ELEVATED EXPRESSION OF MMP-9, AND ITS POTENTIAL IMPLICATIONS IN VARICOSE VEINS

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Abstract:
Background: Varicose veins are twisted, widened veins in the subcutaneous tissues of lower limbs and are often easily visible. The primary cause of varicose vein formation is not clear. An imbalance between Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been implicated in some studies The aim of this study was to study the role of MMP-9 which belong to the subclass of gelatinases of MMPs.
Materials and Methods: 58 male patients in the range of 18-35 years undergoing surgery for varicose veins were selected for the study and compared with normal veins extracted from a similar group undergoing herniorrhaphy for a groin hernia. Immunohistochemistry was performed in these specimens using a kit procured from Biogenex
Results: MMP-9 in all 58 cases in comparison to controls show differential expression between the layers of veins. strong immune positivity of MMP-9 (3+) was predominantly observed in the tunica adventitia in vasa vasorum (14 patients). However, 29 out of 58 patients (50%) did not show any expression in the tunica intima. Hence, our observation of MMP-9 in varicose veins showed high expression in tunica adventitia and tunica media, in comparison to tunica intima.
Conclusion: MMPs play important roles in the biological and pathological processes leading to varicosities. Hence, drugs aiming at the inhibition of MMPs or balancing of MMPs are of significant utility. Future studies should expand on the possibility of hemodynamic and saphenous vein grafts capable of altering biomarkers and biochemical signalling, which can assist vascular remodelling
.This knowledge is critical to developing novel treatment protocols and preventive approaches against varicose veins.

Keywords: MMPs, MMP9, Gelatinase and Varicose veins.

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BACKGROUND

Varicose veins are curved tortuous subcutaneous dilated veins derived from the Latin word – varix means twisted. They appear as green-coloured streaks, which grow up to few centimetres in diameter. Patients usually show signs of itchiness, pulling pain, swelling of the ankles, eczema, and spider veins next to the big varicose veins, with changes in colour around the veins.

Varicose veins are relatively common in Western countries compared to Asia and Africa[1]. Histologically, the veins consist of three layers, the outer most tunica adventitia, the middle layer tunica media, and the inner most tunica intima. The adventitia consists of connective tissue in which collagen fibres are prominent.

Tunica media predominantly consists of elastic tissue or smooth muscle. Some connective tissue is also present. The tunica intima, media, and adventitia can be distinguished, especially in large veins. A clear distinction between intima, media, and adventitia cannot be made in small veins as these veins contain predominantly fibrous tissue [2-4].

The cause of the development of varicose veins has been an enigma wrapped in a mystery. Established risk factors like obesity, hypertension, diabetes, obesity and smoking are potential risk factors. Valvular incompetence occurs as a result of venous dilatation. This concept is regarded as the major cause of varicose veins. Various factors determine the occurrence of the varicose vein, such as exercise, genetics, deep vein thrombosis, contraceptive hormones, pregnancy, people who stand for long hours, and obesity, to name a few. In particular, exercising too much or too less alters blood circulation, contributing to varicose veins [5].

Studies performed on the varicose vein walls have led to several hypotheses. Several studies have reported an increase in smooth muscle. In comparison, others report reduced amounts of smooth muscle due to replacement by connective tissue [6-7]. Varicose veins histology shows fragmentation of the elastic lamellae, loss of circular and longitudinal smooth muscle fibres, and damage to the endothelium.

There has been a lot of focus on the imbalance in the synthesis and degradation of matrix proteins, resulting in the structural weakness of the venous wall resulting in loss of muscle tone [7].

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-containing enzymes that can break down connective tissue; classified into five groups (collagenases, gelatinases, stromelysin, matrilysin, and the membrane-type MMP) based on substrate specificity. Gelatinases such as gelatinase A (MMP-2) and gelatinase B (MMP-9) digest gelatins and are associated with type II fibronectin repeats inserted into the catalytic subunit, bound to laminins, collagens, and gelatins.

Increased blood pressure in the varicose veins can contribute to the overexpression of selected MMPs, affecting the endothelium smooth muscle and extracellular matrix proteins of the vein wall [8-9]. Gelatinases, which include MMP-2 and MMP-9, are responsible for the degradation of the extracellular matrix within the vein wall under both physiological and pathological conditions[10]. The main function of gelatinases involves the degradation of fibres of denatured collagen and other structural components of the extracellular matrix, allowing the migration of cells, including smooth muscle cells[10-11]. MMP-9 is present in large quantities in granules of neutrophils. It plays a major role in the influx of leukocytes to the site of infection or damage to tissue during inflammatory processes[9]. Vascular endothelial growth factor interferes with the integrity of the vascular wall and activates endothelial nitric oxide synthase, which dilates venous vessels and also stimulates the synthesis of MMPs, especially MMP-9.[12-15]

MATERIALS AND METHODS

The study was undertaken after getting approval from the institutional ethics committee, KS Hegde Medical Academy, reference number INST EC/E C/15/2009-10.

Study Design: Case-Control Study

Source of Data: Patients aged 18-35 years, admitted to K.S. Hegde Charitable Hospital, undergoing surgery for varicose veins, and a similar group undergoing surgery for groin hernia. Informed consent was obtained from both groups of subjects.

Inclusion Criteria (Cases):

18-35 years old male

Primary Symptomatic Varicose veins

Ability to give informed written consent

Exclusion Criteria (Control):

Age < 18 years or > 35 years

Inability to give informed written consent

Prior interventions for varicose veins

Eg: Surgery, Sclerotherapy

Immunohistochemistry of MMP-9:MMP-9 expression was analyzed using a kit procured from

Biogenex. The biopsies received from the patients and controls were fixed with 10% buffered formalin for 24 hours. Both the longitudinal and cross-sections from the tissues were further processed in the histokinette. The processing techniques include dehydration, clearing (de-alcoholization), and paraffin-embedding. The fully processed tissues were then embedded in the horizontal surface of Lukard's moulds (L-moulds). The paraffin block was cooled in tap water, trimmed, and prepared for microtome sectioning. Very thin sections (2-4 microns) were taken using a rotary microtome. The sections were floated in the water bath at four degrees Celsius, less than the melting point. The floating tissue was put on the slide and smeared with egg albumin. Slides were kept on the hot plate so that the tissue wax was melted completely, after which the tissue was utilized for the immunohistochemistry staining. The same protocol was repeated for control tissues (normal veins) as well as patient tissue sections.

Protocol

Xylene 1 10 minutes
Xylene 2 10 minutes
100% alcohol-5 minutes
90% alcohol- 5 minutes
70% alcohol- 55 minutes
Running tap water for 2-5 minutes
Antigen retrieval: AR2 solution for 15 minutes at 1degree celsius
Peroxide block (100 µL) for 10 minutes at room temperature
Primary antibody incubation for 30 minutes at room temperature
Buffer wash
Super enhancer (100 µL) for 20 minutes at room temperature
Buffer wash
Polymer HRP (100 µL) for 30 minutes at room temperature
Buffer wash
DAB (to be freshly prepared) - One drop of liquid chromogen in 1ml stable DAB buffer. Add 100 µL to the tissue and incubate for 5 minutes at room temperature. Running tap water
Haematoxylin counterstain (100 µL) for 3 minutes at room temperature
Running tap water
Clearing and mounting
70% alcohol-5 minutes
90% alcohol-5 minutes
100% alcohol-5 minutes
Xylene 1-10 minutes
Xylene 2-30 minutes
Mountin

RESULTS

Table:1MMP-9 expression is increased in the varicose veins compared to controls in the tunica intima

In the present study, 41.4% (n=24) of the patients with varicose veins showed no expression of MMP-9 in the varicose vein. In control subjects, 96.6% (n=56) showed total lack of MMP-9 expression in the normal vein (p value<0.001).

Mild elevation (1+) in MMP-9 level in the varicose vein was observed in 24.1% (n=14) of patients. Mild elevation in the MMP-9 level was also evident in the control group (Hernia Patients' vein) where was observed in 3.4% (n=2) subjects showed mild expression.

Moderate elevation (2+) ofthe MMP level in the varicose vein was observed in 19.0% (n=11) patients. But there was a total lack of MMP-9 expression in the control group.

Very high elevated expression of MMP-9 was observed in 15.5% (n=9) of the patients with varicose veins. But none in the control group showed any positive expression.

Table 2:MMP-9 expression is increased in varicose veins compared to controls in the tunica media

In the present study 36.2%(n=21)of the patients with varicose veins showed no expression of MMP-9.In the control subjects 96.6%(n=56) showed no changes in the MMP-9 level(p value<0.001)

Mild elevation(1+) in MMP-9 was found in 34.5% (n=20)in patients with varicose veins.Only 2 controls which constituted about3.4% of the controls studied showed mild changes.

Moderateelevation(2+)ofMMP-9 was found in 22.4%(n=13) while high elevation(3+) in MMP-9 was found in6.9%(n=4) of the patients. None of the controls showed either moderate or high expression of MMP-9 in the tunica media.

Table3:MMP-9 expression is elevated in varicose veins compared to controls in tunica adventitiaIn the present study, 24.1% (n = 14) of the patients with varicose vein showed no expression of MMP9 in thetunica adventitia. Similarly, 96.6% (n = 56) of the controls showed no expression of MMP–9 in the tunica adventitia.

Mild elevation (1+) in MMP-9 level in the varicose vein was observed in 29.5% (n = 17) patients, whereas in the control group, 3.4% (n = 2) showed mild elevation in the expression of MMP-9 in the tunica adventitia.

Moderate elevation (2 +) in the expression level of MMP-9 in the varicose vein was observed in 20.7% (n = 12) of the patients. But none of the control groups showed any expression of MMP-9 in the tunica adventitia.

High elevation (3+) in MMP-9 level in the varicose vein was observed in 25.9% (n = 15) ofthe patients. But none of the control groups showed any expression of MMP–9 in the tunica adventitia.
Overall, it is significant to notice that the expression levels showed a differential pattern throughout the layers of the veins. More importantly, there was a trend for increased expression of MMP-9 from tunica intima to tunica adventitia within the layers of veins in patients. Accounting for the number of samples taken from the patients showing at least some overall expression (mild to high) to be in ascending order starting from the tunica intima area (34/58; 58.62%), followed by tunica media area (37/58; 63.79%), and then the tunica adventitia area (44/58; 75.86%). This proves that there is considerably higher expression of MMP-9 compared to similar areas within the layers of vein between patients and controls. As such, it can be concluded that MMP-9 could be perceived as a drugg

DISCUSSION

Varicose veins are tortuous veins usually seen in lower limbs. The prevalence of this disease varies widely by geographic location. The highest reported rates are in Western countries. Reports of chronic venous insufficiency prevalence vary from <1% to 40% in females and from 1% to 17% in males. Prevalence estimates for varicose veins are higher, 1% to 73% in females and 2% to 56% in males. These reported ranges in prevalence estimations reflect differences in the population distribution of risk factors, accuracy in the application of diagnostic criteria, and the quality and availability of medical diagnostic and treatment resources [16]. In varicose veins, the valves will become incompetent. These valves with structural changes may be leaky with progressive reflux. The increase in venous pressure leads to structural and functional changes in the vein wall. An increase in vein wall tension increases the expression of matrix metalloproteinases which causes the degradation of extracellular matrix proteins and thereby affects the structural integrity of vein walls [17]. Thrombophlebitis is characterized by inflammation of the endothelial vein wall, inflammation of the valve, and leukocyte infiltration leading to disruption of vein function, and venous thrombosis is a complication of varicose veins [18,19]. Thrombophlebitis may occur in the course of varicose veins, or it may be induced during the treatment of the disease with sclerosing agents [20]. Saphenous vein specimens from patients with CVD have shown increased macrophage infiltration in the vein wall and valves [21].

In a study that compared MMP-1, -2, -3, and -9 in control veins, varicose veins, and varicose veins complicated by thrombophlebitis, the latter showed an elevated content of MMPs in the vein wall and increased MMP-1, -2, and -9 activity. Varicose veins showed increased activity of MMP-9 This agrees with our study, where there was an increase in MMP-9 activity. These marked changes in the content of MMPs and activity in varicose veins, especially those affected with thrombop-hlebitis, could lead to venous tissue remodelling and alterations in the mechanical properties of the vein wall [22]. Studies have shown that increased expression of MMPs can be caused due to mechanical stretch or pressure. Increased venous pressure may increase MMP expression, and MMPs may then affect different wall components and cell types, including extracellular matrix, fibroblasts, vascular smooth muscle cells, and endothelial cells. In the later stages of the disease, severe increases in venous hydrostatic pressure can cause endothelial cell damage, leukocyte infiltration, and superimposed venous inflammation, which are characteristic of advanced stages of chronic venous insufficiency [23-24].

Some studies examined the effects of perfusing saphenous vein segments ex-vivo and demonstrated a decreased expression of MMP-2 and -9 when exposed to venous pressure. But there was a 50% decrease in the expression of gelatinases when veins were exposed to arterial pressure for up to three days. These data suggest that there is an association between hemodynamic changes in venous pressure and shear stress, saphenous vein remodelling, and MMP expression [25].
Type III collagen is essential for the elasticity and distensibility of blood vessels. The alterations in collagen synthesis and collagen type I to type III ratio may affect the vein wall architecture, thus leading to structural weakness, venous dilation, and varicose vein formation. The levels of collagen I and III are co-regulated in fibroblasts, and the addition of collagen III to cultured vascular smooth muscle cells from varicose veins decreases collagen I synthesis [26]. There can be increased, decreased, or unchanged collagen The collagen content is in there is a balance between its biosynthesis and its degradation by MMPs [27].

Marimastat is an inhibitor of matrix metalloproteinase. Some studies have shown that treatment with this compound can cause partial

restoration of type III collagen production in varicose veins [26]. There is a study that demonstrates that in varicose veins, MMPs can be induced by postural changes. This was done by comparing plasma from a brachial vein and lower extremity varicose veins in patients in a standing position after thirty minutes stasis. There was an increase in proMMPs, proMMP-9 in plasma from the varicose vein compared to their levels in an arm vein. The increased proteolytic activity was associated with increased plasma levels of endothelial and leukocyte activation markers, vascular cell adhesion molecule-1, angiotensin-converting enzyme, and L-selectin. This suggests endothelial cell activation, polymorph nuclear cell activation, and enzymatic granule release in varicose veins during periods of postural blood stasis [24, 28]. These observations support the proteolytic role of MMPs.

Other studies have shown that MMP-2 and MMP-9 can inhibit the contraction of rat aorta, which is induced by phenylephrine [29]. But it is unclear how the MMPs cause venous dilation and varicose vein formation. The proteolytic effect of matrix metalloproteinase on the extracellular matrix, degradation of valve leaflets, and weakening of the structure of vein wall is considered to be largely responsible for varicose vein formation [30].

The proteolytic and degenerative effects of MMPs on the extracellular matrix could play a role in the late stages of the formation of varicose veins. On the other hand, the fact that the MMPs are localized near the venous endothelium and vascular smooth muscles raises the possibility that these enzymes could have an additional effect on these cells [29].

There is also a role for MMP-9 in vascular smooth muscle cell migration. Tanshinone IIA inhibits human aortic smooth muscle cell contraction through the inhibition of MMP-9 activity. Tanshinone IIA is a constituent of *Salvia miltiorrhiza* (a plant used in Chinese medicine) [31]. Curcumin has an inhibitory effect on the migration of human aortic smooth muscle cells. This is done by suppressing the MMP-9 expression [32]. In a mouse model of filament loop injury, MMP-9 knockout resulted in a reduction of vascular smooth muscle cell migration [33]. Basement membrane disruption is essential for vascular smooth muscle cell migration [34]. MMPs can degrade the basement membrane, facilitating extracellular matrix-integrin interactions. This, in turn, will lead to the activation of focal adhesion kinases and increased smooth muscle cell migration. MMPs also cause fragmentation of membrane components such as type I collagen [35].

MMPs may also regulate the proliferation of vascular smooth muscle cells. Pre-treatment of human aortic smooth muscle cells with ethanol extract of *Buddleja officinalis* has reportedly caused cell proliferation by suppressing MMP-9 activity [36]. Kosugi et al. analysed the expression of MMP-9 in varicose and non-varicose specimens.

The enzyme was found to be increased, which is in concordance with my study. They found that MMP-9 was localized in the smooth muscle cells in the tunica media. The ratio of immune positive cells for MMP-9 in the great saphenous vein of the groin, a great saphenous vein of the ankle, was significantly higher than that of normal greater saphenous veins of the groin.

So, according to them, MMP-9 may be produced in the Varicose vein wall and degrade elastic lamellae and other extracellular components of the venous wall [37]. However, in a study by Woodside et al. on a similar type of specimen in the same year, it was observed that there was no change in MMP-1 and decreased MMP-9 activity [38].

In our study, MMP-9 on all 58 cases in comparison to controls, show differential expression between the layers of veins. Whereas strong immune positivity of MMP-9 (3+) was observed in tunica adventitia predominantly in vasa vasorum (14 patients), only moderate expression of MMP-9 (2+) was seen in tunica media. However, 29 out of 58 patients (50%) did not show any expression in the tunica intima. Hence, our observation on MMP-9 on varicose veins showed high expression in tunica adventitia and tunica media compared to tunica intima. Since MMP-9 is expressed strongly in vasa vasorum in this study, it can be considered a significant factor in the aetiopathogenesis of varicose veins.

Only 6 controls (10%) showed mild positivity for MMP-9 in this study. Since vasa vasorum had the highest expression of MMP-9 in the varicose veins, there could be a possibility that the blood supply might have been affected, leading to hypoxia and the weakening of the vessel wall. This might, in turn, lead to an imbalance between venous matrix metalloproteinases and TIMP leading to varicose veins. Hence, it can be concluded that weakness in the vessel wall due to vascular ischemia is one of

the primordial causes for the development of varicose veins.

As per the presented data, mild elevation (1+ score) of MMP-9 levels was seen in the control group compared to the subjects who had varicose veins. Although this accounted for only 3.4% (n=2) of the total subjects, we expect this to have been due to the after-effect of concomitant medications or underlying comorbid conditions. Although this remains as an artefact, the results in its totality attest to the significance of elevated expression of MMPs and its connection to the varicose veins.

CONCLUSION

Varicose veins are mistaken to be less important and often neglected as a cosmetic problem. Although, not a cause of disability, varicose veins influence socio-economic societies, and hence can affect the quality of life. Currently, available treatment modalities are invasive by either venous stripping – removing varicose veins by tying at sites of origin and stripping smaller veins by making small perforations along with the limbs. However, these have become obsolete as of now, and newer lesser invasive tactics are currently available. The advent of endovenous ablation techniques provides essential relief to the patients affected, but novel and lesser invasive options are to be expanded. MMPs play important roles in the biological and pathological processes leading to varicosities

Hence, drugs aiming at the inhibition of MMPs or balancing of MMPs are of significant utility. Future studies should expand on the possibility of hemodynamic and saphenous vein grafts capable of altering biomarkers and biochemical signalling, which can assist vascular remodelling. This knowledge is critical to developing novel treatment protocols and preventive approaches against varicose veins.

TABLES AND FIGURES

Table 1: Expression of MMP- control and varicose vein subjects in the tunica intima

Tunica Intima	Group		P value
	Patients Count (%)	Controls Count (%)	
0	24(41.4%)	56(96.6%)	<0.001 vhs
1+Mild	14(24.1%)	2(3.4%)	
2+Moderate	11(19.0%)	0(0.0%)	
3+High	9(15.5%)	0(0.0%)	
Total	58(100.0%)	58(100.0%)	

Vhs-very highly significant (chi-square test)

Table 2: Expression of MMP-9 in control and varicose vein subjects in tunica media

Tunica media	Group		P value
	Patients Count (%)	Controls Count (%)	
0	21(36.2%)	56(96.6%)	<0.001 vhs
1+Mild	20(34.5%)	2(3.4%)	
2+Moderate	13(22.4%)	0(0.0%)	
3+High	4(6.9%)	0(0.0%)	
Total	58(100.0%)	58(100.0%)	

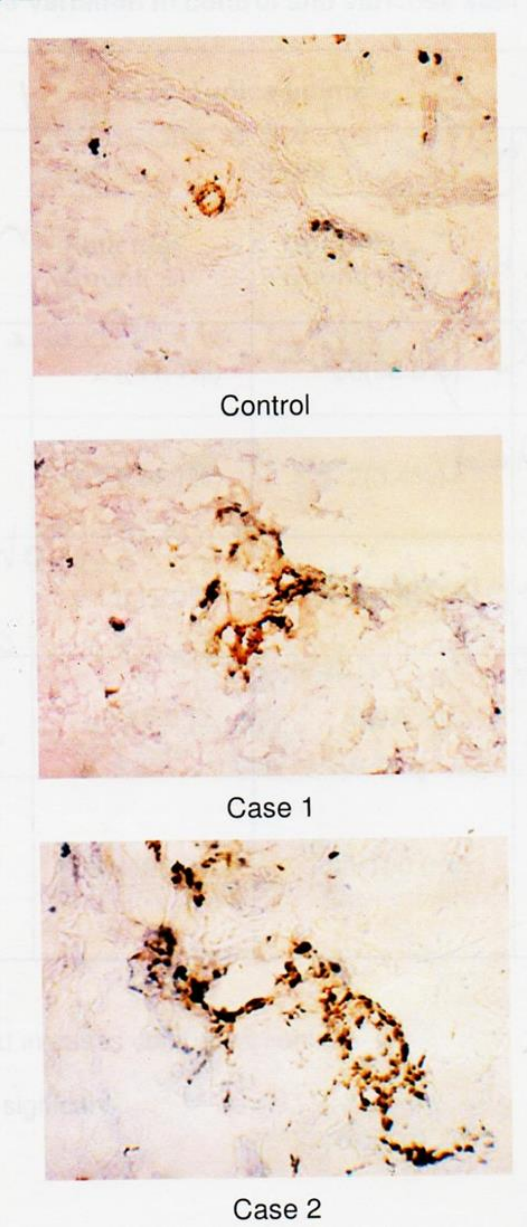
Vhs very highly significant (chi-square test)

Table3: Expression of MMP-9 in control and varicose vein subjects in the tunica adventitia**.**

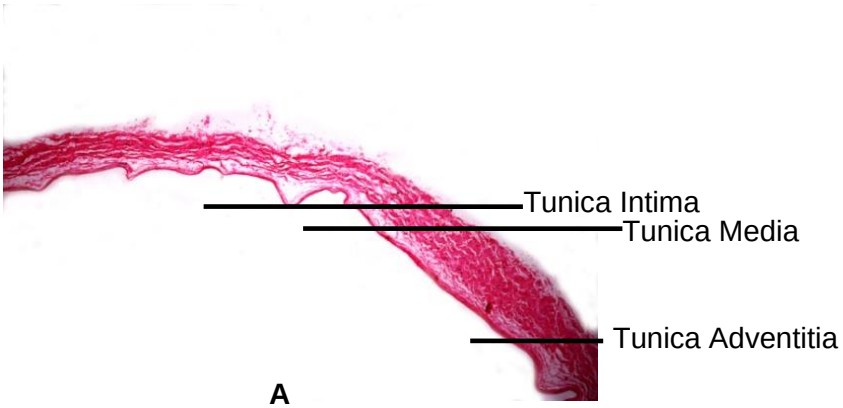
Tunica Adventitia	Group		P value
	Patients Count (%)	Controls Count (%)	
0	14(24.1%)	56(96.6%)	<0.001 vhs
1+ Mild	17(29.5%)	2(3.4%)	
2+ Moderate	12(20.7%)	0(0.0%)	
3+ High	15(25.9%)	0(0.0%)	
Total	58(100.0%)	58(100.0%)	

Vhs-very highly significant (chi-square test)

Fig. 1: Representative immunohisto chemical staining of MMP-9



Immunohistochemistry of mmp-9 in both control and cases.
Control: MildMMP-9 expression in the vasa vasorum.
Cases: Strong MMP-9 expression in the vasa vasorum, tunica adventitia and smooth muscle cells of the tunica media.



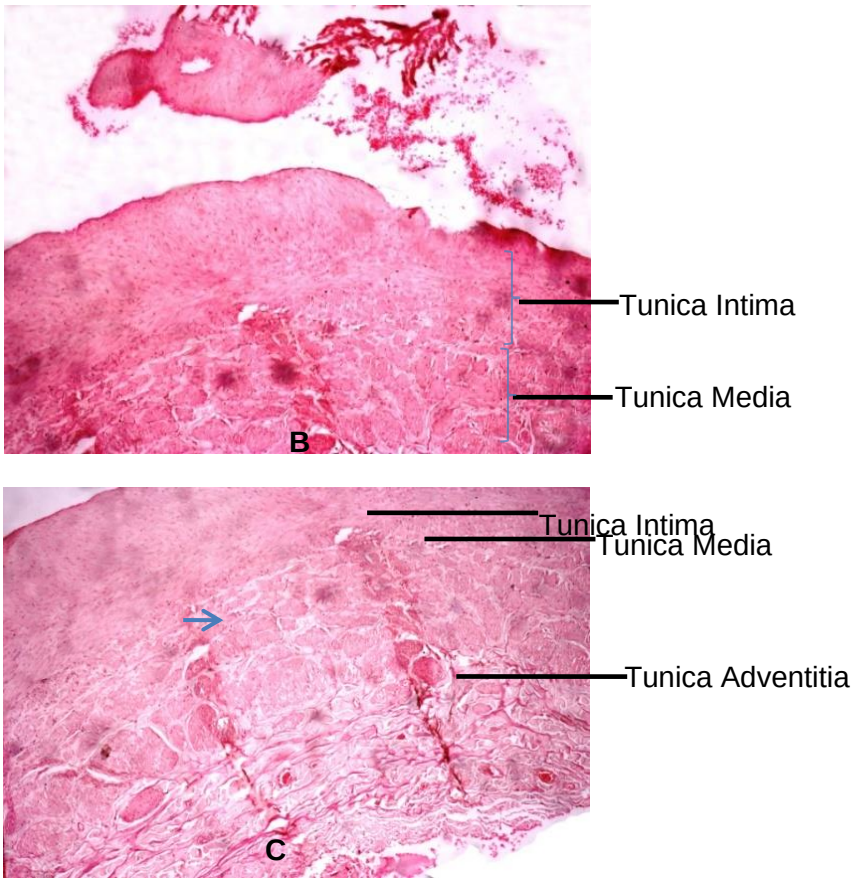


Fig 2: Representative Haematoxylin And Eosin Stain

A. Microphotograph of the normal vein showing tunica intima-media adventitia with thin internal elastic (H & E stain x 10x)
B. Lower power view of VV displaying irregular thickening of the intima and media with replacement by collagen tissue (H & E stain x10)
C. High power view of intima and media displaying irregular thickening and disposition in the intima association with thesplitting of theinner longitudinal muscle layer of the media (H&Estain High power view of adventitia displaying increased collagen disposition in the adventitia (H & E stain x45)

Abbreviations

MMPs-Matrixmetalloprteinases
TIMPs-Tissue inhibitor of matrix metalloproteinases

Competing interests

The authors declare that there are no competing interests

Authors contributions

Dr Remya Vinod: processing the tests and preparation of the manuscript
Dr Sunil Kumar Y:Guiding in test processing and guiding in preparation of the manuscript
Dr Rajesh Ballal: Specimen collection and guiding in manuscript preparation

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Subjects who consented to give the samples

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