Received: 24 May 2022 | Revised: 2 August 2022 | Accepted: 3 August 2022

DOI: 10.1002/ar.25058

FULL LENGTH ARTICLE

The Anatomical Record WILEY

# Ligamentum arteriosum: Muscular and contractile

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**Funding information**
equal opportunity office for mothers in research, Justus Liebig University, Giessen, Germany.; Ghanaian-German Postgraduate Training scholarship from German Academic Exchange Service, or Deutscher Academics Austauschdienst (DAAD

## Abstract

Ductus arteriosus is a muscular artery in fetal circulation, spanning from the bifurcation of the pulmonary trunk to the aortic arch, shunting blood directly from pulmonary circulation into systemic circulation thus by-passing the fluid-filled lungs. Postnatally, it changes name to the ligamentum arteriosum (LA), when a cascade of anatomical and physiological processes leads to its closure. Though the LA has generally been considered as a fibrosed remnant of the ductus arteriosus, anecdotal and contradictory reports still describe the LA as a small muscular artery. We hypothesized the likelihood of contractile muscular elements retainment in this so-called ligament. To investigate this, mediastinum of wild-type mouse, pig, and human LA were subjected to routine and special histological staining, single-immunolabeling, electron microscopy (mouse and pig only), and tension recording of explanted pig LA in organ bath experiments. Contrary to a canonical ligament, the LA was mainly made up of α-smooth muscle actin-positive cells in all three species, confirmed by routine and special histological staining as well as transmission electron microscopy. Myocytes within the LA contracted in response to exogenous noradrenalin (NA). NA-induced precontracted LA relaxed upon administration of the α1-adrenergic blockers (prazosin and tamsulosin). Though the LA does not function in its original capacity as fetal shunt, it is clearly not a passive structure, and may be described as muscular and contractile. The contractile abilities of LA myocytes may act on the two great vessels to which it is attached causing a change in their distensibility.

**KEYWORDS**

contractility, ductus arteriosus, ligamentum arteriosum, smooth muscle, α-smooth muscle actin

## 1 | INTRODUCTION

The ductus arteriosus is the embryonic connection between the pulmonary trunk and the aortic arch which allows oxygenated fetal blood to be shunted from the right ventricle straight into systemic circulation by-passing pulmonary circulation. Although the ductus arteriosus directly connects two elastic arteries, it is a muscular artery (Chuaqui et al., 1977; Toda et al., 1980). The first report of a structure allowing for the passage of blood from the left to the right side of the heart was by Galen in his "De Usu Partium" in the second century when he wrote: "*This is the explanation of the communication of the vena cava with the vein-like artery [i.e., our pulmonary veins and left atrium] during fetal life. In as much as this latter vessel was then acting as the venous supply of the lung, its companion [i.e., the artery-like vein, or our pulmonary trunk and its branches] had to act as the arterial supply of that organ, and nature therefore made a communication between it and the great artery [i.e., the aorta]. As, however, the two vessels were separated by a short distance, a small third vessel [i.e., the ductus] was created by nature to unite them*".

The ductus arteriosus is no longer needed for lung bypass after the newborn takes its first breath thus postnatally, fetal circulation must swiftly make some adjustments to enable transition into extra uterine life without any complications. This often requires the fetal bypass undergoing complete obliteration and fibrosis shortly after birth and the remnant of this by-pass forming the ligamentum arteriosum (LA) (Chiruvolu & Jaleel, 2009; Clyman, 2006; Clyman et al., 1999; Heymann & Rudolph, 1975). This closure/obliteration is both anatomical and physiological in nature and its relevance cannot be downplayed as failure to close is abnormal and leads to patent ductus arteriosus, a congenital condition characterized by diverse disorders within the cardiopulmonary vascular system leading to increased mortality in babies (Friedman & Fahey, 1993; Morton & Brodsky, 2016; Poeppelman & Tobias, 2018).

Physiologically, the overlapping of two processes, that is, increase in the level of arterial oxygen after first breath of baby and significant decrease in prostaglandin E2 leading to inability to sustain prenatal patency leads to the obliteration of the lumen of the ductus. This usually happens hours after birth. Anatomically, there is the formation of cushions by the intima cells, followed by infiltration of the subendothelial space with collagen, fibronectin, and laminin, then the fragmentation of the internal elastic lamina and finally smooth muscle cell migration from the tunica media layer into the subendothelial space. From this point, permanent closure is achieved through structural remodeling and fibrosis of the tunica intima and media (De Reeder et al., 1989; Ho & Anderson, 1979; Yoder et al., 1978). Initial physiological changes together with initiation and maintenance of cascade of the described anatomical occurrence are what ultimately lead to permanent ductus arteriosus closure (Coceani & Baragatti, 2012).

Though the LA has generally been considered as a fibrosed remnant of the ductus arteriosus, there have been contradictory anecdotal reports, reporting the persistence of smooth muscle in the structure (Dohr et al., 1986; Garcia, 1975). Furthermore, no known textbook, publication, or descriptive anatomy brings to bear the gap in knowledge regarding the ultrastructural arrangement of cell layers within the LA or its reactivity to immunostaining. Based on this premise, we hypothesize that LA, the remnant of the fetal muscular artery (ductus arteriosus) which connects the main lung (pulmonary trunk) and main body (aorta) arteries during embryological circulation, is not a mere fibrotic remnant resulting from the complete metamorphosis of the ductus arteriosus but may retain some of its contractile muscular elements. It may be postulated that the contractile abilities of myocytes within the LA, if present may act on the two great vessels to which it is attached thereby causing a change in their distensibility.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics approval statement and samples used

#### 2.1.1 | Animal welfare

This study was carried out in accordance with the recommendations of the European Communities Council Directive of November 24, 1986 (86/609/EEC). The protocol was approved by the local authorities, that is, Regierungspräsidium Giessen, Germany (reference no. 571_M). The body donors sign an official testamentary disposition protected by the *Art. 6 Para. 1 a of the European General Data Protection Regulation* to leave their body to the anatomy institute after death for the training of young doctors, and further training of (specialist) doctors and science (https://www.unigiessen.de/fbz/fb11/institute/anatomie/koerperspende). A formal written approval from the university was also obtained prior to acquisition of pig samples from the commercial abattoir which had an in house veterinary doctor to ensure the right protocol was followed. See Table 1.

### 2.2 | Mouse

Wild-type C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France, Cat#5751862, $n = 8$) were housed under

specific pathogen free conditions (10 h dark, 14 h light) with free access to food and water. The study was carried out in accordance with the recommendations of the European Communities Council Directive of November 24, 1986 (86/609/EEC). The protocol was approved by the local authorities, that is, Regierungspräsidium Giessen, Germany (reference no. 571_M). All samples were taken after mice were killed by inhalation of an overdose of 5% isoflurane (Abbott, Wiesbaden, Germany) and exsanguination through abdominal blood vessels.

## 2.3 | Pig

Mediastinal blocks of pigs were acquired from a commercial abattoir. The abattoir had a qualified veterinary doctor who thoroughly examined the pigs after the slaughter. The pigs were only females ($n = 60$) aged between 6 months to 1 year. The mediastinal blocks were kept on ice and transported to the laboratory in an ice cooler immediately. The LA was dissected and excised from its attachments to the aorta and pulmonary trunk (Figure 1). The samples to be used for organ bath experiments were immediately put in minimum essential medium (MEM, 51200-046, Thermo Fisher, Germany) mixed with 1% penicillin and streptomycin (P4353, Sigma-Aldrich, Germany).

**TABLE 1** Description of sample characteristics

| | Age | Sex | Number |
|---|---|---|---|
| Human | 67–97 years | Males & females | 10 |
| Pig | 6 months to 1 year | Females only | 60 |
| Transgenic mouse | 6–20 weeks | Males & females | 18 |
| Wild-type mouse | 8–30 weeks | Males & females | 20 |

## 2.4 | Human

Human LA from body donors of the anatomy dissection course in the Justus Liebig University was dissected and excised with its attachment to the two major vascular segments to which it is attached. There were six females and four males aged between 67 and 97 years. Registration as a body donor in Germany is only possible from the age of 60. The ligaments were immersed in Zamboni fixative for 1 day to refix them even though they had already been fixed for the dissection course with a fixative (comprising of 10 L of 90% ethanol (57%), 1.3 L of 37% formalin (3%), 0.8 L of phenoxyethanol (5%), and 0.8 L of glycerin (5%)). These were all added to 3 L of water. In four out of 10 samples, there was the presence of a funnel with partial patency, not patent through the

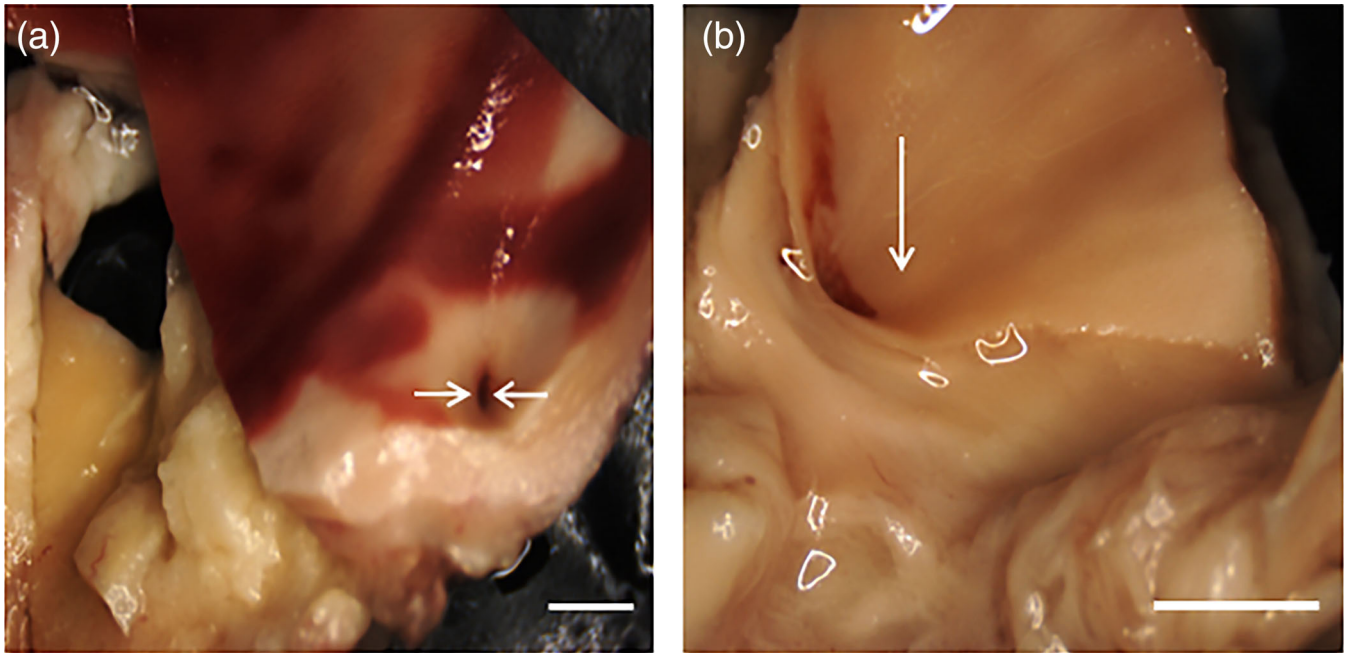


**FIGURE 1** Samples of human LA obtained from dissection course, yet to be subjected to histological processing showing the presence of a funnel (white arrows) with partial patency from the part of the LA connected to the aorta. Scale bars = 50 μm. LA, ligamentum arteriosum

whole LA but from the part of the LA connected to the aorta. This opening therefore could not serve as passage way for blood flow from aortic to the pulmonary side (Figure 1). The embalming process of donors takes between 2 to 4 h after its arrival at the institute. After embalming, the donors usually remain in the anatomy department for 2–3 years, unless in the case of registration as a permanent donor, which stays in the long term. None of the cadavers used were permanent donors and had been embalmed for 3 months prior to the start of the dissection course.

## 2.5 | Routine and special histological preparation and staining (*human samples only*)

For hematoxylin and eosin staining, paraffin-embedded human LA sections were deparaffinized using xylene (VWR Chemicals, France), then rehydrated by passing it through the graded alcohol in decreasing order, that is, absolute alcohol, 96% ethanol, then 70% ethanol (all from the same manufacturer, Sigma-Aldrich, Germany). Slides were then washed, drained, and stained with hematoxylin (Merck, Germany). The slides were washed in distilled water and then dipped in eosin solution (Chroma Gesellschaft GmbH and Co, Germany). Slides were then rinsed with distilled water, passed through graded alcohol in increasing order. The slides were placed in xylene, followed by draining of excess xylene, and finally mounted with distyrene plasticizer xylene (DPX, Sigma-Aldrich, Germany) and cover-slipped.

For Masson Goldner special stain, slides were deparaffinized and rehydrated as described above, then slides were dipped in jar containing "eisenhematoxylin" (Carl Roth GmbH and Co, Germany) stain for 5 min. The slides were removed and washed under running tap water for 15 min. The slides were dipped in Goldner I solution (Ponceau-Säurefuchsin, Carl Roth GmbH and Co, Germany) for 5 min, then in distilled water plus 1% acetic acid for 30 s, then Goldner II (light green or differentiation stain, Carl Roth GmbH and Co, Germany) for 5 min and again in distilled water plus 1% acetic acid (Merck, Germany) for 30 s. Slides were thereafter dehydrated very quickly through 70%, 90%, and absolute alcohol, and cleared in isopropanol (Chem solute, Germany). Finally, slides were mounted with DPX (Sigma-Aldrich, Germany), and cover-slipped.

For Elastica Van Gieson stain, slides were deparaffinized and rehydrated as described above, then the slides were dipped in jar containing resorcin fuchsin (Morphisto, Germany) stain for 15 min. The slides were washed, dipped in hematoxylin (Carl Roth GmbH and Co, Germany), and washed again using tap then distilled water. The slides were dipped again in Van-Gieson-Pikrofuchsin (Morphisto, Germany) and thereafter dehydrated very quickly through 2× 90% and 2× absolute alcohol and cleared in isopropanol (Chem solute, Germany). Finally, slides were mounted with DPX (Sigma-Aldrich, Germany), and cover slipped.

## 2.6 | Cryoprotection (*pig and mouse samples only*) and sample processing for immunofluorescence staining

In mouse, harvested mediastinal blocks containing the LA, and in pig, the LA dissected and excised from its attachment to the aorta and pulmonary trunk, were placed in Zamboni fixative for about 5–6 h. The solution was then replaced by 0.1 M phosphate buffer and changed every hour until fluid around the sample was clear. The sample was then incubated overnight in 18% sucrose in 0.1 M phosphate buffer and kept at 4°C. The samples were cryofixed using an optimum cutting tempereature (OCT) compound tissue tekSakura Finetek, Staufen, Germany) and 2-methylbutane solution (Carl Roth GmbH, Germany) which had been chilled using liquid nitrogen. Samples subjected to processing protocol described above were used for negative and positive control for all immunofluorescent staining.

Frozen mice and pig tissue sections were allowed to air-dry at room temperature and then covered for 1 h with blocking medium (10% normal pork serum, 0.1% bovine serum albumin [BSA], 0.5% Tween 20 in phosphate buffered saline [PBS]) and subsequently incubated with primary antibody directed against α-smooth muscle actin (αSMA) conjugated to fluorescein isothiocyanate (FITC) in PBS with addition of $NaN_3$ + S (0.01% $NaN_3$ and 0.05 M NaCl) in the working dilution and left at room temperature overnight.

Paraffin-embedded human LA sections were deparaffinized using xylene (VWR Chemicals, France), then rehydrated by passing it through the graded alcohol in decreasing order, that is, absolute alcohol, 96% ethanol, then 70% ethanol (all from the same manufacturer, Sigma-Aldrich, Germany). Slides were then washed with PBS and put in a cuvette containing 10 μL of citric acid buffer. The cuvette is then put in a bowl containing 700 mL of aqua and brought to boil in a standard microwave at 700 W for 15–20 min. It is then left to cool down, sections are removed from citric acid and incubated in PBS for 5 min. Sections are dried, covered for 1 h with blocking medium (10% normal pork serum, 0.1% BSA, 0.5% Tween 20 in PBS), and followed through with immunostaining procedure for frozen sample as described prior.

Evaluation and measurements were done using an epifluorescence microscope (BX 60, Olympus, Hamburg,

**TABLE 2** Fluorochrome filter and excitation wavelength in fluorescence microscopy

| Fluorochrome | Color | Excitation filter (nm) | Barrier filter (nm) |
|---|---|---|---|
| FITC | Green | 460–490 | 515–550 |

Abbreviation: FITC, fluorescein isothiocyanate.

Germany or Axioplan 2 imaging, Zeiss, Jena, Germany) equipped with cameras (Olympus DP73 and AxioCam MRm, Germany) connected to Cell Sens Dimension 2.1 and AxioVision Rel 4.8.2 SPZ softwares, respectively. The microscope was equipped with the filter properties below. See Table 2.

Measurements for LA were obtained using scale bars obtained from objective micrometer (Ax0003 OB-M, Japan). Images of interest were printed and imaginary longitudinal and cross-sectional lines intersecting at the center of LA were used as a point of reference to measure the size of the structure. Using a standard bioplastic measuring ruler with reference to scale bar obtained from the micrometer, measurements were taken from its longest axis.

## 2.7 | Electron microscopy (*pig and mouse samples only*)

Mice and pig LA ($n = 8$ for both) were fixed for at least 24 h in 2% paraformaldehyde and 1.5% glutaraldehyde (Merck) in 0.1 M phosphate buffer (pH 7.4). After fixation, specimens were washed in HEPES buffer 0.15 M, pH 7.4 ($5 \times 10$ min), osmicated for 2 h in aqueous 1% osmium tetroxide (Sigma-Aldrich), washed in distilled water, contrasted in 1% uranyl acetate (Merck) overnight, and embedded in epon (Agar Scientific, Essex, UK). Ultrathin sections (between 70 and 90 nm) were cut using an ultramicrotome (Reichert Ultracut E, Leica). The ultrathin sections were viewed using a transmission electron microscope (EM 902 N, Zeiss, Germany) equipped with a slow scan 2K CCD camera (TRS, Tröndle, Moorenweis, Germany) connected to Image SP software version 1.2.8.57 (Unitary enterprise "SYSPROG").

All measurements for LA were conducted using the automatic scale bars obtained with micrographs. For measurement of length and width of dense bands, the ImageJ 1.X software (Schneider et al., 2012) was used.

## 2.8 | Organ bath force recordings (*pig samples only*)

The LA was dissected and excised from its attachment to the pulmonary trunk and aorta and subjected to experiments within 2 h of sample acquisition. Isometric contraction was measured in isolated rings that were mounted between two stainless steel clips in vertical 15 mL organ baths of a computerized isolated organ bath system (Figure 2, ADInstruments GmbH, Heidelberg, Germany).

The chamber was filled with 37°C warm MEM-Medium (Invitrogen Gibco, Oslo, Norway), which was supplemented with 1% penicillin/streptomycin (PAA Laboratories GmbH, Coelbe, Germany) and continuously aerated with a 95% $O_2$/5% $CO_2$ gas mixture. The temperature was held at 37°C by the use of a bath circulator (Thermo Fisher Scientific, Waltham, USA). The upper stainless clip was connected to an isometric force transducer (Power Lab 8.30; ADInstruments GmbH, Heidelberg, Germany). Tissues were equilibrated against a passive load of 1 g for all rings. After this period, samples were adjusted at 0.5 g tension. Changes in the isometric contraction were converted by the transducer into an amplified DC output voltage and assigned to the software LabChart 6 (ADInstruments GmbH, Heidelberg, Germany).

All samples were equilibrated for 10 min until they reached a stable baseline tension. At the beginning of each experiment followed by administration of two successive but cumulative doses of noradrenalin (NA; 100 μM, 500 μM) allowing 10 min between each dose. This was followed by the cumulative administration of alpha1-adrenergic receptor blockers (tamsulosin, 1, 5, 10, 50, 100, 500 μM Cat # PHR1524 and prazosin, 0.1, 0.5, 1, 5, 10, 50 μM Cat # P7791), both purchased from Sigma-Aldrich, Germany to observe if these antagonists would relax the precontracted LA to baseline. Administration of aqua or DMSO (vehicle) in place of prazosin or tamsulosin respectively was used in control samples. Analysis of the half maximal inhibition concentration ($IC_{50}$) in response to NA was calculated using the GraphPad Prism software Version 7. Samples with less than 50% increase in tension from baseline after first dose (100 μM) of NA administration were excluded from the study. Each type of experiment was performed on samples from at least five animals, and the exact number of animals is indicated in the graphs.

## 2.9 | Statistical analysis

Data in graphs depicting time courses or dose responses presented as mean ± standard error of mean (SEM) was used. Statistical analyses were performed using GraphPad Prism software, version 7 (La Jolla, CA, USA). $IC_{50}$ values of antagonist responses were estimated using non-linear regression sigmoidal curve analysis according to the Hill equation. Two tailed *t*-test statistical testing was used to test the differences in length and width of dense bands

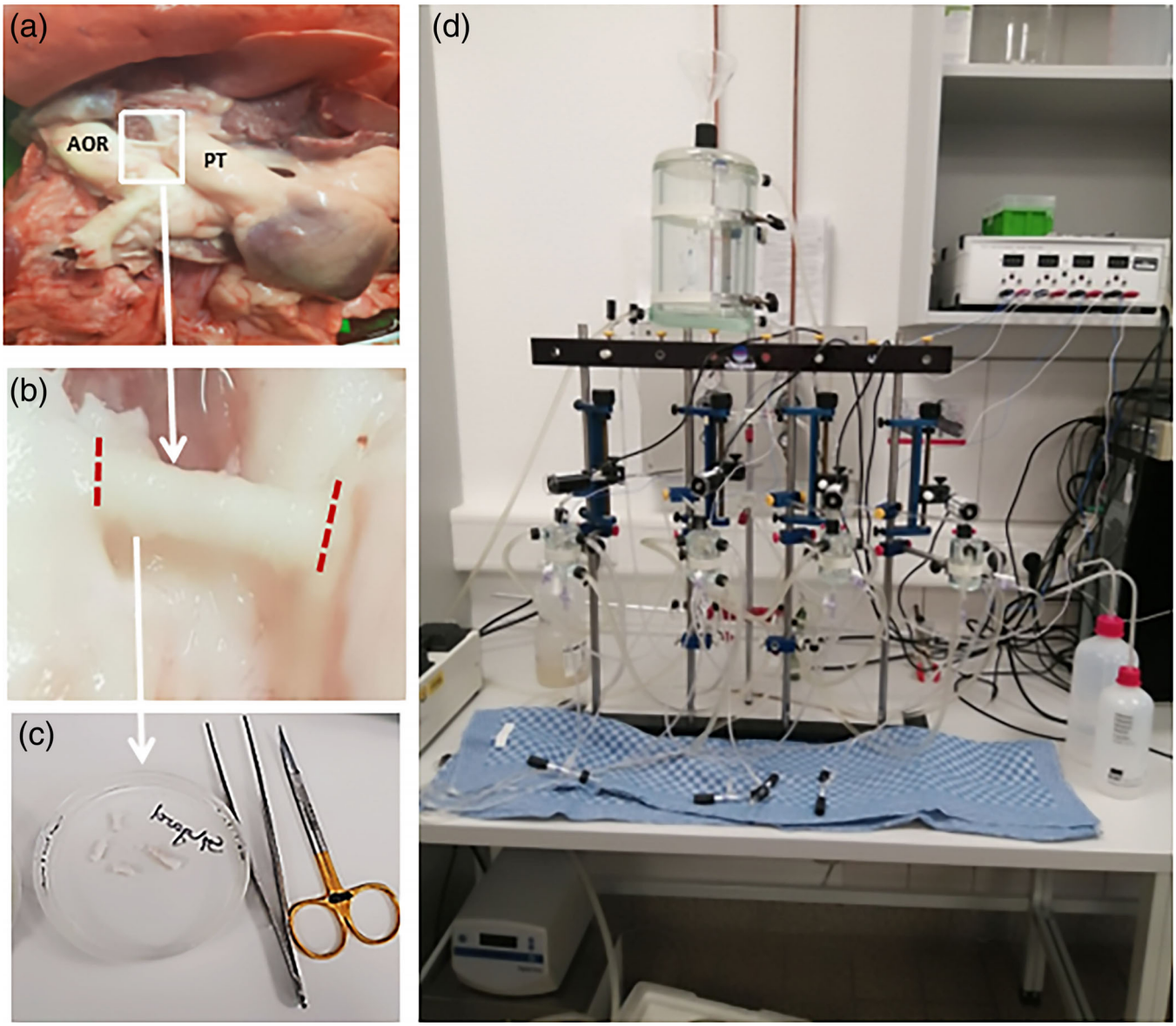


FIGURE 2 Pig LA preparations. (a) Cleaned and dissected LA from pig mediastinal block with attachment to aorta (AOR) and pulmonary trunk (PT) intact. (b) Excision of LA from aortic and pulmonary attachments. (c) Excised LA in medium for organ bath experiments. (d) Organ bath apparatus. LA, ligamentum arteriosum

found in myocytes. Differences were considered as statistically significant when $p \leq 0.05$.

## 3 | RESULTS

### 3.1 | Histological findings of LA morphology

Routine and special histological staining revealed that in cross-section, the walls of the LA could be divided into three distinct layers, namely from outward within, a tunica adventitia, tunica media, and tunica intima (Figure 3). The tunica adventitia is mainly made up of intertwining connective tissue cells, collagen fibers, nerves fiber bundles, and blood vessels. Numerous adipocytes within the adventitia and surrounding connective tissue layer have no clear boundary. The middle layer is sharply contrasted from the adventitia and mainly made up of several layers of compactly arranged myocytes running in different directions, collagen, and elastin (Figure 4). The presence of elastic fibers was confirmed by the Elastica Van Gieson special stain. The innermost layer, had the appearance of the remnant of the tunica intima. There was the presence of an intricate network of collagen making up the core of this layer. In samples obtained from three females aged between 90 and 97 years old, this layer showed calcification and fibrosis at the core (Figure 5).

### 3.2 | Immunohistological findings of LA morphology

#### 3.2.1 | Single-labeling immunofluorescence using antibody against αSMA

Immunofluorescent labeling using FITC-conjugated monoclonal antibody against αSMA revealed immunoreactive smooth muscle cells in the LA. This was present in LA samples sectioned along both its longitudinal and cross-sectional axis. The width of the LA in cross-section averagely measured 150 ± 12 μm (mean ± SEM, $n = 8$). There was no fluorescence of the LA when antibody against αSMA was omitted (Figure 6).

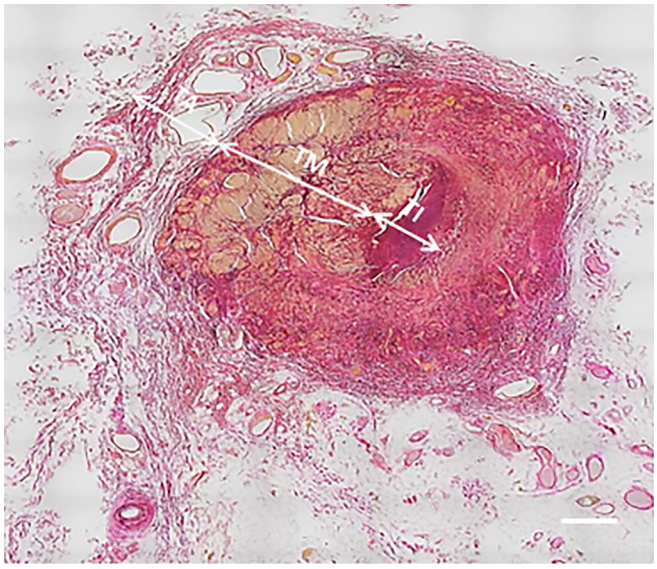


FIGURE 3 Elastica Van Gieson stain of paraffin-embedded human LA in cross-section showing three regions from outward within tunica adventitia and surrounding connective tissue (TA), tunica media (TM), and tunica intima (TI). Color scheme: Red = collagen, yellow = muscle, violet-black = elastic fibers. Scale bar = 300 μm. LA, ligamentum arteriosum

### 3.3 | Ultrastructural findings of LA morphology

In mice, the myocytes observed within LA, though numerous, lacked a consistent orientation and also did not have the typical spindle shape of smooth muscle cells. There was the presence of mononucleated muscle cells with a prominent nucleolus. Observed also were plasma membrane indentations called the caveolae. There was also the presence of cell organelles such as mitochondria and rough endoplasmic reticulum, in the cytoplasm. Additionally, dense bodies were observed within the cytoplasm. There was also the presence of a basal lamina as well as collagen fibrils around the external periphery of these cells. Actin filaments anchored to attachment zones (also called dense bands) on the plasma membrane were present. The dense bands of adjacent myocytes were sometimes found in close apposition with each other forming coupling cell mechanical junction a.k.a. attachment plaques (Hammers & Lee Sweeney, 2018). In some myocytes observed, elastin sheets were seen attaching to the extracellular border of dense bands. Dense bands with extracellular elastin

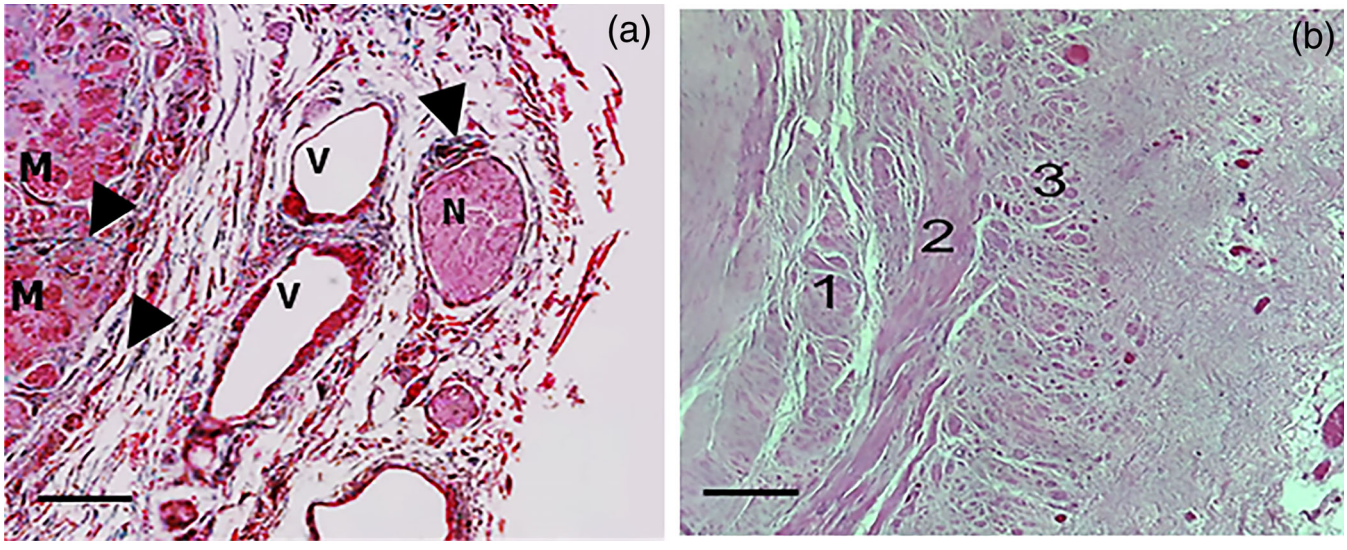


FIGURE 4 (a) Paraffin-embedded human LA stained with Masson Goldner trichrome showing a nerve (N), blood vessels (V), and collagen fibers (black arrow heads) in the adventitial layer. Bundles of myocytes (M) observed in the medial layer. Note the sharp contrast between the medial and the adventitial layer. (b) Paraffin-embedded human LA stained with hematoxylin and eosin showing layers of muscle cells (1–3). Scale bars = 50 μm. LA, ligamentum arteriosum

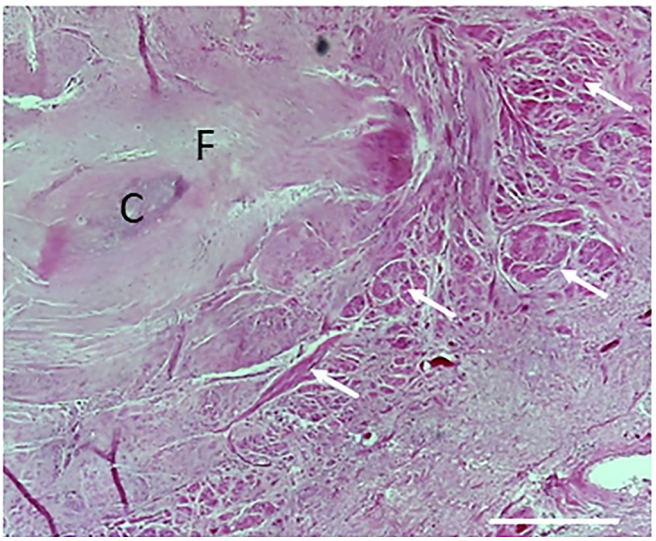


FIGURE 5 Paraffin-embedded human LA stained with hematoxylin and eosin showing a fibrous core (F) with calcification (C). White arrows indicating layers of muscle cells. Scale bar = 100 μm. LA, ligamentum arteriosum

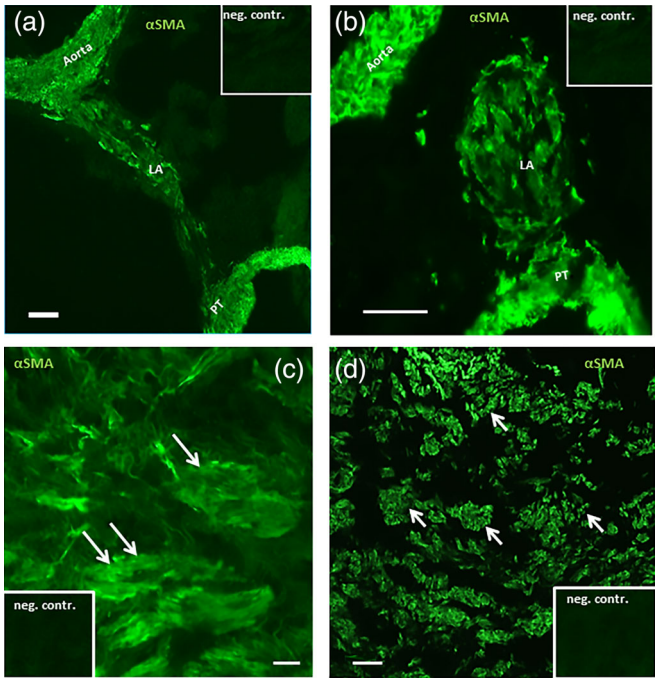


FIGURE 6 Single-immunolabeling of frozen sections from mouse (a and b), human (c), and pig (d) LA. (a) Longitudinal section of the LA appearing between and attached to the aorta and pulmonary trunk (PT) with immunofluorescent αSMA-positive cells throughout its length (green fluorescence). (b) Cross-section of the LA with attachment to the pulmonary trunk (PT) but not the aorta (a). (c) Immunolabeling of LA with immunoreactivity to αSMA (white arrows). (d) Immunolabeling of pig LA with αSMA-positive cells (white arrows). Controls run without antibody against αSMA (neg. Contr.) are shown in the inserts. Scale bars: a, c, d = 50 μm, b = 100 μm. αSMA, α-smooth muscle actin; LA, ligamentum arteriosum

attachment usually appeared more pronounced than dense bands without extracellular elastic attachment. In the LA, the average length of dense bands, parallel to the plasma membrane measured in 60 myocytes from four different samples was 0.4 ± 0.20 μm with a width of 0.049 ± 0.007 μm (mean ± SEM, $n = 60$). Comparatively, the dense bands in the pulmonary trunk were lengthier (0.9 ± 0.09 μm) and also thicker (0.078 ± 0.022 μm, mean ± SEM, $n = 60$) than what was observed in the LA. The difference in length and width between dense bands observed within the LA and pulmonary trunk subjected to a two-tailed *t*-test were statistically significant ($p \leq 0.0001$). Additionally, areas with extracellular elastin abutting the dense bands appeared more pronounced (Figures 7 and 8).

In pig samples, the myocytes I observed revealed the typical appearance of myocytes with pronounced caveolae, dense bands, as well as myofilaments. Mitochondria were present. Some myocytes found here revealed gap junctions indicating intercellular communication. Myocytes were surrounded by a basal lamina (Figure 9).

### 3.4 | Effect of exogenous NA and α1 adrenergic antagonists on pig LA

Pig LA used as treatment and control were subjected to two increasing cumulative doses of NA (100 μm, 500 μm) with 10 min between each dose. There was no pretreatment before the administration of NA. A concentration-dependent contraction was observed. The precontracted LA was further subjected to increasing but cumulative concentrations of prazosin (0.1, 0.5, 1, 5, 10, 50 μM) or tamsulosin (1, 5, 10, 50, 100, 500 μM). All doses, with the exception of 0.1 and 0.5 μM in prazosin and 1 and 5 μM in tamsulosin, showed antagonist effect on the precontracted LA. The subsequent doses produced a consistent relaxation of the LA until it was back to baseline (0.5 g). The control samples also contracted when they were subjected to two different doses of NA administration, however, no relaxation was observed upon administration of aqua or DMSO (vehicle) in place of prazosin or tamsulosin (Figure 10), respectively.

## 4 | DISCUSSION

The LA has generally been described as a fibrosed remnant of the ductus arteriosus (Chiruvolu & Jaleel, 2009). Contrary to reports of the LA being a small fibrous remnant of the fetal ductus arteriosus (Chiruvolu &

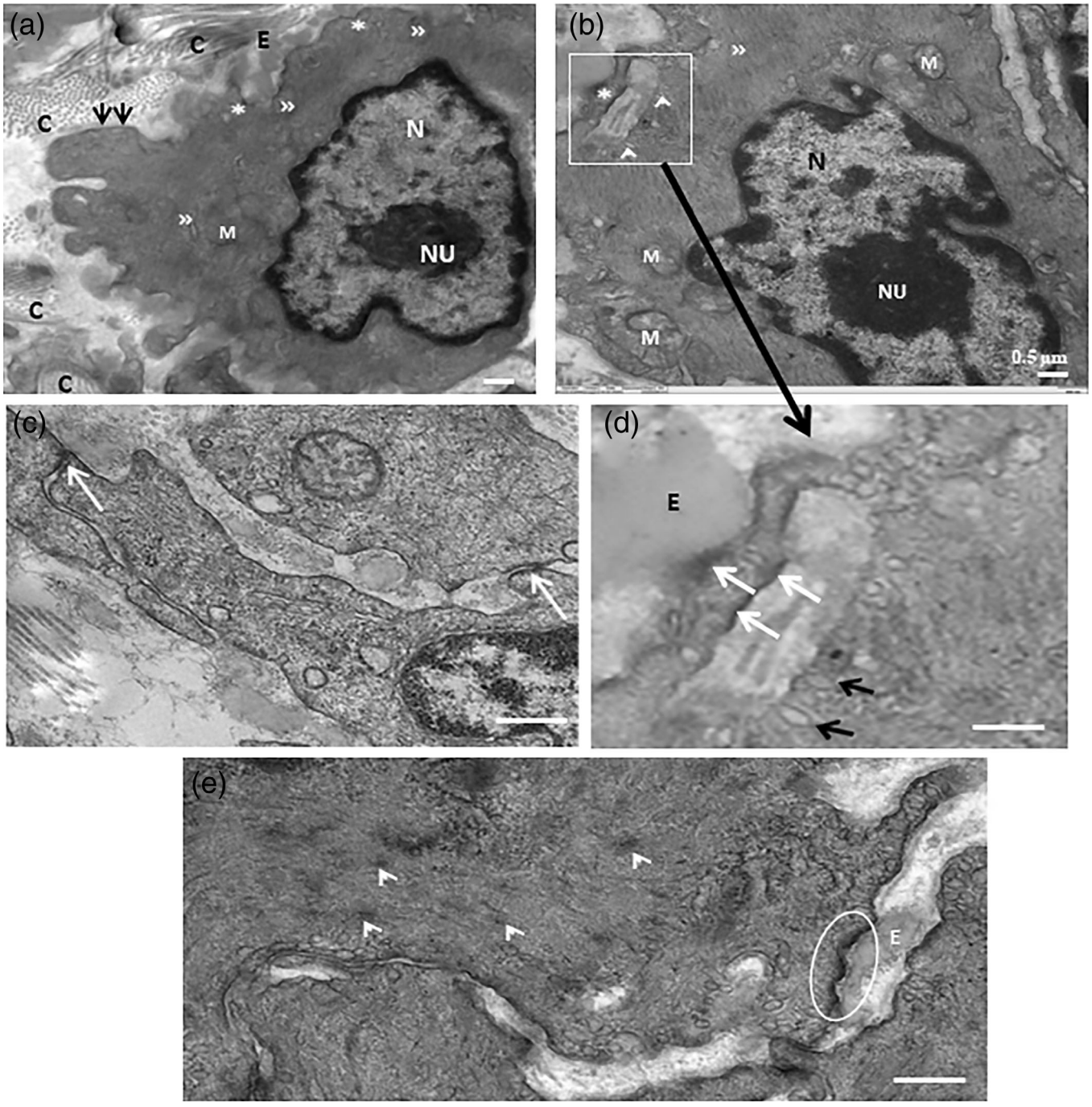


**FIGURE 7** Transmission electron microscopy of murine LA. (a) Smooth muscle cell with heart-shaped nucleus (N) and its nucleolus (NU) in it. The actin filaments (double arrowheads) and attachment zones (white asterisk) are seen. Elastin sheet fragment (E) is attached to the plasma membrane. Collagen fibrils (C) surrounding the myocyte are present. (b) Nucleus (N) and its nucleolus (NU) as well as caveolae (white arrowhead) are present. Attachment zone (white asterisk) and mitochondria (M) are also observed. (c) Region of interaction between two dense bands (white arrows) of two myocytes. (d) Magnification of white insert in panel (b) displaying caveolae (black arrows) and dense bands (white arrows) attached to the plasma membrane. On the opposite, elastin (E) seen abutting a dense band (white arrow). (e) Dense bodies (white arrowhead) within cytoplasm of myocyte. Note a region with elastin (E) anchored to a pronounced dense band (white oval). Scale bars: a = 1 µm, b = 0.5 µm, c–e = 0.25 µm. LA, ligamentum arteriosum

Jaleel, 2009), but in line with the hypothesis, the results from the study show that the structure is mainly muscular. This agrees with the findings by Garcia (1975), who after studying the LA in eight healthy human adults using stereomicroscope dissected Zemper-treated (50%), celloidin-embedded (25%), and paraffin-embedded (25%)

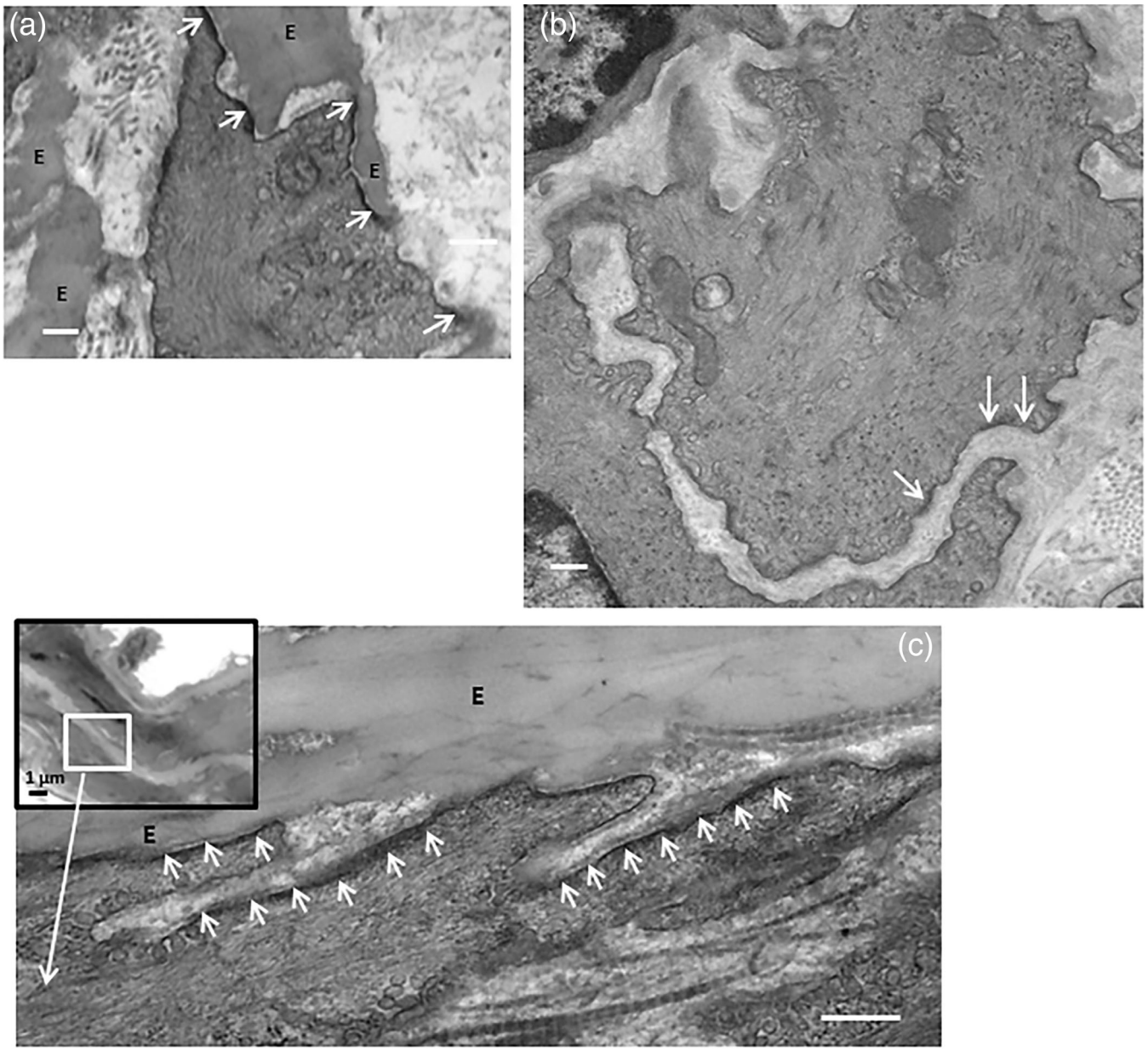


**FIGURE 8** TEM of comparison of dense bands in mice LA and pulmonary trunk. (a) Myocyte within LA medial layer with numerous elastin (E) attachments and well-pronounced dense bands (white arrow). (b) Myocytes within LA with comparatively less-pronounced dense bands (white arrows). (c) Magnification of region of insert (white square) showing dense bands in pulmonary trunk myocyte. Note that not all dense bands are in contact with the elastin lamella (E) present (white arrows). Scale bars = 0.25 µm. LA, ligamentum arteriosum

LA, stained with Axan and "resorcin-fuchsin," described the structure as a smooth muscle. Dohr et al. (1986) also studied the morphology of the LA using routine and special histological stainings in 15 bodies of both sexes aged between 60 and 80 years. The report from the study divided the LA into an outer and inner zone only. In contrast, my study of human LA subjected to routine and special histological stainings to assess the general morphology of the structure revealed that the LA could be distinctively divided into three regions. This corresponded with publications and report with regard to the general histological anatomy of arteries (Pearce & Thomsen, 2000). The present study reported arterial arrangement from outward to innermost layers generally described as, a tunica adventitia, consisting of intertwining collagen fibers, nerve bundles, blood vessels, and elastin fibers embedded in numerous adipocytes were observed. The second region, tunica media, sharply contrasted from the first, was mainly made up of vascular smooth muscle cell (VSMC), collagen, and elastin fibers. The presence of an extensive amount of vascular smooth muscle arranged in circular and longitudinal orientations

within the tunica media authenticated the fact that, majority of the myocytes which make up this layer did not migrate to the intima during closure of the ductus arteriosus. This was contrary to the report by De Reeder et al. (1989) and Yoder et al. (1978) who reported the complete obliteration of the luminal part of the ductus arteriosus by migrated smooth muscle cells from the tunica media into the tunica intima after birth. Additionally, not only do the results from the present study contradict their report on smooth muscle migration, but also on the complete obliteration of the ductus arteriosus. The lumen of the "ligament" was not always completely obliterated. Partial obliteration showing luminal remnants at the aortic end of the LA was observed, though it does not serve as a passage way for blood, because it does not open into the pulmonary trunk. Regardless of whether completely obliterated or otherwise, the core of the LA was sometimes made up of myocytes, although scanty, and cell debris. In samples obtained from three body donors, this layer showed calcification and fibrosis at the core. A study by Bisceglia and Donaldson (1991) using 53 children aged between 5 months and 14 years, 7 (13%) made up of 4 boys and 3 girls, reported calcification of the entire LA during a routine computerized tomography (CT) examination. Wimpfheimer et al. (1996) also studied the same phenomena in 402 adults comprising of 214 women (53%) and 188 men (47%) aged between 18 and 97 years and reported calcification of LA as a common finding, and its frequency increased with age. The results from my study agrees with the findings by Wimpfheimer et al. (1996) evidenced by calcification and fibrosis at the core of human LA obtained from three cadaveric females aged between 90 and 97 years old. Knowledge of juvenile calcification/fibrosis in the LA is essential to avoid wrongful diagnosis of patients or children with mediastinal pathology. Further analysis of whether bodies investigated had cardiovascular disease such as atherosclerosis leading to calcification in the LA

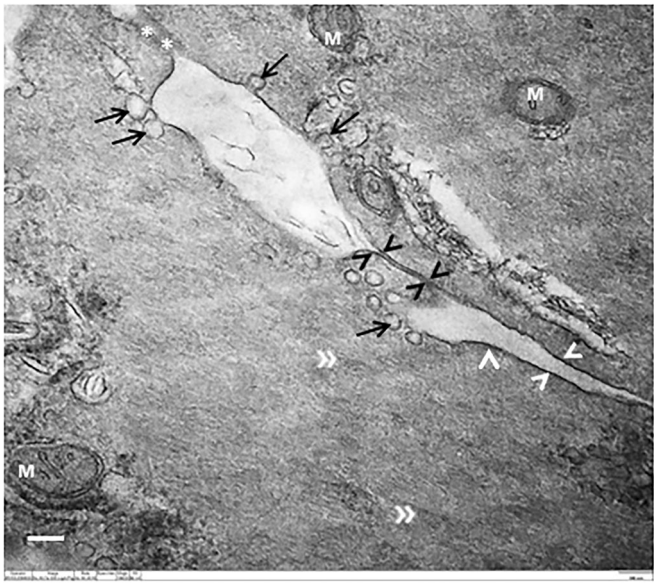


**FIGURE 9** TEM of pig LA. Smooth muscle cell, with actin filaments (double white arrowheads) and dense bands (single white arrowheads). Note the presence of caveolae in the plasma membrane (black arrows) and mitochondria (M). A gap junction (black arrowheads) is connecting two myocytes. A basal lamina (white asterisk) is also present. Scale bar = 0.1 μm. LA, ligamentum arteriosum

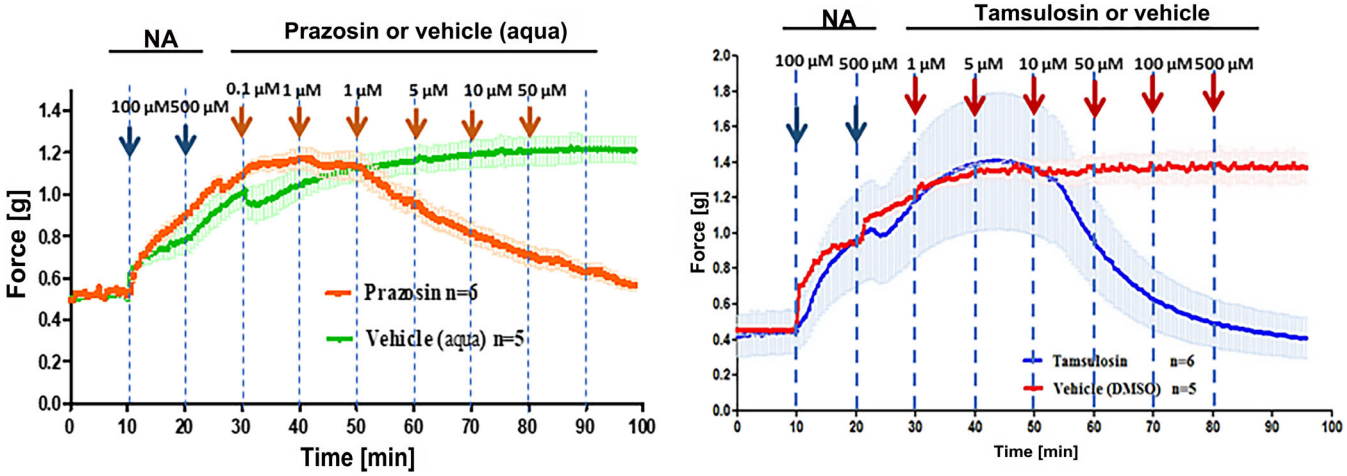


**FIGURE 10** Relaxant effect of prazosin and tamsulosin on pig LA precontracted with NA. There was contraction in both experimental and control samples with NA administration. The LA reported a stepwise relaxation from the precontraction induced by NA after being subjected to increasing cumulative concentrations of prazosin and tamsulosin. In control samples, there was no relaxation observed upon administration of aqua or DMSO in cumulative doses in place of prazosin or tamsulosin, respectively. Arrows indicate point of substance or vehicle administration. LA, ligamentum arteriosum; NA, noradrenalin

was not the focus of the present study, therefore, this was not addressed by further investigation. This general morphology of the LA held true for all species used in the study. No difference was seen in morphology in terms of gender or age. In the human samples for example, the tunica media in the oldest bodies aged 97 years still had the presence of VSMC and this confirms that the VSMC found in the LA is present until senescence. In line with the hypothesis, this confirms that there is the retainment of the muscular elements with contractile abilities in the ductus arteriosus after it becomes the LA.

VSMC make up the majority of cells within the tunica media layer of blood vessels. This layer makes up the bulk of vessel wall in arteries and is usually made up of mononucleated spindle-shaped cells. Its role in determining vessel wall thickness, regulating vessel diameter, differentiating arteries from veins, and maintenance of vessel tone cannot be overemphasized (Tucker et al., 2021). αSMA is expressed in VSMCs (Abberton et al., 1999). Immunofluorescence staining using antibodies against αSMA showed reactivity throughout the whole LA. No known publication has reported immunostaining of LA with antibodies against αSMA. The reactivity was intense and supported results from earlier routine and special histological stainings.

Ultrastructural studies of the ductus arteriosus by Kim et al. (1993) in fetal and neonatal rabbits reported the presence of smooth muscle in the ductus arteriosus. In comparison with the myocytes found in the aorta, they appeared more differentiated, the study concluded. For the first time in the LA, results from transmission electron microscopy (TEM) of wild-type mice and pigs confirmed and supported the presence of myocyte in the LA. About 80% of the myocytes found within the tunica media of the LA in both mice and pigs had the typical morphology of contractile smooth muscle cells. The contractile role of vascular smooth muscle has also been extensively studied. A study by Bond and Somlyo (1982) described the contractile unit in VSMC to consist of dense bodies, the thick and thin myofilaments. These features constituting the contractile unit were all present in the smooth muscle cell observed in this study (Figures 7–9). Furthermore, the presence of dense bands which are believed to enhance mechanical and contractile ability of VSMC was also observed in this study. As described by Galen in his "De usu partium", the two elastic vessels (aorta and pulmonary trunk) are held together by the LA. It is fact that the LA no longer serves in its original capacity as a fetal shunt connecting the two great vessels. Results from this study reports the presence of contractile smooth muscle long after its obliteration and it may be postulated that the contractile abilities of the LA may act on the two great vessels to which it is attached thereby causing a change in their distensibility. It remains to be determined in future studies whether LA might influence impedance/compliance of the vascular segments to which it is attached, which are the best prognostic parameters for patient survival in pulmonary hypertension (Hunter et al., 2008).

## AUTHOR CONTRIBUTIONS

**Benedicta Mensah:** Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (supporting); software (lead); supervision (equal); validation (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead).

## ACKNOWLEDGMENTS

The expert technical assistance of Anika Seipp, Tamara Papadakis, Gerhard Kripp, Martin Bodenbenner-Türich, Ulrich Gardener, and Petra Mermer are gratefully acknowledged. Experiments were conducted at the Institute of Anatomy (Justus Liebig University). I acknowledge the scientific suggestions made by the head of AG Kummer. Open Access funding enabled and organized by Projekt DEAL.

## FUNDING INFORMATION

This project was supported by the Ghanaian-German Postgraduate Training scholarship from German Academic Exchange Service, or Deutscher Academics Austauschdienst (DAAD), Scholarship from equal opportunity office for mothers in research, Justus Liebig University, Giessen, Germany.

## CONFLICT OF INTEREST

The author declares no competing interests.

## DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**How to cite this article:** Mensah, B. (2023). Ligamentum arteriosum: Muscular and contractile. *The Anatomical Record*, *306*(2), 409–421. https://doi.org/10.1002/ar.25058