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From Cell Biology to Tissue Engineering

Morphological and histomorphometric evaluation of the ventral rectus sheath of the rectus abdominis muscle, fascia lata and pectoral fascia. The beginning of a morphological information bank of human fascias

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Summary. The aim of this study was to characterize and compare the morphological and histomorphometric characteristics of the pectoral fascia, fascia lata and ventral rectus sheath. Twenty cadaveric samples of these fascias were analyzed and stained with hematoxylin and eosin, orcein, Van Gieson, Masson's trichrome and Verhoeff's stain (1200 slides in total). Morphological evaluation, semiquantitative, morphometric and microdensitometric analysis of elastic fibers present in each of the tissues and a morphometrical analysis of tissue thickness were performed. The mean value of the pectoral fascia thickness was 612±68.13 μm; 84±246 μm for the fascia lata and 584±92 μm for the ventral rectus sheath. The area occupied by the elastic fibers in the pectoral fascia was 12.24±5.84%; 6.54±3.85% for the fascia lata and 11.11±5.26% for the ventral rectus sheath. There were no statistically significant differences when comparing the mean values between the pectoral fascia and the ventral rectus sheath (p=0.07). There were statistically significant differences when comparing the fascia lata to the pectoral fascia and the ventral rectus sheath (p≤0,001). This study reports other morphological characteristics not described in previous histological studies of the analyzed tissues. The results of the morphometric and densitometric analysis in this study reveal that the fascia lata has the fewest elastic fibers of all the tissues analyzed, and the pectoral fascia has the most. These results will be useful for the beginning of a morphological information bank of human fascias.

Key words: Collagen, Elastic fibers, Fascia lata, Pectoral fascia, Ventral rectus sheath

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Introduction

The term deep fascia refers to any dense fibrous sheath that interpenetrates and surrounds the muscles, bones, nerves, and blood vessels of the body, binding all these structures together into a firm compact mass. Over bones, it is called periosteum; around tendons, it forms the paratendon; around vessels and nerves, it forms the neurovascular sheath; around joints, it strengthens the capsules and ligaments. So the paratendon, the neurovascular sheath, and the periosteum can be considered a specialization of the deep fascia, not only because they are in continuity with it, but also because they have the same histological features (Stecco et al., 2013a,b).

The deep fascia has been largely studied in recent years due to its involvement in a wide range of pathologies including iliotibial tract syndrome, low back pain, myofascial pain, compartment syndromes, fibromyalgia, Dupuytren's contracture and plantar fasciitis or fasciosis (Benjamin, 2009; Alfonso-Rodríguez et al., 2014; Satoh et al., 2016). Recent research shows that there are differences between the fasciae of the limbs and of the trunk. In particular, the deep fasciae of the trunk are usually formed of a single layer of undulated collagen fibers that are continuous/adherent with intramuscular septa, and intermixed with many elastic fibers, whereas the deep fasciae of the limbs are formed of two or three sub-layers of parallel collagen fiber bundles of densely packed collagen bundles, interspersed with thin layers of areolar connective tissue and able to slide over the underlying muscle, sharing only a few myofascial connections with it (Stecco et al., 2008). Nonetheless, our research group believes that the morphological characteristics of these tissues have yet to be described in a definitive manner.

It is increasingly evident that the fasciae may play important roles in venous return (Caggiati, 2000), dissipation of tensional stress concentrated at the sites of entheses (Benjamin et al., 2008), etiology of pain (Langevin et al., 2001; Langevin, 2006), interactions among limb muscles (Huijing et al., 1998; Huijing, 1999; Huijing and Baan, 2001; Yucesoy et al., 2006) and movement perception and coordination (Vleeming et al., 1995, 1996), due to their unique mechanical properties and rich innervation.

Several studies have morphologically analyzed human fascia and connective tissue from various body regions in patients, and compared them with healthy controls, in order to detect possible differences in composition (type 1 collagen, type 3 collagen, elastic fibers, cellular elements, etc.) and to associate them with certain conditions. Such studies have been performed on the anterior and posterior laminae of the rectus sheath, fascia transversalis and interfoveolar ligament (patients with inguinal and/or anterior abdominal wall hernias) (Pans et al., 1999; Quintas et al., 2000; Fachinelli et al., 2011; Szczesny et al., 2006, 2013), periesophageal ligaments and diaphragmatic pillars (patients with gastroesophageal reflux disease and hiatal hernia) (Curci et al., 2008; Fei et al., 2009), endopelvic fascia, sacrouterine ligaments, cardinal ligaments and anococcygeal ligaments (patients with uterine prolapse, urinary incontinence and prostate diseases) (Ewies et al., 2003; Söderberg et al., 2004; Klutke et al., 2008; Salman et al., 2010; Chen and Yen, 2011; Julio Junior et al., 2015; Jin et al., 2015), dura mater and pericranium (encephalic and spinal dura mater surgeries) (Chauvet et al., 2010), thoracolumbar fascia (patients with chronic back pain) (Bednar et al., 1995), temporalis fascia (patients undergoing tympanoplasty) (Shenoi, 1982), perirectal fascia (Kraima et al., 2015), auricular cartilage (Ito et al., 2001), cricoarytenoid articulation (Kawamoto-Hirano et al., 2015) ankle retinacula (ankle sprain) (Stecco et al., 2010), plantar fascia (patients with plantar fasciitis, Achilles tendon diseases and patients with ankle or foot pain) (Stecco et al., 2013a,b) as well as fetal tissue donors (Kinoshita et al., 2013; Blasi et al., 2014).

We consider it important to conduct thorough morphological studies of all human connective tissues, to create an information bank that is useful for all basic and clinical scientists, who require healthy control groups for morphological comparison of individuals with certain diseases, whose etiology may involve a change in the components of the extracellular matrix of the tissue of interest. The aim of this study was to determine and compare the morphological characteristics of the pectoral fascia, fascia lata and ventral rectus sheath.

Materials and methods

Study design

This is a morphological, observational, cross-sectional, descriptive, comparative and blinded study.

Biological material

Samples were obtained from 20 previously embalmed male cadavers with an age range of 24 to 66 years (mean: 38±14 years) and an average body mass index of 23.18±2.44 kg/m^2. The fixation and embalming technique used in the present study preserve the structure of living tissue with no alteration from the living state. Tissues which have been fixed should not change shape or volume during any of the subsequent procedures. Additionally, this technique protects the tissue against disruption during embedding and sectioning and leaves the tissue in a condition which subsequently allows clear staining of sections (Suvarna et al., 2013). The samples were collected in blocks of 5x5 cm of pectoral fascia, fascia lata and ventral rectus sheath, resulting in two samples per cadaver (Fig. 1). None of the cadavers used in this study showed high body mass index, evidence of traumatic injuries, diseases, obvious abnormalities such as diastasis of the rectus abdominis muscles, hernias of the anterior abdominal wall, inguinal or femoral hernias or abdominal/ thoracic visible scars. It has been found that these conditions may alter the normal histology of the tissue analyzed in this study (Fachinelli et al., 2011; Szczesny et al., 2006, 2013).

Ethical considerations

This study was approved by the Ethics and Research Committee of the Faculty of Medicine, and the registration number was AH13-002. The authors have no conflict of interest and no financial or commercial gain from the realization of this study.

Dissection technique

Full thickness specimens were obtained. Dissections for obtaining samples were performed according to the following protocols:

Pectoral fascia and ventral rectus sheath

To obtain these two tissues three incisions were made. First, a superficial, longitudinal incision was made along the midline of the body from the level of the sternoclavicular joint to the pubic symphysis, followed by two transveral incisins. The upper transverse incision was made along the lower edge of both clavicles uniting in the midline and the lower transverse incision was made at the level of the anterior superior iliac spines.

For the pectoral fascia, the skin was removed in order to display the subcutaneous tissue and the superficial fascia. Later, the superficial fascia was removed and the deep fascia was exposed. 5x5 specimens were taken from the inferior third of the pectoral region in both sides of the trunk (because previous studies have shown that this is the thickest part of the fascia, reporting no morphological differences between different regions of the pectoral fascia) (Stecco et al., 2009), along the midclavicular line. The samples were as far as possible carefully separated from the pectoralis major muscle. For the ventral rectus sheath, the skin was removed in order to display the subcutaneous tissue and the superficial fascia. Later, the superficial fascia was removed and the ventral rectus sheath was exposed, 5x5 samples were obtained from a point located 3 cm above the superior border of the umbilicus and at another point located 2 cm below the inferior border of the umbilicus for laboratory analysis (these points were established based on the methodology of a previous article, with the purpose of further comparison) (Fachinelli et al., 2011).

Fascia lata

In the thigh, three incisions were made; a vertical incision was made along the anterior region of the thigh, from the anterior superior iliac spine to a point located 2 cm above the superior border of the patella, and then two transversal incisions were made. The upper transverse incision was made along the inguinal ligament position and the inferior was made at the level of the superior border of the patella; then the subcutaneous planes were highlighted in a similar way to the thorax dissection. Specimens of 5x5 cm were taken from the distal third of both thighs, along the line of the rectus femoris muscle.

Morphological analysis

The samples were accurately oriented and mounted on cardboard to avoid deformation artifacts. All the specimens were immediately preserved in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.2-7.4 and then embedded in paraffin. Longitudinal and cross-histological sections were obtained at 4-μm thickness (each specimen was prepared by slicing parallel and perpendicular to the specimen surfaces), the slices were stained with hematoxylin and eosin (H&E) to assess the cellular components and general tissue. The cross-sections were stained with orcein to identify the elastic fibers. Masson's trichrome was used to identify collagen fibers. Van Gieson's trichrome was used to distinguish cell nuclei, collagen fibers, and muscle fibers; and a histochemical staining technique (Verhoeff's stain + Van Gieson's trichrome) was used to identify nuclei, elastic fibers, collagen fibers, and muscle fibers. A total of 1200 slides were analyzed.

Morphological analysis of the slides was performed by three blinded specialists with a PhD. in Morphology; these specialists evaluated the tissue characteristics; the morphological organization of the collagen and elastic fibers; the presence of septae or areolar connective tissue; the number of layers in each specimen; the orientation and arrangement of the fibers; the presence of fibroblasts, arterioles, capillaries, venules, lymphatic vessels, nerves, mast cells, myofibroblasts, adipose tissue or muscle fibers and any other elements observed in the tissue (Bhattacharya et al., 2010). A Nikon Eclipse 50i light microscope was used, magnified between 10x

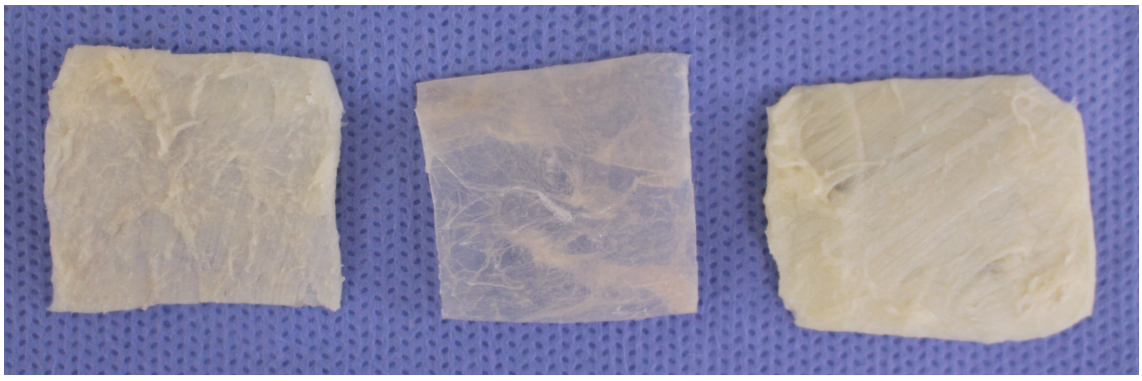

Fig. 1. Examples of blocks of tissue samples used for the study. From left to right: **A)** pectoral fascia, **B)** fascia lata and **C)** ventral rectus sheat.

and 40x, as well as an image analysis system that included NIS-elements AdVanced Research software, Digital Sight DS-2Mu, and the Image J program, version 1.49 (National Institutes of Health).

Histomorphometric analysis of tissue thickness

Using the same software, measurements were taken to determine the thickness of each tissue studied. These measurements were taken from samples of tissue sliced in an axial manner, stained with Van Gieson's stain and amplified 10x, taking into consideration the mean value of 3 measurements (center and external areas of the tissue).

Morphometric analysis of elastic fibers

The elastic fibers were evaluated using a cross-semiquantitative scale proposed by our research group in a previous study (Morales-Avalos et al., 2016). The scale was classified as follows: absence of fibers (-), slim and thin fibers (±), few thick fibers thin to medium (+), thin fibers in moderate quantity (++), medium fibers or thick in moderate amount (+++) and thick and abundant fibers (++++). The values obtained from every sample analyzed are presented in a comparison table. The immunohistochemical visualization of elastic fibers can be achieved using a monoclonal antibody specific for elastin (Midwood and Schwarzbauer, 2002). Otherwise, orcein staining is a reliable dye solution to identify these elastic fibers in laboratory analysis (Henwood, 2003). Additionally, a microdensitometric analysis was performed to quantify the proportion of elastic fibers in the samples of interest. This analysis was performed in the following manner: High-resolution digital images of ten consecutive sections from the orcein-stained slides were obtained with high dry objective lenses (40x). The color parameters, hue distribution, saturation, and luminance were established in the capture software and were the same for all the images obtained. The images were analyzed with Image J (Image J is a public domain, Java-based image processing program developed at the National Institutes of Health, USA, Version 1.49) to quantify via microdensitometry, the amount of elastic fibers in pectoral fascia, fascia lata and ventral rectus sheath. From the images obtained, the dark-red hue of the elastic fibers was manually selected, and the program (with the detection threshold of the control sample pre-calibrated) converted the interval color to grayscale and the other tissue components to white. Then, the processed images were automatically analyzed to determine the percentage of the area and intensity of the density (Int Dent) of elastic fibers in each sample. This analysis was performed in triplicate.

Statistical analysis

The values obtained from the microdensitometry quantification were captured in IBM SPSS Statistics Version 21.0 for Windows XP (Chicago, IL, USA) and the average and standard deviation of the thickness of each of the tissues and the presence of elastic fibers was

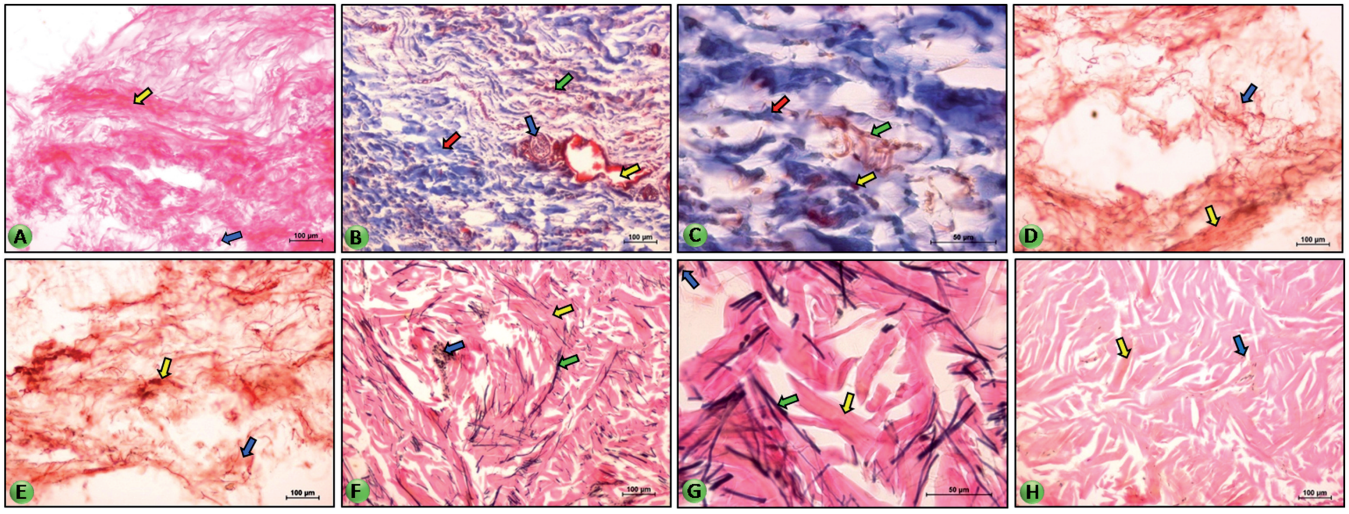

Fig. 2. Morphologic characteristics of the pectoral fascia. **A.** Micrograph showing collagen fibers arranged in parallel undulated bundles (yellow arrow), fibroblast nuclei (blue arrow). **B.** Micrograph showing blood vessels (yellow arrow), nerve bundles (blue arrow), elastic fibers (green arrow) between the collagen fibers (red arrow). **C.** Higher magnification micrograph of collagen fibers (red arrow), elastic fibers (green arrow), fibroblast nucleus (yellow arrow). **D and E.** Micrographs of elastic fibers arranged in parallel beams (yellow arrows) or irregularly (blue arrow). **F and G.** Micrographs showing elastic fibers (green arrow) between collagen fiber bundles (yellow arrow), nuclei of cells from the wall of a blood vessel (blue arrow). **H.** Bundles of collagen fibers stained with Van Gieson technique (yellow arrow), fibroblast nuclei (blue arrow). A, H&E stain; B and C, Masson's trichrome; D and E, Orcein; F and G, Verhoeff's stain; H, Van Gieson's trichrome. Paraffin-embedded, light microscopy.

determined. Parametric correlation tests (two-tailed student's t test) were performed to determine the significance of differences between the average values of the thickness of each of the tissues and the elastic component between the pectoral fascia, fascia lata and ventral rectus sheath. A value of p≤0,05 was considered significant.

Results

Pectoral fascia

In the slides stained with H&E, and Masson trichrome and Van-Gieson's stain, it was observed that the pectoral fascia is formed by a thin layer of connective tissue formed by multiple thin bundles of undulated collagen fibers with a parallel arrangement between them (Fig. 2A-D,H), the fibers are arranged in a transverse arrangement with respect to the underlying muscle fibers; the inner portion of the fascia is in direct contact with the muscle fibers and intramuscular septa of the pectoralis major muscle. It was observed in all cases that some muscle fibers penetrate into the temporal fascia. Collagen fibers are intermingled with elastic fibers, clearly visible with orcein stain and Verhoeff's; these were arranged in an irregular manner without any identifiable pattern with respect to collagen fibers. These elastic fibers are an intermediate to coarse size/thickness and dispersed in large amounts in the whole of the pectoral fascia. The elastic fibers were equally distributed throughout the fascia (Fig. 2E-G). In the slides stained with H&E, among some collagen fibers, we observed few fibroblast nuclei with basophilic staining, which were flattened, elongated, and oriented parallel to collagen. The vasculature was present throughout the tissue. The presence of septa within the pectoral fascia or any portion of adipose tissue was not found.

Fascia lata

In sections stained with H&E, Van Gieson's stain and Masson's Trichrome, it was observed that the fascia lata is composed of a regular dense connective tissue and collagen fibers organized in undulated and thick bundles that formed highly organized sheets in three concentric sub-layers of parallel collagen fiber bundles of densely packed collagen bundles presenting an undulating arrangement (3A-C,F). In each layer, the fibers are parallel to each other. Each layer was separated from the

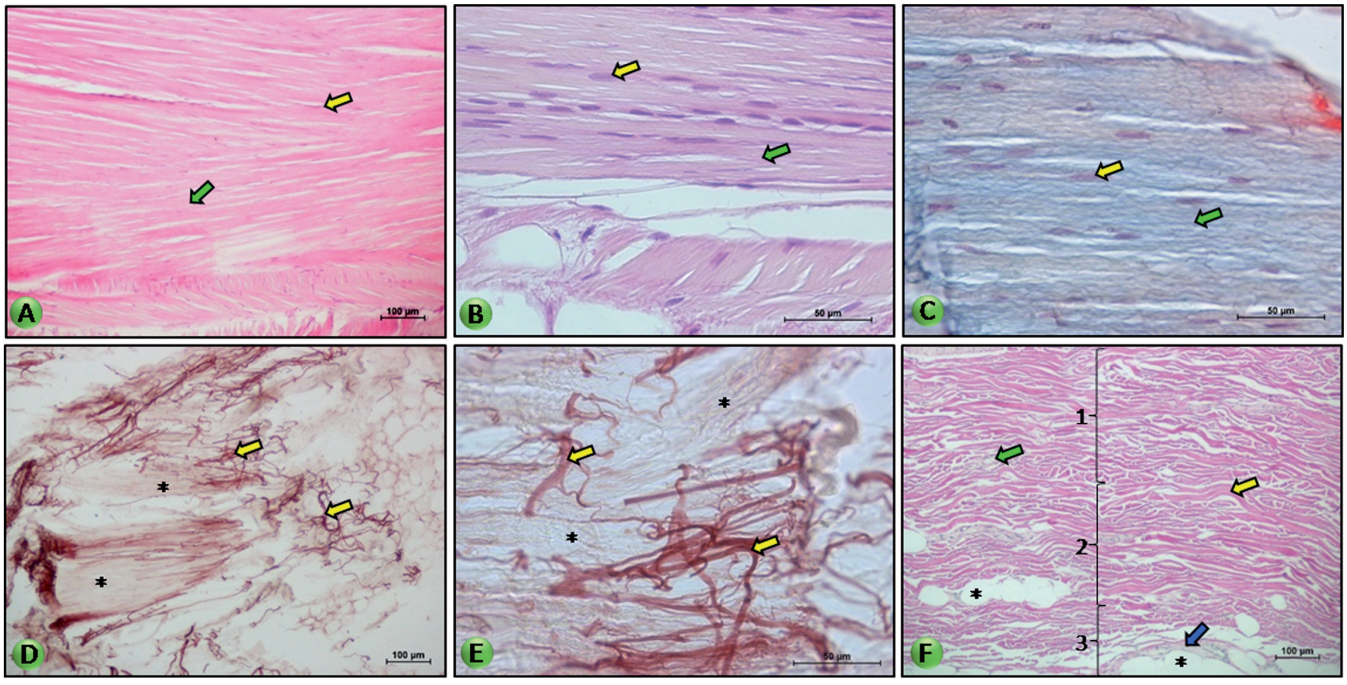

Fig. 3. Morphologic characteristics of fascia lata. **A.** Micrograph showing regular dense connective tissue composed of collagen beams arranged in parallel manner (green arrow), fibroblast nuclei (yellow arrow). **B and C.** Amplification of the fiber bundles of densely packed collagen (green arrow) with fibroblast nuclei between them (yellow arrow). **D and E.** Micrographs showing the elastic fibers in the tissue periphery irregularly organized (yellow arrows) with respect to the collagen fiber bundles (asterisks). **F.** Micrograph showing the three layers of the fascia lata listed in superficial to deep order (numbers), made up of undulated beams of collagen fibers (yellow arrow) with unilocular adipose tissue (asterisks) and blood vessels (green arrow). The presence of undulated collagen fibers grouped in bundles in the deepest portion (blue arrow) is further noted. A and B, H&E stain; C, Masson's trichrome; D and E, Orcein; F, Van Gieson's trichrome. Paraffin-embedded, light microscopy.

adjacent one by a thin layer of loose connective tissue. Adjacent layers show different orientations from their collagen fibers. In 2 cases it was possible to identify within the fascia lata well-defined bundles of muscle fibers. At the internal and external surfaces of the fascia lata two thin laminae (mean thickness: 42±4 μm) of undulated collagen fibers, not grouped into bundles, were present (indeed the more internal and external layers are small undulated formed by collagen fibers and many elastic fibers embedded in abundant extracellular matrix). Among some collagen fibers, there were observed some fibroblast nuclei with basophilic staining, which were oriented parallel to collagen. In the slides with orcein and Verhoeff's stain it was possible to identify very thin elastic fibers in small proportion in the entire tissue analyzed (Fig. 3D,E), but the distribution of these fibers was irregular, having a higher concentration of elastic fibers in the external and internal portions of the fascia lata and in the junction points between the sublayers of the tissue (in the areas occupied by loose areolar tissue where change was evident in the orientation of the collagen fibers), elastic fibers that neither followed a particular direction nor were oriented in any characteristic pattern with respect to collagen; they had a spiculated "web"-shaped pattern. It was possible to demonstrate a large number of arterioles and capillaries interspersed with collagen fibers. The distribution of nerves in the fascia lata was not homogeneous; on the external part the nerve tissue are abundant. In contrast, the median layers are composed of two to three layers of parallel collagen fiber bundles with few nerve fibers. It became evident that there is a vast quantity of adipose tissue in the external surface of the tissue, which was lacking in the other areas.

Ventral rectus sheath

The anterior layer of the ventral rectus sheath is composed of dense wavy collagen bundles, oriented in a regular longitudinal way, with the presence of extracellular matrix between these bundles. The number of collagen bundles varies from sample to sample (range 8-12, mode 9). Ground substance can be seen between the fibroblasts and collagen fibers are plentiful (Figs. 4A-D,G-H). Elastic fibers observed were of an intermediate thickness, well defined and in a moderate amount, distributed throughout the tissue, but most of them were arranged at the periphery of the collagen bundles, usually in a longitudinal direction (Fig. 4E-F). In some samples it was possible to identify the presence of unilocular adipose tissue in the external surface and muscle fibers in the inner surface of the fascia.

Morphometric analysis

The mean value of the pectoral fascia hickness was 612±68.13 μm; 1384±246 μm for the fascia lata and 584±92 μm for the ventral rectus sheath. There are no statistically significant differences when comparing the mean values between the pectoral fascia and the ventral

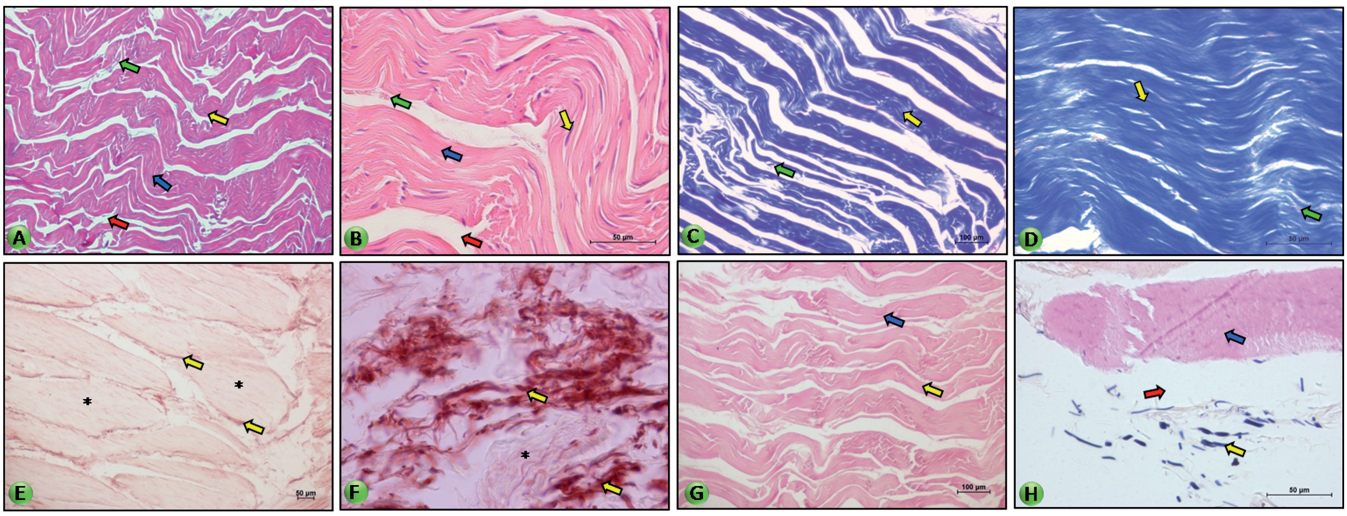

Fig. 4. Morphologic characteristics of the previous sheet of the rectus sheath. **A and B.** Micrographs showing undulated bundles of collagen fibers arranged in a parallel manner (blue arrow) with ground substance (red arrow) and blood vessels (green arrow) including fibroblast nuclei (yellow arrow). **C and D.** Micrographs showing bundles of collagen fibers stained with Masson's trichrome method (yellow arrows), fibroblast nuclei (green arrows). **E and F.** Micrographs highlighting the arrangement of irregularly organized elastic fibers (yellow arrows) at the periphery of the collagen fiber bundles (asterisks). **G.** Micrograph of collagen fibers bundles stained with Van Gieson's method shows the characteristic red color (yellow arrow), fibroblast nucleus (blue arrow). **H.** Micrograph of the elastic fibers (yellow arrow) immersed in spaces where the fundamental substance used to be (red arrow) on the periphery of the collagen fiber bundles (blue arrow). A and B, H & E stain; C and D, Masson's trichrome; E and F, Orcein; G, Van Gieson's trichrome; H, Verhoeff's stain. Paraffin-embedded, light microscopy.

rectus sheath (p=0.64). On the other hand, there were statistically significant differences when comparing the fascia lata to the pectoral fascia (p≤0.001) and the fascia lata to the ventral rectus sheath (p≤0.001) (Fig. 5A).

Semiquantitative and quantitative analysis of elastic fibers

The values obtained from the semiquantitative analysis of the elastic fibers are shown in Table 1. Under light microscopy, we observed that the elastic fibers of pectoral fascia were thick and abundant (+++). The elastic fibers in the fascia lata were slim and thin to medium thickness (+) and in the ventral rectus sheath there were thin fibers in moderate quantity (++). This pattern was observed in the transverse and longitudinal sections.

In the microdensitometric analysis of each tissue, the area occupied by the elastic fibers was determined in the pectoral fascia and showed a value of 12.24±5.84%, while fascia lata reached a value of 6.54±3.85%, and the ventral rectus sheath reached a value of 11.11±5.26%. There are no statistically significant differences when comparing the mean values between the pectoral fascia and the ventral rectus sheath (p=0.07). On the other hand, there are statistically significant differences when comparing the fascia lata to the pectoral fascia (p≤0.001) and the fascia lata to the ventral rectus sheath (p≤0.001) (Fig. 5B). The intensity of the density values was 24846870±13535363 for the pectoral fascia, 22544074± 8587782 for the fascia lata and 34375402±2346660 for the ventral rectus sheath. Significant differences are evident when comparing statistically all tissues together (p≤0.001) (Fig. 5C).

Discussion

The fibrous components of the extracellular matrix are classified into three types of fibers: collagenous, reticular and elastic (Ushiki, 2002). The fibroelastic system, with uniform distribution of forces, keeps the resiliency adapted to the demands of local tissues

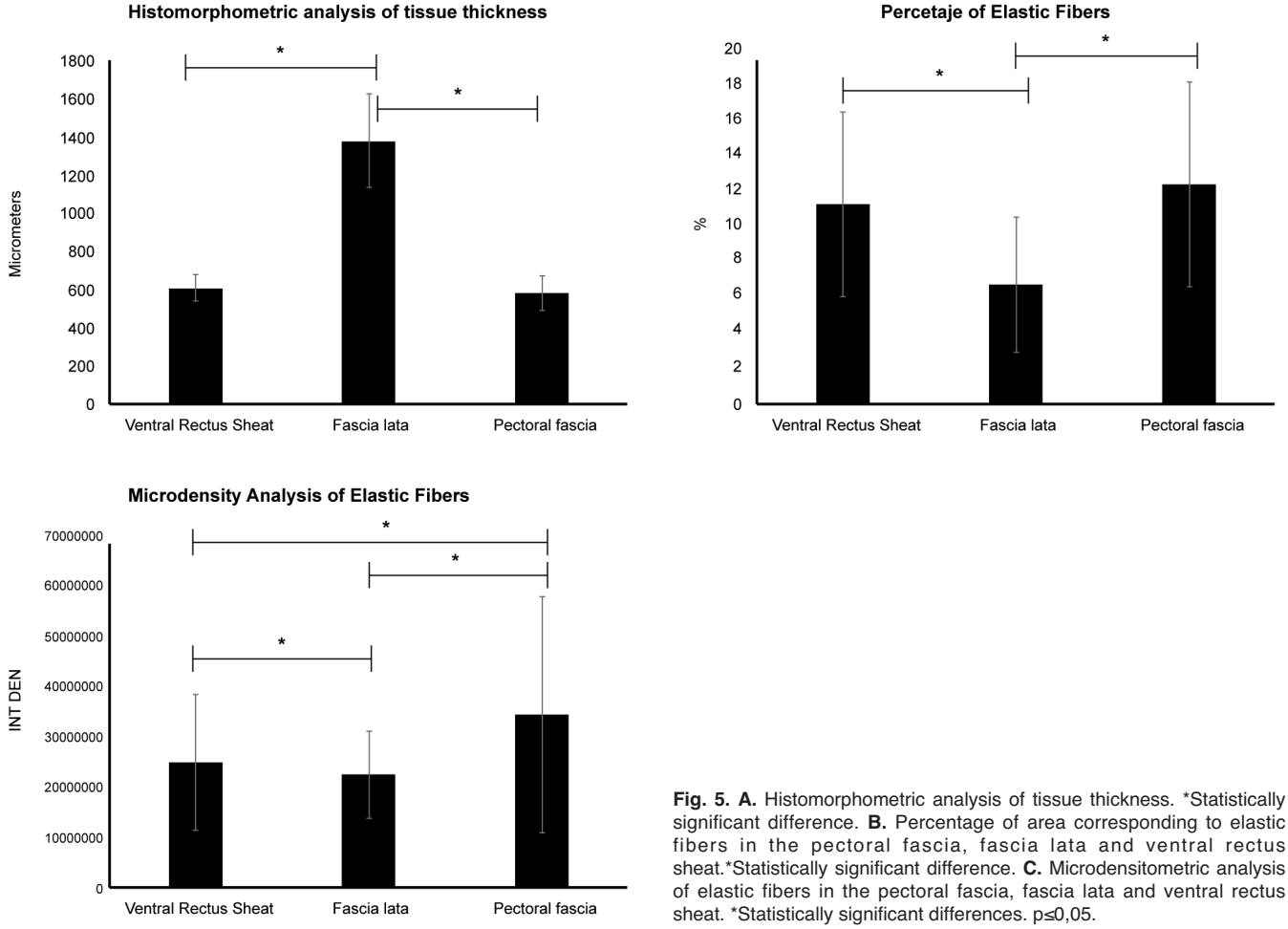

Fig. 5. A. Histomorphometric analysis of tissue thickness. *Statistically significant difference. **B.** Percentage of area corresponding to elastic fibers in the pectoral fascia, fascia lata and ventral rectus sheat.*Statistically significant difference. **C.** Microdensitometric analysis of elastic fibers in the pectoral fascia, fascia lata and ventral rectus sheat. *Statistically significant differences. p≤0,05.

(Ushiki, 2002). The elastic fibers are responsible for elasticity and resiliency (capacity to recover initial form) in tissues of many vertebrate species (Czirok et al., 2006), and play an important role in the healing of dermal lesions (Zheng et al., 2006). Collagen is the major component of the extracellular matrix. Therefore, it has a more native surface, which favors cellular attachment as well as being chemotactic to cells. Furthermore, collagen substrates can modify the morphology, migration and in certain cases the differentiation of cells. These properties of collagen emphasize its significance in tissue regeneration (Postlethwaite et al., 1978; Kleinman et al., 1981). The collagen fibers increase the strength of the connective tissue, while elastin fibers bring elasticity and allow the tissue to return to its previous shape after deformation (Szczesny et al., 2012).

Advances in knowledge of the physiology of elastic tissues help understand the pathogenesis of elastic tissue disorders. Many acquired disorders have not yet been well understood because few cases have been reported. Such disorders include elastoderma, linear focal elastosis and late-onset focal dermal elastosis (Milewicz et al., 2000; Kielty et al., 2002; Lewis et al., 2004; Thomas et al., 2008). Our research group considers particularly important the analysis of the elastic component of connective tissue due to its possible implications for some pathologies.

Pectoral fascia

The pectoral fascia is a thin lamina of connective tissue, relatively rich in elastic fibers, and it is firmly adhered to the underlying muscle (Stecco et al., 2009). The pectoral fascia is attached to the clavicle and sternum and covers the pectoralis major muscle; it is continuous inferiorly with the fascia of the abdominal wall and laterally with the fascia of the back. The pectoral fascia can be successfully dissected bluntly along the subfascial plane and varies in thickness from 0.2 to 1.14 mm (Jinde et al., 2006). In the present study, the thickness of the pectoral fascia was found approximately at the middle of that range. Stecco et al., (2009a,b) determined that the thickness of the pectoral fascia in the inferior thorax region was 579±42 μm, very similar to those found in our study results

Stecco et al., (2009a,b) performed a macroscopic and microscopic analysis of fascia from the regions of the anterior thorax in six cadavers (4 males, 2 females, mean age of 69 years). The following stains were used: H&E, Van-Gieson's for the elastic fibers and Mallory-Azan for the collagen fibers. The histological study revealed that the pectoral fascia appears to be formed by undulated collagen fibers and some elastic fibers in an irregular mesh. This study found that an epimysial fascia, or epimysium, is not discernible between this deep fascia and the underlying muscle and that the pectoralis major muscle does insert directly onto the pectoral fascia itself. Undulated collagen fibers, arranged more or less transversely with respect to the underlying muscle, form the pectoral fascia. An elevated number of elastic fibers were evident with the Van Gieson's stain. These fibers form an irregular mesh.

The estimated percentage of elastic fibers in that study, with respect to the collagen fibers, could be approximately 15%. In the present study the percentage of elastic fibers was slightly lower (12%). However, the results of the intensity of the signal emitted by the elastic fibers in the microdensitometric analysis, suggest that the thickness and density of the fibers in the pectoral fascia is high relative to the other two tissues studied.

Stecco et al. (2009a,b) performed a morphological comparison of the fascia lata and the pectoral fascia in 6 fresh cadavers using H&E, Van Gieson's and Mallory-Azan techniques, and performed a quantification of elastic fibers based on analysis of photographs of the slides treated with Van Gieson's stain. The study found that in the pectoral fascia an epimysial fascia, or epimysium, is not distinguished between this fascia and the underlying muscle, but many muscular fibers of the pectoralis major muscle are in continuity with the pectoral fascia itself. Undulated collage fibers, set at right angles to underlying muscular fibers, form the pectoral fascia. At Van Gieson's stain the elastic fibers are 14±1.2% and the collagen fibers are 86±1.2% (Stecco et al., 2009a,b). The results of our study broadly

Table 1. Morphological and semiquantitative analysis of the presence of elastic fibers in pectoral fascia, fascia lata and ventral rectus sheat.

Tissue	Hematoxylin and Eosin		Orcein stain		Verhoeff's stain	
	Axial Section	Longitudinal Section	Axial Section	Longitudinal Section	Axial Section	Longitudinal Section
Pectoral fascia	DRCT, muscle fibers	DRCT, muscle fibers	+++	+++	+++	+++
Fascia lata	DRCT, adipose tissue	DRCT, adipose tissue	+	+	+	+
Ventral rectus sheath	DRCT, adipose tissue muscle fibers	DRCT, adiposetissue musclefibers	++	++	++	++

DRCT. Dense regular connective tissue. DICT. Dense irregular connective tissue. LCT. Loose connective tissue. Absence of fibers (-), slim and thin fibers (±), few thick fibers thin to medium (+), thin fibers in moderate quantity (++), medium fibers or thick in moderate amount (+++) and thick and abundant fibers (++++).

extend those obtained by these two previous authors.

Fascia lata

Stecco et al. (2008) performed a histological study of the fascia lata in 72 samples from 6 fresh cadavers. Their studies showed that the deep fascia of the thigh is thinner in the proximal region and thicker near the knee (mean thickness 926±156 μn), this result was similar to the one found in the present study. Similarly, the study found that the fascia is composed of two or three layers of parallel collagen fiber bundles, presenting an undulating arrangement, form all the considered fasciae. Each layer was separated from the adjacent one by a thin layer of loose connective tissue that allows the different layers to slide one on the other. Adjacent layers show different orientations of their collagen fibers. In that study, some well-delimited bundles of muscular fibers were evidenced inside the fascia lata in one subject. On the external and internal sides of all of the considered fasciae, two thin layers (mean thickness 23 μm) of fibroelastic tissue were present. In the deep fascia of the inferior limb, the elastic fibers were evident only in the loose connective tissue between the different fibrous layers and in the periphery of the fasciae. Furthermore, numerous vessels followed rather tortuous paths through the different collagen layers of the muscular fascia. They had a mean caliber of 102.15±34 μm. Nerve fibers were found in all specimens of the deep fascia. They are particularly numerous around vessels, but are also distributed homogeneously throughout the fibrous components of the fascia. The bigger nerves are usually surrounded by loose connective tissue, while the small nerve fibers are connected to the collagen

The results of this study showed that the deep fasciae of the limbs could be divided into two components: the more internal and external layers show characteristics similar to that of the epimysial fascia, while the median layers show an aponeurotic aspect. Indeed the more internal and external layers are formed by small undulated collagen fibers and many elastic fibers embedded in abundant extracellular matrix, disposed in one thin lamina and rich in nerve fibers. In contrast, the median layers are composed of two to three layers of parallel collagen fiber bundles with few nerve fibers. Thin layers of loose connective tissue separate adjacent layers, allowing the single layers to slide one on the other (Stecco et al., 2008).

Stecco et al. (2009a,b) performed a morphological comparison of the fascia lata and the pectoral fascia in 6 fresh cadavers using H&E, Van Gieson's and Mallory Azan techniques as well as a quantification of elastic fibers based on analysis of photographs of the slides stained with Van-Gieson's staining. The study found that the deep fascia of the femoral region is a lamina of connective tissue similar to an aponeurosis. The collagen fibers form thick fibrous bundles arranged in two or three layers. In each layer, the fibers are parallel to each other, whereas, from one layer to the adjacent one, the orientation of the fibers varies. A thin lamina of loose connective tissue separates each layer. In one anatomical specimen, well-defined bundles of muscular fibers were found between these connective laminae (in the present study this was found in 2 specimens). At the internal and external surfaces of the fascia lata two thin laminae (mean thickness: 23±4 μm) of undulated collagen fibers, not grouped into bundles, were present. At the Van Gieson's stain, elastic fibers are present only in these two thin external layers, whereas they were lacking between the aponeurotic layers of the fascia lata. The histological stains demonstrate that the epimysial fascia (or epimysium) is a mono-layered structure formed by connective tissue and elastic fibers (Stecco et al., 2009a,b; Szczesny et al., 2012). In the present study elastic fibers were identified in the middle portion of the fascia lata, and we hypothesize that the difference between these studies is due to the increased sensitivity that orcein staining and Verhoeff's staining have for identification of elastic fibers. We think the use of these stains in future studies of connective tissues represents a better choice for the analysis of the elastic component.

Ventral rectus sheath

The rectus abdominis muscle sheath (RAMS) is considered representative of an individual's fasciae (Rath et al., 1996; Pans et al., 1999). Szczesny et al. (2013) performed a histological and immunohistochemical analysis of the concentration of elastin in the anterior sheath samples of the rectus sheath in obese patients and in healthy controls. They analyzed a total of 39 samples (20 obese patients and 19 healthy controls) with H&E staining and a monoclonal antibody directed against elastin, determining that in healthy controls the tissue consisted of dense and regular bundles of collagen fibers and only in some samples there was presence of adipose tissue. In the specimens obtained from the morbidly obese, the density of the fibers was lower and their architecture was disrupted; the bundles were thinner and less regularly arranged. Most photographs show adipose tissue infiltrating the structure of the fascia. On the immunohistochemical analysis, the area occupied by the elastin was significantly higher in healthy controls (8-15%) compared with obese patients (3-5%) (Szczesny et al., 2013). The results of our study (11.11%) are very similar to those reported by these authors in healthy control samples.

Fachinelli et al. (2011) performed a histological and immunohistochemical analysis of 30 biopsies from the linea alba aponeurosis in patients between 20 and 60 years of age who suffered anterior abdominal wall hernias and 30 fresh frozen cadavers who did not present any signs of degenerative diseases, hernias, scars or abdominal wall trauma as a control group. One section was stained with orcein and the other was submitted to immunohistochemistry with a monoclonal antibody to elastin. Digital images of 5 random fields of each slide were analyzed and the results between the two age

groups generated (20-40 years old and 41-60 years old) were compared. The results of the two techniques employed showed that there is a greater amount of elastin (35%) in patients with hernias compared with the control group, and that there are significant differences regarding gender in the patient group (higher results in women), with no differences between genders in the control group. Regarding age, no significant difference with either technique was demonstrated. In patients, the elastic fibers showed a fragmented, thickened and winding shape. In the control group, the elastic fibers were more defined and thinner (Fachinelli et al., 2011).

Axer et al. (2001) studied 12 anterior rectus sheaths (divided into 14 anatomic segments in cranio-caudal manner) stained with eosin and analyzed by confocal microscopy for 3D viewing, they observed three layers of oblique collagen bundles and muscle fibers underneath it. Similarly, this study's histomorphometric analysis determined that the ventral rectus sheat gradually becomes thicker in cranio-caudal sense, with values between 400-700 microns (Axer et al., 2001). These results are within the range of those obtained by the present study.

Szczesny et al. (2006) studied a total of 10 samples of rectus sheath from male patients undergoing inguinal hernia repair (n=5) and control group of patients undergoing open appendectomy (n=5) by using H&E techniques, Masson trichrome and electron microscopy. The results of this study showed alterations in the architecture of the connective tissue within the RAMS of patients with primary inguinal hernia (PIH). The structure of the connective tissue in the study group differed significantly from the control group. The alterations concerned both the direction and thickness of collagen fibers and the elastin content. In the unaltered tissue of patients from the control group, both collagen and elastic fibers are visible. Masson's staining enabled examination of tissue architecture. The structure of collagen is visible, as well as the positioning of elastin fibers. The collagen fibers form a regular pattern, and the elastin fibers are plentiful and clearly visible. Observations showed the elastin-to-collagen ratio of healthy tissue to be 1:2. The collagen fibers are regular and aligned longitudinally, forming tight bundles. The arrangement of the elastic fibers is regular as well, and they are abundant. Ground substance can be seen between the fibers. Healthy tissue contains little ground substance. In patients with primary groin hernia, the arrangement of the collagen fibers was found to be irregular, in most places chaotic. Collagen was less abundant than in healthy specimens. Little or no elastin was found. Ground matter was found to be overabundant in the connective tissue of the RAMS of hernia patients, and it infiltrated the spaces between the collagen fibers in place of the elastin fibers (Szczesny et al., 2006). Currently, our working group is conducting research studies in other human fascias with the methodology of this study with the aim of creating a morphological information bank of human fascias.

Conclusions

This study reports morphological characteristics not previously described in previous histological studies of the analyzed tissues. The results of the morphometric and densitometric analysis in this study reveal that the fascia lata has the fewest elastic fibers of all the tissues analyzed, and the pectoral fascia has the most.

The present study analyses in an exhaustive manner the morphological characteristics of the studied tissues with an innovative combination of techniques which allows a full description of the cellular and tissular components. We believe this type of study could be extrapolated to any connective tissue of the human and animal anatomy.

The information obtained in this study will be useful to scientists in need of healthy controls for morphological comparison between individuals with particular pathologies in which the etiology could be related to an alteration of the extracellular matrix components.

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