cord injury (orange arrow). Note the thin rim of spared tissue connecting both spinal cord stumps (green arrow). (C) Sagittal magnetic resonance imaging (MRI) scan performed 21 months after the injury. The posttraumatic gap increased its size to 8 mm because of progressive spinal cord degeneration, seen as hyperdense myelopathic “caps” adjacent to the focus of injury. (D) Axial view of the area of spinal cord transection showing a 2-mm-thick tissue connecting both spinal cord stumps (orange arrow). frFSE: fast relaxation fast spin echo;

Fig. 2 Imaging of head, nasal cavity and cells to be transplanted.

(A) Coronal computed tomography (CT) scan of the head. An almost total obliteration of the nasal cavity and paranasal sinuses due to sinusitis is apparent. Black arrow, affected ethmoid sinuses. (B) Coronal CT scan performed after the operation of endoscopic anterofrontoethmosphenoidectomy, showing an improved air flow through the sinuses. (C) Photo taken from the operating microscope, showing the region of left olfactory groove with the posterior part of the olfactory bulb (long arrow), olfactory tract (asterix) and crista galli (short arrow). (D) Microphotographs of p75 low affinity nerve growth factor receptor positive (p75/NGFR⁺) bulbar olfactory ensheathing cells

(OECs; green) and fibronectin (FN)-positive olfactory nerve fibroblasts (ONFs; red), taken before cell transplantation, on the 11th day of culture. Hoechst: blue nuclear staining. Scale bar=100 μ m.

Fig. 3 Area of the spinal cord injury

(A) A virtual 3D-model of the spine and area of spinal cord injury. The spinal cord stumps in yellow, the posttraumatic CSF-containing gap in blue; rim of tissue connecting stumps in dark red. For each spinal cord stump the schematic grid for cell microinjection consisted of 4 rows (black lines), 2 mm apart. White dots, the sites of planned cell microinjection in the posterolateral sulcus (blue lines) and in the rim of tissue connecting the stumps. p-proximal spinal cord stump, d-distal stump; (B,C,D)

Intraoperative microscopic images of the area of spinal cord transection. (B) Initial view of the spinal cord after opening of the dura mater. The area of spinal cord transection was covered with yellowish scar tissue (asterisk). Arrow indicates an adhesion between the scar and dura. (C) View of the spinal cord after myeloadhesiolysis and resection of the intraparenchymal scar. This maneuver led to an increase of the initial gap from 8 to 10 mm. Asterisk, the rim of spared tissue connecting both stumps; arrow, a thoracic spinal nerve. Arrowhead, the dura of the ventral surface of the spinal canal, which confirmed that the spinal cord stumps were totally disconnected in this area. (D) View of the spinal cord gap reconstructed with 4 implanted strips of nerves (2 strips were placed ventrally and 2 strips dorsally), before their fixation with fibrin sealant.

Fig. 4 Summary of the smell perception test performed for the right and left nostril pre- and postoperatively. The maximal achievement that can be assigned for each tested side was 6 points. Note that after removing the left olfactory bulb the patient did not totally lose olfaction on the left but remained hyposmic.

Fig. 5 MRI Studies of the spinal cord at set time points after treatment.

MRI studies performed at 5 weeks (A,B), 5 months (C,D,E) and 17 (F,G,H) months after the spinal cord operation. Orange arrows, implanted sural nerve strips. (A) On the sagittal T2-weighted image the nerve implant appeared as a hypodense structure that reconnected effectively the sectioned spinal cord stumps. (B) Coronal scan of the same area showing that after contrast administration the nerves were hyperdense, which confirms their good vascularisation. Implant size was 1.3 cm x 0.7 cm. T2-weighted sagittal (C) and coronal scans (D) after 5 months confirmed the integrity of the nerve grafts with the host tissue. (E) Axial T2-weighted scan shows that the nerve grafts (orange arrow) filled most of the surface connecting the spinal cord stumps, while the rim of spared tissue was hardly visible (blue arrow), when compared with preoperative MRI scans (Fig. 1D). (F) Sagittal T2-weighted image at 17 months showed a hyperdense area of mild spinal cord degeneration that was present in both pre-as well as all postoperative studies (green arrows). (G) The sural nerve autografts retained their size when compared with the early MRI study. (H) At 17 months post-surgery, the nerve grafts still were the dominant structure found in the area of spinal cord reconstruction (orange arrow). The spared tissue was also identifiable (blue arrow).

Fig. 6 Scans of spinal cord tractography. (A) An 8-mm-gap of continuity of diffusion tracts was observed preoperatively. (B) 5 weeks after the operation the tracts of water diffusion crossed through the area of implanted nerves (orange arrow) and the rim of spared tissue connecting the stumps (blue arrow). (C) At 17 months, a gap of 1.8 cm was present. Orange arrow points out either thin tracts at the level of the implant or an artefact.

Fig. 7 Spinal cord scar. A. Scar composed of peripheral nerve fibers and fibrous connective tissue without central nervous system (CNS) tissue, hematoxylin and eosin (HE) staining. B. Immunohistochemical expression of S100 protein confirming aligned clusters of Schwann cells with typical ovoid nuclei within the scar. C. Lack of immunohistochemical expression of epithelial membrane antigen (EMA) excluding presence of meningeal tissue. D. Negative immunohistochemical staining for glial fibrillary acidic protein (GFAP) proving no glial components. E. Immunohistochemical expression of neurofilament (NF) typical for nerve fibers (scale bar=50 μ m). We assumed that these are the central branches of dorsal rootlets lying in the scarred area F. Immunohistochemical expression of vimentin corresponding with connective tissue.

Fig. 8 Summary of the results from the Light Touch and Pin Prick tests. The observed improvement in these tests in the period after the 10th postoperative month were statistically significant ($p < 0.05$) when compared to the preoperative state and the first 10 months post-surgery. There was also more evident recovery of superficial

sensation in the right lower extremities (LE) when compared with the left. ASIA: American Spinal Injury Association.

Fig. 9 Graphs showing the results from motor tests of the LE. A voluntary muscle activity could be observed in both the left and right LE, but was more evident after the 10th postoperative month and achieved higher Medical Research Council (MRC) scores in the left one.

Fig. 10 Chart showing the changes in the ASIA score in the postoperative period.

ASIA Score increased to B around 6 months and C around 11 months.

Fig. 11 Pre- and post-operative comparison of motor evoked potentials and electromyographic recordings.

Comparison of motor evoked potential recordings (MEPs; upper traces) and electromyographic recordings (EMGs; lower traces) performed pre- (A,B) and postoperatively (C-1 month post-surgery, D- 5 months, E-8 months, F-11 months, G-14 months, H-17 months). Recordings were performed from the left LE. Note different amplifications of recordings and increase of MEPs amplitudes recorded from both rectus abdominis and rectus femoris muscles (spinal efferent transmission recovery) as well as the improvement of motor units activity in rectus abdominis and rectus femoris muscles observed in EMG recordings. The calibration bars of amplification (vertical, in millivolts or microvolts) and time base (horizontal, in milliseconds) for all MEP and EMG recordings are shown in A.

Fig. 12 Variability of the motor evoked potentials (MEP) duration, latency and amplitude parameters, recorded at stages during the neurophysiological assessment of the left rectus femoris muscle. There was no muscle response to motor cortex activation preoperatively. Note the continued muscle response after the 5th postoperative month, and the improvement in spinal cord efferent transmission, registered as a gradual decrease of the latency and duration of the potential. A smaller MEP-response of the right rectus femoris muscle (shown only numerically in Table 5) was present at 5-11 months but was not observed in the following 2 trials.

Fig. 13 Schematic diagram showing the proposed pattern of fiber regeneration.

The injury consisted of a complete transection with 8mm separation over the left 3/4ths of spinal cord at the vertebral T9 level resulting in complete loss of motor and sensory function from and including the spinal L1 segment downwards. Cultured OEC/ONFs were microinjected into the lateral parts of the upper and lower stumps adjacent to the injury and into the rim of non-functional spared tissue on the right. Four strips of sural nerve (green) were used to bridge the gap between the stumps over the remaining 3/4ths of the cord.

Within 5 months, motor recovery (red) had started around the left hip (L1/2 cord segments) and superficial sensation (SS, green) over dermatomal levels S3-5 (sacral sparing). By 12 months motor recovery on the left had extended to the leg (L2,3) and deep sensation (DS, blue) had returned on both sides down to the feet (L5/S1). On the right side of the body there was a slight but much inferior and incomplete recovery of motor control, while there was a substantial recovery of SS. In contrast, on the left there was a predominant motor recovery but minimal recovery of SS. The patient has

been restored from complete paraplegia to a condition resembling an incomplete Brown-Sequard syndrome.

Together with the neurophysiological data this is consistent with regeneration of descending motor control (corticospinal) fibers (red) across the injury and their progressive descent with time on the left side of the distal cord. Similarly the primary fibers carrying SS (green) from the right side of the body, which cross below the injury level, have also regenerated across the injury site and made contact with local spinothalamic relay neurons on the left side of the cord above the injury. The fibers of DS (blue) seem to have been able to cross the medial cord regions (the dorsal columns) which were bridged by sural nerve strips alone, but in the absence of local relay neurons in the cord, the DS fibers require a longer time for the greater distance of regeneration needed for sensation to reach the brain. Unlike the progressive downward extension of the motor fibers, the ascending, sensory fibers from all caudal levels are severed at the level of the injury and there is no preferential recovery of upper versus lower segmental fibers.

The ability of motor and SS fibers to cross the injury requires both OEC/ONFs and sural nerve bridging. There is an indication that DS fibers may have regenerated across sural nerve bridges without OEC/ONFs. Infiltrating the non-functional residual tissue on the right of the cord with OEC/ONFs, but without interposed peripheral nerve tissue gives a much inferior motor and SS result.

Table 1. Parameters of cell microinjection.

volume of single injection	velocity of cell injection	total number of injections	number of injection sites	total volume	total number of grafted cells	percentage of OECs in the cell suspension
0.5 μ l	2 μ l/min.	96	24	48 μ l	500,000	16%

OECs: olfactory ensheathing cells.



Table 2. Summary of the values of fractional anisotropy (FA).

FA values were measured in the spinal cord above and below the lesion epicenter.

Levels 0.5 cm and 2 cm refer to the proximal spinal cord stump, whereas levels -0.5 cm and -2 cm to the distal stump. preop.-preoperatively

Date	+2 cm	+0.5 cm	-0.5 cm	-2 cm
preop.	0.305	0.266	0.366	0.415
preop.	0.397	0.399	0.424	0.468
1 month	0.480	0.323	0.327	0.545
5 months	0.407	0.287	0.504	0.487
8 months	0.316	0.377	0.436	0.574
17 months	0.363	0.280	0.419	0.476

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Table 3. Summary of the achievement from selected rehabilitation exercises.

Roman numerals refer to the month. Positive numbers show the value of applied load (in grams). Negative numbers show the value of load relief. Minus sign indicates that the patient was unable to perform the exercise.

1. Forward bends from a supine position - a test of abdominal muscle strength. In this case the patient was initially holding a bar connected with a suspended load and started the exercise from an angle of 30°. Starting from 9 months after surgery he could bend forward without any help (w.h.), beginning the movement from a completely supine position (0°).
2. Hip extension - an exercise testing trunk muscles and gluteal muscles and performed in a supine position with suspended straight legs. Preoperatively the patient was able only to initiate a simultaneous downward movement of both legs when blocked in long braces. This leg movement was triggered by the use of trunk musculature, but further movement was prevented by increased spasticity and muscle paralysis. After surgery he was able to perform a full range of hip extension without leg immobilization in braces. In addition the patient became able to perform the same exercise with his legs suspended separately (Nr 3, right leg; Nr 4, left leg). The left leg was able to overcome much higher loads than the right.
5. Leg abduction in a sitting position.
6. Leg adduction in a sitting position.
7. Drawing of suspended knees towards the abdomen – using hip flexors (iliopsoas muscles and rectus femoris).
8. Cycling in supine position - test of hip flexors and knee extensors (quadriceps muscle). The feet are placed into a rotor and the patient is trying to perform alternating pedaling movements. This could be performed first at 5 months post-surgery with the use of high load relief. In the period after the 12th month the patient could cycle without any support. The left leg was the dominant one in this exercise.
9. Knee flexion in a prone position - test for the posterior group of lower

extremities (LE)-muscles (semitendinosus, semimembranosus, biceps femoris and gastrocnemius, innervated by the sacral spinal cord segments). There was no improvement in this muscle group.

Nr.	VII-XII 2011	I-III 2012	o p e r a t i o n	V- VII 2012	VIII-XII 2012	I- III 2013	V-VIII 2013	IX-XII 2013
1	-8750	-8000		-1000/-1000	-1000/-1000	w.h.	w.h.	w.h.
2	10350	8100		7050	8300	-	7500	9050
3	-	-		-	-	4200	3990	3900
4	-	-		-	-	4200	4750	5100
5	-	-		-	-	-	0	1000
6	-	-		-	-	-	2500- 5000	8750
7	-	-		-	-	-3000	-3000	-3000
8	-	-		-	-11450	-5500	w.h.	w.h.
9	-3000	-3250		-2700	-3600	-4200	-4200	-4100



Table 4. Summary of the most important correlations found during the performed tests of neurological and physiotherapeutic achievement.

Variable	Spearman coefficient	<i>p</i> -level
ASIA motor score - right & ASIA motor score – left	0.83	<0.01
Leg adduction - left & Leg adduction – right	0.92	<0.01
ASIA light touch - left & ASIA light touch – right	0.80	<0.01
ASIA pin prick - left & ASIA pin prick – right	0.83	<0.01
ASIA motor score - left & Walking Index	0.93	<0.01
ASIA motor score - right & Walking Index	0.85	<0.01
ASIA light touch - right & Walking Index	0.68	0.05
ASIA pin prick - right & Walking Index	0.71	0.03
FIM & Walking Index	0.93	<0.01
Leg adduction - right & Ashworth spasticity - right	-0.72	0.03
Leg adduction - left & Ashworth spasticity – left	-0.73	0.02

ASIA: American Spinal Cord Injury Association; FIM: Functional Independence Measure.

Table 5. Motor-evoked potentials, electromyographic recordings, and electroneurographic findings over time.

Comparison of parameters in motor evoked potentials induced with the magnetic field at cortical level (MEP; values of amplitudes, latencies and durations are shown, respectively), electromyographic recordings (EMG; values of amplitudes are shown) from abdominal and lower extremity muscles during attempts of their maximal contractions as well as electroneurographic findings (ENG; values of amplitudes and latencies are shown) in motor fibers transmission of peroneal nerves. They were recorded bilaterally at eight stages of observation (preop1, preop2, 1 month, 5 months, 8 months, 11 months, 14 months and 17 months).

Nr: non recorded.



Recording sites (muscle)	Preoperative recordings												Postoperative recordings											
	1 st trial						2 nd trial						3 rd trial						4 th trial					
	Motor evoked potential (MEP) parameters																							
Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		
Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	
rectus abdominis rectus femoris	3000	2000	16.4	16.0	26.3	25.2	2500	2500	16.4	16.3	37.1	25.7	1000	3500	13.9	13.8	60.0	65.6	4000	4500	15.1	15.0	66.4	69.5
	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Electromyography (EMG) parameters																								
Amplitude (μV)						Amplitude (μV)						Amplitude (μV)						Amplitude (μV)						
Right			Left			Right			Left			Right			Left			Right			Left			
rectus abdominis rectus femoris extensor digiti	200			200			150			150			100			100			1800			2000		
	nr			nr			nr			nr			100			200			200			250		
	nr			nr			nr			nr			nr			50			nr			50		
Electroneurography (ENG) of peroneal nerves parameters																								
Amplitude (μV)		Latency (ms)		Amplitude (μV)		Latency (ms)		Amplitude (μV)		Latency (ms)		Amplitude (μV)		Latency (ms)		Amplitude (μV)		Latency (ms)		Amplitude (μV)		Latency (ms)		
Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	
1000	1000	12.3	14.5	1000	1000	12.4	15.2	1000	1000	14.1	15.8	nr	500	nr	14.2									
Postoperative recordings (continued)																								
5 th trial						6 th trial						7 th trial						8 th trial						
Motor evoked potential (MEP) parameters																								
Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		Amplitude (μV)		Latency (ms)		Duration (ms)		
Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	
rectus abdominis rectus femoris	4000	4000	14.5	14.4	55.3	58.1	2800	3000	17.4	16.6	50.5	54.1	3500	4000	20.1	20.0	48.3	44.2	2000	3000	17.0	17.1	42.1	41.7
	50	150	29.5	29.0	68.0	68.7	50	150	28.9	24.8	66.9	64.0	nr	300	nr	23.4	nr	64.7	nr	100	nr	21.4	nr	44.0
Electromyography (EMG) parameters																								
Amplitude (μV)						Amplitude (μV)						Amplitude (μV)						Amplitude (μV)						
Right			Left			Right			Left			Right			Left			Right			Left			
rectus abdominis rectus femoris	1800			1800			1000			1000			2000			1800			700			700		
	200			300			150			200			200			200			150			150		

extensor digiti	50		50		nr		50		nr		50		50		100	
Electroneurography (ENG) of peroneal nerves parametrs																
	Amplitude (μ V)		Latency (ms)		Amplitude (μ V)		Latency (ms)		Amplitude (μ V)		Latency (ms)		Amplitude (μ V)		Latency (ms)	
	Right	Left	Right	Left												
extensor digiti	nr	250	nr	16.1	nr	200	nr	16.6	nr	300	nr	15.9	100	3000	16.8	15.5

Abbreviation: nr-non recorded



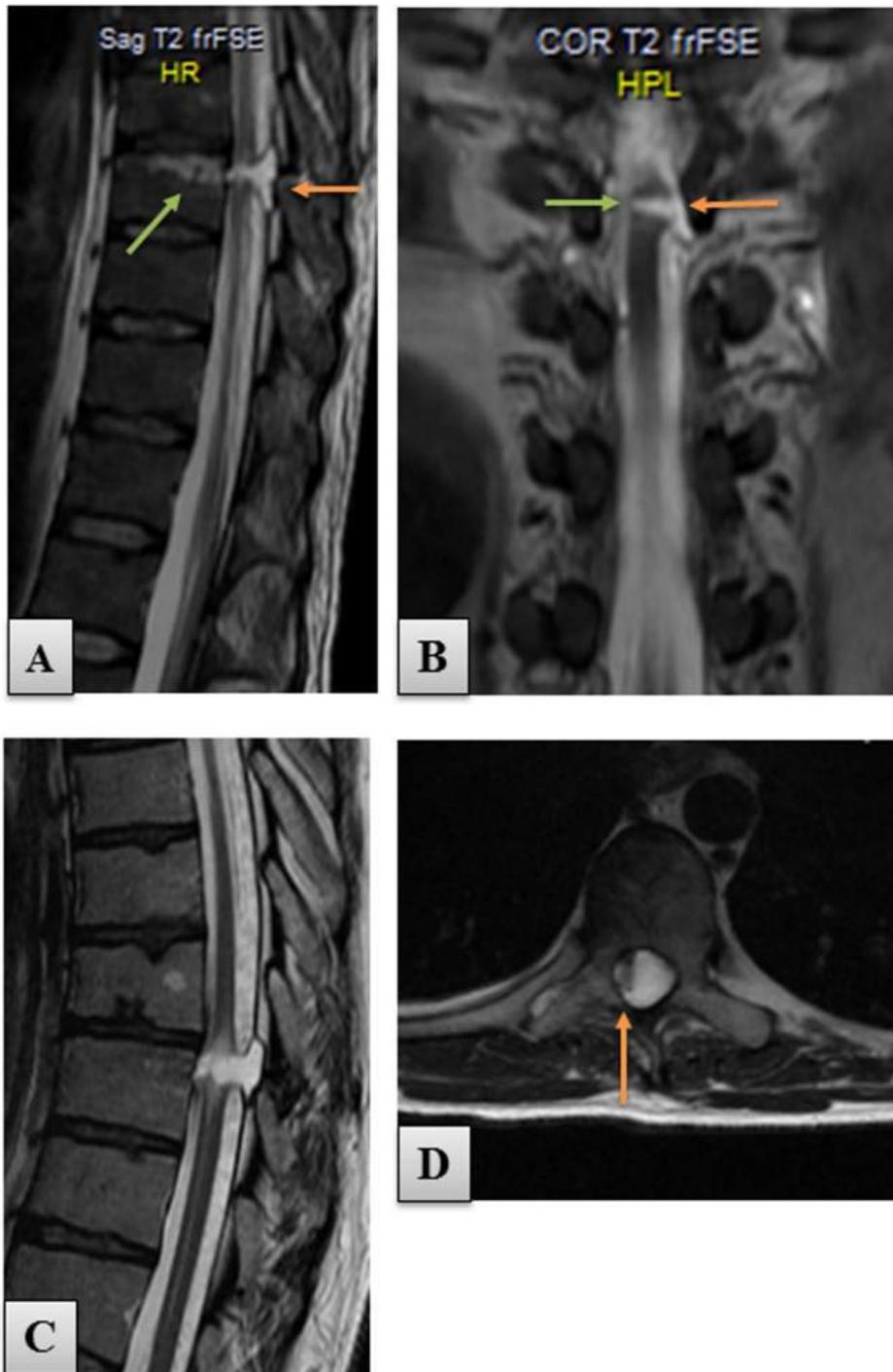


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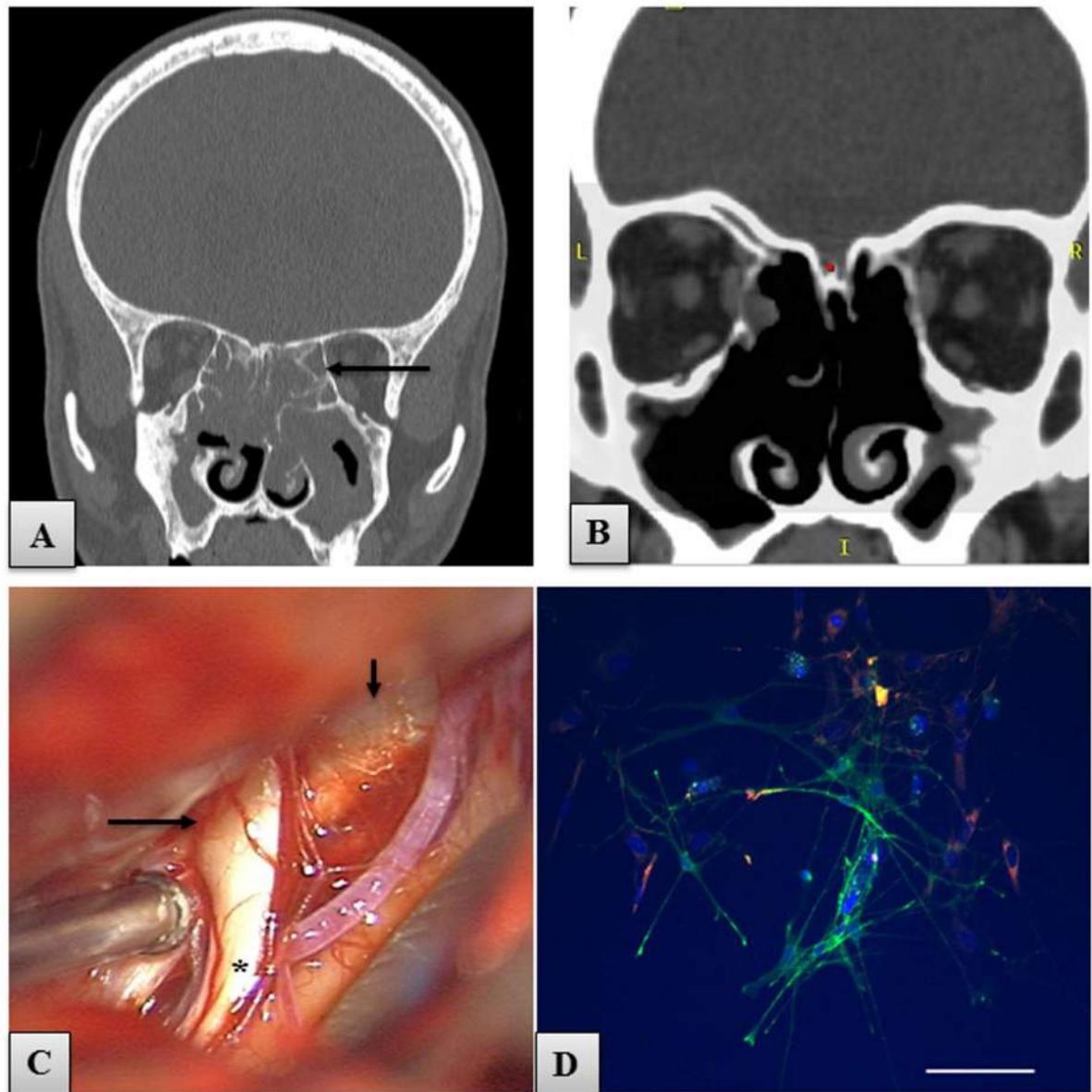


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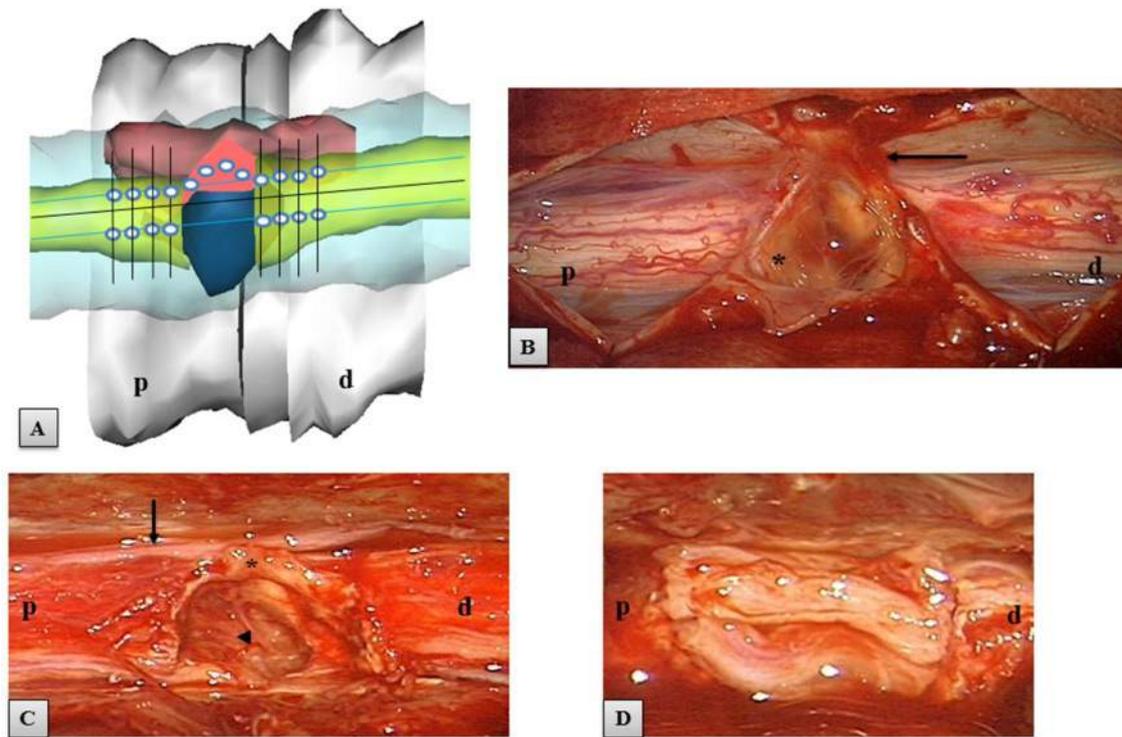


Figure 3:

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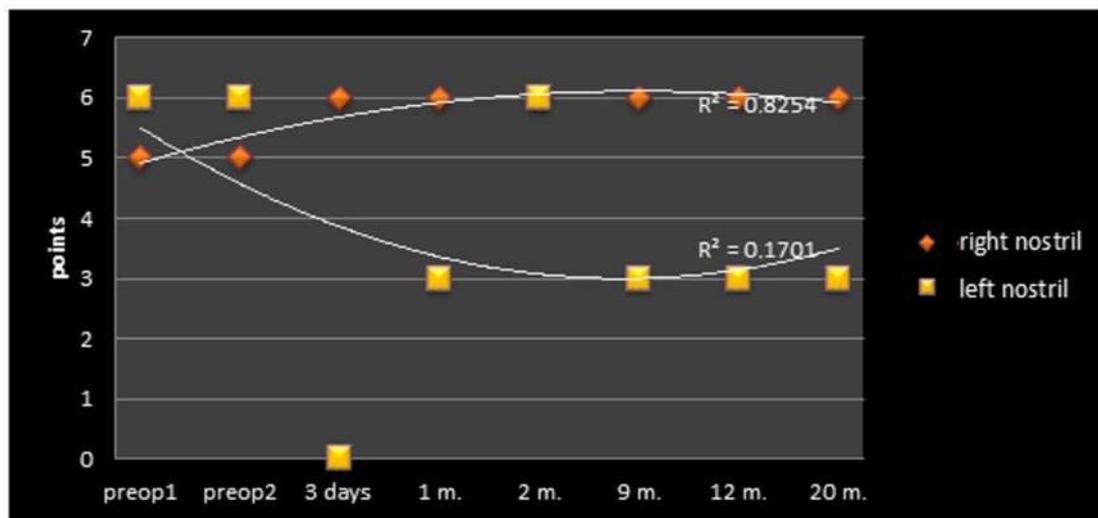


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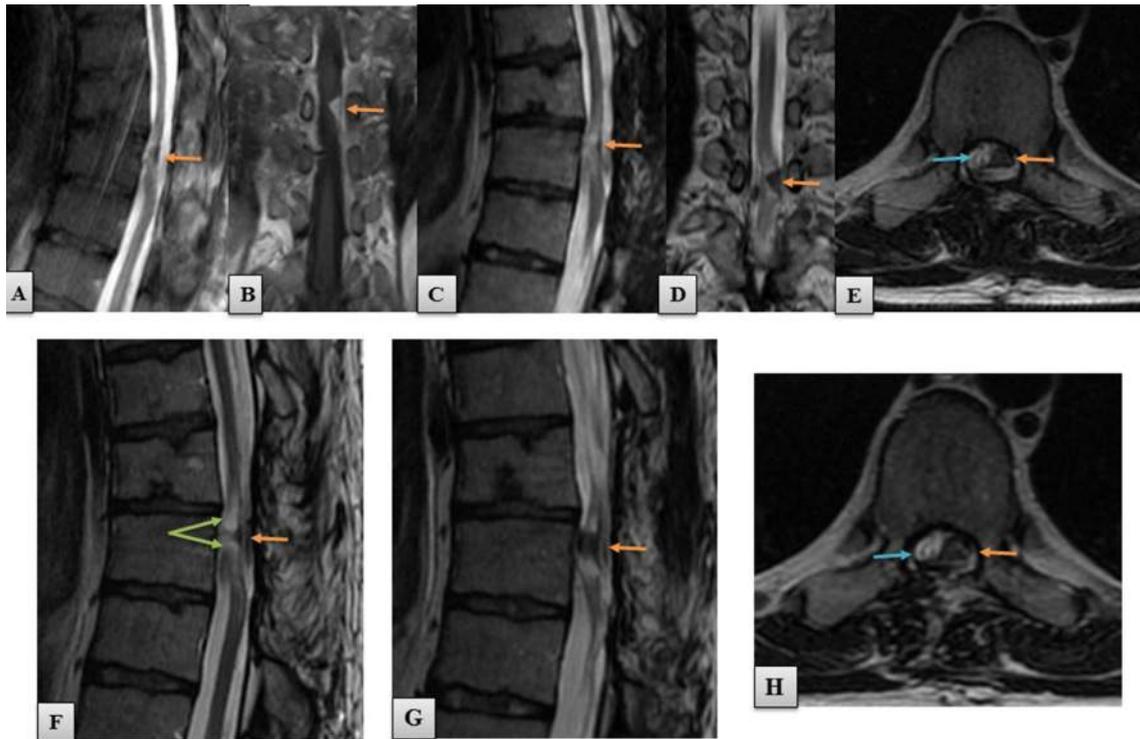


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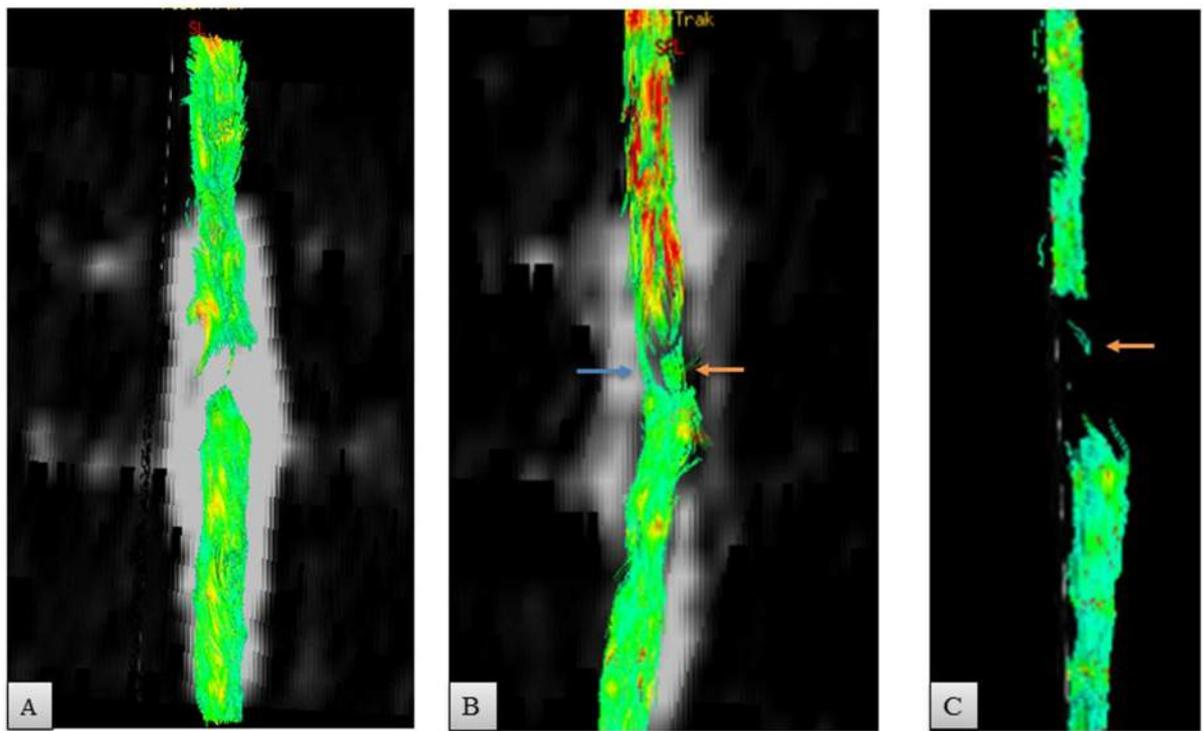


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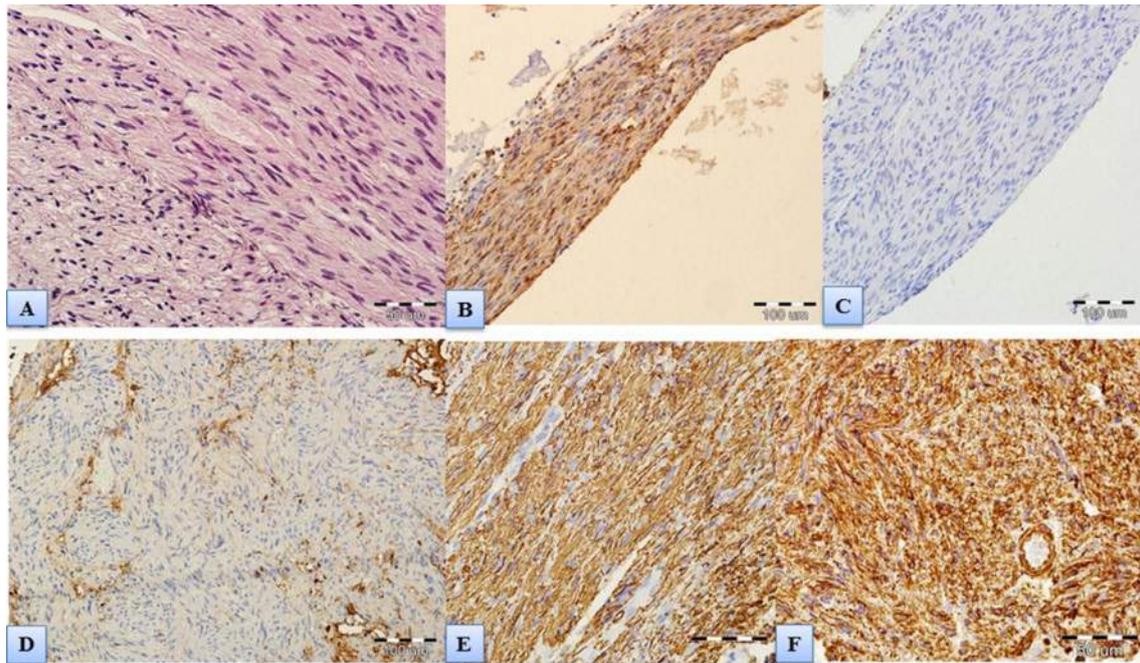


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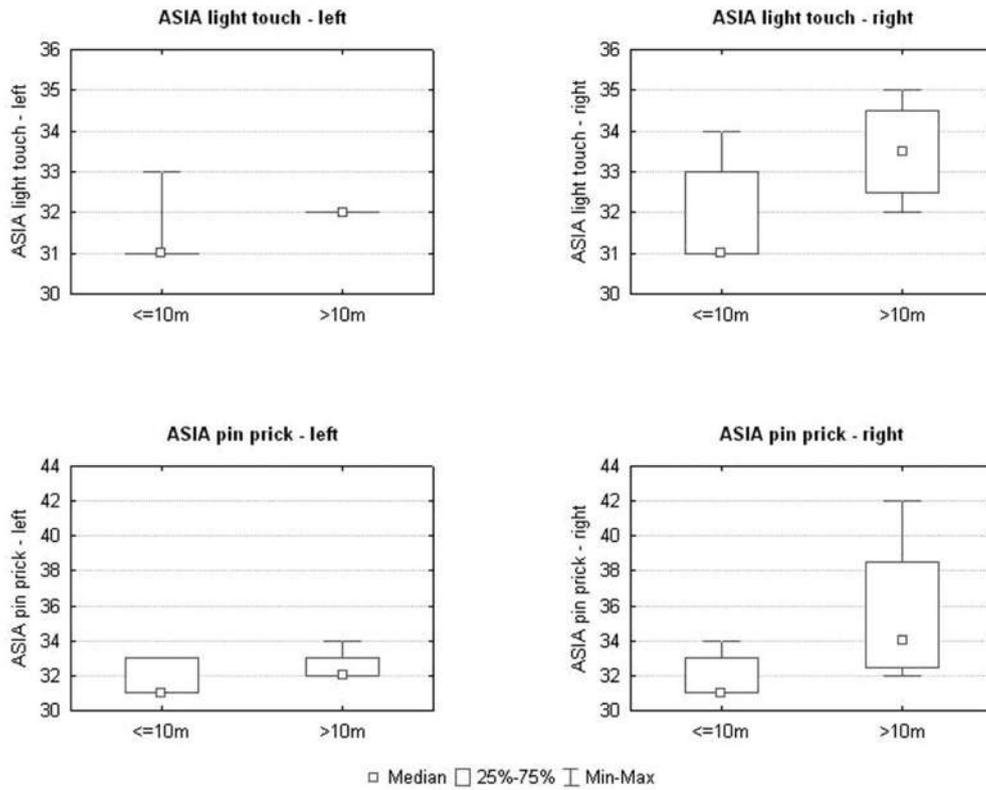


Figure 8:

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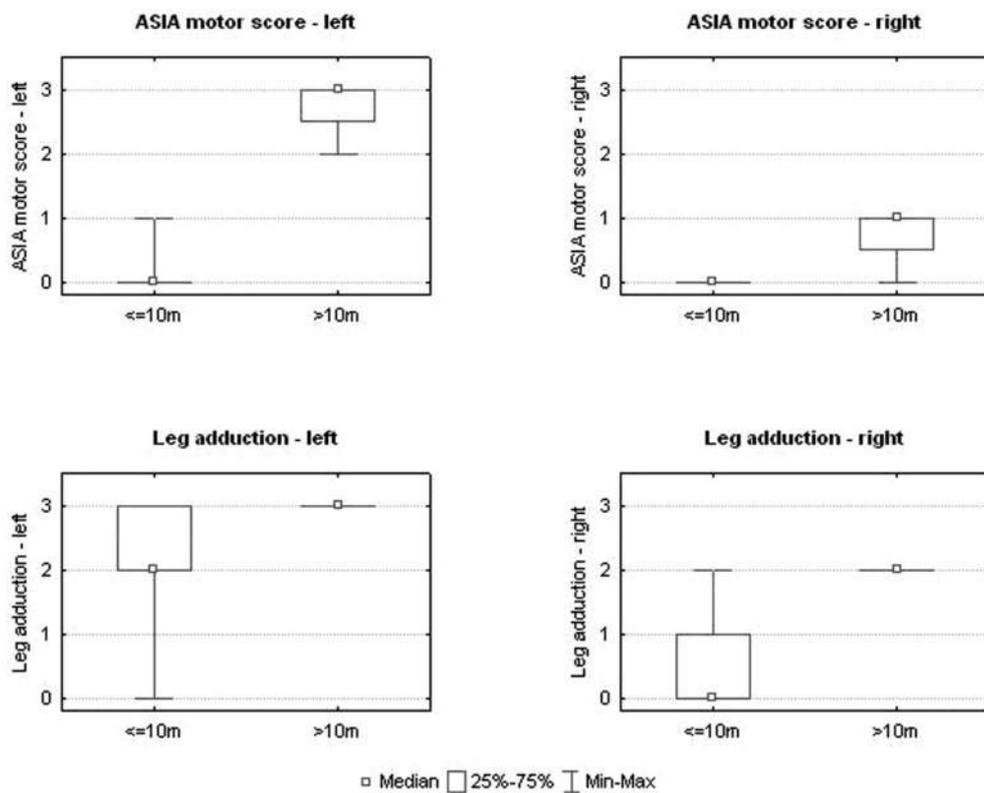


Figure 9:

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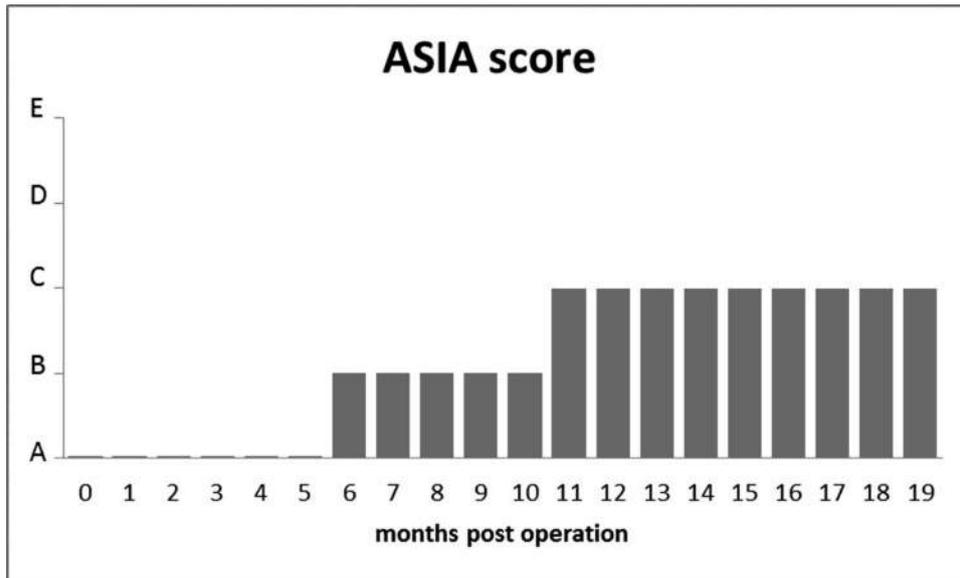


Figure 10:

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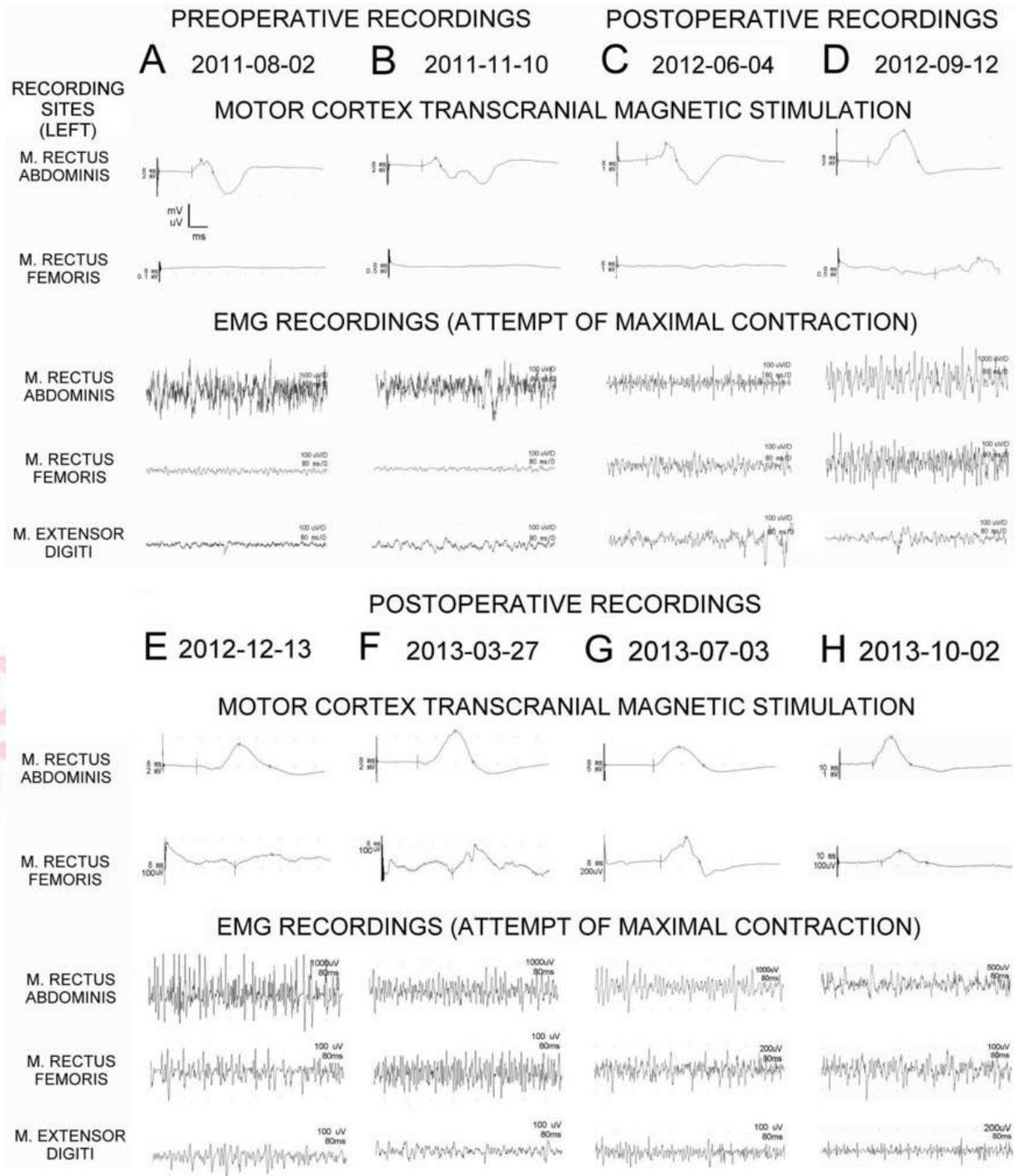


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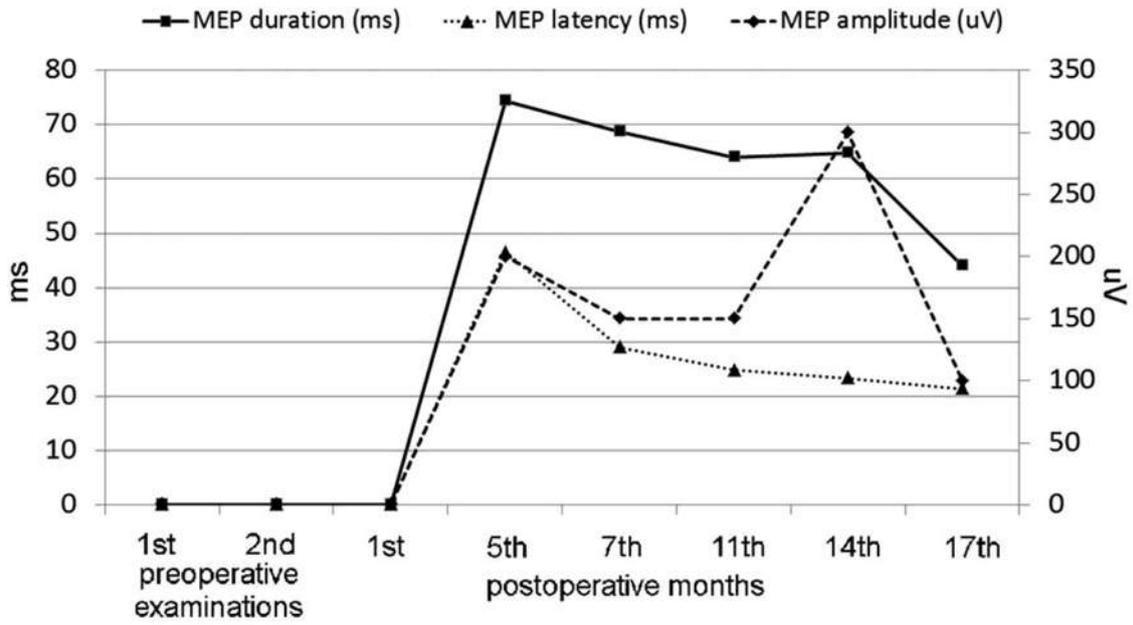


Figure 12:



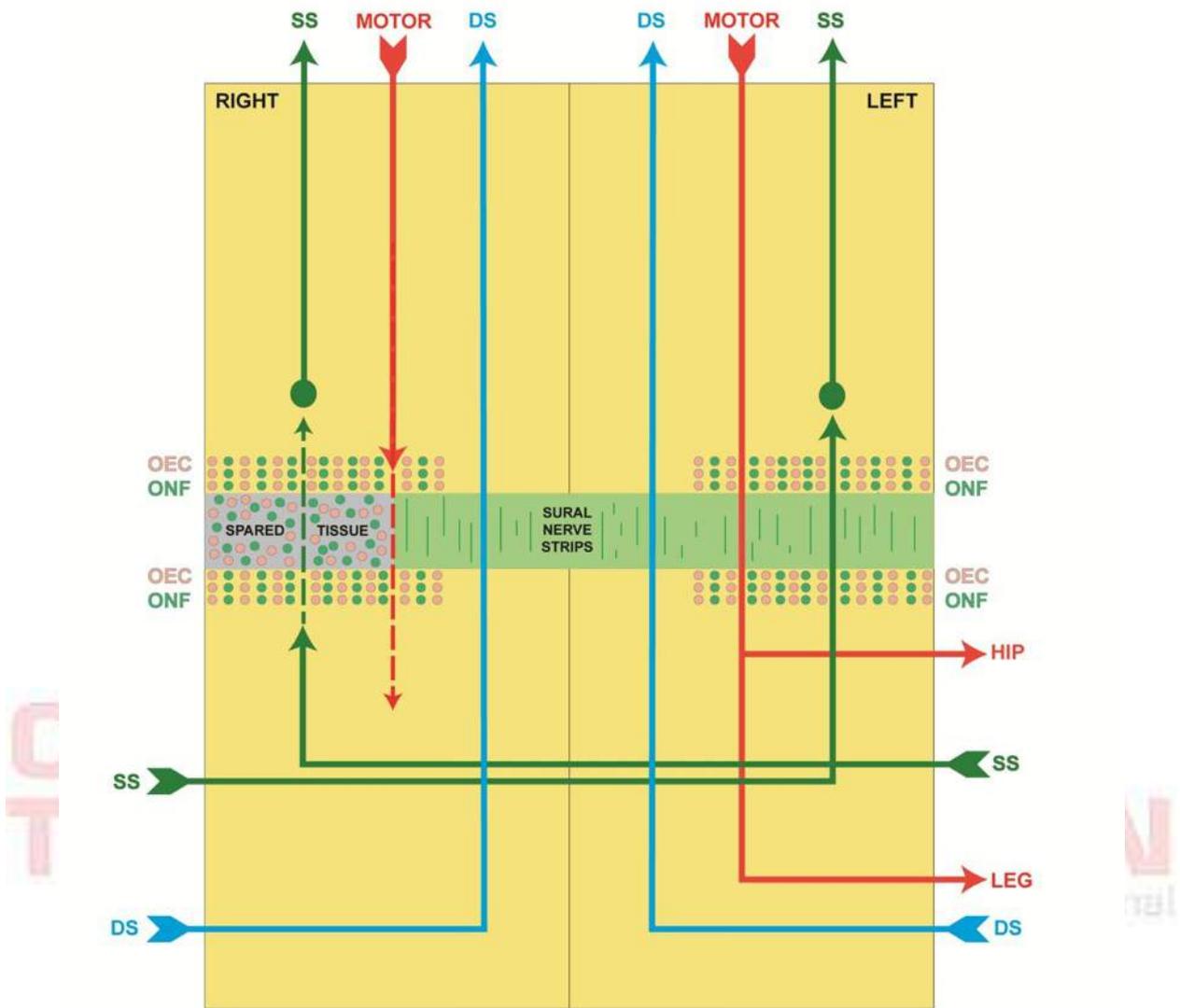


Figure 13: