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Study of biochemical substrate and role of metalloproteinases in fascia transversalis from hernial processes

J. M. BELLO´ N, J. BUJA´ N, N. G. HONDUVILLA, F. JURADO, M. J. GIMENO, J. TURNAY* N. OLMO* & M. A. LIZARBE* Department of Morphological Sciences and Surgery, Faculty of Medicine, University of Alcala´, Madrid, Spain, and *Department of Biochemistry and Molecular Biology, University Complutense, Madrid, Spain

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Abstract. The aim of this study was to examine the fascia transversalis (FT) from patients with direct and indirect hernia in an attempt to identify possible differences between each type of hernia. FT samples were obtained from 36 patients presenting inguinal hernia (23 indirect hernia and 13 direct hernia) who underwent surgery. We have analysed the ultrastructure of the fascia surrounding the hernial lesions, the proline and lysine hydroxylation in the tissue, the type I–type III collagen ratio and the presence of metalloproteinases. We have not detected ultrastructural differences in the collagen fibrils from FT in direct and indirect hernias. However, the interfibrillar matrix was more abundant in direct hernias, showing abundant electron-dense particles. No differences in proline hydroxylation were observed between each type of hernia. A small decrease in lysine hydroxylation was detected in patients with direct hernia. Enzyme-linked immunosorbent assays (ELISAs) showed no statistically significant differences in the type I–type III collagen absorbance ratios. Immunohistochemistry revealed no differences in the expression of matrix metalloproteinase-1. FT from patients presenting direct hernia showed a very strong staining vs. metalloproteinase-2 when compared with that observed in indirect hernia.

Keywords. Extracellular matrix, fascia transversalis, hernia, metalloproteinases.

Introduction

Inguinal hernia is still one of the most common targets of surgical treatment. Approximately 700000 surgical procedures are carried out each year to treat this pathology [1]. But the aetiology of inguinal hernias remains unclear. In addition to an individual predisposition, numerous other predisposing factors appear to be involved. The integrity of the abdominal wall is dependent on the oblique orientation of the inguinal canal, a sphincter-like structure of the inguinal ring, and the fascia transversalis. Obesity, obstructive pulmonary disease, hyperplasia of the prostate, ascites, pregnancy and constipation are regarded as predisposing factors because of chronically raised intra-abdominal pressure. The aetiological impact of the processus vaginalis on the development of inguinal hernias has not yet been clarified. Although all children with an indirect hernia present an open processus vaginalis, not all those with this process will develop a groin hernia. Anyway, the majority of inguinal hernias represent a defect of the fascia transversalis [2].

It is well known that the extracellular matrix plays an important role in several physiological and pathological processes, showing its heterogeneous composition between tissues and species. Collagens, the most abundant and widely distributed proteins in the body, are the major structural components of the extracellular matrix [3]. Several connective tissue diseases have been related to an abnormal collagen metabolism, including changes in the type I to type III collagen ratio, disorders in the chain synthesis or hydroxylation degree of collagen chains, as well as abnormal fibril formation or interaction with other extracellular matrix components [4–6].

It has been suggested that changes in collagen could be involved in the origin of hernias [7,8]. In any case, the examination of the possible changes in collagen has focused on anatomical structures, such as the anterior rectus sheath [9] or even the skin [10] of patients with hernias, there being few reports dealing with the study of the fascia transversalis.

For this reason, we have examined the fascia transversalis in patients with direct or indirect hernias in an attempt to correlate ultrastructural aspects with changes in the collagen molecules of such tissues. Parameters such as the number and diameter of the collagen fibrils, hydroxylation degree and collagen types have been considered. We have also focused on metalloproteinases as the enzymes responsible for the degradation of extracellular matrix components. These enzymes are involved in other disorders, such as arterial aneurysms and connective tissue diseases [11,12], but there are no previous reports showing their role in a possible degradation of fascia transversalis in hernia formation.

Patients and methods

Tissue samples were obtained from patients presenting

Correspondence: J.M. Bello´n, Department of Morphological Sciences and Surgery, Faculty of Medicine, University of Alcala´, Crta. Madrid-Barcelona Km 33.6-28871 Alcala´ de Henares, Madrid, Spain.

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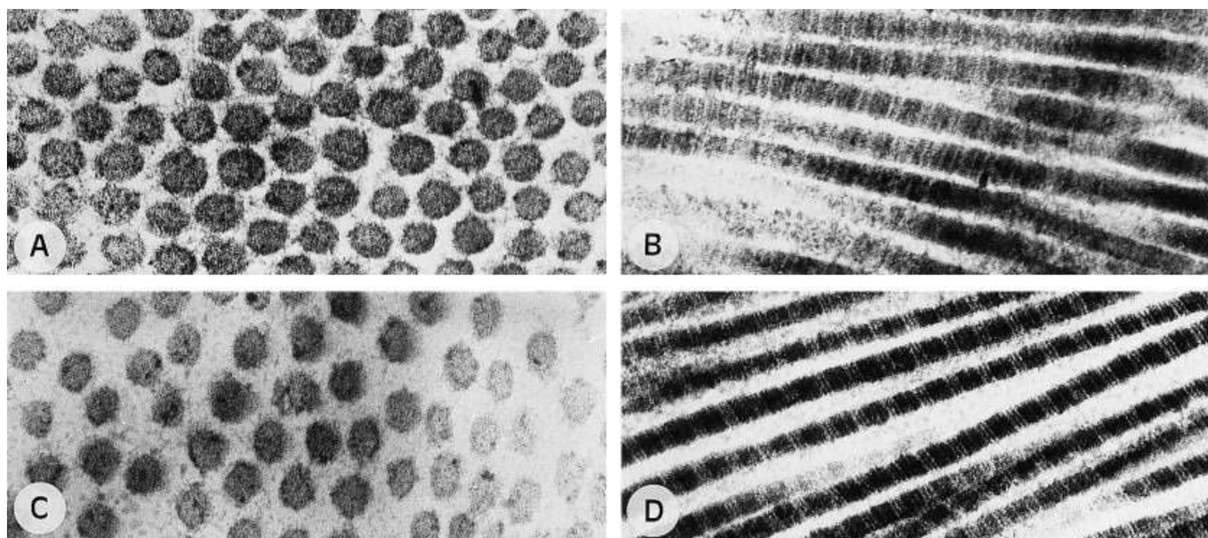

Figure1. Transmission electron micrographs of fascia transversalis from direct (A and B) and indirect (C and D) hernias. Transversal sections of collagen fibrils (A and C) were used to determine fibril diameter and density. Longitudinal sections (B and D) show the characteristic collagen fibril periodic band pattern. Staining by lead citrate and uranyl acetate. Original magnification 85000.

inguinal hernia who underwent surgery for hernia repair. Fascia transversalis samples were taken intraoperatively from the posterior wall of the inguinal canal. All samples were taken from patients who only presented with primary inguinal hernia. The following groups were established: (I) patients with indirect hernia (*n* ¼ 23); and (II) patients with direct hernia (*n* ¼ 13). The ages of the patients ranged between 20 and 60 years (mean age 44 years). According to this parameter, the patients were further divided into two subgroups: 20–40 years and 41–60 years.

Immediately after surgery, fascia transversalis samples were placed in sterile culture medium (minimum essential medium; MEM) and stored at 48C for their transfer to the laboratory, where they were processed using light microscopy (immunohistochemistry) and transmission electron microscopy. Samples selected for biochemical studies were frozen and kept at –208C until use.

The study was approved by the local ethics committee and informed consent was obtained from each patient.

Immunohistochemical study

For the immunohistochemical studies, samples were embedded in paraffin and cut into 5-mm slices using a microtome (Microm, Barcelona, Spain). Two different monoclonal antibodies to metalloproteinases were used as primary antibodies: MMP-1 (interstitial collagenase; The Binding Site, Birmingham, UK) and MMP-2 (gelatinase B; Biogenesis, Sandown, USA). To detect the antigen–antibody reaction, the peroxidase–antiperoxidase (PAP) technique was followed, using the rabbit/ mouse rapid staining kit (Sigma Chemical, St Louis, MO, USA). The chromogenic substrate used in this kit was 3-amino-9-ethyl carbazole in *N*,*N*-dimethylformamide.

Samples were examined in a microscope (Zeiss, Jena, Germany), and an image analyser (Microm) was used to quantify the intensity of the antigen–antibody interaction.

Ultrastructural study

The samples destined for ultrastructural study were fragmented into small sections, fixed in 3% glutaraldehyde, placed in Millonig buffer (pH 7˙3) and post-fixed in 2% osmium tetroxide. Dehydration was performed with slow water replacement by a graded series of acetone solutions and embedded in Araldite for thin cuts. Afterwards, they were contrasted with lead citrate and observed under a Zeiss 109 transmission electron microscope.

Biochemical study

Tissue samples for amino acid analysis were washed exhaustively with 50mmolL^{-1} sodium phosphate (pH 7˙4) containing 0˙15molL^{-1} NaCl (phosphate-buffered saline; PBS), followed by two washes with distilled water, and were allowed to dry off at 608C. Dried tissue samples (0˙5–2mg) were hydrolysed for 24h at 1088C with 0˙5mL of constant boiling 5˙7 N HCl containing 0˙1% (w/v) phenol in vacuum-sealed tubes. Hydrolysate samples were further evaporated to dryness at 608C and 50mb. Amino acid analyses were performed on a Beckman 6300 amino acid analyser using norleucine as internal standard [13,14]. The Hyp–Pro and Hyl– Lys molar ratios were determined from the amino acid analysis data.

Tissue specimens for collagen extraction, obtained as described above, were washed with PBS and cut into 2- to 3-mm pieces. Collagens were solubilized from the fascia using limited pepsin digestion in 0˙5molL^{-1} formic acid

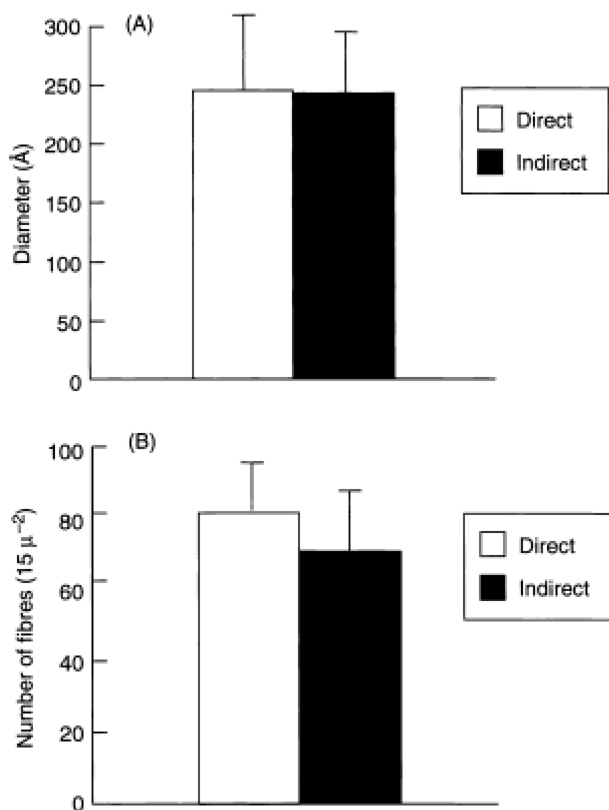

Figure2. Quantification of the diameter (A) and density (B) of collagen fibrils in fascia transversalis from direct and indirect hernias.

at an enzyme-to-tissue weight ratio of 1:100 for 48h at 48C, with constant shaking [15]. The samples were then centrifuged at 18000*g* for 45min at 48C. The supernatants containing the solubilized collagens were dialysed against 0˙1 N acetic acid.

Biotin-labelled goat anti-type III and anti-type I collagen, both from Southern Biotechnology Associates (Birmingham, UK), have been used to determine the relative proportions of type I and type III collagens in the preparations obtained by limited pepsin digestion of the tissues by an enzyme-linked immunosorbent assay (ELISA). Collagen preparations were conveniently diluted in PBS and were coated on the bottom of 96well polystyrene plates (Costar Europe, Badhoeredorp, The Netherlands). Control wells were prepared with only bovine serum albumin. Binding of antibodies was visualized with HRP-labelled streptavidin using 1,2-phenylenediamine as substrate. The absorbance values were recorded in an automated ELISA plate reader at 492nm (Titertek Uniskan II; Flow Lab, UK).

Collagen preparations were analysed using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), according to the method of Laemmli [16]. Standard type I and type III collagens were prepared from fetal calf skin by saline extraction and fractional salt precipitation, as described previously [15,17]. Samples were dissolved in 0˙05molL^{-1} Tris-HCl, pH 6˙8, containing 2molL^{-1} urea, 0˙1% (w/v) SDS, 5% (v/ v) b-mercaptoethanol and 0˙1% (w/v) bromophenol blue.

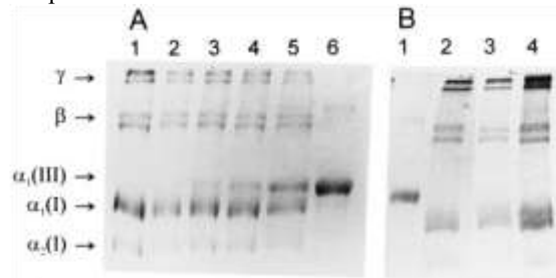

Figure3. Electrophoretic analysis of collagen obtained from fascia transversalis. Collagen was solubilized from the tissue by limited pepsin digestion as described in Methods. (A) Electrophoresis of standard collagen type I (1) and type III (6), and mixtures of both at different type I–type III collagen weight ratios: (2) 95:5; (3) 90:10; (4) 80:20 and (5) 50:50. (B) Electrophoretic band pattern of pure collagen type III (1) and collagen extracted from fascia transversalis of patients affected by indirect (2) and direct hernias (3,4).

The a_1(I) chains were resolved from a_1(III) reduced chains on a 7% acrylamide gel containing 3˙6molL^{-1} urea, as described previously [18]. Protein bands were visualized by Coomassie blue staining.

Statistical analysis

Statistical analysis was made using the Mann–Whitney *U*-test for comparisons between groups.

Results

Ultrastructural study

Fascia transversalis is basically composed of a small number of fibroblasts embedded in an abundant fibrillar extracellular matrix. Specimens from indirect hernias (Fig.1A and B) showed a greater uniformity in the diameter of the collagen fibrils and also presented the characteristic periodic collagen band pattern. The ultrastructure of the collagen fibrils from direct hernias did not differ from that of the other group. However, the interfibrillar matrix was more abundant. This matrix is made up of small electron-dense bundles distributed irregularly throughout the interfibrillar space (Fig.1C and D).

When the two hernia groups were compared, the mean diameter of the fibrils (Fig.2A) did not differ significantly (370–420A˚; *P*>0˙05). Moreover, the analyses performed to determine the number of fibrils per field (Fig.2B) showed no significant differences in this parameter even when various age groups were compared.

Biochemical analysis

The percentage of collagen in the different tissue samples was determined on the basis of the amino acid composition, as described previously [5,14]. No significant differences were observed among the two

groups of hernias studied. When the percentage of proline hydroxylation was evaluated in the whole population of patients, no differences were observed.

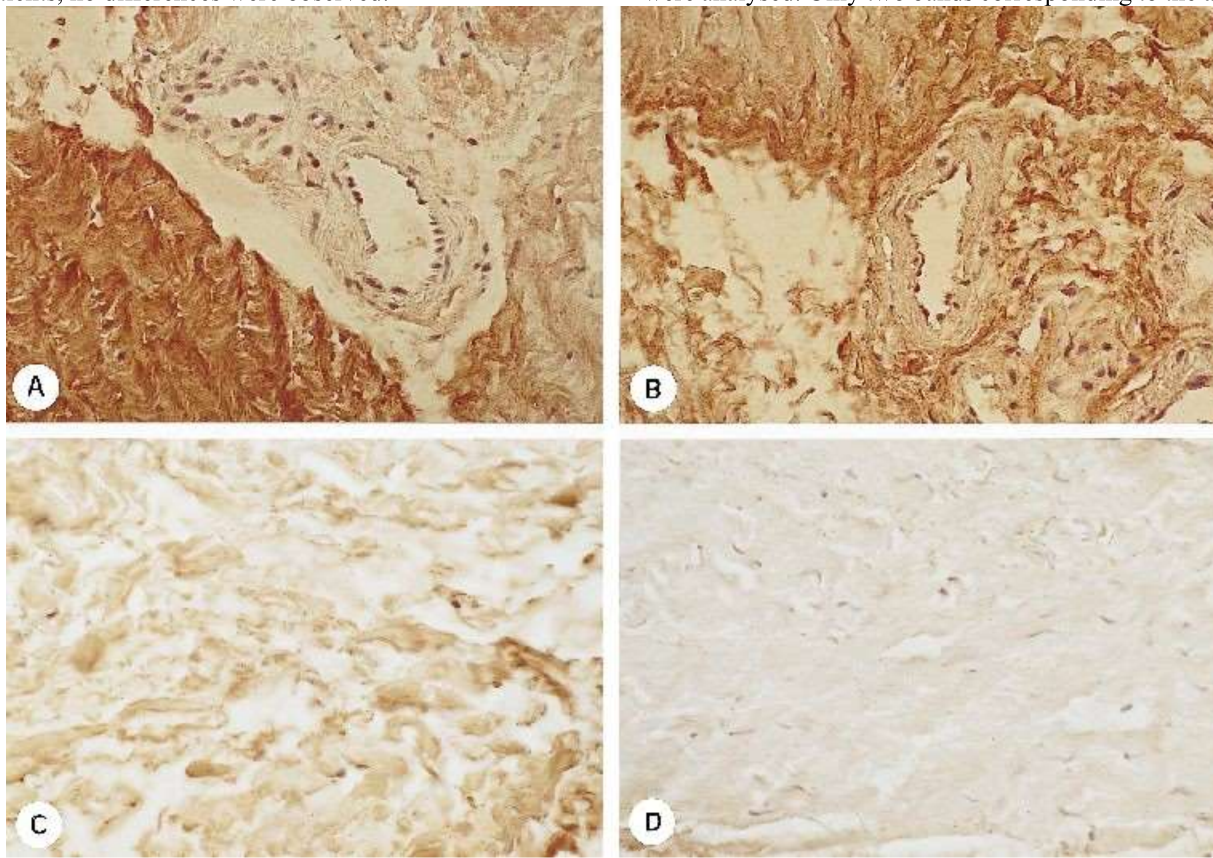

Figure 4. Immunohistochemical staining of fascia transversalis from direct (A, C) and indirect (B, D) hernias with antibodies against MMP-1 (A, B) and MMP-2 (C, D).

Table 1. Proline and lysine hydroxylation in fascia transversalis

		Direct hernia		Indirect hernia		
Age of patients	*n*	Hyp/Pro	Hyl/Lys	*n*	Hyp/Pro	Hyl/Lys
20–40	3	0˙811+0˙043	0˙770+0˙057	14	0˙179+0˙009	0˙190+0˙050
41–60	10	0˙712+0˙043	0˙795+0˙037	9	0˙221+0˙044	0˙228+0˙039

n, Number of patients.

the two groups of hernia is shown in Fig.3B. No a_1(III) collagen chain was observed when the tissue samples were analysed. Only two bands corresponding to the a_1(I) and a_2(I) chains were observed. Other protein bands of higher molecular mass corresponding to the b and g components of collagen were also detected. The a_1(III) chain of type III collagen is not visualized under the experimental conditions used.

However, a small decrease in the hydroxylation of lysine in fascia transversalis from patients with direct hernia was detected. A more significant difference was observed when patients aged 41–60 years old were considered (Table 1).

Pepsin-digested tissue samples were used to determine the collagen types present in the fascia. The a_1(III) collagen chain can be distinguished from the a_1(I) chain by SDS-PAGE in the presence of 3˙6molL^{-1} urea [18]. The electrophoresis of standard type I and type III collagens, in which the relative quantity of each one was modified, is shown in Fig.3A. Moreover the a_1(III) chain can be detected even when the ratio of type I–type III collagen is 95:5. The electrophoresis of the pepsin-solubilized collagens from fascia transversalis of

In order to check the presence of type III collagen and the possible differences in the type I–type III collagen ratio between fascia transversalis in direct and indirect hernias, an ELISA test was performed. Positive reaction against type I and type III collagen was observed. Data were processed and type I–type III collagen absorbance ratios of 3˙6 (SD¼1˙2) and 5˙4 (SD¼2˙7) were obtained from direct and indirect hernias respectively. The statistical analysis of these values does not reveal a significant difference (*P*<0˙05).

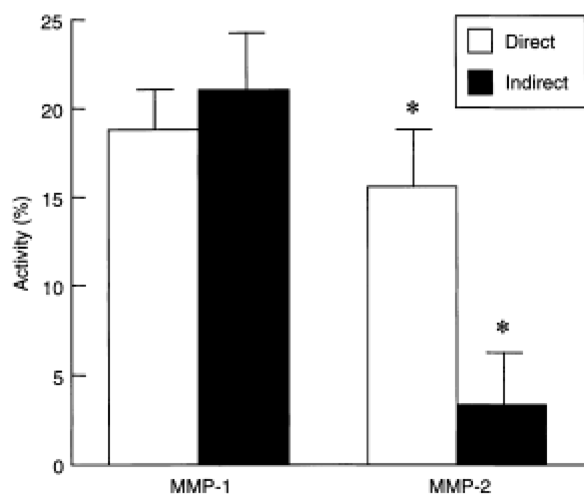

Figure 5. Quantification of metalloproteinase immunostaining in fascia transversalis. Sections were stained with anti-MMP-1 or anti-MMP-2 antibodies and the intensity of the reaction was evaluated directly under the microscope using a Microm image analysis system. Data are expressed in arbitrary units. $P < 0{\cdot}001$.

Immunohistochemistry

To test whether the alterations in the extracellular matrix of fascia transversalis from direct vs. indirect hernias could be a result of a matrix degradation, we have analysed the presence of two matrix metalloproteinases (MMPs).

No significant differences were observed in the MMP1 staining of fascia transversalis from direct hernias when compared with that of the indirect hernias (Fig.4A and B). The extracellular matrix was intensely labelled and the fibroblasts presented marked positivity. Interestingly, the endothelium of the vessels showed intense positivity.

A strong reaction vs. MMP-2 was observed in the fascia transversalis of direct hernias in contrast to the poor reaction detected in that of indirect hernias. The distribution of this enzyme was homogeneous throughout the extracellular matrix. Positive reaction was also observed surrounding the cell periphery in the tissue samples from direct hernias (Fig.4C and D).

In each case, the quantification of the immunological reaction was performed by image analysis. Figure 5 shows the above-mentioned differences in the expression of MMP-2 staining, which was always significantly lower in indirect hernias.

Discussion

Our results show that the collagen fibril ultrastructure presents no abnormal features concerning their density and diameter that could justify the appearance of the herniation processes. These observations partially agree with the results of Wagh *et al.* [8], who found no differences among the different types of hernias. Nikolov & Beltschev [19] carried out an ultrastructural study of fascia transversalis in senile patients with direct hernias, finding dysplastic collagen fibrils of variable diameters and non-uniform profiles, as well as a marked collagenophagia in fibroblasts containing collagen vacuoles in different stages of degradation. They also reported the existence of matrix vesicles among the collagen fibrils. We have detected these abnormalities only sporadically and, more frequently, in samples from patients in the 41– 60 year age group. These observations suggested that an aging factor [20] could be responsible for these alterations rather than a specific process for direct hernias. It can only be pointed out that direct hernias show a greater accumulation of electron-dense material, which could reflect an active process of matrix degradation.

It has been suggested that the underlying defect may involve the process of hydroxylation of the amino acids, proline and lysine, in the collagen molecule [9]. The hydroxyl groups of hydroxyproline are essential in the formation and for the stability of the triple helix of collagen. Lysine hydroxylation is involved in the establishment of the intra- and intermolecular collagen cross-linking and glycosylation. Our results suggest that, in the fascia transversalis of both direct and indirect hernias, there are no changes in the percentage of proline hydroxylation. Wagh *et al.* [8] have also reported a lack of differences in the hydroxylation degree of proline in direct and indirect hernias in the rectus sheath. In the present study, we have detected a general decrease in lysine hydroxylation in fascia transversalis from direct hernias compared with indirect hernias, which is more significant in the 41–60 year age group, the subpopulation with the greatest incidence of this type of hernia. This could indicate that there are changes in the collagen cross-linking or in the glycosylation of the collagen molecule, which could affect its interaction with other components of the matrix, finally leading to changes in the elastic and mechanical properties of the tissue.

We have not been able to detect type III collagen in any of the samples analysed by electrophoresis. However, we know by ELISA that this type of collagen is present in the tissue. A number of studies have demonstrated that several tissues present heterotypic collagen fibrils, in which type I collagen is associated with type III or type V collagen [21,22]. Moreover, the existence of covalent lysine-derived cross-links between type I and type III collagen molecules has been shown [23]. Thus, type III collagen could be present in the fascia but in a very low proportion, and probably covalently crosslinked with type I collagen. This could explain the apparent absence of free a_1(III) chain in the electrophoresis. Some authors [10,24] report an increase in type III collagen expression with a parallel decrease in the type I–type III collagen ratio, associated with the development of inguinal hernias. However, these studies were not performed in tissue, but in fibroblast cultures obtained from the skin of patients undergoing herniation. Our results from studies performed directly with tissue samples show no significant differences in the type I– type III collagen ratio in the fascia transversalis between each type of hernia.

In order to study the matrix degradation, the presence

of two metalloproteinases, MMP-1 or interstitial collagenase and MMP-2 or gelatinase A, was assessed. The reported substrates for MMP-1 are types I, II, III, VII, VIII and X collagens and gelatin [25,26]. We have not found significant differences in the expression of this interstitial metalloproteinase (MMP-1) in fascia transversalis from direct and indirect hernias. Thus, considering these results and those concerning the ultrastructure of collagen fibrils, it seems that the pathological processes that lead to the clinical development of direct or indirect hernias are neither clearly attributable to defects in the interstitial collagen fibrils nor to their degradation. The MMP-2 (72-kD matrix metalloproteinase II) is an enzyme that degrades types IV, V, VII, X and XI collagens, gelatin and other components of the matrix, such as elastin, fibronectin and the proteoglycan core protein [25,26]. High levels of MMP-2 are associated with lysis of basement membranes and metastatic invasion of tumour cells [27]. In contrast to the similar staining obtained for the MMP-1, the results obtained with MMP-2 demonstrated that this enzyme is highly expressed in direct hernias when compared with the indirect ones. The increased staining for MMP-2 has not been reported previously in the literature in this type of tissue, although it has been detected in degenerative or aggressive processes involving the arterial wall in aortic aneurysms [28,29], in degenerative processes affecting cartilage [30] and in a variety of pulmonary disorders [27]. An excess of this enzyme could reflect an overall proteolytic effect [27]. This observation led us to focus on the role of tissue lability in the development of direct hernias. MMP-2 could be active on collagen type V, which is found in tissues in which type I collagen is present and appears to participate in fibril development. However, the results indicate that there is no significant change in the fibril diameter and general appearance, although electron microscopy studies of direct hernias reveal the presence of clusters of electron-dense bodies in the interfibrillar spaces that could be compatible with a greater degenerative activity. Other potential substrates for MMP-2 among the components of the matrix could be considered, such as fibronectin and elastin or the proteoglycan core protein. Changes or defects in any of these matrix molecules may also alter the tissue architecture, resulting in the impairment of the proper assembly of the components and modifying the mechanical properties of the tissue.

Thus, we can conclude that, in our study on the pathological process of hernia development in fascia transversalis, we have detected (a) no ultrastructural differences in the collagen fibrils; (b) no significant differences in the type I–type III collagen absorbance ratio or in the proline hydroxylation; only a decrease in the lysine hydroxylation degree was detected in direct vs. indirect hernias (more significant in the fascia transversalis of patients aged 41–60 years old presenting direct hernia); and (c) no alteration in the expression of the interstitial collagenase (MMP-I) was detected but, in direct hernias, we observed a significant increase in the MMP-2 expression.

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