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Original Article

Histotechnological and socio-epidemiological evaluation of aorta aneurysmal and atheromatous lesions of in humans

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Abstract: Introduction: Aneurysms and atheromatous process are prominent pathological entities, commonly associated with significant morbidity and mortality. The present study aimed to evaluate socio-epidemiological and histomorphometric aspects in aortas impaired by aneurysmal and atheromatous process. Methods: Anatomical pieces from abdominal and thoracic aorta from cadavers presenting dissecting aneurysm and atheromatous process underwent histopathological, morphometric, ultrastructural and molecular procedures, altogether with a socio-epidemiological survey. Results: A higher prevalence of aneurysmal and atheromatous process was observed in men over women. Histopathological analysis identified that most cases presented collagen and elastic fragility. Morphometric analysis revealed that comparing the collagen fibers, the average number of the aneurysmal group pixels was lower than the control group. In ultrastructural analysis, dissecting aneurysm showed a rupture and fiber loss of uniformity which made up the vessel, above all of the collagen and elastin. Molecular analysis was unable to pinpoint mutations in sequences obtained from our samples. Conclusions: The atheromatous and aneurysmal process prevailed in men, with considerable collagen and elastic fragility in the aneurysmal group, however no polymorphism was detected in samples.

Keywords: Aorta, dissecting aneurysm, histopathology

Introduction

Aneurysms are important pathological entities, commonly associated with important morbidity and mortality rates, especially in recent decades. These entities are circumscribed dilation found in arteries or in cardiac chambers, characterized by progressive focal dilatation of the vessel wall, involving three layers: medium, intimate and adventitia, which it may evolve to a rupture or dissection [1]. It is considered an aneurysm when the vessel diameter is greater than 3 cm or 1.5-fold its original diameter [2]. It was demonstrated that, according to Laplace's law, the diameter augment of the aneurysm increases the surface of the aorta wall, producing an injury expansion, leading to the rupture [3].

The dissecting aortic aneurysm is characterized by the sudden and acute development of a laceration in the inner layer, which exposes directly the medial layer of the vessel. The blood penetrates into the middle layer separating it lengthwise, and therefore dissecting its wall. The space is filled up with blood between the dissected layers from the aortic wall make up a false lumen [4]. Thus, the aorta dissection can be understood as the delamination of its walls, which runs a virtual space between the adventitia and intima, being considered as a rare situation, of emergency heart surgery and potentially fatal due to the high risk of rupture [5-8].

The aorta artery can also be impaired by lipidic striae that can evolve in the long term, to atherosclerotic plaques. These plaques may leadi to atherosclerotic disease [9]. Thus, atherosclerosis is a chronic disease that affects the peripheral and central blood vessels, and it could be considered an inflammatory active

condition, whose lesions have long and gradual evolution [10]. Inflammatory cells are involved in the pathogenesis of atherosclerotic plaques rupture, through the weakening of the fibrotic plaque due to high activity of enzymes produced by these cells, which degrade the extracellular matrix [11-13]. This process is enhanced by the presence of cardiovascular risk factors such as smoking, alcohol consumption, sedentary lifestyle and fat excessive consumption, making the atherosclerotic disease one of the main causes of morbidity and mortality in adults all over the world [14, 15].

The thoracic aorta aneurysms that lead to an acute dissection are responsible for significant mortality, being classified as a rare situation of emergency heart surgery and potentially deadly, due to the dissection or rupture high risk of and it is in many cases associated with a wide range of conditions including hypertension such as Marfan syndrome (MFS) or syndrome of Loeys-Dietz 1 [5-8]. Approximately 20% of patients with abdominal aortic aneurysms (AAA) have a positive family history for aneurysms, suggesting a genetic predisposition for AAA in those families [16]. Soon, hereditary factors play an etiological role in the thoracic aortic aneurysm and dissection, with a number of specific genes which are to predispose to this condition [17]. Previous studies carried out in aorta dissecting aneurysm patients demonstrated that an association between mutations found in genes fibrillin-1 (FBN1) and receptor of transforming growth factor beta-1 (TGFBR1) and the various clinical manifestations of the disease [18]. Such gene encodes the protein fibrillin-1, which is a structural macromolecule present in all connective tissues. FBN1 is the gene involved in the Marfan syndrome, a hereditary disease of the connective tissue whose main characteristics include the thoracic aortic aneurysm and dissection [19].

Based on these data, the present manuscript had the objective of evaluating the main histomorphometric, epidemiological, ultrastructural and molecular aspects in aorta arteries impaired both by the atheromatous process and the aneurysm.

Materials and methods

Place of experiment and ethical aspects

Tissue samples were only obtained after the consent of the responsible-legal from the cadaver, through the informed consent form. Cadavers (n = 33) were necropsied from the Deaths Verification Service, organ from the Department of Health from Pernambuco State located in the department of Pathology from the Federal de University (UFPE), thourghout the year of 2014. This research was approved from the Research Ethics Committee from the UFPE, according to letter no. 133/2010.

Aortas processing

After collection, the anatomical pieces were soaked into buffered formalin at 10%, in a final volume with 20-fold the volume of the material. Histological processing was performed in a time-window of up to 72 hours. In parallel, the intensity of atherosclerosis was analyzed macroscopically, and classified as mild, moderate or severe. The aortas were evaluated with the support of a standardized scale from 0.0 to 12.0 cm, being considered mild from 0.1 to 4.0 cm, moderate to 4.1 to 7.0 cm and severe from 7.1 to 12.0 cm. The atheromatous plaques extension was used as a reference for scoring the degree of involvement of the aortas studied [20].

Histochemical study

For this analysis, 15 fragments were obtained for dissecting aneurysm and atherosclerosis. Fifteen aortic samples with no anatomical changes (n = 15) were obtained for negative control purposes. After the correct setting of tissue in 10% formalin, histological sections were obtained (4 μm) through horizontal microtome Yamato (Japan) and then mounted on histological slides previously identified, in a total of n = 106 slides. These slides were subjected to hematoxylin-eosin (HE) staining batteries for histopathological analysis of the aortic tissue, Orcein and Picro-sirius for elastic and collagen fibers analysis, respectively.

Histopathological analysis

The stained tissues were submitted to histopathological analysis by selecting 10 fields on each slide, where it was evaluated the inflammatory profile, focal points of necrosis, hemorrhage, attempted repair, distribution of glycosaminoglycans, through the characteristic patterns of each dye which was used in the procedure. Images recording were performed through a capture system with a camcorder camera connected to a microcomputer and in turn to an optical microscope.

Through the program of image capture Motic image Plus 2.0, it was made a preliminary selection and storage of areas of interest in which were subsequently evaluated morphometrically using the software GIMP 2.0.

Ultrastructural analysis

For this analysis, fragments were obtained from 4 cases, being 2 from the negative control aorta artery and 2 with dissecting aneurysm, fractioned and fixed in glutaraldehyde (GA) 2.5% + Paraformaldehyde (PFA) 4% + cacody late buffer 0.1 M, pH 7.4, in 2 hours or overnight at 4°C, each sample was then washed with the same buffer 3 times for 10 minutes each. Samples were post fixed in (OsO_4) 2% + calcium chloride to 5 mM and Potassium ferrocyanide 0.8% in cacody late buffer for 1 hour in a dark camera. After this process, samples were washed with distilled water for 10 minutes, was and then transferred to a permeable basket, to be used in the equipment for critical point.

Samples were dehydrated in ascending series of acetone 30%, 50%, 70%, 90%, 15 minutes each and 100% three times, 20 minutes each. Samples were transferred to the critical point apparatus, where a number of replacements with carbon dioxide (CO_2) were performed. After this phase, the drying was performed using the critical point method, it was made the removal of the dry part and mounted on Stub (catalytic microscope), in this phase it was performed the gold cover, and then the sample was observed in scanning electron microscope, through the analysis in scanning electron microscopy (**Figure 4C** and **4D**).

Molecular analysis

For this analysis, anatomical parts from the abdominal and thoracic aorta were evaluated of cadavers aged between 55 to 96 years with dissecting aneurysm and atheromatous (n = 33). All obtained samples were sectioned at around 1 cm immediately after the death, with a sterile scalpel and placed in deep freezer at -80°C prior to performing the procedure. Out of the 65 exons present in the FBN1 gene (N_M000138.4), the exon 2 was selected for molecular analysis, and exon 1 (out of 7 present in the gene) was selected for the study of TGFBR2 gene. The PCR primers and reaction details are found elsewhere [18]. DNA extractions were performed individually, using the DNeasy_Blood_&_Tissue_kit (Qiagen®), following the manufacture's protocol. After extraction, each DNA sample was quantified in a Nano_drop2000c (Thermo_Scientific®) spectrophotometer and subsequently stored at -20°C.

Each PCR reaction contained 200 μM of each dNTP, 1.5 mM $MgCl_2$. 10 pmol of each primer, 1 U Taq polymerase (Invitrogen) and approximately 20 ng of DNA template. The reactions were performed in a T3 Professional® (Biometra) programmed as follows: a cycle of 94°C for 5 min; 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 2 s; and a final cycle at 72°C for 10 min. The amplified products were separated and identified by electrophoresis in agarose gel, stained with ethidium bromide and photographed on a U.V. transilluminator.

PCR products were cleaned up, using the illustra GFX PCR DNA and Gel Band Purification® kit (Amersham Pharmacia Biotech) and subsequently quantified in Nanodrop_2000c® (Thermo Scientific). These purified products were submitted to sequencing reactions in the capillary sequencer ABI capillary 3100 (Applied Biosystems). Both strands from each sample were sequenced. Sequences obtained were edited, analyzed and aligned with the program CodonCode Aligner program v. 3.7.1.

The identity of each of the sequence was confirmed through the BLAST tool, which allows the comparison of sequences from the present study with other previously deposited in the NCBI database (National Center for Biotechnology Information).

Statistical analysis

Epidemiological and Clinical Aspects: Information relating to gender, age, anatomical parts of aorta and related diseases were obtained by analyzing the medical records of patients. Statistical analyzes were performed with SPSS version 13.0 and software Epi-Info version 7.0. For the evaluation of the differences between means, it was used t-Student test for unmatched data. The level of significance in the decision of the statistical tests was 5.0%.

Histotechnological analysis: The quantification of protein fiber (in pixels) in the wall for each type of aorta lesion was determined with

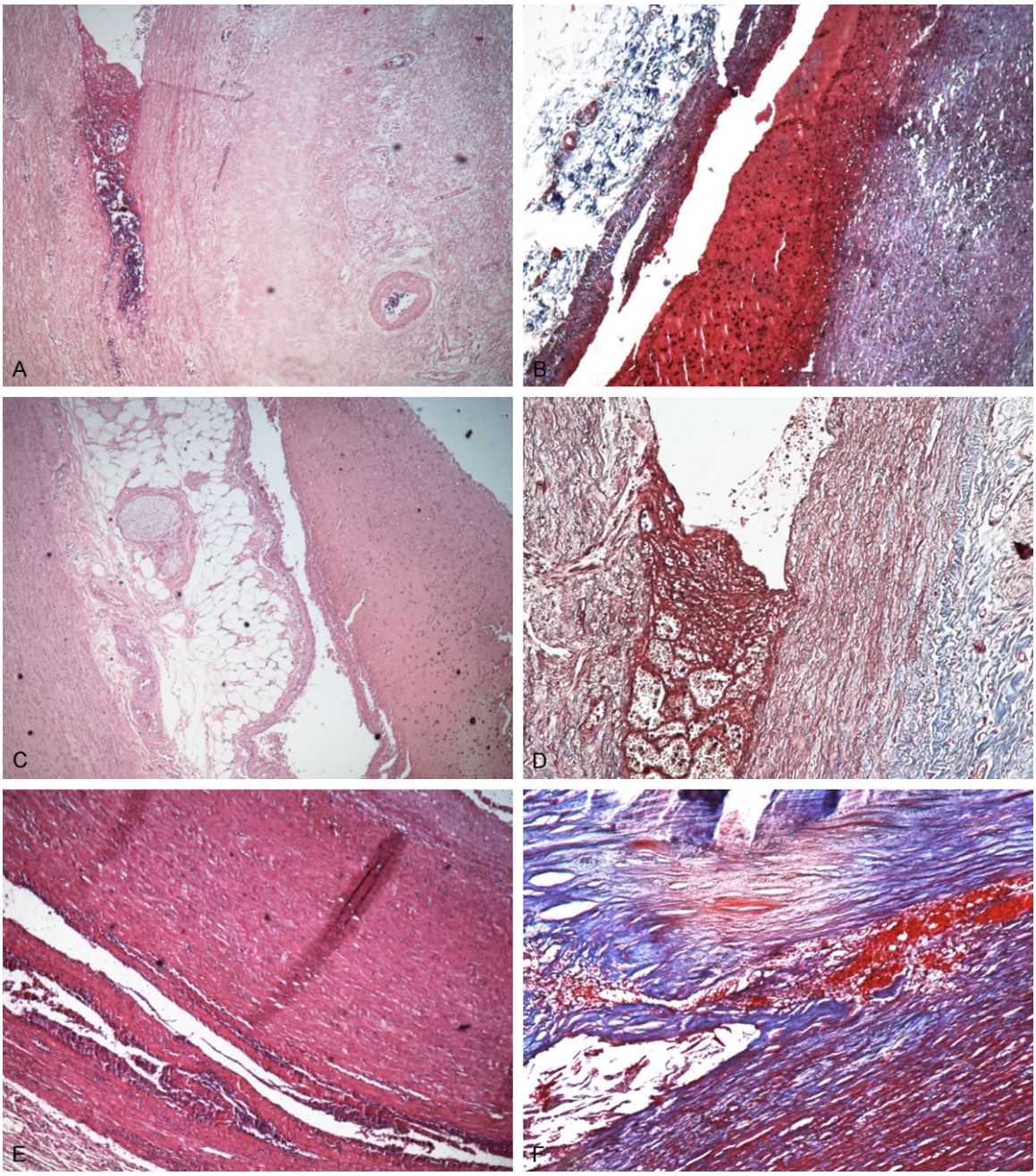

Figure 1. Histological aspects of aortic dissecting aneurysm cases. A. Presence of blood collection in H.E staining in 4×; B. Presence of blood collection in Masson's trichrome staining in 4×; C. False lumen formation in H.E staining in 4×; D. Formation of the false lumen in 4× Masson trichrome staining; E. False lumen formation and blood collection in H.E stain in 4×; F. Formation of false lumen and blood collection in 4× Masson trichrome staining.

Software GIMP 2.0. Data from the digital morphometric study were analyzed using the paired Student's t test with significance level of 5% ($P < 0.05$) by means of the GraphPad PRISM® 5.0 software.

Molecular analysis: The software OriginPro8 (USA) was used for the statistical analysis and data were expressed as mean ± standard deviation. Continuous quantitative variables related to sequence of the gene FBN1 and TGFBR2 were tested regarding the character of normality using the Kolmogorov-Smirnov test. Comparisons analyses were performed using parametric statistic test of Tukey ($P < 0.05$) through SigmaPlot (USA).

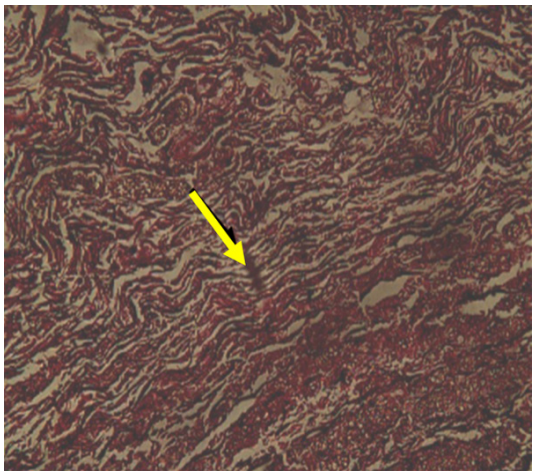

Figure 2. Arrow indicating the disorganizations and irregularity of the elastic fibers in aneurysmal aorta of corpses in orcein staining (orcein 100×).

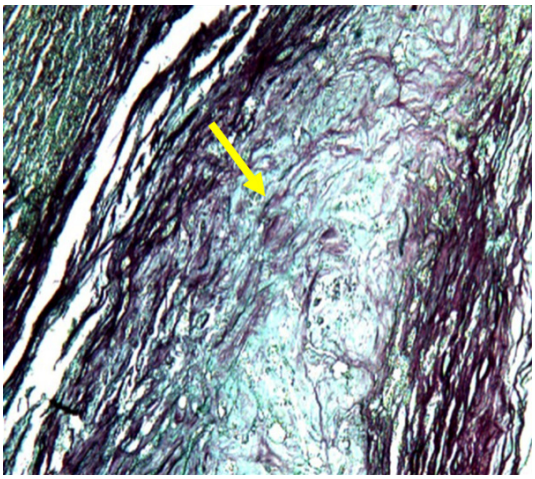

Figure 3. Arrow indicating the histologic aspect of the aorta artery affected by an atheromatous plaque. Stain of (Picro-sírius 100×).

Results

Epidemiological aspects

Anatomical parts (n = 30) were obtained from the abdominal and thoracic aorta from cadavers aged between 55 to 96 years with dissecting aneurysm and atheromatous process (n = 15), and samples from the aorta with no anatomical changes (n = 15) as a negative control. Out of the 15 dissecting aneurysm aortas, 60% (9/15) belonged to males, as opposed to 40% (6/15) to the female sex. Grouping all phenotypes, 11 cases (73.33%) had as background the hypertension, followed by 13 cases of smoking (86.66%), 8 cases of alcoholism (53.33%), 1 of diabetes (6.66%) and four cases of heart disease (26.66%) (**Table 1**).

In (n = 13) 86.6% of all cases studied exhibited smoking, as a personal history, followed by hypertension (n = 11) 73.3%. Out of 60% of the cases of dissecting aneurysms that occurred in men (n = 6) cases 66.66% was hypertensive, (n = 9) cases 100% of smokers (n = 5) cases 55.55% of alcoholic, (n = 1) case 11.11% of diabetic, (n = 2) cases 22.22% of cardiac patient. The average age among men was 71.2 years, whereas the average age among women was 74.1 years, and of these 83.3% with hypertension (**Table 1**).

Histotechnological analysis

Out of the (n = 15) aortas impaired by dissecting aneurysm, (n = 13) cases 86.66% exhibited collagen and elastic fibers fragility, fatty deposits and the presence of foamy cells, in addition to a sharp tearing of the vessel with the presence of the false lumen and blood infiltration (**Figure 1A-F**).

The atheromatous impairment was also checked at a macroscopic level in the arteries selected in the study, presenting itself in a general way with intensity from moderate to intense. The necrosis was present in these (n = 13) dissecting aneurysm cases, indicating a deterioration and destruction of the aortic tissue, especially due to the presence of a column of blood at the site where there was a tearing.

In our study (n = 11) cases, 73.33% presented, by orcein staining, a disorganization and fragmentation of the fibers (**Figure 2**).

Through Picro-sirius staining, (n = 11) cases 73.33% exhibited the presence of the fragmentation of collagen fibers all over the tissue, weakly birefringent and greenish, besides the presence of fat deposit (**Figure 3**).

The morphometric study, based on the analysis of image selected in advance, allowed comparing the average distribution of collagen and elastic fibers in the connective tissue.

From the morphometric data, comparing the collagen and elastic fibers, no statistically significant differences were found, although the average quantity of collagen fibers of the aneurysm group was lower compared to the control group while the elastic fibers group was greater than the control group (**Table 2**).

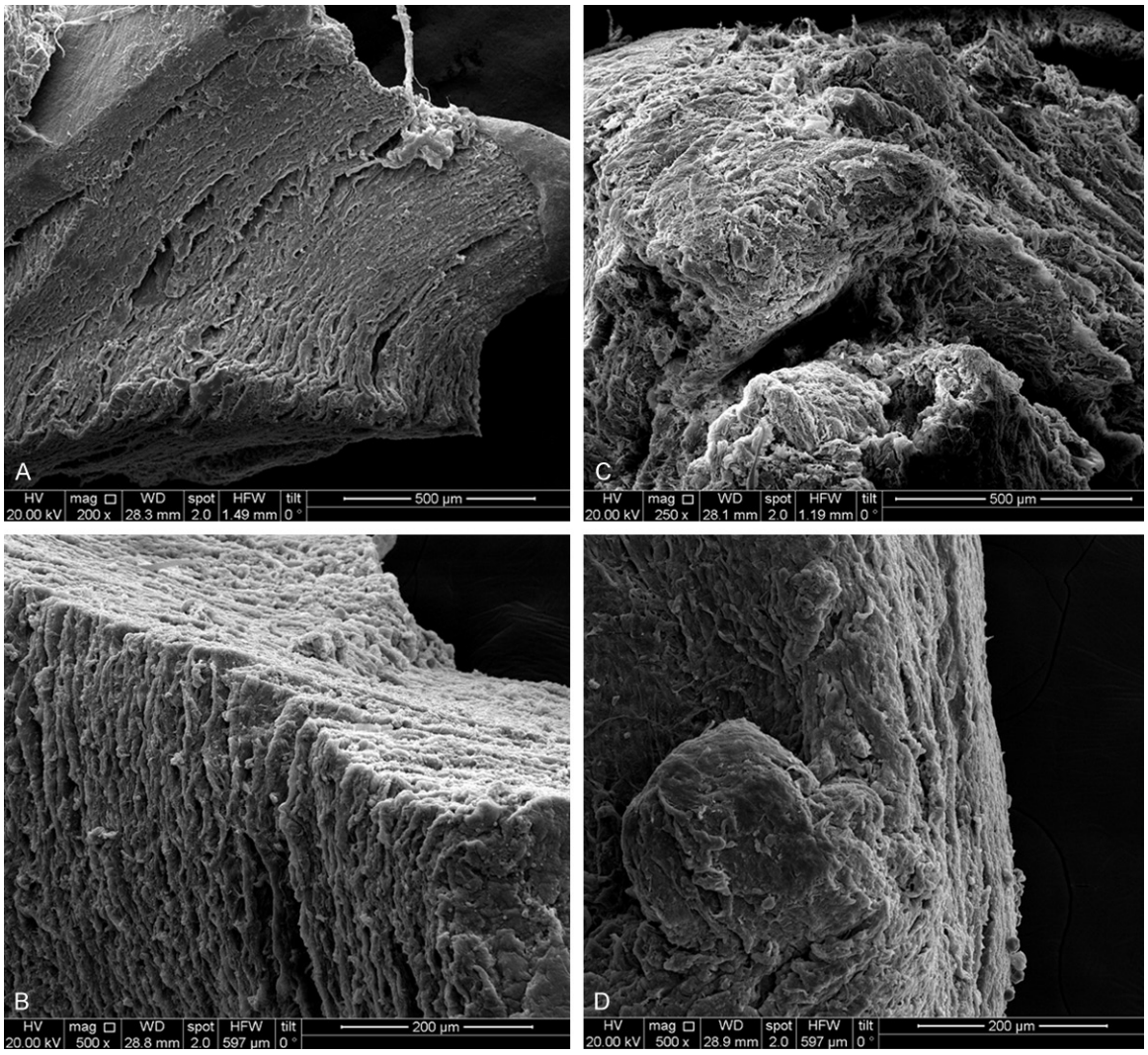

Figure 4. Ultrastructural analysis of normal and aneurysmal aortas. In (A and B) it is observed the arrangement of the layers of the aortic wall with the uniform provision of collagen and elastic fibers. In dissecting aneurysm there is rupture and loss of uniformity of fibers resulting in fragility of the vascular wall (C and D).

Ultrastructural analysis

In normal aorta it is observed the uniform provision of collagen and elastic fibers (**Figure 4A** and **4B**). In dissecting aneurysm there are rupture and loss of uniformity of fibers which make up the vessel, above all, the collagen and elastin, resulting in the vascular wall fragility, events that trigger the dissection of the vessel wall (**Figure 4C** and **4D**).

Molecular analysis

Sequences obtained here from the FBN1 gene were aligned among themselves and with the FBN1 reference sequence o (NG_008805.2). As the complete gene FBN1 presents approximately 245 kb, only the nucleotides between the positions 37.583 and 37855 bps were used for the alignment of this study (Data not shown).

The purified DNA specimens from 1 cm of the biological material presented concentrations ranging from 20 to 400 ng/µl. This variation may have been a reflection of two biases: the conservation status of each sample and the amount of biological material contained in the fragment sectioned of the aorta. The amplification of the genes FBN1 and TGFBR2 generated a fragment of 272 bp and 299 bp, respectively corresponding to the expected sizes of the frag-

Table 1. Epidemiological profile regarding sex, age and co-morbidities of corpses affected by dissecting aneurysm

Cases	Sex	Age	CO-morbidities
Case 1	Male	75	Hypertension, diabetes, smoking, alcoholism, cardiac patient.
Case 2	Female	80	Hypertension
Case 3	Male	62	Hypertension, smoking, alcoholism
Case 4	Male	82	Hypertension, smoking, alcoholism
Case 5	Male	76	Smoking
Case 6	Male	63	Hypertension, smoking
Case 7	Female	96	Hypertension
Case 8	Male	76	Smoking
Case 9	Female	62	Smoking, alcoholism
Case 10	Female	64	Hypertension, smoking, alcoholism, cardiac patient.
Case 11	Male	61	Hypertension, smoking, cardiac patient.
Case 12	Male	91	Smoking, alcoholism
Case 13	Male	55	Hypertension, smoking, alcoholism
Case 14	Female	64	Hypertension, smoking, alcoholism, cardiac patient.
Case 15	Female	79	Hypertension, smoking

Table 2. Average distribution* of collagen and elastic fibers in aneurysmal aortas of corpses

Group	Connective tissue (Fibers)**	
	Collagen	*Elastic*
Aneurysm	110331.8 ± 7254	119164.9 ± 7365
Control	156592.1 ± 8534	92251.6 ± 3014
	Value of $P = 0.122$	Value of $P = 0.179$

*Pixel values (total area per field = 12234 μm^2).
**Mean ± standard deviations (paired t test, $P < 0.05$).

ments. After sequencing, the sequences obtained from the fragments from the gene FBN1 were edited and aligned with each other along the sequence of reference gene FBN1 (NG_008805.2). As the total size of gene FBN1 is approximately 245 kb, only the nucleotides between the positions 37.583 and 37855 bps were used for the alignment of this study. The alignment showed that, regardless of the biological sample under study, all of the sequences obtained for this fragment were monomorphic. Besides monomorphic among themselves, it was not also possible to observe mutations in our sequences when compared to the reference sequence of the gene. Unfortunately, the sequences obtained for the fragment concerning the gene TGFBR2 did not exhibit satisfactory quality and were therefore excluded from the final analysis.

Discussion

The general mean age in our casuistry was 72.4 years in line with recent data from the literature that indicate a incidence peak of aortic dissection in the sixth and seventh decades of life. In addition to that, there is a higher prevalence of men affected by dissecting aneurysm [21, 22]. In Brazil, incidence studies of abdominal aneurysm, but information on mortality is still scarce. A study carried out in São Paulo-Brazil revealed a significant increase in mortality rates by standardized aneurysm and aortic dissection, from 1985 to 2009, in which the rate of mortality by dissecting aneurysms and ruptures were 2.86 for men and 2.19 in women by 100,000 inhabitants [23].

The change of connective tissue present in the aortic wall plays an important role in the development of aneurysms because the elastic and collagen fibers are the major determinants of mechanical properties of the aorta. Necrosis is an evident process in the middle layer on the aortas impaired by dissecting aneurysm [3].

Atherosclerosis has been directly associated with the degeneration of the median layer in aortic aneurysms, leading to a dissection condition [24]. According to histopathological findings, a study confirms that the aneurysm is an insidious chronic disease that corresponds to

an extension segment of the vessel wall, including its three layers, where the middle layer forms a false light which can be seen in histological preparations [25].

According to the literature, the elastic fibers, given this context are fragmented and irregular and in some areas absent with large areas of basophilic accumulation and an apparent reduction in the number of smooth muscle cells [25]. In another recent study, the morphological comparison of aneurysmal and non-aneurysmal aortas showed that at the aneurysmal wall presents a rupture of the linearity of elastic fibers in the middle layer and of the collagen structure both in the tunica media and adventitia. In addition, several inflammatory cells were located around the peri-vascular spaces of the vasa vasorum [3].

In the literature the Picro-sirius staining method is applied to the histopathological diagnosis of collagenolysis due to the fact that this technique detects morphologically, not only the presence of intact bundles of collagen, but also fragmented ones. Regarding the aneurysmal process there are drastic morphological changes in bundles of collagen exhibiting themselves disorganized and weakly stained. One of the most important histologic characteristics of aneurysmal tissue is the elastic fibers fragmentation and a decrease in the concentration of elastin during the aneurysm growth until its eventual rupture [3].

The collagen component is responsible for the physical strength of the wall that would also suffer degradation, especially of metalloproteinases. The fact of this component fibrillate being in a smaller quantity could explain the easier fragility of the aortic wall in this region. The blood pressure constantly applied on this wall, already weakened and consequently presenting a lower resistance to mechanical forces, could have resulted in tearing and dissection [25]. The elastin and collagen alterations are reflections of the consequence of the production of proteases by the artery wall cells, such as the smooth muscle cells in the media layer, fibroblasts and inflammatory cells. Proteases and, namely, metalloproteases (MMP), are closely bounded with aneurysm [26].

Studies of electronic microscopy addressing the main characteristics at the ultrastructural level of the dissecting aneurysm are scarce. According to studies, the aorta histological structure with atherosclerosis showed fragility in terms of lamellar construction on its wall [27].

Because of the high rate of mortality associated with acute dissection of the ascending aorta being approximately 40% to 50%, it is crucial not only to identify patients with aortas and amplified ascending ones in order to try and slow down the growth using medical treatments, but also to intervene surgically in an elective way before dissection occurs [28]. The knowledge of the likely dissection risk for a given condition and aorta diameter is primordial. The surgery is usually recommended in cases in which the diameter exceeds 5.0 to 5.5 cm; however, a large proportion of dissections may occur in smaller diameters, and even in patients without dilatation [29].

The study of the gene FBN1 provides information that enables healthcare genetically customized and provides the identification of new mutations responsible for the aortic pathology [17]. Recent results have demonstrated that the detection of genes can contribute to discover the real cause of the abdominal aorta aneurysm and help to identify precisely relatives at risk [30]. The genetic predisposition for aortic aneurysm was established, and the discovery of genes in affected families has identified several major categories of genetic modifications [6].

In patients with Loeys-Dietz syndrome, which is caused by mutations in the transforming growth factor, in the genes type I or II receiver (TGFBR1 and TGFBR2), dissections may occur with little aortic growth [28]. Up to now, the genes identified in the TAAD have mainly been those associated with the smooth muscle maintenance with contractile function, including the TGFBR2 autosomal dominant [28]. Individuals have already been identified with mutations in the gene TGFBR2 featuring the aneurysm fusiform type. In families who also have members with AAAs, screening for AAAs is also recommended. Ultimately, identification of defective genes means that only family members who shelter a mutant gene are the ones who must be submitted to surveillance for such aneurysms [31].

Conclusions

Over the epidemiological data, there was a greater prevalence of men affected when compared to women in line with several studies published; being the smoking 86.6% followed by hypertension with 73.3% the most relevant personal history. The atheromatous process is related in many cases with the dissecting aneurysm of the aorta, because, through the macroscopic study that was conducted, we observed impaired aorta arteries both with atheromatous process and the aneurysm.

In dissecting aneurysm there are ruptures and loss of uniformity of fibers which make up the vessel, above all, the collagen and elastin, resulting in a fragility of the vascular wall, i.e., out of the (n = 15) aortas affected by dissecting aneurysm, 86.66% had fragility of the collagen and elastic fibers besides a sharp tearing of the vessel with the presence of the false lumen and infiltration of blood. 73.33% exhibited through orcein and Picro-sirius staining a disorganization and fragmentation of the elastic and collagen fibers, respectively.

We also observed that the hemorrhage and the dissection process are present in most of the cases evaluated in addition to necrosis areas, a fact that we found in the histopathological and morphometric study. In the latter we quantified the average value of pixels by comparing two types of staining. We verified that in the aneurysm group the mean values in pixels per area was close, revealing a provision characteristic of collagen and elastin concerning the aneurysmal process.

Unlike in the control group we observed a variation in the average number in pixels and this fact can be attributed to the staining marking at the time of the histological preparation. Comparing the two groups concerning the collagen it can be observed closer proximity to the value of $P < 0.05$ compared with the elastic fibers. Even in the case of collagen fibers, the average amount of pixels of the aneurysm group was lower comparing with the control group. On the other hand, when it comes to the elastic fibers, the average amount of pixels of the aneurysm group was lower than the control group.

Regarding the ultrastructural study, we found that at the normal aorta there is uniform arrangement of the collagen and elastic fibers, while at the dissecting aneurysm rupture and loss of uniformity of fibers occurred, especially the collagen and the elastin.

The final result of the molecular analysis demonstrated the absence of mutations among the sequences obtained from our samples. In addition, the comparison between the reference sequence and our sequences also showed the monomorphism of this fragment. The alignment of the sequences revealed that the fragment analyzed exhibited no polymorphisms, since so many of the normal individuals and patients showed the same nucleotide sequence. There is intention to sequence the TGFBR2 gene.

Disclosure of conflict of interest

None.

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