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ORIGINAL ARTICLE

Diabetes mellitus is associated with increased elastin fiber loss in ligamentum flavum of patients with lumbar spinal canal stenosis: results of a pilot histological study

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Abstract
Purpose Lumbar spinal canal stenosis (LSCS) is associated with fibrosis, decreased elastin-to-collagen ratio, and hypertrophy of the ligamentum flavum (LF). Diabetes mellitus (DM) is known to cause metabolic disturbances within the extracellular matrix in multiple tissues. These alterations may play a major role in the severity of clinical symptoms of LSCS affecting diabetic patients. We aimed to examine the hypothesis that DM may contribute to the LF changes seen in patients with LSCS.
Methods The study cohort included 29 patients: 23 with LSCS (10 with DM vs. 13 without DM) as well as six patients with lumbar disc herniation (LDH). Surgical LF specimens were retrieved for histological assessment. Morphologic quantification of confocal microscopy images using fast Fourier transform analysis allowed us to compare anisotropy and elastin fiber orientation between groups.
Results There was a significant positive correlation between fasting plasma glucose values and degree of elastin degradation ($r = 0.36$, $p = 0.043$). The diabetic patients with LSCS showed a significantly greater loss of elastic fibers (2.3 ± 0.9 vs. 1.5 ± 0.55, $p = 0.009$), although fibrosis was shown to be similar (1.44 ± 0.7 vs. 1.43 ± 0.88, $p = 0.98$). There was no significant difference in the degree of calcification in the LSCS group between patients with and without diabetes (1.71 vs. 2.05%, $p = 0.653$). Fiber orientation was found to be less homogenous in the LSCS compared with the LDH group, although not significantly affected by DM.
Conclusions The present study points to a significant contribution of DM to the loss of elastin fibers that occurs in the LF of patients with LSCS.

Keywords Ligamentum flavum · Elastin · Diabetes mellitus · Lumbar spinal stenosis

Introduction

Lumbar spinal canal stenosis (LSCS) is characterized by narrowing of the spinal canal with nerve root impingement, resulting in radiculopathy or pseudoclaudication. It results from intervertebral disc herniation and degenerative changes in the posterior structures of the lumbar spine, such as hypertrophy of the facet joints and ligamentum flavum (LF), and is more common in elderly patients. As the LF mostly covers the posterior and lateral parts of the lumbar spinal canal, its role in the pathogenesis of LF is crucial [1]. However, the underlying mechanism remains unclear. The LF is composed of elastin and collagen fibers in a 2:1 ratio. The elastin fibers provide elasticity to the ligament, and the collagen fibers provide stiffness and stability [2, 3]. LF thickness is an age-dependent and gender-independent phenomenon, and is correlated with disc degeneration, aging, BMI, LSCS, spinal level involved, and the presence of disc herniation [4, 5].

Several cross-sectional studies of patients diagnosed with spinal stenosis and degenerative disc disease, found that the

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spinal stenosis group had a significantly higher rate of diabetes mellitus (DM), suggesting that DM may be a predisposing factor for the development of LSCS [6, 7]. Cui et al. found that LF from diabetic LSCS patients was significantly thicker than from non-diabetic LSCS patients and the thickness was correlated with the expression of matrix metalloproteinase 13, which is known to degrade fibrillary collagens, including type I, II, and III collagens, into gelatin [8].

Purpose

We hypothesize that DM may contribute to the alterations in fiber microarchitecture found in LF of LSCS patients, ultimately leading to the development of clinical LSCS. The purpose of the present study was to determine the following: (1) Does LF derived from patients with DM and LSCS show higher fibrosis, elastin degradation or degree of calcification than non-diabetic LSCS patients? (2) Is the elastin fiber microarchitecture different between the two populations?

Materials and methods

Patients

The study cohort consisted of patients who were diagnosed with LSCS and underwent lumbar decompression surgery during a 12-month period at a tertiary university-affiliated medical center. The inclusion criteria were: (1) clinically diagnosed LSCS with symptomatic neurological claudication, and (2) evidence of LSCS on imaging studies [magnetic resonance imaging (MRI) or high-resolution computerized tomography (CT)]. Exclusion criteria were: revision surgery, trauma, malignant disease, or systemic inflammatory disease.

The study group was designed to include diabetic patients with LSCS, and compare it with a control group of non-diabetic LSCS patients. A third group included adult patients diagnosed with a single-level lumbar disc herniation (LDH) and neither LSCS nor diabetes per imaging studies and laboratory results, who underwent discectomy during the same period at the same hospital. The LDH group served to test the assumption that tissue derived from patients without LSCS has less fibrosis and elastin degradation, and as a general control for baseline measurements. The study was approved by the institutional review board of Rabin Medical Center (approval number 0414-11-RMC, September 2013). Informed consent was obtained from all of the patients prior to index procedure.

The cohort included an overall number of 29 patients. The study group consisted of ten patients with LSCS and diabetes [LSCS DM(+)] and the control group included 13 without diabetes [LSCS DM(−)]. All the diabetic patients had type II diabetes, nine patients were treated with oral antidiabetic medications and one patient with continuous Insulin pump. The mean duration of diabetes was 12.9 ± 7.5 years (range 4.5–30 years), with six out of the ten patients having a long-standing disease of greater than 10 years' duration. glycated hemoglobin A1c (HbA1c), an indicator of the average blood glucose concentrations over the preceding 2–3 months, was used as an integrated measure of the quality of glycemic control [9, 10]. The last measured HbA1c of all the diabetic patients was documented, with 6/10 patients who had diabetes with good glycemic control of HbA1c ≤ 7%) and 4/10 had diabetes with poor glycemic control (7% < HbA1c < 9%). The LDH group included six patients treated surgically for a single-level LDH, with LF obtained during their index procedure.

Preoperative evaluation

Prior to surgery, all patients completed the Oswestry Disability Index (ODI) [11], which measures functional impairment, and the self-report Swiss Spinal Stenosis (SSS) [12] questionnaire, which measures symptom severity, physical functional status, and patient satisfaction. According to our departmental practice, computed tomography (CT) or magnetic resonance imaging (MRI) scans of the lumbar spine were obtained in all cases to support the diagnosis and identify the involved spinal levels. Midpoint LF thickness was measured from axial T1-weighted magnetic resonance image at the facet joint level of the operative lesion. Measurements were made by two of the authors (SS, ES) blinded to the clinical information and were then averaged.

Patients with LSCS were identified as diabetic or not diabetic according to history and supporting blood chemistry. Fasting plasma glucose in venous blood was measured preoperatively in all cases.

Tissue acquisition and preparation

The operating surgeons (N.O., D.S.) obtained tissue from the central portion of the LF (entire layer) to minimize tissue damage and optimize the harvest of the ligament only. The LF was obtained from the same anatomical area, either from the right of the left inter-laminar space for the spinal stenosis and the disc herniation patients. After all epidural fat was removed, the tissue specimens were washed with saline to remove blood and bodily fluid contaminants. All specimens were transported under sterile conditions to the laboratory within 20 min of excision. The harvesting and processing procedures were identical in all patients.

The specimens were fixed in 4% formaldehyde in phosphate-buffered saline and prepared for paraffin embedding. Twenty consecutive sections, each 4-μm thick, were cut on a microtome and treated with the appropriate stains:

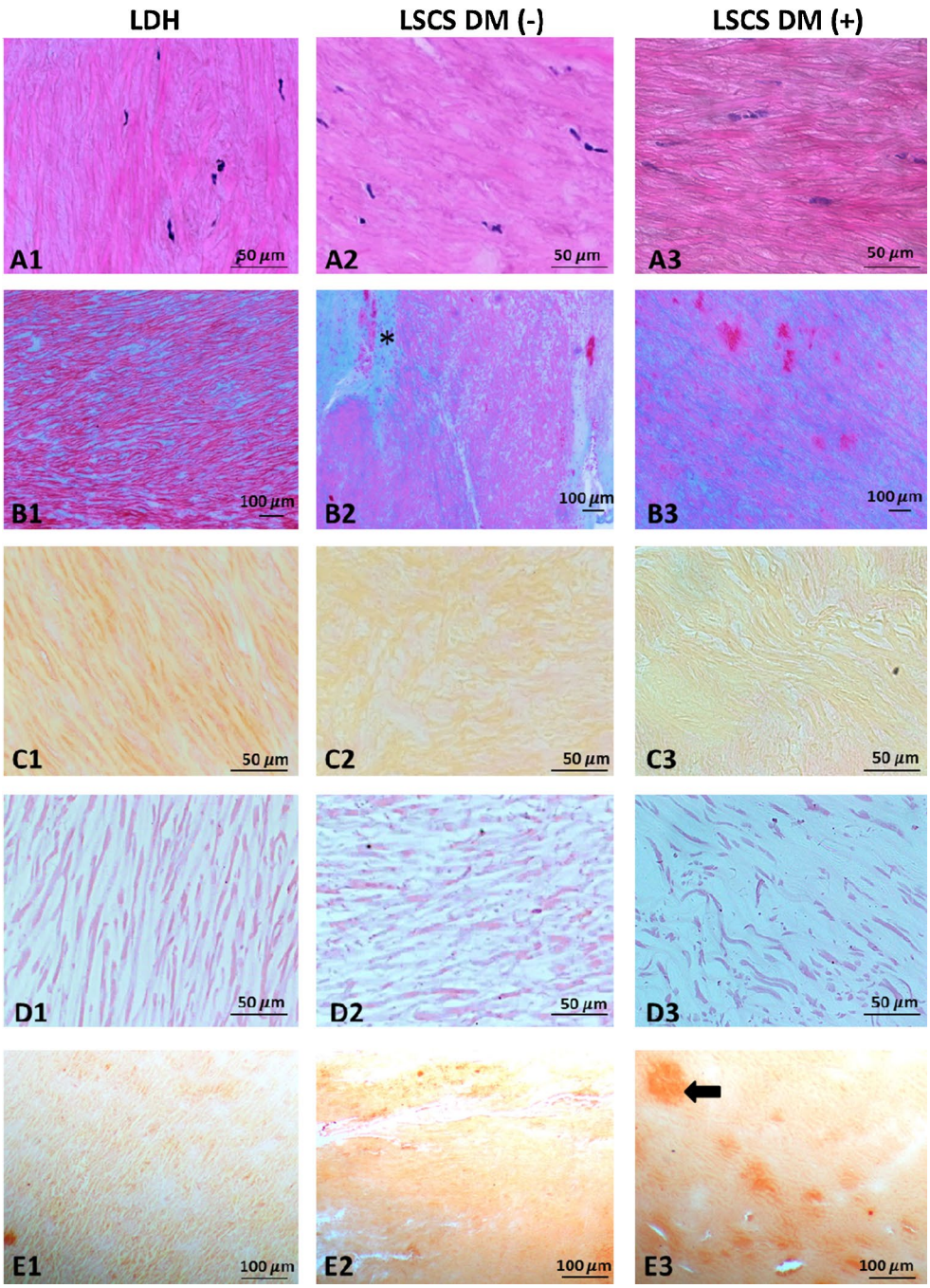

Fig. 1 Histology sections stained for different techniques are corresponding to the three groups [lumbar disc herniation (LDH), spinal stenosis with no diabetes [LSCS DM(−)] and spinal stenosis with diabetes LSCS DM(+)]. Each column contains light microscopy images obtained from a single patient and from the same section, scale bar shown on the right bottom corner. **a** Hematoxylin–eosin (H&E) stain was used to evaluate the tissue morphology and the degree of elastin degradation. **b** Masson's trichrome stain was used to demonstrate tissue fibrosis. Larger areas of fibrosis (asterisk) are noted in images B2 and B3. The elastic fibers are dyed in pink and the collagenous fibers (fibrosis) are dyed in blue. **c** Elastica van Gieson for detection of fibrous tissue. The elastic fibers are dyed in orange and the collagenous fibers (fibrosis) are dyed in pink. **d** Orcein stain for elastic fibers. **e** Alizarin Red stain for calcium deposits (arrow)

hematoxylin–eosin, to evaluate tissue morphology; Masson trichrome, to demonstrate tissue fibrosis (collagen fiber appears turquoise, elastin fiber appears red); elastica van Gieson and orcein, to visualize elastic fibers; and Alizarin Red, to identify calcium deposits (Fig. 1). A light microscope (Nikon, Japan) was used to view and photograph the specimens.

Fibrosis and elastic fiber loss grading

Four cross-sections of each specimen were randomly selected for histologic analysis and graded independently by three of the authors (S.S., E.S., and D.B.), all of whom were blinded to the patients' identity and group. The severity of fibrosis (based on Masson trichrome staining) was graded on four-point scale, according to the guidelines set by Sairyo et al. [13] 0—normal tissue showing no fibrotic region; grade 1—fibrosis of less than 25% of the entire area; 2—fibrosis of 25–50% of the entire area; grade 3—fibrosis of 50–75% of the entire area; 4—more than 75% fibrosis (Fig. 2). The same method was used to evaluate elastin fiber loss (based on elastica van Gieson and Orcein staining). Average grades of fibrosis and elastin fiber loss were calculated for every patient.

Confocal microscopy

To demonstrate the elastic fibers, hematoxylin–eosin-stained sections were viewed under a multi-photon confocal microscope (Leica TCS SP8, Leica Microsystems, Buffalo Grove, IL), as reported in earlier studies of elastin research [14, 15]. The distribution and organization of the fibers were analyzed by auto fluorescence (channel RXD1, wavelength 525 ± 25 nm) under 600× magnification (Fig. 2). Digital images were obtained from each sample for further image processing.

Analysis of elastin fiber orientation

To study the elastin microarchitecture, the confocal microscopy images were processed with two-dimensional fast Fourier transform based image analysis, a well-established approach for describing the overall orientation of fiber networks [16]. In this manner, we were able to assign a numerical value to the degree of fiber alignment in each specimen. Fiber alignment was analyzed with Fiji/ImageJ (NIH, USA) software [17] using two different plug-ins: the Orientation J [15] plug-in, to describe the orientation and isotropy properties of regions of interest in each image; and the Directionality plug-in (http://fiji.sc/wiki/index.php/Directionality) to describe the mean orientation and angular dispersion of the fibrils. Three statistical measurements were generated for each confocal microscope image: Amount—sum of the histogram from center-minus SD to center-plus SD divided by the total sum of the histogram; Dispersion (°)—standard deviation of the Gaussian curve, and Coherency—bounded between 0 and 1, with 1 indicating highly oriented structures and 0 indicating isotropic areas. Three different cross-sectional images from separate areas of the specimen were used for directionality measurements.

Statistical analysis

The statistical analysis was performed with SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL). Categorical bivariate analysis was conducted using Chi-square and Fisher's exact test where appropriate, and a p value < 0.05 was considered significant. Data is presented as mean ± standard deviation, unless otherwise stated. Pearson correlation was used to determine significant relationships of histologic parameters and directionality with demographic and clinical data. Statistical analysis was conducted using IBM SPSS software (Chicago, IL) with a two-tailed alpha of 0.05.

Results

The demographic data and functional scores of the LSCS DM(+) and DM(−) groups are shown in Table 1. The LDH patients did not significantly differ from the LSCS patients, with regards to age, gender or BMI (Table 2). The LSCS DM (−) and LSCS DM(+) groups mean fasting glucose level was significantly higher in the LSCS DM(−) group than in the LSCS DM (91.3 ± 6.4 vs. 124.2 ± 27, $p = 0.0001$). Compared to the LDH patients, the patients with LSCS had higher mean scores on the MODS ($p = 0.18$) and significantly higher scores on the SSSS ($p = 0.01$), with no significant difference for either instrument within the LSCS group, between the diabetic and nondiabetic patients (MODS 52.6 ± 22.4 vs. 54 ± 12.4, $p = 0.91$; SSS 66 ± 14 vs. 71.8 ± 6.6, $p = 0.44$). Hemoglobin A1C concentration at the last measurement was 7.13 gr% in the diabetic group (range 6.1–8.6%). Analysis of the preoperative MRI and CT scans revealed that the 80% of the LSCS DM(+) group and 84% of the LSCS DM(−) group had more than one spinal level of involvement ($p = 0.77$). L4–L5 level was involved in every patient with spinal stenosis in our cohort.

LF fibrosis and elastin fiber loss grades

A total of 116 cross-sections were evaluated by confocal microscopy (29 samples × 4). Fibrosis was significantly more abundant in patients with LSCS compared to LDH (mean grade 1.43 ± 0.76 vs. 0.053 ± 0.08, $p < 0.001$). No significant difference was found between the LSCS DM(+) and LSCS DM(−) groups (mean grade 1.44 ± 0.7 vs. 1.43 ± 0.88, $p = 0.98$) (Fig. 3). There was a significant positive correlation between mean SSS score and fibrosis grade (Pearson correlation $r = 0.559$, $p = 0.038$).

A significantly higher degree of elastin fiber loss was found in the LSCS DM(+) group than the LSCS DM(−)

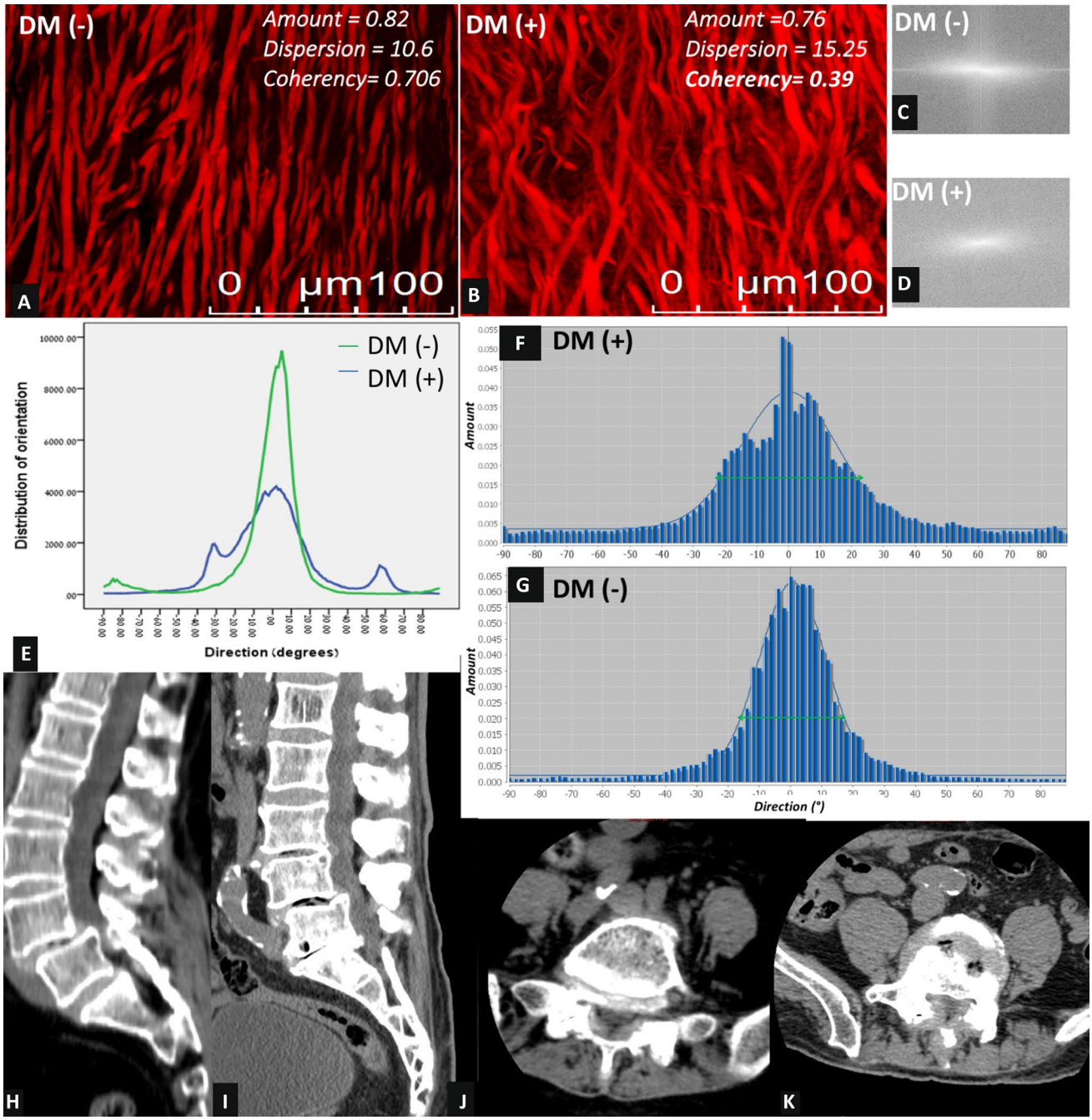

Fig. 2 Confocal microscopy images (×600) of **a** a 74-year-old non-diabetic female with spinal stenosis [DM(−)] and **b** an 81-year-old male patient with spinal stenosis and diabetes [DM(+)], scale bar shown on the right bottom corner. Directionality parameters (Amount, Dispersion, and Coherency) for each image are shown in the textbox. **c**, **d** represent fast Fourier transform frequency images for the confocal microscopy images, **a** and **b** respectively, composed of grayscale pixels that are distributed into a pattern that can be used to measure the degree of fiber alignment present in an original data image. Fast Fourier transform alignment plots are shown in **e** for the patients depicted in **a** and **b**: the green curve representing the fiber orientation in the DM(−) patient shows a narrower distribution, with a higher maximal count of fibers in the same main direction, compared with the DM(+) patient, represented by the blue curve. The same results are shown in **f** and **g** where histograms of DM(−) and DM(+) patients (respectively), indicating the amount of fibers in a given direction (from − 90° to 90°), peaking at the preferred orientation. **h**, **j** represent computerized tomography sagittal and axial (L4–5 level) sections, respectively, from the same DM(−) patient. Similarly, **i** and **k** represent sagittal and axial (L4–5 level) sections, respectively, from the same DM(+) patient. The imaging studies reveal significant narrowing of the spinal canal affecting L4–L5 and L5–S1 levels, in both patients

Table 1 Bivariate analysis of patient demographics, characteristics, functional scores, and histologic measurements between diabetic LSCS patients and non-diabetic LSCS patients

	DM – LSCS ($n = 13$)	DM + LSCS ($n = 10$)	p value
Age (mean)	61.9 (SD 14)	63.0 (SD 11.5)	0.846
Gender			0.855
Male	6 (46%)	5 (50%)	
Female	7 (53%)	5 (50%)	
BMI (mean)	26.7 (SD 3.9)	29.4 (SD 6.4)	0.24
SSSS	71.8 (SD 6.6)	66 (SD 14.1)	0.44
MODS	54 (SD 12.4)	52.6 (SD 22.4)	0.91
Fasting glucose (mg/dL)	91.3 (SD 6.4)	124.2 (SD 27)	< 0.001
LF width (mm)	5.12 (SD 1.9)	4.73 (SD 0.72)	0.75
Hb A1C (gr%)	N/A	7.13	
Number of levels involved			0.77
1	2 (15.3%)	2 (20%)	
2	9 (69%)	5 (50%)	
3	1 (7.6%)	1 (10%)	
> 3	1 (7.6%)	2 (20%)	
Sairyo score	1.44 (SD 0.7)	1.43 (SD 0.88)	0.98
Elastin fiber loss	1.5 (SD 0.55)	2.3 (SD 0.90)	0.009
Calcifications	2.05 (SD 1.7)	1.71 (SD 1.78)	0.65
Directionality analysis			
Dispersion	11.14 (SD 3.19)	14.02 (SD 15.3)	0.12
Amount	0.67 (SD 0.09)	0.63 (SD 0.1)	0.33
Coherency	0.37 (SD 0.18)	0.32 (SD 0.11)	0.44

LSCS lumbar spinal canal stenosis, *LF* ligamentum flavum, *BMI* body mass index, *SSSS* Swiss spinal stenosis core, *MODS* Modified Oswestry Disability score

Table 2 Bivariate analysis of patient characteristics and histologic measurements between LSCS and LDH patient groups

	LSCS ($n = 23$)	LDH ($n = 6$)	p value
Age (years)	62 (SD 12.7)	52 (SD 7)	0.06
Gender (M; F)	11; 12	2; 4	0.663
BMI	27 (SD 5.2)	25 (SD 3.1)	0.184
MODS	53.5 (SD 15.2)	40.6 (SD 19.2)	0.18
SSSS	69.6 (SD 9.5)	54 (SD 10.1)	0.01
Sairyo score	1.43 (SD 0.76)	0.053 (SD 0.082)	< 0.001
Elastin degradation	1.8 (SD 0.83)	0.69 (SD 0.57)	0.003
Calcifications (%)	1.9 (SD 1.7)	0.66 (SD 0.34)	0.003
Directionality analysis			
Dispersion	12.3 (SD 4.4)	9.7 (SD 2.3)	0.18
Amount	0.65(SD 0.09)	0.7 (SD 0.034)	0.228
Coherency	0.35 (SD 0.15)	0.48 (SD 0.7)	0.05

LSCS lumbar spinal canal stenosis, *LDH* lumbar disc herniation, *BMI* body mass index, *ODI* Modified Oswestry Disability score

group (mean grade 2.3 ± 0.9 vs. 1.5 ± 0.55, $p = 0.009$) (Fig. 3). Mean fasting blood glucose values were significantly and positively correlated with elastin fiber loss grade ($r = 0.37$, $p = 0.048$).

LF fiber directionality

A total of 87 cross-sections were evaluated by confocal microscopy (29 samples × 3). Mean coherency coefficient was higher in LDH patients compared with LSCS patients (0.48 ± 0.7 vs 0.35 ± 0.15, $p = 0.05$), indicating a more homogenous fiber alignment. The LSCS DM(+) had the lowest coherency coefficient of all groups (0.32 ± 0.11). In comparison between DM(−) and DM(+) LSCS patient groups, the later had a lower coherency coefficient (0.32 ± 0.11 vs 0.37 ± 0.18), a lower amount value (0.63 ± 0.1 vs 0.67 ± 0.09) and a higher dispersion value (14.02 ± 15.3 vs 11.14 ± 3.9), although none of these measurements demonstrated statistical significance ($p = 0.44$, $p = 0.33$, $p = 0.12$, respectively). A positive correlation was noted between the Amount and Coherency parameters (Pearson's correlation $r = 0.6$, $p < 0.001$). Fasting plasma glucose levels also correlated positively with the fiber dispersion. However, this correlation was only marginally significant ($r = 0.34$, $p = 0.052$).

BMI at time of surgery, was found to be negatively correlated to coherency ($r = -0.386$, $p = 0.038$) and positively correlated to dispersion ($r = -0.475$, $p = 0.009$).

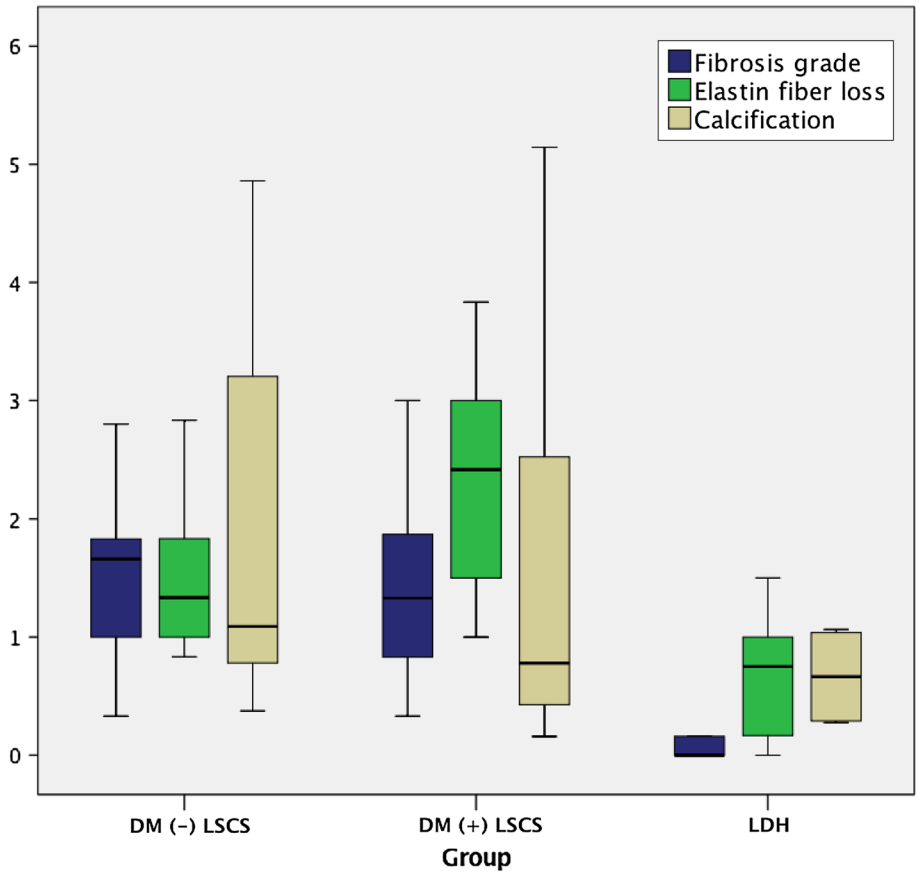

Fig. 3 Box plot presenting mean and SD values for fibrosis (Sairyo grading), degree of calcification and elastin degradation for patients with lumbar spinal stenosis with diabetes [LSCS DM(+)], lumbar spinal stenosis without diabetes [LSCS DM(−)], and lumbar disc herniation (LDH)

Figure 2 shows an example of findings from confocal microscopy analysis, comparing two patients: a LSCS DM(+) patient and a LSCS DM(−) patient.

LF degree of calcification

In the whole cohort, calcifications were measured using ImageJ on Alizarin Red-stained slides, and occupied an average of 1.66% of the cross-sectional area of the LF. There was a significantly higher degree of calcification in the LSCS group compared with the LDH group (1.9 ± 1.7% vs. 0.66 ± 0.34%, $p = 0.003$). However, there was no significant difference in the degree of calcification between the LSCS DM(+) and LSCS DM(−) groups (1.71 ± 1.78 vs. 2.05 ± 1.7%, $p = 0.6$). No correlation was found between the degree of calcification and patients' age by Pearson correlation.

Discussion

The LF in the lumbar region is rich in elastic fibers. Whose principal components are elastin and fibrillin [18]. The pathologic processes involved in LF hypertrophy include fibrocartilaginous changes due to the proliferation of type II collagen, ossification, calcium crystal deposition, degeneration of collagen and elastin fiber, and chondroid metaplasia of the ligament fibroblasts [1–3, 19–21].

With age, the elastin-to-collagen ratio decreases, resulting in decreased elasticity and increased stiffness or fibrosis [2]. It has been postulated that the loss of elasticity may cause the hypertrophied LF to fold into the spinal canal, leading to compression of the dural sac [1–3]. The tissue disorganization and decreased levels and degeneration of the elastic fibers are accompanied by an increase in collagen levels. There is also an increase in the expression and activity of various molecules, including matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs), platelet-derived growth factor-BB (PDGF-BB), connective tissue growth factor (CTGF), bone morphogenetic protein (BMP), and inflammatory cytokines [8, 22–27].

We analyzed the morphological and geometric changes in the LF in subjects with clinical and radiographic evidence of LSCS and compared several parameters between diabetic and nondiabetic patients. This is the first study, to our knowledge, to examine the microarchitecture and fiber orientation in the LF using confocal microscopy. Previous LSCS studies using light microscopy showed that the parallel order of the elastic fibers in the LF is lost [21]. Our analysis demonstrates

that there is a higher degree of loss of parallelism in LSCS patients compared with LDH patients, as reflected by the lower coherency of the fiber arrangement. In the whole cohort, the degree of calcification was 1.66% of the slide cross-sectional area on average, and calcifications were more abundant in the LSCS patients compared with LDH patients. This result is in line with previous studies. Schrader et al. [21] found calcifications in 35 out of 38 LSCS patients, with an average area of 0.17% (range 0–3.8%), and a much lower percentage in their control group.

Diabetes is a multiorgan disease that affects many types of connective tissues, including bone and cartilage. The changes that occur in the extracellular matrix of diabetic patients may differ from the normal aging process in two important ways: an increased nonenzymatic cross-link of proteins by sugar glycosylation at lysine residues and a decreased rate of proteoglycan synthesis [28]. Only a few studies linked diabetes and LSCS [7, 28, 29]. Other studies demonstrated a protective effect of spinal decompression surgery on diabetic patients with LSCS, since successful surgery may improve the level of physical activity and thus facilitate glycemic control [30, 31]. Nonetheless, the literature with regards to the histologic changes in the LF that may be attributable to DM in LSCS is still scarce. Cui et al. showed an increased expression of matrix Metalloproteinase 13 (MMP13) in DM(+) patients with LSCS, and demonstrated using light microscopy that elastin fibers were more disorganized and focally lost [8]. Still, the exact impact of diabetes on the pathophysiology of LSCS needs further clarification. We found a significantly increased elastin fibers loss in diabetic patients, through repeat measurements from different microscopic areas of the specimens. Elastin fiber loss was positively correlated to fasting plasma glucose. These findings are plausible, as elastin degradation was found to play a key role in several microvascular and macrovascular complications of diabetes affecting other organs, including retinopathy [32], nephropathy [33] and macrovascular disease [34].

On analysis of directionality, we found lower coherency and higher dispersion in diabetic patients, though the difference was not statistically significant. Another important finding that can be indirectly related to diabetes was the correlation between BMI and fiber alignment as expressed by lower coherency and increased dispersion demonstrated in patients with higher BMI. This finding is interesting in light of the recently published data, suggesting that obese and overweight patients are at a higher risk of developing LSCS [35].

We acknowledge several limitations of our study. First, our cohort of participants is small and the results should therefore be interpreted with caution. However, our groups of DM(+) and DM(−) did not defer significantly with regards to age, gender, number of levels involved, radiologic measurements and functional scores, and were therefore suitable for comparison. We also tried to mitigate the effect of the small sample size by performing multiple measurements on different areas of the specimen. Second, the present study is focused on histology alone, and the molecular alterations including the expressions of certain types of collagen and elastin have not been explored.

Conclusions

The present study points to a contribution of DM to the loss of elastin fiber that occurs in LF in LSCS. Further research of the molecular changes underlying this process may lead to the development of new therapies for LSCS in this population.

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Author contributions All authors have demonstrated (1) substantial contributions to research design, or the acquisition, analysis or interpretation of data; (2) drafting the paper or revising it critically; (3) approval of the submitted and final versions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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