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Laboratory Study

# New Insights from Immunohistochemistry for the Characterization of Epidural Scar Tissue

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**Background:** The association between epidural fibrosis and recurrent symptoms after lumbar spine surgery remains a matter of debate in scientific literature and the underlying pathophysiological mechanism has not been clearly elucidated.

**Objective:** To investigate the presence of nerve fibers and the expression of osteopontin in epidural fibrous tissue after lumbar surgery in humans.

**Study Design:** Laboratory study of human tissue samples.

**Methods:** Twenty-four patients with persistent or recurrent low back and/or leg pain after lumbar spine surgery, in whom no relevant findings were present on magnetic resonance imaging (MRI) besides epidural scar tissue, were submitted to epiduroscopy. Biopsy samples of epidural scar tissue resting in the posterior epidural and periradicular space were obtained from 15 patients, using an endoscopic grasping forceps, in locations where the stimulation with the tip of a Fogarty consistently reproduced pain. Biopsy samples were processed for examination under optical and transmission electron microscopes and under a fluorescence microscope after incubation in primary antibodies against beta3-tubulin or against osteopontin.

**Results:** Optical and transmission electron microscopy revealed a homogeneous fibrous tissue rich in collagen and lacking nerve fibers. No immunofluorescence was present in any of the samples immunoreacted against beta3-tubulin. In the samples immunoreacted against osteopontin, a punctate signal was detected around the collagen fibers.

**Limitations:** Being a human study, there was no control group, so it is not possible to determine the contribution of osteopontin in the formation of epidural fibrosis and its relation to the patients' symptoms. Additional animal studies are needed to investigate these issues.

**Conclusion:** Rather than direct stimulation of nociceptors in the epidural scar tissue, other factors should relate epidural fibrosis and recurrent symptoms after lumbar spine surgery. Osteopontin seems to play a role in the formation of epidural fibrosis.

**Key words:** Osteopontin, failed back surgery syndrome, epidural fibrosis, immunofluorescence, beta3-tubulin, epiduroscopy, nerve fibers, lumbar surgery

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Notwithstanding all the advances in diagnostic methods and surgical techniques, 8% to 40% of patients who undergo lumbar disc surgery experience inadequate pain relief after the primary procedure (1) and just over 60% return to work (2,3). Even in a very selected and usually highly motivated group such as professional athletes, 11% do not return to play after a microscopic lumbar discectomy

(4). The return to work rate is even lower in workers' compensation cohorts, and a recent paper concluded that only half of the patients who underwent lumbar spine surgery returned to work and as few as 14% returned to pre-injury duties (5).

The term "failed back surgery syndrome" has been coined to designate a range of different situations after lumbar spine surgery, in which the end result fell short of the expectations of the patient and the surgeon (6,7). Possible causes of these persistent symptoms are multiple and diverse, from errors in the preoperative evaluation and surgical indication, complications from surgery (dural or nerve injury, infection, postoperative hematoma), recurrence or development of new pathology (disc herniation, spinal stenosis, adjacent segment degeneration, spondylolisthesis and instability, pain originating from the zygapophyseal or sacroiliac joints), epidural fibrosis, and so on (8-10).

Postoperative epidural fibrosis can be defined as non-physiologic scar formation at the site of surgical access into the spinal canal, in intimate vicinity to and around the origin of the radicular sheath (11). The association between epidural fibrosis and recurrent symptoms after lumbar spine surgery is a matter of a long-standing debate in scientific literature and has been reviewed in a recent publication (12). While some authors advocate an association between epidural fibrosis and clinical outcomes (10,11,13-15), others deny it (16-19).

Epidural fibrosis is regarded as deriving from the invasion of postoperative hematoma by fibroblasts originating from adjacent periosteum and paraspinal muscles, leading to the formation of a dense fibrous tissue (20-22). This fibrous tissue replaces the epidural fat and, unlike the latter, can cause adhesions between the dura mater, nerve roots, and surrounding structures, leading to compression, tethering, or stretching of the nerve structures (10,23-25). These mechanisms may be the cause of persistent back and radicular pain after surgery (24,26).

A recent study yielded the presence of unmyelinated and small myelinated nerve fibers in the periradicular adhesive tissue in patients with lumbar disc herniations (27). Moreover, recent experimental studies performed in rats showed a positive correlation between the existence of epidural fibrosis and changes in nerve conduction detected by electrophysiological studies (28,29).

Osteopontin (OPN) is a SIBLING (small integrin-binding ligand N-linked glycoprotein) protein named in 1986 (30). It was first identified in cortical bone (31) but is currently known to be synthesized by a broad range of cells, including fibroblasts (32). OPN is upregulated in human organ fibrosis including the lung, heart, kidney, liver, and muscle (33-39).

A recent experimental study in a rat peridural scar model showed that OPN plays an important role in the formation of epidural fibrosis (29). In the study, the animals that underwent laminectomy not only presented a marked OPN expression in the epidural fibrous tissue, but also in the thickened dura mater and dorsal root ganglion (DRG) neurons. The increase in percentage of OPN positive DRG neurons and a decrease in the amplitude of the somatosensory-evoked potentials in postlaminectomy animals corroborate an intrathecal response to epidural scar formation.

Since there are no references in the literature regarding the innervation of the epidural scar tissue in the absence of disc herniation and to the expression of OPN in human postoperative epidural fibrous tissue, this study was designed to investigate these issues.

## Methods

The present study was conducted in a University Hospital (Centro Hospitalar São João / Faculty of Medicine of the University of Porto, Portugal). The study protocol was approved by the Ethics Committee and every patient included provided a voluntary, written informed consent.

### Patients

Twenty-four patients with persistent or recurrent low back and/or leg pain after lumbar spine surgery were included. All of them had a magnetic resonance imaging scan (MRI) and dynamic x-rays of the lumbar spine excluding recurrent disc herniation, spinal stenosis, spondylolisthesis, infection, or any other specific diagnosis as the cause for the symptoms. In all patients, MRI yielded more or less extensive contrast-enhancing epidural soft tissue consistent with fibrous granulation tissue adjacent to the dura mater and/or nerve root sheet (Fig. 1).

### Inclusion and Exclusion Criteria

Patients included were over the age of 18 years and reported low back pain and/or lower extremity pain lasting a minimum of 6 months, with a VAS (Visual Analogue Scale) score (40) greater than or equal to 5/10, unresponsive to conservative management including, at least, medication and a rehabilitation program.

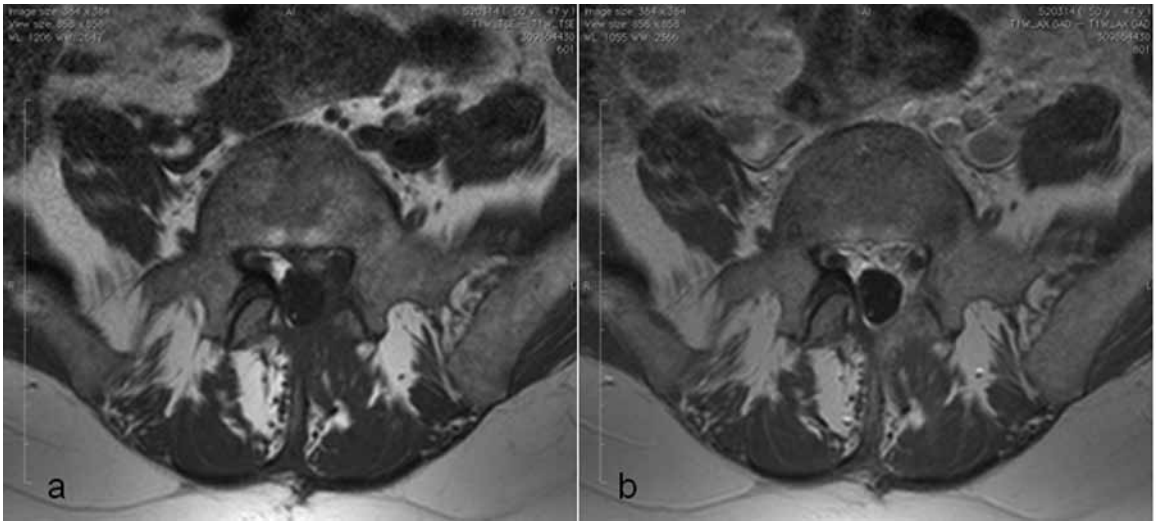


Fig. 1. *Axial views of a T1-weighted without (a) and with (b) contrast enhancement postoperative MR image of a patient who underwent L5S1 microdiscectomy through a left side approach, showing scar tissue on the left epidural space and surrounding the left S1 nerve root.*

Intracranial hypertension, coagulopathy, ocular hypertension, retinopathy, renal failure, cerebrovascular disease, pregnancy and lactation, sepsis, infection in the region of sacral hiatus, major psychiatric disturbance, cauda equina syndrome, congenital or acquired disturbances of the sacral anatomy that could interfere with the progression of the endoscope, and a past history of allergic reactions to contrast dye, local anesthetics, or corticosteroids were considered exclusion criteria.

## Epiduroscopy

All patients underwent epiduroscopy under local anesthesia and mild sedation, performed by a single surgeon (PP) in a sterile operating room. The procedures utilized fluoroscopy, and participants were in the prone position. After local anesthesia of the region, the epidural space was accessed through the sacral hiatus using an 18G Tuohy needle, confirmed by injection of non-ionic contrast. A short length of a guidewire was then inserted in the sacral hiatus to guide the insertion of a dilator surrounded by a plastic sleeve used to insert the flexible, steerable, sterile epiduroscope (Resascope®, MRT – Medical Device Manufacturer s.r.l., Italy) into the epidural space. The epiduroscope was slowly advanced using small boluses of physiological saline solution flushed in the epidural space under direct visualization until pathological areas of fibrosis or adhesions between the dura mater and epidural structures were reached. Then the tip of a 3F Fogarty catheter (Edwards Lifesciences Corporation, USA) was used to probe the epidural structures, looking for concordant pain with the patient's usual one (epidural pain provocation test) (41). Biopsy samples of epidural scar tissue resting in the posterior epidural and periradicular space were obtained in 15 patients, using a 1 mm flexible endoscopic grasping forceps (Karl Storz GmbH, Germany), in locations where the stimulation with the tip of the Fogarty consistently reproduced pain. These samples were collected only in patients in whom excision of the scar tissue with a biopsy forceps was deemed the appropriate method of adhesiolysis.

## Biopsy Tissue Processing

Immediately after obtaining the biopsy samples through epiduroscopy, the specimens underwent fixation in 4% paraformaldehyde in 0.1M phosphate buffer for one to 2 hours, followed by inclusion in paraffin wax and sectioning. After deparaffinization and hydration, 5-micrometer thick contiguous sections were processed in 3 groups. In group 1 the specimens were stained with hematoxylin-eosin and the slides were examined under an optical microscope. In group 2 the samples were incubated in primary antibody against beta3-tubulin produced in mouse (Abcam, Cambridge, UK), and in group 3 in primary antibody against OPN produced in rabbit (Abcam, Cambridge, UK), both di-

luted 1:4000 in phosphate-buffered saline containing Triton X-100 for 24 hours at 4°C. Primary antibodies were detected by immunofluorescence with secondary antibodies anti-mouse labeled with Alexa Fluor 488 (group 2) or secondary antibodies anti-rabbit labeled with Alexa Fluor 568 (group 3). The reactions were controlled by parallel processing of human tissues known to contain nerve fibers (group 2) and OPN (group 3). The observation and image capture were performed in a Zeiss Axiovision Z1 fluorescence microscope.

In 2 of these patients additional biopsies representative of the same fibrous tissue were collected. The samples were fixed in 1% glutaraldehyde in 0.1M phosphate buffer for 2 hours and post-fixed in 2% osmium tetroxide for one hour in the same buffer. After inclusion in Epon 812 resin, ultrathin sections were obtained in a Reichert Ultracut S microtome, stained with lead citrate and uranyl acetate and examined in a Jeol transmission electron microscope (group 4).

Table 1. *Patient demographics.*

| | |
|---|---|
| Number of patients | 15 |
| Gender (M/F) | 9/6 |
| Age, mean ± SD (years) | 45.1 ± 7.7 |
| Duration of symptoms, mean ± SD (months) | 30.6 ± 27.0 |
| Time since previous surgery, mean ± SD (months) | 61.3 ± 43.2 |
| VAS, back pain, mean ± SD | 7.0 ± 2.2 |
| VAS, leg pain, mean ± SD | 6.5 ± 2.7 |
| ODI, mean ± SD (%) | 42.8 ± 13.5 |

*SD - standard deviation*

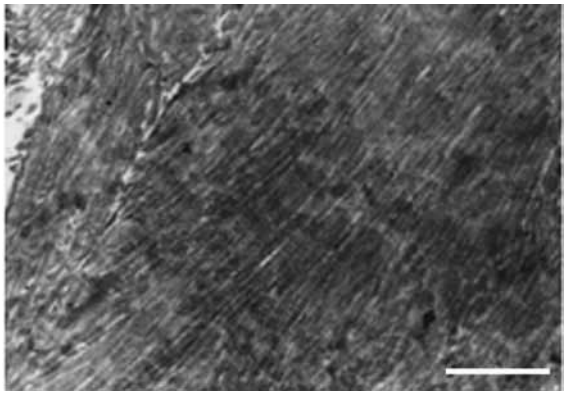

*Fig. 2. Transmission electron microscope photograph showing collagen fibrils with typical striped pattern. Calibration bar: 0.5 micrometers.*

### Additional Tissue Samples

In 2 other patients who underwent transforaminal lumbar interbody fusion for isthmic spondylolisthesis, samples of annulus fibrosus and ligamentum flavum excised during the surgery were collected, after obtaining consent from the patients. The samples were processed as described above for OPN detection. The rationale for this analysis was to assess if OPN was present in connective tissues in the vicinity of epidural fibrosis.

### Statistical Analyses

All data were entered in an MS Excel database. Basic statistics were calculated for patient demographics and clinical data. The results are presented as means +/- standard deviations. Given the uniformity of the results of immunohistochemical studies, statistical analysis was not performed.

## Results

Demographics and clinical data from the patients included in the present study are shown in Table 1. The mean score on the Oswestry Disability Index (ODI), reported on a 0 to 100 scale where 0 = minimal disability and 100 = maximal disability, was 42.8, corresponding to a level of severe disability (42,43). Pain in lower back region (VAS, back pain) or lower extremity (VAS, leg pain) was marked by the patient on 10-cm VAS, where 0 = minimal pain intensity and 10 = maximal pain intensity (40). Both VAS and ODI scores refer to the condition of the patient on the day before intervention.

Hematoxylin-eosin stained slides examined under an optical microscope showed a homogeneous pinky fibrous tissue with abundant eosinophilic fibers and very few cells. Electron microscopic studies depicted collagen fibrils with a typical striped pattern. No nerve fibers were detected on electron microscopy images (Fig. 2).

No immunofluorescence was present in any of the samples incubated in primary antibody against beta3-tubulin (Fig. 3). The reaction was controlled with positive staining in parallel processing of human bladder samples previously demonstrated to show positive reactions for beta3-tubulin (44).

In all the samples incubated in primary antibody against OPN, punctate immunoreactivity was detected in the vicinity of fibrillar structures, corresponding to collagen fibers seen on electron microscopy, thereby confirming the presence of OPN in epidural scar tissue (Fig. 4). The reaction was controlled with human cancellous bone tissue samples, which showed diffuse immunofluorescence staining for OPN.

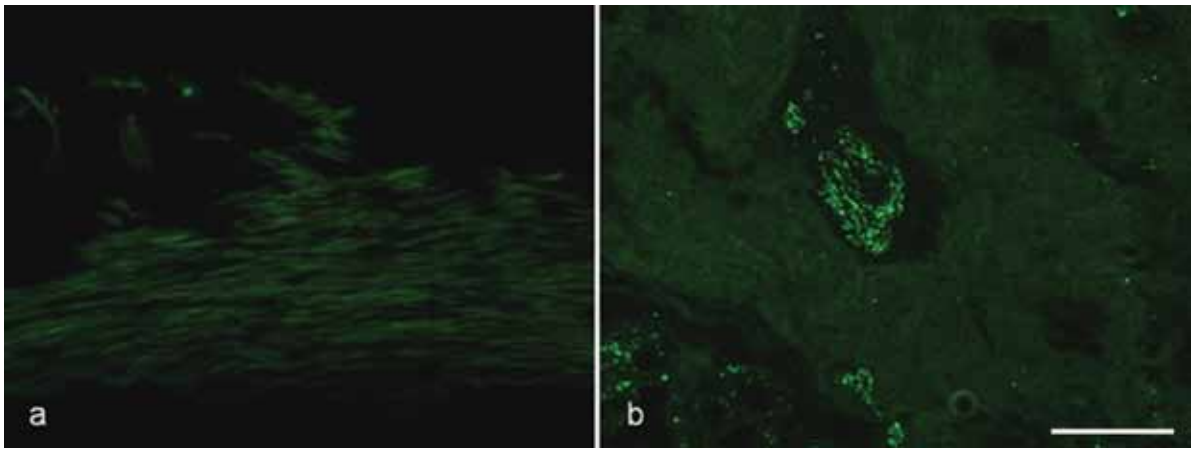


Fig. 3. *Microphotographs obtained in a fluorescence microscope from samples incubated in primary antibody against beta3-tubulin produced in mouse and secondary antibodies anti-mouse labeled with Alexa Fluor 488 (green). a) Epidural scar tissue sample, showing no immunofluorescence. b) Human bladder tissue sample, showing immunofluorescence staining for beta3-tubulin, reflecting the presence of nerve fibers. Calibration bar: 100 micrometers.*

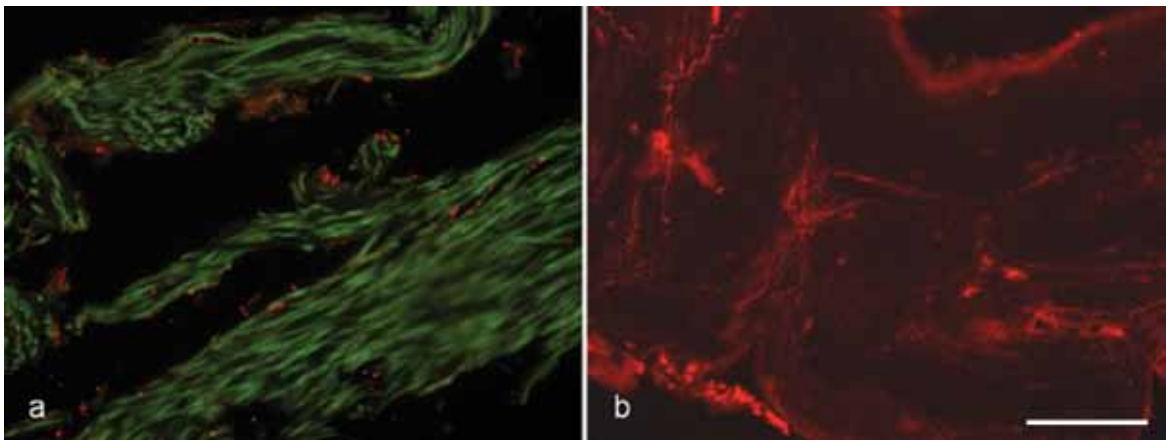


Fig. 4. *Microphotographs obtained in a fluorescence microscope from samples incubated in primary antibody against osteopontin produced in rabbit and secondary antibodies anti-rabbit labeled with Alexa Fluor 568 (red). a) Epidural scar tissue sample, showing punctate immunoreactivity around collagen fibers, confirming the presence of osteopontin. b) Human cancellous bone tissue sample after decalcification, showing diffuse immunofluorescence staining for osteopontin. Calibration bar: 100 micrometers.*

In the samples of annulus fibrosus and ligamentum flavum obtained from patients who underwent transforaminal lumbar interbody fusion no immunofluorescence staining was detected.

## Discussion

Intervertebral discs, zygapophyseal joints, sacroiliac joints, spinal ligaments, muscles and fascia, dura mater and nerve root sheets, and vertebrae are recognized causes of low back and leg pain both in non-operated and operated spines (25,45-50). However, even after a systematic investigation using interventional techniques, the etiology of chronic low back pain cannot be identified in at least 13% to 19% of patients (51,52).

When a patient has persistent low back pain and/or leg pain after a lumbar spine surgery, or when pain reemerges after a pain-free interval, a comprehensive investigation of the underlying cause is mandatory. If the advocated approach to persistent pain is to assume that the problem causing the pain was not addressed

by surgery and thus to investigate the reason thereof, in the event of symptom recurrence after a pain-free period, a scrutiny for a relapse or development of new pathology is mandatory. In either case, 20% to 36% of these patients may present epidural fibrosis as the only remarkable finding (53). Although the implication of epidural fibrosis in recurrent symptoms seems more straightforward, the presence of adhesions between the neural structures and the walls of the vertebral canal or the encasement of the dura mater and nerve roots may be one reason for no improvement after a lumbar surgery.

The mechanism most frequently cited to explain the relationship between the recurrence of pain after lumbar spine surgery and epidural fibrosis is the tethering of the dura mater and the nerve roots to the surrounding structures (10,24). Other mechanisms mentioned in the literature are changes in the perineural microcirculation (54) and expression of pro-inflammatory cytokines, namely IL-1ß and IL-6, in the fibrous tissue (12,55).

An alternative hypothesis to correlate epidural fibrosis with recurrent pain could be the direct stimulation of primary afferent nociceptors (56) in the scar tissue, as occurs in pain originating in the intervertebral discs, zygapophyseal joints, or sacroiliac joints (45). Nerve growth into the epidural scar tissue, as occurs into the diseased intervertebral discs with annulus tears (57), could constitute a pathomorphological substrate for recurrent pain after lumbar spine surgery, and explain the difficulty in treating these patients and the modest results of the various types of surgical interventions (58,59).

The class III beta-tubulin isotype is widely regarded as a pan-neuronal marker (60). The current study did not detect immunoreactivity in the biopsy samples obtained from epidural adhesions using a monoclonal antibody against beta3-tubulin, thus excluding the presence of nerve fibers in the specimens. It is noteworthy that in all patients the biopsy samples were collected in areas where the stimulation of the epidural adhesions with the Fogarty triggered a similar pain to the patient's usual one. So, even in painful areas of epidural fibrosis, regardless of their location, we could not find nerve fibers.

This result does not replicate the findings by Kobayashi et al (27), who demonstrated the presence of nerve fibers in the epidural fibrous tissue surrounding lumbar disc herniations. A possible explanation for this discrepancy is that the fibrous tissues examined in the 2 studies are not identical. Indeed, in the publication by Kobayashi et al the investigated tissue was located in the anterior epidural space, surrounding herniated disc fragments and adjacent to the outer annulus fibrosus, which is an anatomical structure known to be innervated (61). Likewise, the posterior longitudinal ligament, which is closely related to herniated disc material, is also richly innervated by branches of the sinuvertebral nerve (62). In contrast, samples collected in the current study were located in the posterior epidural and periradicular space, and therefore away from disc material and the well innervated structures within the anterior spinal canal.

Schuetze (41) hypothesized that direct irritation of a meningeal branch of a spinal nerve could be responsible for pain provocation by a heat impulse emitted by a laser beam used during epiduroscopy (epidural laser pain provocation test). On such report, 73.3% of the 120 patients with failed back surgery syndrome had a positive pain provocation test, but the author does not specify in how many of these patients the result was due to stimulation of fibrous epidural scar tissue. Moreover, the author states that the scarred areas identified via epiduroscopy were not regularly sensitive to pain.

By the abovementioned, the present study does not support the hypothesis of direct stimulation of nociceptive fibers in the epidural fibrous tissue as a cause of pain in patients with a history of lumbar spine surgery.

OPN participates in the formation of collagen fibrils during tissue remodeling, macrophage and neutrophil migration, angiogenesis, and wound healing (63). Mice lacking a functional OPN gene have shown an alteration of collagen fibrillogenesis leading to small diameter collagen fibrils and matrix disorganization (64). In humans, overexpression of OPN has been reported in idiopathic pulmonary fibrosis (33), interstitial fibrosis in diabetic kidney (34), and alcoholic liver disease (36).

The present research demonstrated the presence of OPN at the periphery of collagen fibers in samples of human postoperative epidural fibrosis, irrespective of the location where the scar tissue was obtained, either in the posterior epidural space or in the posterior periradicular space. This result is in line with the findings from the animal research conducted by Brzezicki et al (29), which highlighted the importance of OPN in the formation of epidural fibrosis and in the neural response to the presence of the scar tissue.

It seems to us noteworthy that OPN was not detected in neighboring tissues, including the annulus fibrosus of the intervertebral disc and the ligamentum

flavum. This result suggests that OPN does not have a ubiquitous distribution in these tissues, and that its presence can be directly related to the pathophysiology of wound healing and scar formation. Accordingly, these data reinforce the possibility that OPN may have an important role in the formation of postoperative epidural fibrosis and, hence, with the symptoms related thereto.

A recent critical review on the peridural membrane of the spinal canal was published (65). A possible implication of adhesions between this membrane and other spinal contents to the development of spinal pain was suggested. Anatomical descriptions of the peridural membrane diverge substantially among publications. Like the authors, we also found frequent epidural septa of connective tissue during lumbar epiduroscopies. Although the thickness and extent of these layers vary among patients and spinal levels, they are usually thin and limited and we never found a continuous membrane. The tissue samples analyzed in this study were taken in areas of dense scar tissue, painful to manipulation. Nonetheless, we can acknowledge that postoperative epidural fibrosis may form along the anatomical pathway of the peridural membrane and its attachments to the spinal canal contents.

From the findings of this study, one might hypothesize that if the synthesis, expression, or action of OPN after a spine surgery could be locally inhibited, then the formation of epidural fibrosis could probably be reduced as well as the incidence of symptoms connected therewith. Blocking OPN expression in a skin wound model resulted in decreased formation of granulation tissue and fibrosis (66). On the other hand, it should be noted that OPN stimulates the production of IL-12 and inhibits the production of IL-10 by macrophages, thereby promoting the generation of a T helper 1 (Th1) cytokine pattern by immune and structural cells (67), which is known to favor tissue repair with restoration of its normal architecture (68). Moreover, OPN also seems to have a neuroprotective effect after stroke (69), spinal cord (70), and peripheral nerve injury (71). Thus, more research is needed to determine if the knockdown of OPN in a postlaminectomy fibrosis model would have a beneficial effect and potentially a clinical application.

The strengths of this study include the use of human material obtained from patients with the pathology under investigation. The study population is fairly homogeneous and well characterized. The techniques of obtaining the biopsies and tissue processing are uniform and reproducible. The results are very consistent. This paper documents for the first time, the absence of beta3-tubulin expression and the expression of OPN in human postoperative epidural scar tissue.

The main limitation of the present study is the lack of a control group, since fibrosis is a pathological tissue. Although many patients who underwent lumbar spine surgery and have postoperative MRI scans showing epidural fibrous granulation tissue are asymptomatic and, thus, could be used as a control group, we did not consider it appropriate to propose an invasive procedure in asymptomatic individuals only for research purposes. Moreover, regarding the part of the study related to beta3-tubulin detection, the search of nerve fibers in the epidural fibrous tissue in this population would be more relevant if it had been positive in the studied patient group who had symptoms. However, investigating the presence of OPN in asymptomatic individuals with epidural fibrosis would be relevant, since it could help to clarify if this protein is only associated with the formation of fibrosis or if it has a role in the onset of symptoms related thereto.

## Conclusion

This immunohistochemical study demonstrates diffuse OPN immunoreactivity around collagen fibers, suggesting a role of OPN in scar formation. However, beta3-tubulin reactivity was not seen within epidural scar tissue, suggesting that epidural scar does not contain nociceptive fibers that could explain the source of pain associated with epidural fibrosis.

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