3 Alterations of the Extracellular Matrix of the Connective Tissue in Inguinal Herniogenesis

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3.1 Introduction

Inguinal hernia is still one of the most frequently performed procedures by general surgeons. Their socio-health and labor costs are important. In the USA, this pathology has a cost of approximately three billion dollars a year [1]. Its etiology and pathogenesis are complex, with multiple factors contributing to its development, including individual predisposition and some congenital alterations such as peritoneal-vaginal duct persistence. Positive familial susceptibility to inguinal hernia development has been demonstrated, suggesting the role of genetic contribution in the etiology of the disease, but site-specific familial factors might exist [2]. Studies of families with inguinal hernia propose a genetic trait for both primary and recurrent inguinal hernias [3]. Mutations in different collagen genes have been recently suggested to be associated with the development of inguinal hernia [4], and four novel inguinal hernia susceptibility loci have been identified, showing an important role for two of these genes (EFEMP1 and WT1) in connective tissue maintenance and homoeostasis [5].

From a general point of view, the integrity of the abdominal wall at the level of the inguinal region depends on the oblique orientation of the inguinal canal, a sphincter-like structure that forms part of the deep inguinal ring and the transverse fascia (TF) [6]. The latter structure, which constitutes the posterior wall of the inguinal canal, is the one that finally prevents hernia formation and, in a special way, direct hernias. Some authors [7], after performing mechanical studies, attribute to the integrity of TF, a containment mechanism that would prevent the formation of both direct and indirect type hernias.

The development of hernias at the abdominal wall level and its recurrence has been shown to occur more frequently in patients with connective tissue disorders, not to mention some other important factors such as smoking [8]. It has been suggested that defective connective tissue metabolism is involved in the pathogenesis of both the indirect and the direct types of inguinal hernia. In diseases with connective tissue alterations, the incidence of inguinal hernia is higher, such as patients with aortic aneurysm, Marfan and Ehlers-Danlos syndromes, cutis laxa, osteogenesis imperfecta, and congenital dislocation of the hip [9, 10].

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G. Campanelli (ed.), *The Art of Hernia Surgery*, https://doi.org/10.1007/978-3-319-72626-7_3

An important database study [11] including a large number of patients operated on inguinal hernia has been published some years ago showing that patients with direct or recurrent inguinal hernias are at a higher risk of developing ventral hernia repair compared to patients with indirect inguinal hernia. This fact was supported by previous studies demonstrating that the more important connective tissue alterations are found in these patients suffering direct or recurrent inguinal hernia, suggesting future research that reveals the specific alterations of the connective tissue.

This review paper aims to collect the experience and previous results of our group in the study of the constituents of the abdominal wall extracellular matrix of connective tissue, in the development of inguinal herniogenesis.

3.2 Role of the Extracellular Matrix in Hernia Pathology

The extracellular matrix is a complex integrated system developing a structural network that supports and surrounds cell populations within the connective tissue. It includes a set of tissue fibers such as collagen and elastic fibers that are present in variable amounts depending on the structural needs or function of the connective tissue. In addition this matrix contains a variety of proteoglycans, multiadhesive glycoproteins, and glycosaminoglycans that constitute the ground substance.

The mechanisms of the development of the inguinal hernia involve changes in the expression of different components of the extracellular matrix detectable at the TF level, such as collagen turnover (collagen I/III ratio) and metalloproteinases (MMPs). In the same way, the elastic component that forms part of the extracellular fibrillar matrix may contribute to the development of this pathology.

The biological factors, proposed by the Read group, involved in the development of hernia have gained acceptance in recent years, conferring a particularly relevant role to metabolic factors in the development of inguinal hernia [12–14]. Other groups such as Jansen et al. [15] have located inguinal hernias in the context of a condition generated by an abnormal composition of the extracellular matrix.

Patients with inguinal hernia show some alterations in collagen metabolism and significantly altered collagen types I/III ratios [16, 17], but few data are known about the elastic component of the extracellular matrix and factors involved in tissue remodeling that may affect the metabolism of elastin.

The extracellular matrix is a very complex integrated system, responsible for the mechanical properties of the connective tissue. The different constituents of the matrix interact with each other, and any alteration of one of them may lead to a disorganization of the extracellular matrix and the development of different pathologies such as inguinal hernia (Fig. 3.1).

Among the most studied different constituents of the extracellular matrix in relation to hernia pathology, collagen and MMPs are found. However, it has been demonstrated by our group that other soluble mediators, such as certain growth factors or enzymes related to the cross-linking of the matrix fibrillar proteins, may be altered in patients suffering from hernias [18, 19].

Following, we will review the most studied extracellular matrix constituents in relation to the pathology of inguinal hernia, with special emphasis on the findings obtained by our research group.

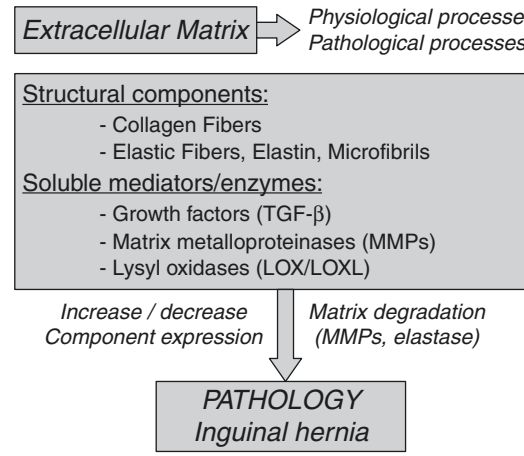

Fig. 3.1 Scheme of the different constituents of the extracellular matrix as a complex integrated system and its disorganization in the development of different pathologies such as inguinal hernia

3.2.1 Collagen Fibers

Collagen is the main and most abundant fibrillar protein in the extracellular matrix. This protein is mainly synthesized by connective tissue fibroblasts. In the process of collagen formation, important hydroxylation reactions occur at the intracellular level to form hydroxylysine and hydroxyproline, which are essential in the synthesis process and confer stability to the collagen molecule. This molecule is formed by three polypeptide α chains assembled at the intracellular level in the form of a triple helix. It is secreted into the extracellular matrix in the form of procollagen, which after a process of cleavage of its terminal uncoiled ends by procollagen peptidases, will become tropocollagen, assembling in the form of collagen fibril in the extracellular space [20]. This cross-linking is mediated by enzymes of the family of lysyl oxidases that promote the formation of highly resistant covalent bonds between lysine and hydroxylysine residues and provides strength and stability to collagen fibril, which are highly organized polymers that further associated with each other to form larger collagen fibers. Some groups [21] have shown a decrease in proline hydroxylation in TF accompanied by a significant decrease in the content of proline and hydroxyproline in the rectus sheath [22] of patients with direct inguinal hernia, indicating a compromised collagen stability at the level of the fascia.

The α chains that constitute the helix are not all the same; to date at least 42 types of α chains encoded by different genes have been identified. There are more than 28 genetically different types of collagens that have been categorized on the basis of the combination of α chains [23]. Type I collagen is the most resistant, widely distributed in the human body, including the fascia, the integumentary system, the ligaments, and the fibrous tissue. Type III collagen is found in small amounts in the same tissues and in greater proportion in the initial stages of tissue repair and wound healing [8, 23]. Type I confers mainly tensile strength, while type III is related to a temporal matrix during the tissue remodeling process. Therefore, a change in the ratio of collagen in favor of the type III would results in a loss of resistance of the structures involved.

Several studies reported an imbalance between type I collagen and type III collagen [16, 17]. When the collagen content in tissue samples is analyzed, the result is frequently quantified by the ratio of collagen type I:III. This collagen ratio has been found that was significantly decreased in the TF of patients with indirect inguinal hernia compared to controls [24]. In contrast, other studies have shown an increase in type III collagen but do not report statistically significant differences in the collagen I: III ratio in TF between patients with inguinal hernia and controls [21, 25]. Other authors [7] have shown that TF of patients with direct hernia shows higher levels of immature type III collagen and that the total amount of collagen is lower in direct hernia than in indirect hernia [26]. Ultrastructural studies using transmission electron microscopy have focused on the study of the collagen and interfibrillar matrix of the connective tissue of patients with this pathology, showing the absence of alterations in the diameter of the collagen fibers in the TF of patients with inguinal hernia [27].

Our group [21], examining the TF ultrastructure of patients with direct and indirect hernia, observed that there were no differences in the uniformity of collagen fibrils nor in their characteristic banding pattern; however, the interfibrillar matrix was more abundant in direct hernias, showing a large amount of small particles with high electrodensity (Fig. 3.2). In this same work, and by using biochemical studies, the degree of hydroxylation of the lysine and proline, essential in the process of synthesis and stability to the molecule of collagen, was analyzed. No differences were observed in proline hydroxylation in different types of hernia, and only a small decrease in lysine hydroxylation was detected in patients with direct hernia of more than 40 years (Fig. 3.3). The ratio of collagen type I:III studied by immunoenzymatic analysis did not show statistically significant differences between controls and patients with hernia pathology (Fig. 3.4).

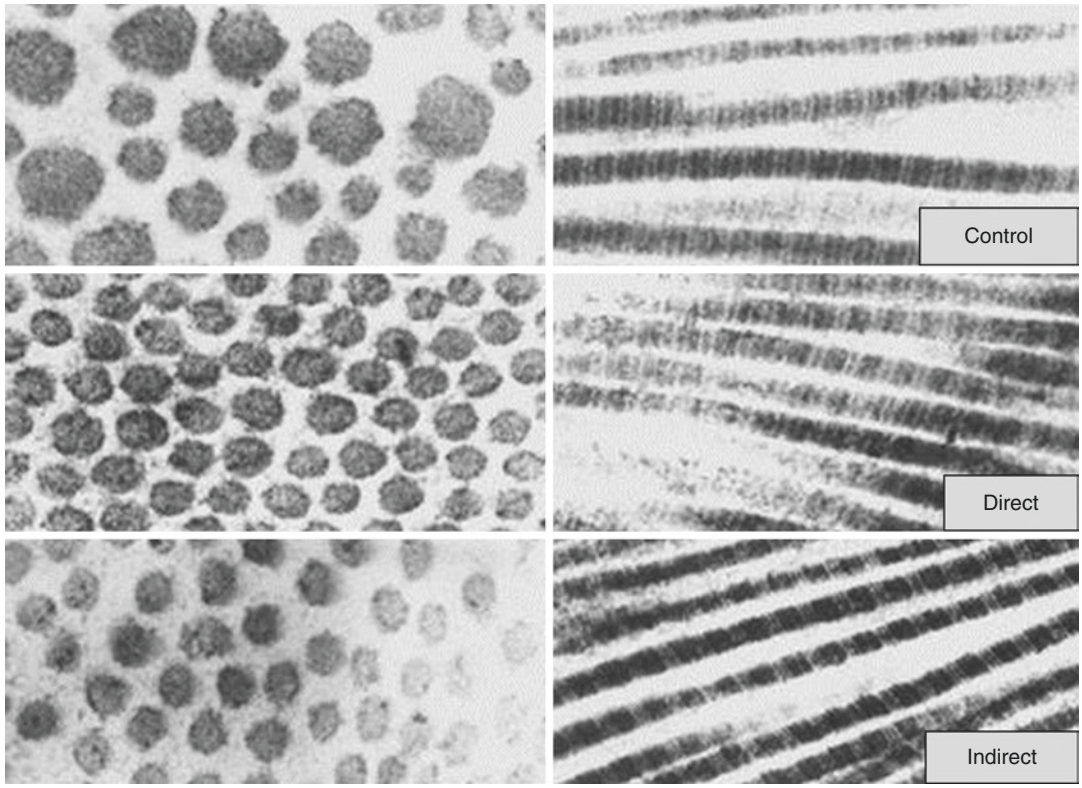

Fig. 3.2 Transmission electron microscopy images of the connective tissue of the transversalis fascia showing absence of ultrastructural alterations of the collagen fibers in the different study groups. Lead citrate and uranyl acetate staining (Magnification 85,000×)

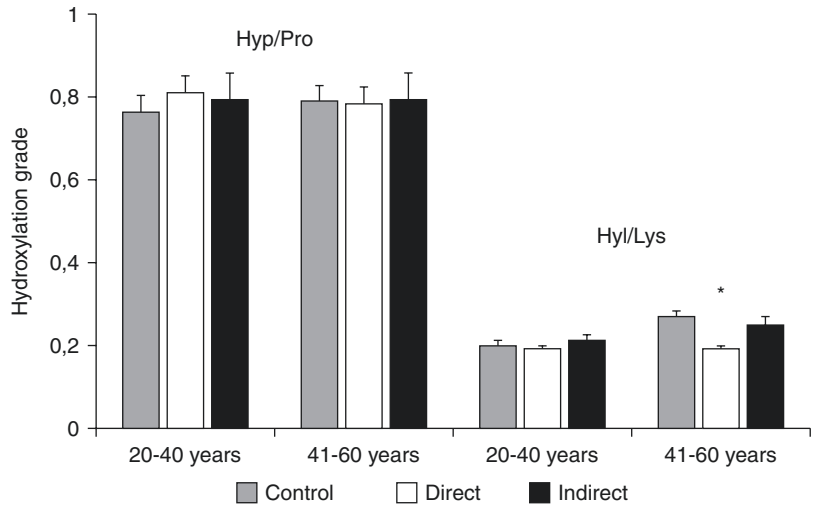

Fig. 3.3 Hydroxylation of proline and lysine in the transversalis fascia of the control groups and direct and indirect inguinal hernias, depending on the age factor of the population. A significant decrease in lysine hydroxylation was observed in the direct hernias of the older group compared to the rest of the study groups ($^{*}p < 0.05$) (Hyp/Pro, Hydroxyproline/Proline ratio; Hyl/Lys, hydroxylysine/lysine ratio)

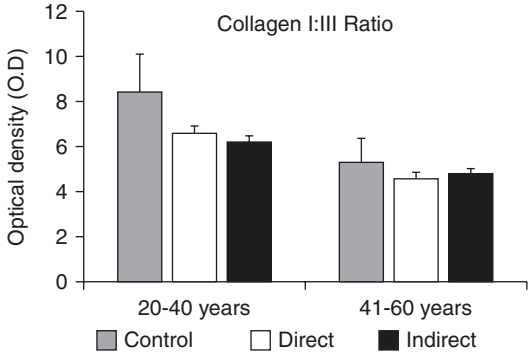

Fig. 3.4 Collagen I:III ratio observed in the different study groups, taking into account the age factor of the population. No significant differences were observed between the different groups of patients (*OD* optical density)

3.2.2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc dependent important proteins involved in extracellular matrix remodeling, which is subjected to a constant dynamic balance between its synthesis and degradation by the action of these enzymes. The MMPs are known to regulate the synthesis and degradation of collagen, but also of many other components of the extracellular matrix, such as proteoglycans, elastin, fibronectin, etc. There are about 23 different types of human MMPs that are grouped into collagenases, gelatinases, stromelysins, matrilysins, membrane MMPs, and other MMPs [28]. The classical MMPs include collagenases like MMP-1, MMP-8, and MMP-13, involved in the degradation of type I, II, and III collagens, and the MMP-2 and MMP-9 gelatinases involved in the degradation of denatured type IV collagens and proteoglycans. However, MMP-2 is also capable of degrading native type I, II, and III collagens [29, 30]. Collagenases and gelatinases are probably the most important MMPs in relation to hernia formation.

In general MMPs are expressed at very low level; however, their expression may be induced as a consequence of different pathological mechanisms. Pro-inflammatory cytokines, growth factors, and hormones are important regulators of MMPs expression. The proteolytic activity of these enzymes, latently secreted, is mainly controlled by the activation of tissue inhibitors of MMPs known as TIMPs [29, 31]. It has been shown that doxycycline administration, as MMPs inhibitor, results in significantly improved strength of repair fascial interface tissue along with a remarkable increase in collagen I, II, and III ratios [32, 33].

Experimental studies performed by different groups have shown that there are no significant differences in the levels of the MMP-1, MMP-9, and MMP-13 enzymes in the TF of patients with direct or indirect hernia compared to controls [34, 35]. Unlike other studies that found significantly higher values of MMP-1, MMP-2, and MMP-9, in inguinal hernia cases [36]. Other authors have found significantly elevated levels of MMP-2 in patients with direct inguinal hernia compared to indirect hernia or control, accompanied by a significant decrease in their inhibitor TIMP-2 [37, 38].

The degradation of the extracellular matrix by the effect of MMPs on the TF has also been the objective of our investigations. Four different types of MMPs (MMP-1, MMP-2, MMP-3, and MMP-9) were analyzed by our group in tissue sections, using immunohistochemical techniques with specific monoclonal antibodies. However, we found only significant differences in the protein expression of MMP-2 [21], where a significant overexpression of the enzyme was observed in the direct hernias of the young patients group with respect to the rest of the groups (Fig. 3.5).

After this study we carried out a second in vitro phase [39], using fibroblasts, in order to check whether the overexpression of MMP-2 observed in tissue was maintained in the cultured cells obtained from TF. The results obtained with immunocytochemical, immunoblotting, and zymography techniques corroborated that MMP-2 would be involved in the degradation process of the TF matrix in patients with direct hernia. The persistence of alterations in MMP-2

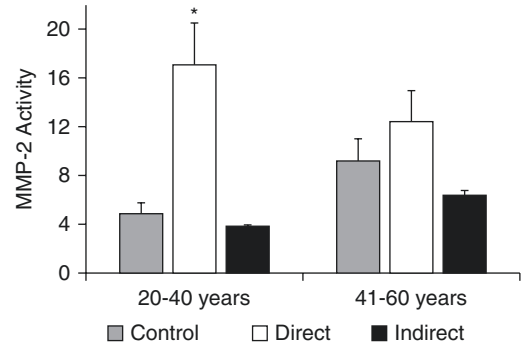

Fig. 3.5 Immunohistochemical staining images for the detection of MMP-2 in tissue sections of transversalis fascia. An increase in expression may be observed in the group of direct inguinal hernia (Magnification 200×). Quantification of MMP-2 activity in the different study groups, depending on the age of the population. A significant ($^{*}p < 0.05$) increase in expression was observed in direct hernias in the group of patients younger than 40 years

levels in cell cultures seems to suggest a genetic defect or irreversible change as the origin of this pathology rather than environmental factors that may later be involved in the development of the disease (Fig. 3.6). These works were the first in the literature to implicate MMP-2 in the pathogenesis of a type of hernia, namely, direct hernia in patients under 40 years of age, where this hernia is often bilateral.

Our research group has even more previous experience [40] related to MMP-2 and its modulators, using human skin biopsies obtained from patients suffering inguinal hernia repair. In this study immunocytochemical and immunoblotting techniques were used in intact tissue and fibroblast cell cultures, as well as zymography techniques to analyze the degradative activity of MMP-2. These results indicate an overexpression of the active form of MMP-2 in the group of direct hernias that could point to an abnormal systemic metabolism as a risk factor for the development of this type of hernia.

3.2.3 Growth Factors

Cytokines or growth factors like TGF-β (transforming growth factor beta) are involved in remodeling processes of different types of tissues. TGF-β is a multifunctional secretion protein that regulates many aspects of cellular function, including cell proliferation, differentiation, and metabolism of the extracellular matrix, [41] through its binding to specific cellular receptors. Five different isoforms have been described, and three of them are found in all species of mammals. TGF-β1 is most widespread and is a 25,000 Kd molecular weight homodimeric protein composed of two identical 12.5 Kd proteins linked by a disulfide bridge [42, 43]. A wide vari-

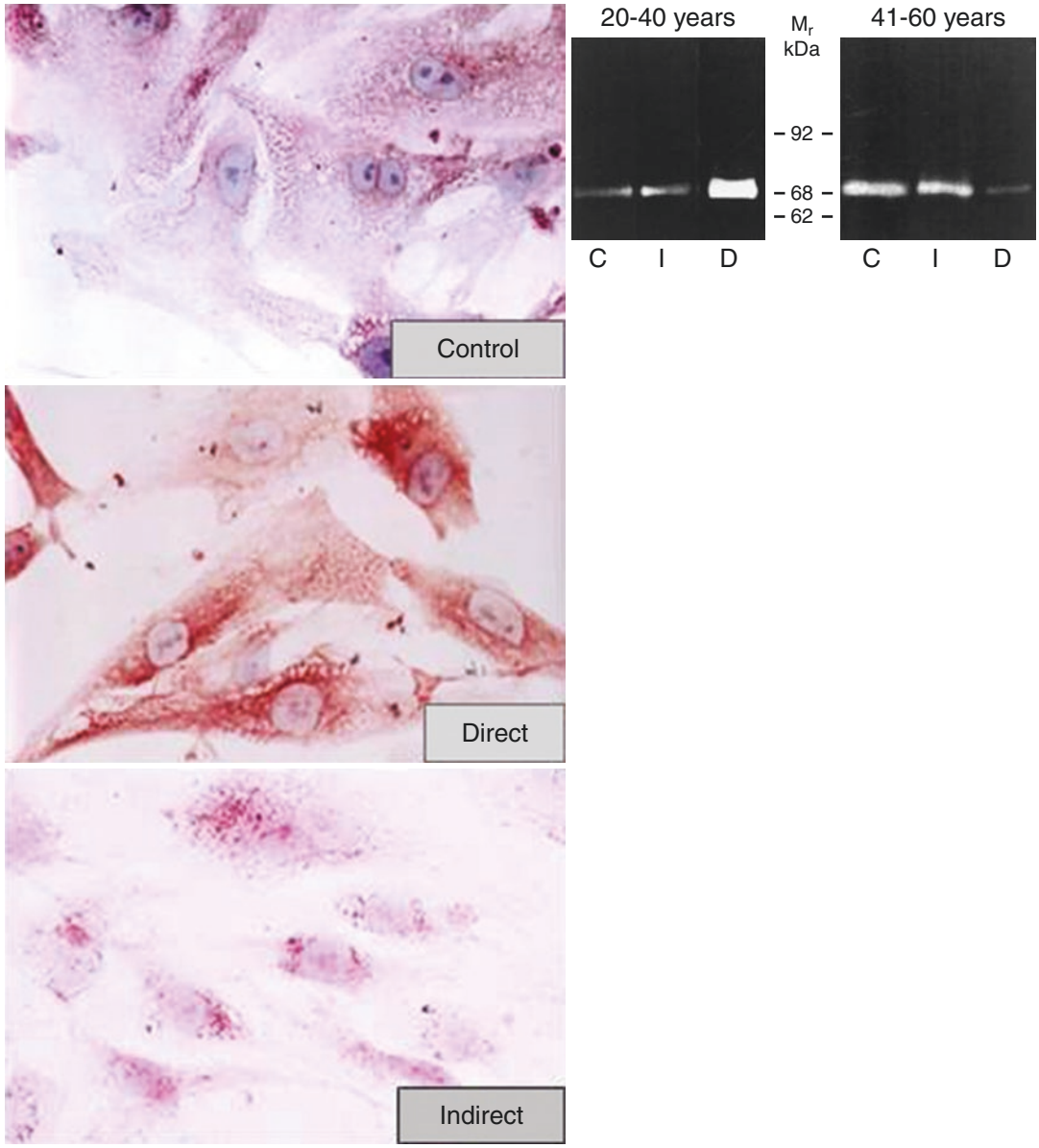

Fig. 3.6 Images of fibroblasts obtained from the transversalis fascia of the different groups of patients, submitted to immunocytochemical techniques for the detection of MMP-2. Higher levels of the enzyme were observed in the group of direct hernias (Magnification 1000×). Gelatinolytic activity determined by zymography techniques in the different study groups, showing an increased degradative band in the group of direct hernias of the younger age group (*C* control, *I* indirect hernias, *D* direct hernias, *Mr* molecular weight)

ety of potential clinical applications have been suggested for this growth factor, including increased scar tissue, control of chronic inflammation associated with fibrosis, and suppression of autoimmune diseases. TGF-β is a pleiotropic factor that can stimulate, inhibit, or modulate cellular events in a time- and concentration-dependent manner. It is a crucial peptide in the control of healing, attracting cells to the wound, but especially promoting the subsequent deposition of collagen and matrix [42]. It has also been identified as a potent modulator of MMPs

expression. Some authors have stated that this growth factor regulates the expression of MMP-2 in several cell types such as fibroblasts and endothelial cells [44, 45].

Our group has carried out different studies in order to evaluate the expression of different growth factors in tissue affected by inguinal hernia [18] and on the integration tissue after the implantation of different types of prosthetic materials in hernia repair [46]. Accordingly, a protein analysis of the distribution and levels of the active and latent form of TGF-β1 was performed, using immunohistochemical and western blot techniques. No significant differences were found in the expression of the latent form of TGF-β1 (LAP-TGF-β1); however, the results of our study indicated an overexpression of the TGF-β1 active form in TF of young patients with direct inguinal hernia (Fig. 3.7). This overexpression of TGF-β1 correlated with the previously described overexpression of MMP-2, in the same group of patients, which could be interpreted as an attempt to counteract the process of degradation of the extracellular matrix observed in this type of hernia.

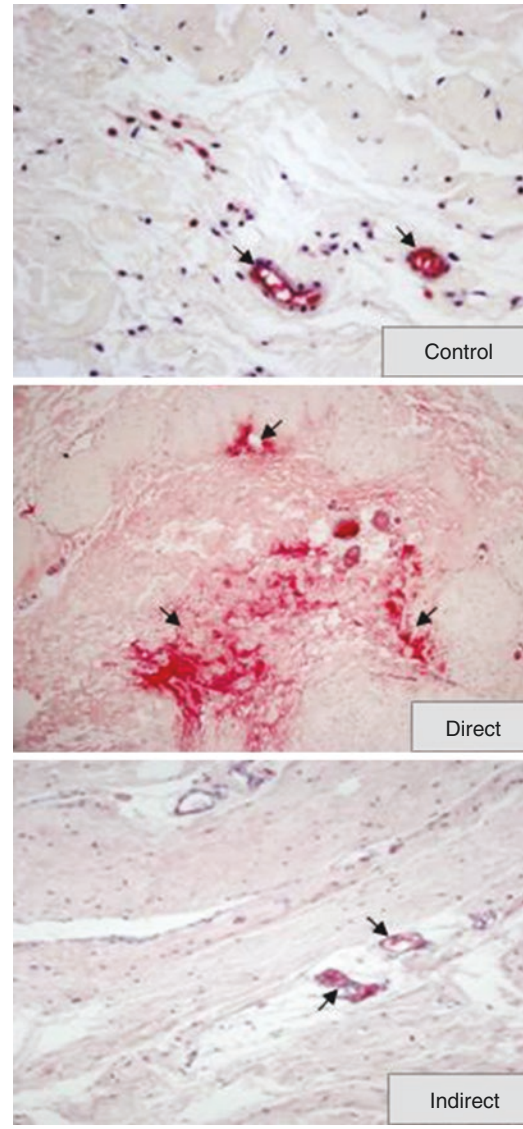

Fig. 3.7 Histological images of the immunohistochemical technique performed on tissue sections of transversalis fascia of healthy patients and patients with direct and indirect inguinal hernias to detect active MMP-2. Overexpression of active enzyme levels on the tissue corresponding to patients with direct hernia can be observed (Magnification 200×)

3.2.4 Elastic Fibers

Elastic Fibers are large fibrillar extracellular matrix structures that provide recovery to tissues undergoing repeated stretching. Elastic fibers are formed by two main components, elastin and microfibrils, that are assembled in a spatial and temporal certain way [47]. Elastin is encoded by a single gene and is the main constituent of the mature fiber. This polymer with a molecular weight of 72 kDa with great capacity of expansion is formed through the cross-linking of tropoelastin (TE) monomers on a support of microfibrils which consist mainly of fibrillin [48] but also associated with proteins such as fibulins, microfibril-associated glycoproteins (MAGPs), and EMILIN-1 [47]. In this crosslinking process, the enzyme lysyl oxidase (LOX) plays a key role. LOX is a family of copper-dependent enzymes that play a critical role in the cross-linking of different extracellular matrix proteins. Some authors [49] have proposed a selective role for LOXL-1 (lysyl oxidase like-1) in the metabolism of elastin, by which elastin deposition is stabilized in a spatially defined manner, as a prerequisite for the formation of functional elastic fibers [50]. One of the most important degradative enzymes of the

elastic system is elastase, which is capable of degrading elastin and elastic fibers, which together with collagen determines the mechanical properties of the connective tissue.

Structural alterations in elastic fibers, related to age, including a considerable reduction in the number of microfibrils leading to a loss of tensile strength and elasticity of transverse fascia tissue have been previously described [51]. This fact could explain the high incidence of inguinal hernia observed from the 50 to 60 years of age.

As we have already mentioned, patients with inguinal hernia show some abnormalities in collagen metabolism and alterations of the MMPs system [16, 17], but there is not much knowledge about the elastic component of the extracellular matrix and the factors involved in tissue remodeling that could affect the elastin metabolism.

Therefore, some studies that aimed to examine in the TF affected by inguinal hernia, the expression of the elastin precursors, tropoelastin (TE), LOXL-1, the enzyme responsible for the cross-linking of elastin polymer and elastase, the main enzyme that causes the degradation of elastin, were performed. Protein analysis techniques such as immunohistochemistry and western blot were used, as well as molecular biology techniques for gene expression analysis. A deficiency in the metabolism of elastin was demonstrated in patients with inguinal hernia that could contribute to the failure of TF [19]. This deficiency was reflected by the insufficient production of LOXL-1 (Fig. 3.8), which plays a selective role in elastin cross-linking, as well as by the overproduction of elastase, one of the most important enzymes involved in the degradation of the elastic component. The findings indicated similar

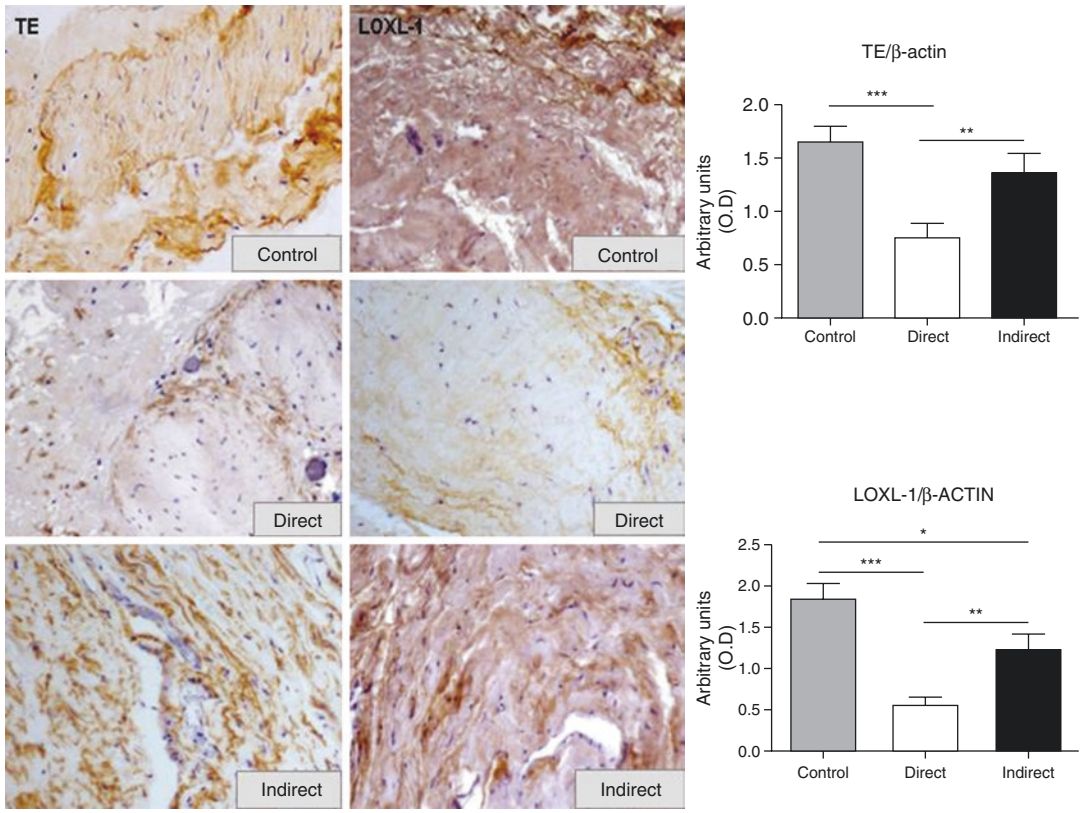

Fig. 3.8 Immunohistochemical detection and levels recorded in the different study groups revealed by western blot analysis of TE and LOXL-1 on transversalis fascia tissue (Magnification 200×). Significantly lower levels were detected in both constituents for the direct hernia group compared to the rest of the groups ($^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$)

amounts of mRNA encoding for TE in fibroblasts isolated from TF from patients with direct and indirect inguinal hernia. But messenger levels for LOXL-1 showed significantly decreased expression in cell cultures obtained from patients with direct inguinal hernia.

Both elastic fiber fragmentation and reduction of its number in spite of an increase in the extracellular matrix have been observed by other groups [52], in patients with hernia. Other studies have reported a decrease in the total amount of elastic fibers in connective tissue in remote locations to the site of the hernia, such as the rectus sheath, supporting the theory of a global connective tissue disorder [53].

3.3 Discussion

Throughout all this review, we have been able to verify in inguinal herniogenesis that the TF is formed by a connective tissue with an altered extracellular matrix, mainly in those patients with direct inguinal hernia. The ultrastructural analyses did not show alterations in the density and diameter of the collagen fibers that justify the formation of hernias [21]. Other groups, according to these findings have reported similar results [54], but some of them have observed some alterations that have been attributed to the age factor and not to the hernia condition [51]. Hydroxylation of the amino acids proline and lysine of the collagen molecule is an essential process in the formation and stabilization of the collagen triple helix. Our results showed no proline hydroxylation differences, as did other authors [22] in patients with hernia. However, a significant decrease in lysine hydroxylation was observed in direct inguinal hernia of patients of the older age group. This could indicate alterations in the cross-linking of collagen that could affect the interaction with other components of the extracellular matrix [18].

Alterations in the collagen I:III ratio have been described by some authors [16, 55], in contradiction with our group that has not demonstrated significant differences in this ratio in TF between different types of hernias. A literature review [8] performed by the group of Henriksen, on collagen alterations in abdominal wall hernia, states that there is evidence of a significant increase in type III immature collagen with respect to mature type I collagen, resulting in the corresponding loss of biomechanical resistance of the repair area. It suggests that these alterations may be due to variations in the process of synthesis, maturation, or degradation of the collagen matrix by MMPs, in combination with other processes or independently. The authors of this review conclude that both the development of primary hernia and its recurrence are associated with a decrease in the collagen I:III ratio.

After the study involving the collagen component, our interest was centered in the analysis of different MMPs. We found only significant differences in the expression of MMP2, whose main substrates are different types of collagens and other extracellular matrix components such as fibronectin, elastin, and proteoglycans [56]. Our results with MMP-2 demonstrated that this enzyme is overexpressed in direct hernias at the tissue level and in cell cultures obtained from the TF of these patients [21, 39]. These results were corroborated by investigations of other groups showing an increase in MMP-1, MMP-2, and MMP-9 in inguinal hernia, stating that these enzymes play a very important role in the development of this pathology [36].

Other groups [57] have subsequently shown dysregulation of the extracellular matrix degradation process in patients with inguinal hernia, showing a significant increase of MMP-2 and 9, accompanied by a decrease in their endogenous inhibitors (TIMPs). The results of this study suggest problems in collagen metabolism that could be the underlying pathophysiological mechanism of inguinal hernia formation.

There is scarcely any bibliography to analyze the importance of growth factors in the development of inguinal hernia. TGF-β1 has been described as an important modulator of MMPs [41]. In our study overexpression of TGF-β1 was correlated with the overexpression of MMP2 in patients with direct hernia. Other authors have shown selective regulation of MMP-2 by TGF-β1 in transcriptional and posttranscriptional lev-

els in fibroblast cultures [58]. Other research work [59], according to this regulation, maintain the possibility that under the pathophysiological conditions, the digestion of the extracellular matrix by the MMPs could induce the TGF-β-mediated tissue reaction released by the connective tissue. All these results are in agreement with our findings in the TF of patients with hernia pathology.

In a model of experimental hernia in rat, some authors [60] have shown that the local application of this growth factor does not increase the biomechanical resistance of the abdominal wall. However, another research group [61], also using an experimental rat model, states that treatment with TGF-β2 prevents the development of hernias, stimulating the mobilization of macrophages and fibroblasts, as well as an increase of collagen deposition in the wound area.

Regarding the elastic component, a genetic mutation has been described by the group of Junqueira et al. [62] involving the elastic tissue and its dysfunction at the TF level. Our studies have shown a disorganization and reduction in the number of elastic fibers in the TF of patients with direct inguinal hernia, which corresponded with the minimal expression of LOXL-1, which would prevent normal cross-linking of TE and with the greater expression of elastase, which degrades the elastic components. These results emphasize the importance of LOXL-1 to avoid the loss of elasticity of tissues in which elastic fibers are essential for the correct functionality.

According to our results, other groups [52] have also observed in inguinal hernia both elastic fiber fragmentation and reduction of its number with an increase in the extracellular matrix. A decrease in the total amount of elastic fibers in connective tissue of remote locations to the site of the hernia have been also reported, supporting a global connective tissue disorder [53]. Conversely, some studies [19] have shown a significant increase of elastic fibers in the fascia of patients with direct inguinal hernia. Other papers using immunohistochemical evaluation showed no statistically significant differences in the amount of elastic fibers and collagen I and III among patients with inguinal hernia when compared with subjects without hernia [63].

There are very few published reports in the literature relating inguinal hernia to the analysis of the enzymes involved in elastin and collagen cross-linking. These include a study by Kayaoglu et al. [64] in which significant lower plasma and hernia sac copper levels were detected in patients with direct hernias than those with indirect hernias. Given that copper is an essential cofactor for lysyl oxidase, the authors proposed that patients with direct hernia could show impaired collagen and elastin synthesis because of the deficient activity of LOX. Other studies [65] evaluating copper and zinc levels in hernia formation have showed significantly lower tissue levels compared to control, which might reflect excessive consumption or dysfunction of lysyl oxidase as playing a role in the etiology of hernias.

The amounts of collagen and elastic fibers in the TF determine its tensile strength and elasticity. Significant biomechanical changes in the TF of patients with hernia have been reported by Pans et al. [7] Some other authors [66], according to our results and in a search for possible relationship between hernia and abdominal aneurysm, have described elevated levels of elastase and significantly higher prevalence of inguinal hernia in these patients with aneurysm suggesting systemic fiber degeneration. Other authors [67], also in agreement, have reported significantly higher circulating serum elastinolytic activity in patients with direct hernia.

Taking into account our findings and those of other authors, in relation to the biological factors involved in herniogenesis, we could conclude that the different elements of the connective tissue extracellular matrix play an important role in the genesis of inguinal hernias, and especially in one type, the direct hernia.

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