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# Down-regulation of lysyl oxydase-like in aging and venous insufficiency

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**Summary.** Background: Elastin expression is higher in tissues where elastic fibres are essential for the correct maintenance of function such as blood vessels. Elastin expression usually diminishes with age, however, it may be re-expressed in response to injury or repair processes. Some authors attribute the characteristic loss of elasticity of the varicose vein to a drop in the population of smooth muscle cells in the media layer. A reduction in elastin has been observed in chronic venous insufficiency, but little is known about some of the factors involved in elastin synthesis such as lysyl oxidases. The aim of this study was to examine the in vivo expression of the elastin precursor, tropoelastin (TE), and lysyl oxidase-like 1 (LOXL1), a cross-linking enzyme responsible for elastin polymer deposition. The effects of age on these expression patterns were also evaluated. Methods: Saphenous vein segments were obtained during surgery from organ donors (controls, n=20) and subjects with venous insufficiency (varicose veins, n=20). Both these groups were subdivided according to subject age into <50 years (n=10) and ≥ 50 years (n=10). Control and varicose vein tissue specimens were immunolabelled using anti-tropoelastin and anti-LOXL1 antibodies and also subjected to Western blot analysis. Results: Our results indicate that the levels of these markers of elastin synthesis (LOXL/tropoelastin) in the vein wall diminish in a significant way ($p<0.05$) with the age factor. Excluding the age factor, LOXL1 was significantly decreased in the varicose condition ($p<0.05$). In the younger pathological population they showed an inverse relationship (LOXL decreased, tropoelastin increased). Conclusions: The already established reduction in elastin in the varicose condition may be related, at least in part, to the decreased LOXL1 levels observed here. These events could reduce spontaneous reticulation of elastin and the partial loss of tissue elasticity in this group of patients.

**Key words:** Varicose vein, Chronic venous insufficiency, LOXL1, Tropoelastin, Elastin synthesis

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## Introduction

Varicose veins are a common problem mainly affecting the adult population, with an incidence that increases with age. Although the aetiology of this condition is not well understood, several theories have been put forward to explain the transformation of a normal vein into a varicose or insufficient vein. The dilatation and tortuosity observed in varicose veins show evidence for continuous wall remodelling associated with the affected smooth muscle cells and extracellular matrix of the varicose vein wall (Sansilvestri-Morel et al., 2002). The extracellular matrix acts as a structural framework and is essential for the functional properties of vessel walls (Jacob et al., 2001). Some of the components mentioned in the literature that are modified in the varicose condition are constituents of the extracellular matrix, such as collagens, elastin and proteoglycans (Gandhi et al., 1993; Venturi et al., 1996; Jacob et al., 2001; Leta et al., 2002). Several of the changes observed in connective tissue are thought to reflect variations in the levels of MMPs, the enzymes involved in regulating and maintaining the extracellular matrix that degrade extracellular matrix molecules, proteoglycans, elastin and different types of collagens (Matrisian, 1990; Woessner, 1991; Buján et al., 2000). Venous insufficiency in varicose disease has been

associated with depleted elastic fibre contents (Gandhi et al., 1993; Porto et al., 2002), impairing the key role of elastic fibres in maintaining vessel tone.

Elastin expression is higher in tissues where elastic fibres are essential for the correct maintenance of function such as blood vessels. This expression diminishes with age in most adult animal blood vessels, although it may be re-expressed in response to injury or repair processes. The protein elastin is formed through the reticulation of tropoelastin monomers over a framework of fibrillin-rich microfibrils (Mecham and Heusar, 1991), and latent transforming growth factor-beta binding proteins (LTBPs) (Saharinen et al., 1999; Shipley et al., 2000).

Some authors attribute the characteristic loss of elasticity of the varicose vein to a drop in the population of smooth muscle cells in the media (Lengyel and Acsady, 1990). Other authors have found decreased contractility in varicose veins showing an important role of smc receptors of noradrenalin, endothelin-1 and angiotensin-II in maintaining the normal contractility of the tissue (Barber et al., 1997; Rizzi et al., 1998). Indirect biochemical studies have also demonstrated a decrease in the elastin content of the affected vein wall (Andreotti et al., 1978; Maurel et al., 1990; Mecham and Heusar, 1991; Gandhi et al, 1993; Lethias et al., 1996; Niebes, 1996; Porto et al., 2002). Others have also noted that in the varicose condition there are more elastic fibres during the initial stages of the disease (Kirsch et al., 1999), and that these decrease as it advances, thus explaining the rigidity of the varicose vein wall.

Lysyl oxidase (LOX), a copper-containing amine oxidase, belongs to a family of enzymes that oxidize primary amine substrates to reactive aldehydes. LOX is traditionally known for its extracellular catalysis of lysine-derived cross-links in fibrillar collagens and elastin. The identification of four other LOX-like (LOXL), members of this family (LOXL 1-4) indicates the possibility that these functions are performed in both intracellular and extracellular environments by new individual members of the LOX amine-oxidase family. Structural similarities in highly conserved copper-binding and lysyl-tyrosylquinone cofactor sites between LOX and LOX-like proteins may reflect similar amine oxidase activities. However, specific novel functions, such as a potential role in cell adhesion and cell growth control, are determined by other conserved domains such as the cytokine receptor-like domain, which is shared by all LOXs, and by multiple scavenger receptor cysteine-rich (SRCR) domains present in LOXL2 and LOXL3 (Csiszar, 2001).

In a recent study (Buján et al., 2003), we documented changes in the expression levels of elastic components and their proteolytic enzymes that suggest a higher turnover of these components in affected areas of varicose veins.

While a reduction in the level of elastin has been already established, little is known about the factors involved in tissue remodelling that could affect elastin levels. The aim of this study was to correlate the in vivo expression of the elastin precursor tropoelastin with lysyl oxidase-like 1 (LOXL1), a cross-linking enzyme responsible for elastin polymer deposition. Expression levels were also correlated with subject age.

## Materials and methods

### *Patients*

Forty specimens of saphenous vein were obtained during surgery from organ donor extraction (control) or varicose vein surgery. Informed consent to participate in this study was obtained from all of the subjects.

The specimens were first visually inspected to check for the presence of damaged areas in the vein wall. Given that the molecular and morphological changes that occur during ageing have not been well established, we divided the study population into two groups according to whether the subjects were <50 years or >50 years of age as follows:

Group I control (n=20). This group was comprised of 10 vein specimens harvested from patients under 50 years (mean age 38.4±5.8, range 36-45 years) and a further 10 specimens from patients aged 50 years or over (mean age 71.2±10.6, range 57-89 years). These segments of saphenous vein were obtained from organ donors, with no history of venous insufficiency or proven reflux during organ extraction surgery.

Group II varicose veins (n=20). This group was comprised of 10 vein specimens harvested from patients under 50 years (mean age 39.4±7.8, range 26-46 years) and a further 10 specimens from patients aged 50 years or over (mean age 60.7±9.4, range 52-70 years). Segments of saphenous vein were obtained at the time of extraction from patients with primary venous insufficiency and clinically confirmed reflux. The CEAP classification, based on clinical, etiologic, anatomic, and pathophysiologic data, was applied previously to the venous extraction. All varicose veins used in the study were classified as type 2 (C2).

Immediately after procurement, the specimens were placed in sterile culture medium (MEM) and stored at 4ºC for their transfer to the laboratory, where they were divided into two fragments, one fragment was processed for light microscopy (immunohistochemistry) and the other fragment was frozen at -80°C until Western blot analysis.

### *Immunohistochemical analysis*

For immunohistochemical analysis, vein specimens were fixed in Bouin's solution, embedded in paraffin and cut into 5 µm slices using a microtome (Microm, Barcelona, Spain). The sections were then deparaffinated, hydrated and equilibrated in PBS buffer (pH 7.4). We used a rabbit polyclonal anti-tropoelastin antibody (1:500) (donated by Dr. Mecham) and a rabbit monoclonal anti-LOXL1 antibody (1:200) (donated by

Dr. Sommer). The antigen-antibody reaction was detected by peroxidase-labelled avidin-biotin procedures. The chromogenic substrate contained diaminobenzidine (DAB). Nuclei were counterstained with Carazzi haematoxylin. After immunostaining, the tissue sections were examined under a light microscope (Zeiss, Jena, Germany).

### *Western blot analysis*

Vein specimens selected for the Western blot analysis were frozen at -80°C until use. The tissue samples were cut into small pieces, immersed in the extraction buffer (0.1m $K_2HPO_4$ pH7.7, 0.15 M NaCl) with protease inhibitor and milled using a grinder (Universal Mühle M20, Ika Industries). Once milled, the resultant solutions were centrifuged at 15000g for 30 minutes at 4°C, the supernatants with the soluble proteins were recovered and the pellets were resuspended in 0.016 M $K_2HPO_4$ pH7.8, 4M Urea and protease inhibitor. After centrifugation at 15000g 4°C for 30 min the urea soluble proteins were recovered in fresh Eppendorf tubes. The proteins were then separated by electrophoresis on a 14% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) under reducing conditions, according to the modified method of Laemmli.

Equal aliquots (20 µl) containing approximately 10mg of protein, were diluted in sample buffer (50 mM Tris-base, pH 6.8, containing 50% glycerol, 0.125% bromophenol blue, 15% sodium dodecyl sulphate in the presence of 10% 2-beta-mercaptoethanol) and were heated at 100°C for 5 minutes before loading. A broad-range pre-stained SDS-PAGE standard (Bio-Rad, Richmond, CA, USA) served as a molecular weight marker. After 2 h of electrophoresis at 100V, the separated proteins were transferred to a nitrocellulose membrane (Bio Rad Laboratories, Hercules, CA, USA) at 210 mA for 2 h at room temperature. The membranes were blocked overnight at 4°C with 5% dry milk in phosphate-buffered saline (PBS1x) using 0.05% Tween 20. The following primary antibodies were applied to the blotted membranes for 2 h at room temperature: anti-tropoelastin (1:3000) and anti-LOXL1 (1:500). The secondary antibody anti-rabbit IgG-horseradish peroxidase (Sigma, St. Louis, MO, USA) (diluted 1:10000) was incubated with the membranes for 1.5 h at room temperature. The blots were developed using the SuperSignal Westpico Chemioluminescent kit (Pierce, Rockford, IL, USA). Positive bands were visualized on X-ray film.

Mouse anti-ß actin antibody (Calbiochem, Inc. La Jolla, CA, USA) was used as a loading control. Western blot bands were quantified using the Scion Image program. The intensities of TE and LOXL1 bands were normalized with those of ß-actin bands. Results were expressed as the mean±SE of the ten experiments. Statistical analysis was performed with the Graph Pad Prism program using the Student's t-test. The level of significance was set at $p<0.05$.

## Results

### *Tropoelastin*

Immunostaining of the vein wall from the younger control group showed TE staining in the luminal surface of the intimal layer, homogeneously distributed around smooth muscle cells of the medial layer (Fig. 1a) and in the adventitia. The older group showed little and heterogeneous expression for TE. The staining in this group was restricted to little areas in the intimal layer, among the parcels of smooth muscle cells (Fig. 1b) and to some areas in the adventitia closed to the elastic fibres. In insufficient veins, intense TE staining was detected in the hyperplasic intimal areas and homogeneously distributed in the extracellular matrix of the medial layer (Fig. 1c) in the younger group. However, in patients over 50 years the TE expression was confined to specific areas in deep medial layers close to adventitia (Fig. 1d). Staining was also observed in hyperplasic intimal areas in this group of patients.

Western blot analysis of tissue extracts revealed no significant differences in tropoelastin (TE) between the healthy and insufficient vein wall (Fig. 2a), when we ignored the age of the patients. However, significant differences were observed when the samples were grouped by age of the individuals. Fig. 2b shows that the presence of TE in the tissue samples was lower in specimens from the older age group in both healthy and chronic venous insufficiency ($p<0.05$). Increased tropoelastin levels were observed in specimens from young patients with varicose veins ($p<0.05$) when compared with the young control group (Fig. 2b). However, when the older groups were compared no significant differences were found ($p>0.05$).

### *LOXL1*

LOXL expression was detected in all layers of the vessel wall in control samples. The most intense staining occurred around smooth muscle cells, in the extracellular matrix of the vessel media, particularly in healthy veins from the younger age group (Fig. 3a), the older group showed a significant decrease in the LOXL expression (Fig. 3b). Varicose vein specimens showed less expression in both age groups; little labelling was observed in the endothelium, media and adventitia. Staining was less intense in specimens from patients over the age of 50 years (Fig. 3c,d).

Western blotting found statistically significant differences in LOXL1 levels between the two age groups (Fig. 4b) ($p<0.01$). Differences in LOXL1 Levels were also detected when control and varicose vein groups from younger individuals were compared (Fig. 4b). In contrast to what was found for TE, differences in LOXL1 levels between control and varicose vein groups continued to be significant when age was not taken into

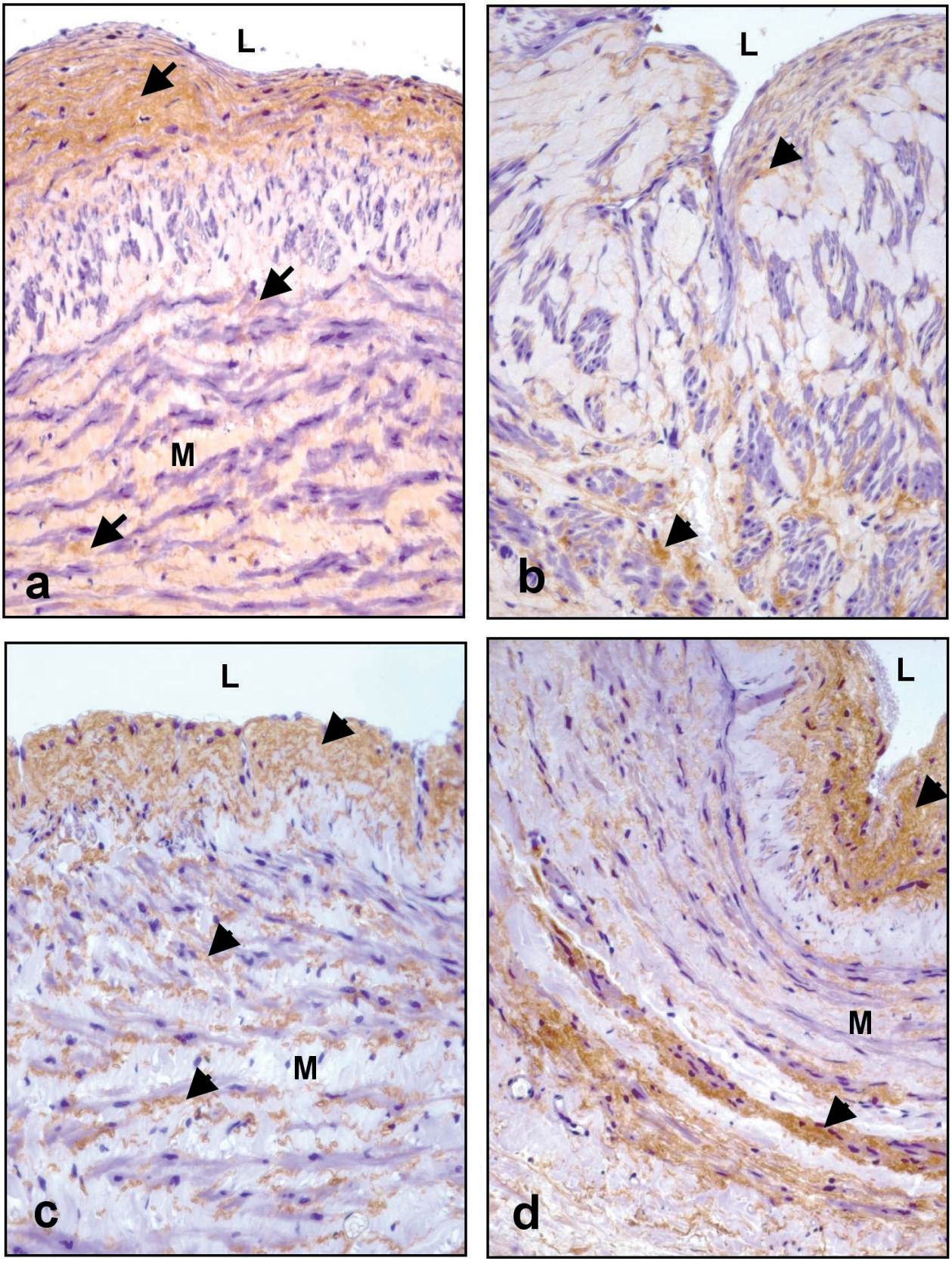


**Fig. 1.** Immunohistochemical staining for tropoelastin in the vein wall. **a.** Control specimens from subjects under 50 years (x 320). Scarce TE expression was detected around smooth muscle cells in control specimens from subjects over 50 years (**b**) (x 320). Varicose veins from subjects younger than 50 years (**c**) showed increased expression in the intimal and medial layers (x 320). (**d**) Varicose vein from patients over 50 years (x 200) (arrows: tropoelastin staining, L: lumen; M: medial layer).

account ($p<0.05$) (Fig. 4a).

## Discussion

Lysyl oxidases are copper-dependent monoamine oxidases secreted by fibrogenic cells, such as fibroblasts and smooth muscle cells, which catalyse the oxidative deamination of lysyl residues in collagen and elastin (Smith-Mungo and Kagan, 1998). This process is followed by spontaneous covalent cross-linking, which is essential for the structural integrity and function of connective tissue. Mammalian genomes have up to five potential LOX family members encoding the prototypic LOX and LOX-like proteins (Kagan and Li, 2003).

A selective role for LOXL1 in elastin but not collagen metabolism has been proposed, based on desmosin and hydroxyprolin levels, which represent elastin and collagen cross-links, respectively (Liu et al., 2004). The authors of this study reported significantly lower desmosin levels in several tissues of a LOXL1 mutant, whereas hydroxyprolin levels remained unchanged.

The main function of LOXL1 seems to be to stabilize elastin deposition in a spatially defined manner, a prerequisite for the formation of functional elastic fibers. LOXL1 converts tropoelastin into a lysyl-deaminated form, and this "activated" tropoelastin associates with other activated tropoelastins or deposits onto the existing polymer through coacervation (Vrhovski and Weiss, 1998) followed by spontaneous covalent cross-linking. It has also been established that LOXL1 but not LOX is specifically targeted to sites of elastogenesis (Liu et al., 2004), showing that LOXL1 was intimately associated with the elastic lamina whereas LOX was broadly distributed.

The previously established reduction in the level of elastin itself in varicose veins can be attributed to some extent to the LOXL defect observed here, whereby its levels in vivo decrease with age. Other factors including enzymes that degrade elastin, such as elastase and MMPs, are also thought to be involved in reducing elastin expression in the varicose condition (Buján et al., 2000, 2003), also contributing to the degradation of the extracellular matrix and to the progression of the disease.

In general, when we excluded the age factor, the expression of tropoelastin observed here in our varicose vein specimens did not vary significantly with respect to the control veins. Hence, elastin reduction was not the result of decreased synthesis of the monomeric form of this amorphous polymer. The expression of tropoelastin was inversely correlated with the LOXL level, such that if LOXL levels fell, tropoelastin is accumulated. This led to its significantly higher expression in varicose vein specimens taken from the younger patients compared to control veins, and a corresponding decrease in LOXL expression. Both factors (TE/LOXL) were in vivo affected by the age of the patients, with lower levels expressed in the older age group, probably accounting for the lack of differences between varicose and healthy veins in terms of TE and LOXL contents detected in this group of elderly subjects.

It would be interesting to perform complementary cell culture studies, in which we were able to check the

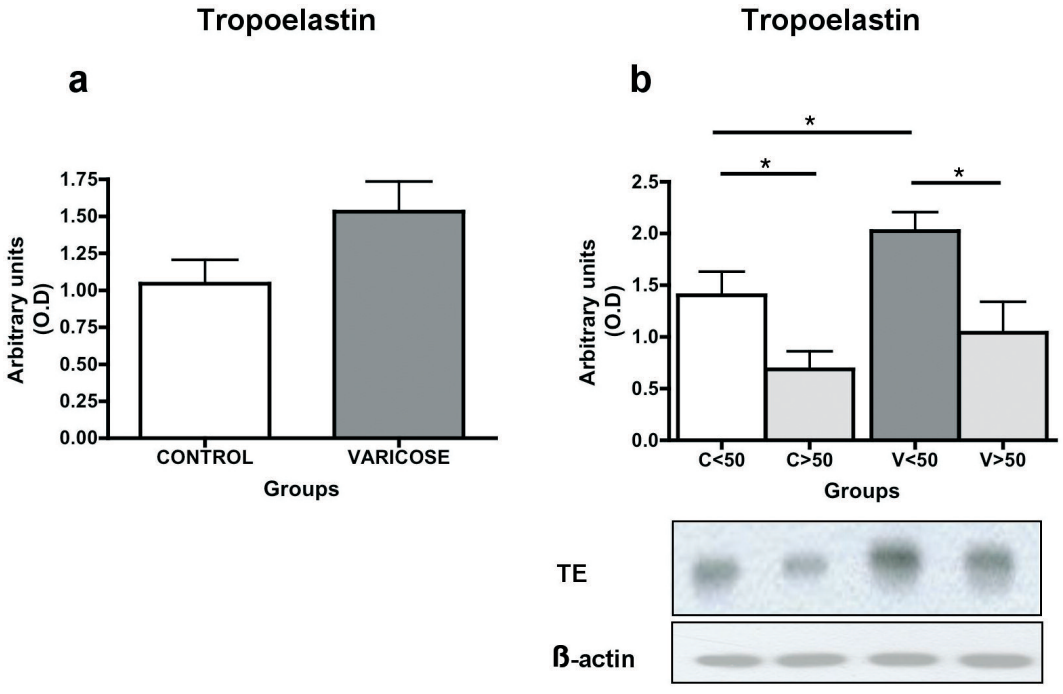


**Fig. 2.** Western blotting analysis for tropoelastin. Densitometric analysis of tropoelastin (**a**) regardless of age. When the age factor was examined (**b**), tropoelastin showed a significant decrease with age (*$p<0.05$) and differences emerged in specimens from the younger subjects between healthy and varicose veins (*$p<0.05$). Mouse anti-ß actin antibody was used as a loading control.

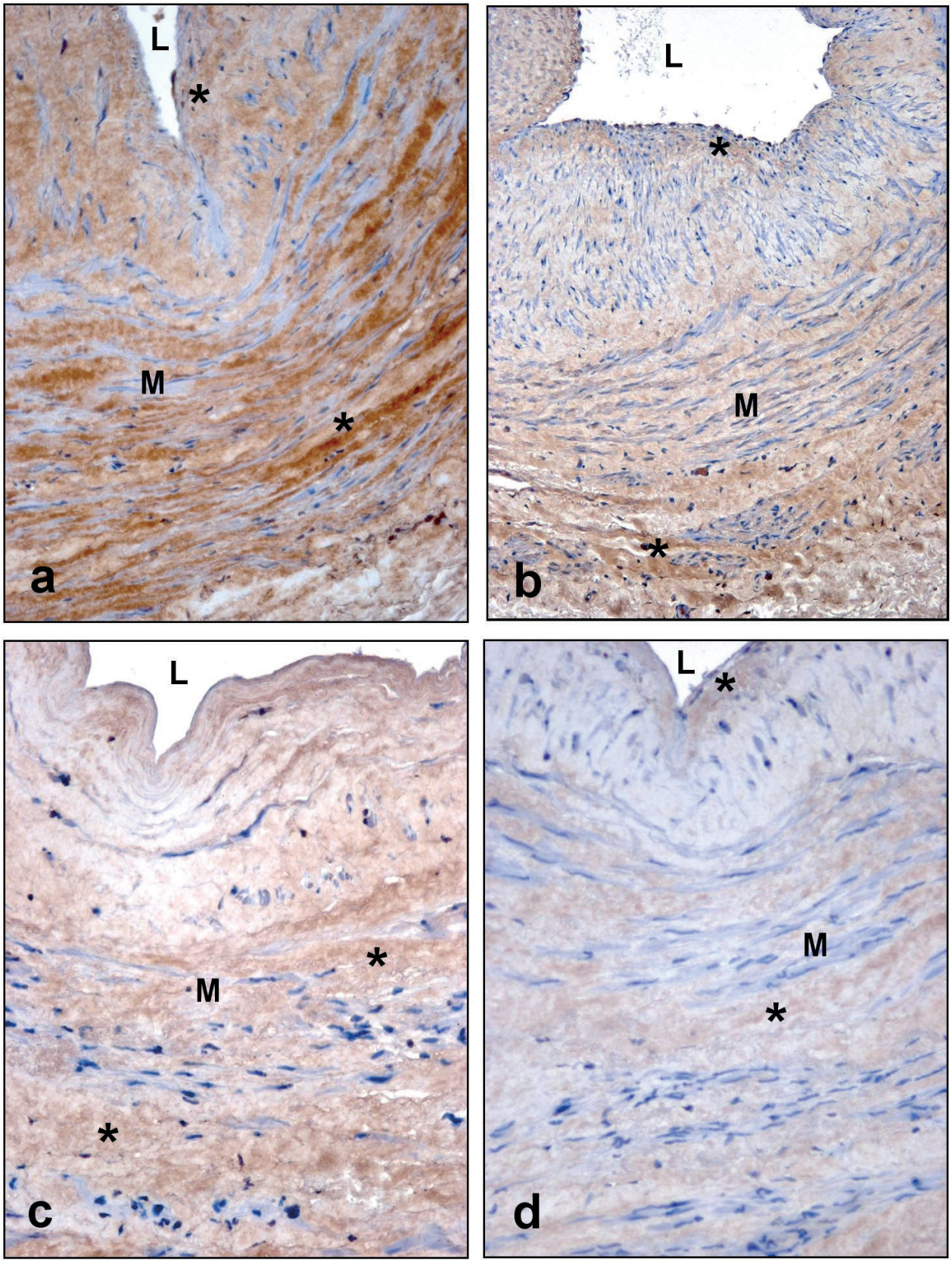


**Fig. 3.** Immunohistochemical staining for LOXL1 in the vein wall. Healthy veins from subjects in the younger age group (**a**) showed most intense labelling for LOXL (*) (x 250), contrasting with the least amount of staining noted in the rest of the groups. **b.** Control specimens older than 50 years (x 100). **c.** Varicose veins under (x 320) and over (**d**) 50 years (x 320). L: lumen; M: medial layer.

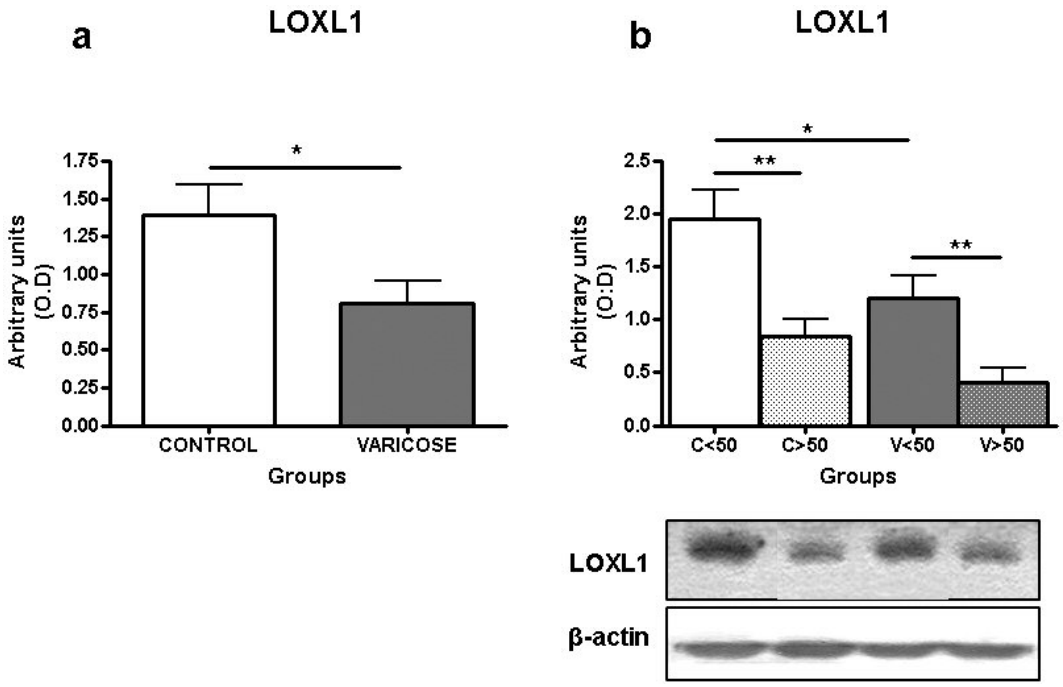


**Fig. 4.** Western blotting analysis for LOXL1. Densitometric analysis of LOXL1 (**a**) regardless of age. When the age factor was examined (**b**), LOXL1 showed a significant decrease with age (**p<0.01) and differences emerged in specimens from the younger subjects between healthy and varicose veins (*p<0.05). Mouse anti-ß actin antibody was used as a loading control.

permanent or reversible nature of the smooth muscle cell alterations observed. The use of cell cultures ensured that all the cells were under the same environmental conditions, permitting us to determine whether the changes shown in vivo persisted under such controlled conditions.

In summary, this study confirms that markers reflecting the synthesis and stability of the vein wall elastic component (LOXL/tropoelastin) diminish with age. This possibly gives rise to a defective form of elastin (non-reticulated) consistent with a functional loss of elasticity, which is one of the characteristics of the aging process.

In chronic venous insufficiency (varicose veins), the stratification of patients by age enabled us to demonstrate the inverse relationship between LOXL and tropoelastin in the young population, in which tropoelastin accumulates when LOXL decreases. The greatest TE expression was observed in the veins of young people with venous insufficiency. This increase could be a reaction mechanism designed to compensate for the loss of elasticity and tone suffered by the vein wall as the insufficiency starts to develop.

These results emphasize the importance of LOXL1 as a gene target to avoid the characteristic loss of elasticity of the varicose vein, in tissues where elastic fibres are essential for the correct maintenance of function such as blood vessels.

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