Eur J Vasc Endovasc Surg **11**, 230–237 (1996)

Assessment of Wall Structure and Composition of Varicose Veins with Reference to Collagen, Elastin and Smooth Muscle Content

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Objectives: *To compare collagen, elastin and smooth muscle contents of varicose and control long saphenous veins.*
Design: *Collagen, elastin and muscle were estimated stereologically using random sampling and histological staining.*
Materials: *Varicose vein samples were collected from nine patients (mean age 52 years, range 34–64 years) undergoing vein stripping, sample sites being saphenofemoral junction and knee. Control samples were taken from five patients (mean age 58 years, range 38–76 years) presenting for femoral-popliteal bypass at equivalent levels.*
Methods: *Veins were fixed, sectioned transversely, and stained with Picric Acid Sirius Red. Analysis of samples was performed using point and intersection counting on vertically projected images.*
Results: *Using two way analysis of variance tests, varicose saphenous veins had significantly larger wall areas ($p < 0.01$) and higher amounts of collagen ($p < 0.01$). Collagen content and wall area were significantly larger proximally compared to distally in both control and varicose veins ($p < 0.05$) with a higher content of smooth muscle and elastin in varicose veins proximally compared to distally ($p < 0.05$). There was no difference in wall thickness or elastin content between the two groups.*
Conclusions: *This suggests that varicose veins are a dynamic response to venous hypertension and are not thin walled structures as previously thought.*

Key Words: Collagen; Elastin; Muscle; Varicose vein.

Summary

This study compares the collagen, elastin and smooth muscle contents of varicose and control long saphenous veins estimated stereologically using a random sampling regime. Varicose saphenous vein samples were collected from nine patients (mean age 52 years, range 34–64 years) undergoing vein stripping operations, sample sites being the saphenofemoral junction and knee. Control samples were taken from five patients (mean age 58 years, range 38–76 years) presenting for femoral-popliteal bypass operations at equivalent levels. All veins were fixed, embedded in wax, sectioned transversely, mounted and stained with Picric Acid Sirius Red for differential staining of collagen and elastin. Analysis of samples was performed using point and intersection counting on vertically projected images bearing a calibrated test grid square for $\times 40$ and $\times 200$ magnification. Results show, using two way analysis of variance tests that varicose saphenous veins had significantly larger wall areas ($p < 0.01$) and higher amounts of collagen ($p < 0.01$) compared to controls. Collagen content and wall area were significantly larger proximally compared to distally in both control and varicose veins ($p < 0.05$) and there was a higher content of smooth muscle and elastin in varicose veins proximally compared to distally ($p < 0.05$). There was no difference in wall thickness or elastin content between the two groups. This suggests that varicose veins are a dynamic response to venous hypertension and are not thin walled structures as previously thought.

Introduction

Varicose veins affect up to 20% of people in the Western World.[1] Roughly 10% of those affected have complications such as superficial thrombophlebitis,

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1078–5884/96/020230 + 08 $12.00/0

pigmentation, lipodermatosclerosis, haemorrhage, ulceration and increased risk of deep vein thrombosis. This common venous disorder presents a large cost to National Health Services and a socioeconomic problem. A clearer understanding of varicosis may help us to effect better forms of treatment or prevention.

Why varicose veins occur in some individuals and not others is unknown. Genetic factors are thought to play a role whilst other theories are based on abnormalities in vein wall or valve structure.[2–7] Vein wall distensibility is controlled by collagen, elastin and smooth muscle. Smooth muscle in the tunica media is responsible for wall tone, which is influenced by autonomic nerves and circulating stimulants. Passive tone is provided by collagen and elastin. Loss of tone in varicose veins could be due to defects in these wall components.

The weak wall hypothesis proposed that the primary fault is a general defect of vein walls in the lower limb.[8] The theory proposes that this defect in wall structure and composition is the cause of varicosis. Varicose veins appear dysplastic and vulnerable to pressure-induced dilation.[5] They dilate in response to normal pressures and valvular incompetence develops as the cusps spread apart. Others propose a reduction or deficiencies in vein valves[9] but Cotton[8] showed that the site of venous dilation is often below valves and, if valves were incompetent, the resulting heightened hydrostatic pressure should cause the sinuses above the valves to dilate.

A characteristic finding in varicose veins is localised "lateral blowouts". If primary valve failure had occurred, the resulting back pressure would be evenly distributed to the vein wall producing a uniform dilation. Cotton[8] concluded that the origin of varicose veins could not be attributed to descending valvular incompetence originating at the saphenofemoral junction.

The natural response to over-filling is an increase in tone which will return blood to deep veins through competent perforators above and below the leak.[6] A normal vein wall when distended from below is capable of withstanding considerable back pressure.[10]

The weak wall theory is the most favoured due to the above objections to valve failure theories. Primary wall weakness explains why varicosities are often found below competent valves.[6]

Recent histological studies performed on varicose vein walls have led to several hypotheses. Contradictory evidence exists on the connective tissue concentration of varicose veins. Several workers have found collagen decreases in varicose veins.[11–13] Andreotti *et al.*[12] also found a significant decrease in elastin with an increase in total sugars and soluble non-scleroproteins. They proposed that a primary defect in the amount of collagen and elastin supporting an endothelium from the outside would lower resistance to venous pressure. In contrast, Rose and Ahmed[6] and Maurel *et al.*[14] have shown that varicose vein walls have a higher than normal collagen content whereas Travers *et al.*[15] found no difference in the ratio of collagen to total protein between control and varicose veins.

Contradictory evidence also exists on the pathology of smooth muscle in varicose veins. Several studies have reported an increase in smooth muscle, or its activity[11,16,17] whereas others report reduced amounts of smooth muscle due to replacement by connective tissue.[6,16] Rose and Ahmed[6] suggested that separation of muscle cells by fibrous infiltration prevents them from acting as a unified whole, with subsequent alterations in wall tone leading to pathological dilation. Other studies[18,19] have failed to demonstrate any difference in the wall muscle content of control and varicose veins.

Because of the conflicting reports on the collagen, elastin and muscle contents of varicose veins, this study aims to look at the composition and structure of control and saphenous varicose vein walls by a combined histological and morphometric approach. Some of the uncertainties concerning the composition of the vein wall may be due to arbitrary sampling which may not reflect sufficiently the natural differences within veins. Moreover, the practice of expressing protein content relative to dry weight may be misleading if absolute wall mass alters in varicose veins (the so-called "reference trap"[20]). To overcome these deficiencies, this investigation quantifies wall dimensions and composition at different sites along and across veins. Due to variation in wall constituents along the length of the long saphenous vein,[21] longitudinal and transverse vein samples were drawn using a randomised sampling protocol.

Materials and Methods

Sample collection

Varicose saphenous vein samples were collected from nine patients (age range 34 – 64 years, mean 52 years) with primary varicosis of, and undergoing stripping operations on, the long saphenous vein. All patients had clinically demonstrable venous reflux. Samples were taken from proximal (near to the insertion into the femoral vein) and distal (at the knee) regions.

Control samples were taken from five patients (age range 38 – 76 years, mean 58 years) with no history of varicosis and no clinically demonstrable reflux, presenting for femoral-popliteal bypass operations.

Sections of vein from control and varicose patients were immediately fixed in 10% buffered formalin, processed, wax-embedded, sectioned and mounted on glass microslides. Picric Acid Sirius red staining was used for differential staining of collagen and elastin, staining collagen deep red, elastin black, and background tissue, which would be mostly muscle, yellow.

Stereological analyses

The sections analysed consisted of proximal and distal regions of control and varicose specimens. A Wild microscope was set up which projected the image of each microslide specimen onto a vertical viewing screen. The scale of linear magnification of the projected image was calibrated at low and high power (objective lenses $\times 40$ and $\times 200$ respectively) using a 10mm stage graticule. To check that there was no image distortion, both vertical and horizontal distances across the graticule were measured on the magnified image. For flat projection, these distances should be equal.

A grid lattice consisting of 2cm $\times$ 2cm squares was employed for morphometry of the areas and relative volumes of wall components. The grid was randomly placed over the projected image of the vein wall and the numbers of test points of the grid that overlaid the sectional images of the wall and lumen were counted. Provided that grids are positioned randomly, the point totals provide unbiased estimates of sectional areas (for a review, see Mayhew[22]). The size of the grid and the linear magnification are constants. The relative numbers of points overlying different components also provides unbiased estimates of the fractional volumes which they occupy in the vein wall.[22]

At low power, estimates were made of the transverse sectional areas of the whole vein wall (excluding adventitia) and of the lumen. The adventitia was not included because this is damaged during surgical intervention and is artefactually inconstant in amount. All other data were gathered from high-power projections. For this purpose, we sampled sectors of the vein wall in a systematic random fashion.[23] A test circle divided into 12 sectors was superimposed on the projected image so that their centres were coincident. Three sectors per slide were chosen using the lottery method to obtain a random number between 1 and 12 (the number corresponding to a sector and, hence, to part of vein wall lying within it). This provided the first sector and systematic sampling determined the position of the other two sectors to be studied. The three areas of vein wall were contained within sectors 120° apart.

Point totals were obtained for the collagen, elastin and muscle content in the intima, media and whole wall by recording red, black and yellow areas respectively. Point counts on the sectors of each vein were totalled and a percentage of the different wall components was then obtained for the intima, media and whole wall. The proportions of collagen, elastin and muscle in the intima, media and whole wall were multiplied by intimal, medial and wall areas respectively, to obtain the actual amounts of wall constituents in different compartments of the vein wall.

Wall thicknesses were measured at right angles to the tunics from random start points generated by the sectors. Thicknesses were recorded at the boundaries and middle of each sector to obtain an average. Knowing the linear magnification, actual thicknesses (uncorrected for shrinkage errors) were calculated. As with sectional areas, these are valid for comparative purposes provided that processing distortion errors are equivalent.

Statistics

Two-way analysis of variance tests were used to compare varicose and control veins and proximal and distal regions for all the variables measured.[24] The interaction term generated by this test made it possible to investigate whether or not varicosis exerted preferential effects at proximal or distal sites. Paired *t*-tests were used to assess intra-individual variation, i.e. to compare proximal and distal regions within veins. All data were handled using the Unistat Statistical Package (Unistat Ltd, London, U.K.) on a Viglen Genie Mini Tower 4DX266 PC.

Results

Two-way analyses of variance failed to demonstrate any significant interaction effects between group (control *vs.* varicose) and site (proximal *vs.* distal). Generally speaking, control veins did not show any regional (site) variation. There were no significant regional differences in the sectional areas of the intima, media, whole wall or lumen. Also, there were

Table 1. Summary morphometric data on the thickness and elastin composition of vein walls. Values are group means (SEM)

Control		Varicose	
Proximal	Distal	Proximal	Distal
Thicknesses (mm)			
Intima			
0.05 (0.01)	0.11 (0.03)	0.08 (0.02)	0.06 (0.02)
Media			
0.49 (0.05)	0.38 (0.08)	0.56 (0.06)	0.40 (0.06)
Wall			
0.54 (0.06)	0.50 (0.08)	0.64 (0.06)	0.46 (0.07)
Elastin Areas (mm^2)			
Intima			
0.31 (0.07)	0.35 (0.04)	0.59 (0.13)	0.43 (0.07)
Media			
1.03 (0.37)	0.64 (0.14)	1.79 (0.36)	0.87 (0.13)
Wall			
1.34 (0.43)	0.99 (0.17)	2.39 (0.47)	1.30 (0.22)
Elastin Proportions (%)			
Intima			
57.0 (8.93)	58.8 (9.89)	42.4 (5.80)	52.0 (8.12)
Media			
28.1 (2.87)	27.2 (3.02)	21.6 (2.51)	22.3 (3.77)
Wall			
31.7 (2.75)	32.6 (1.99)	24.4 (2.90)	31.1 (4.64)

no significant differences in collagen, elastin and muscle content of these wall compartments between proximal and distal regions.

Vein wall thicknesses

Using two-way analyses of variance, there was no significant difference in intimal, medial or wall thickness of varicose veins compared to controls (Table 1). Nor were there any significant regional differences in intimal or whole wall thickness. However, proximal media was significantly thicker than distal ($p < 0.05$).

Using paired *t*-tests to compare proximal and distal regions separately in varicose and control veins, we detected no differences in intimal, medial or wall thicknesses.

Vein wall areas

Figure 1 shows the cross-sectional areas of the vein walls and lumen. Analyses of variance revealed that varicose veins had significantly larger areas of media ($p < 0.01$), intima ($p < 0.02$) and total wall (media + intima, $p < 0.01$) compared to controls. In both control and varicose groups, significaantly larger areas were seen in proximal regions of the media ($p < 0.05$) and wall ($p < 0.05$) compared to distal ones.

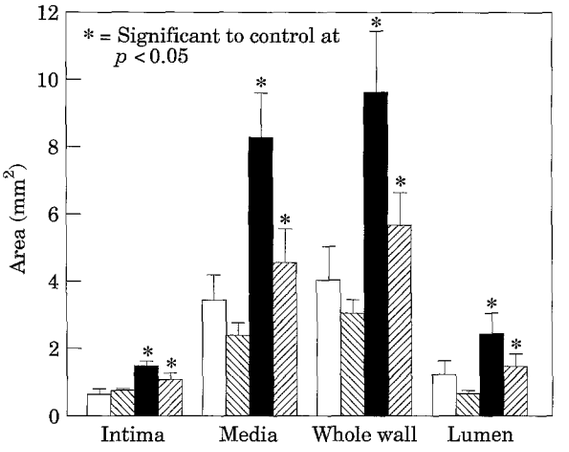

Fig. 1. Areas of control and varicose veins. (□) control proximally; (▨) control distally; (■) varicose proximally; (▨) varicose distally.

A significantly larger lumen area ($p < 0.05$) was seen in varicose veins compared to controls (Fig. 1). However, there was no difference in luminal area between proximal and distal sites.

Using paired *t*-tests to compare proximal and distal regions in the same veins, there were significantly larger wall and medial areas ($p < 0.01$ in both cases) in proximal regions of varicose veins, but no variation in intimal and lumen area was found.

Component contents

(a) Collagen Relative and absolute amounts of collagen are illustrated in Fig. 2. Using analyses of variance, significantly higher amounts of collagen were seen in the wall ($p < 0.01$), media ($p < 0.01$) and intima ($p < 0.05$) of varicose saphenous veins. Moreover, significantly higher levels of collagen were found in the proximal *vs.* distal media in both groups ($p < 0.05$). There was a higher proportion of collagen proximally in the intima ($p < 0.05$), media ($p < 0.05$) and whole wall ($p < 0.02$) of varicose saphenous veins compared to controls (Fig.2a) and a higher percentage of collagen distally in the media of varicose saphenous veins ($p < 0.05$) compared to controls (Fig.2b). Using paired *t*-tests to assess proximal and distal variation, we detected significantly higher collagen contents in the proximal wall ($p < 0.01$) and media ($p < 0.02$), but no difference in the proximal intima of varicose veins.

(b) Muscle There was relatively more smooth muscle in the media ($p < 0.05$) of controls compared to varicose

saphenous veins, (Fig. 3) but there was more smooth muscle overall due to the larger area in varicose veins (Fig. 3a). However, there was no difference in intimal muscle content. No significant difference in smooth muscle content of intima, media or whole wall was found between the proximal and distal regions of the two groups. Paired *t*-tests revealed that proximal regions contained higher amounts of smooth muscle in the wall and media ($p < 0.02$ in both cases). No differences in the muscle content of the intima were found in varicose saphenous veins.

(c) Elastin Except for higher quantities in the proximal media ($p < 0.02$), no significant group, region or interaction effects were demonstrable for elastin content or concentration (Table 1). Using paired *t*-tests, a higher elastin content proximally was found in the media ($p < 0.05$) but not in intima or whole wall.

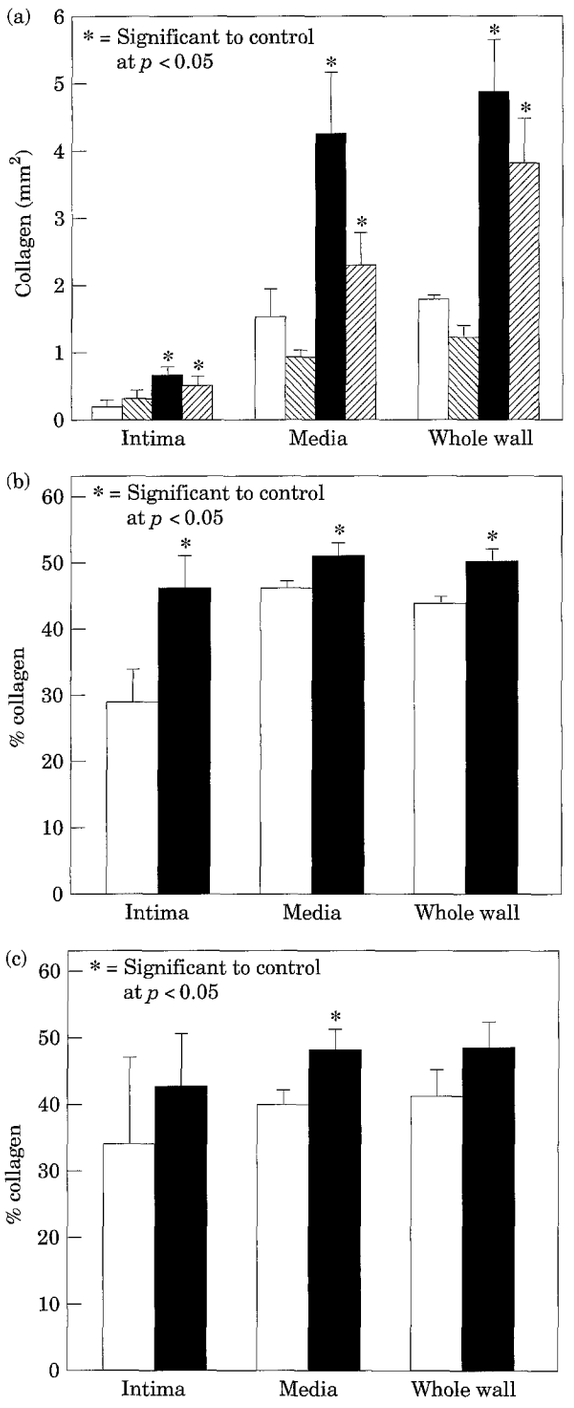

Fig. 2. (a) Collagen content in varicose and control veins. (□) control proximally; (▨) control distally; (■) varicose proximally; (▨) varicose distally. (b) % collagen proximally in control and varicose veins. (□) control; (■) varicose. (c) % collagen distally in control and varicose veins. (□) control; (■) varicose

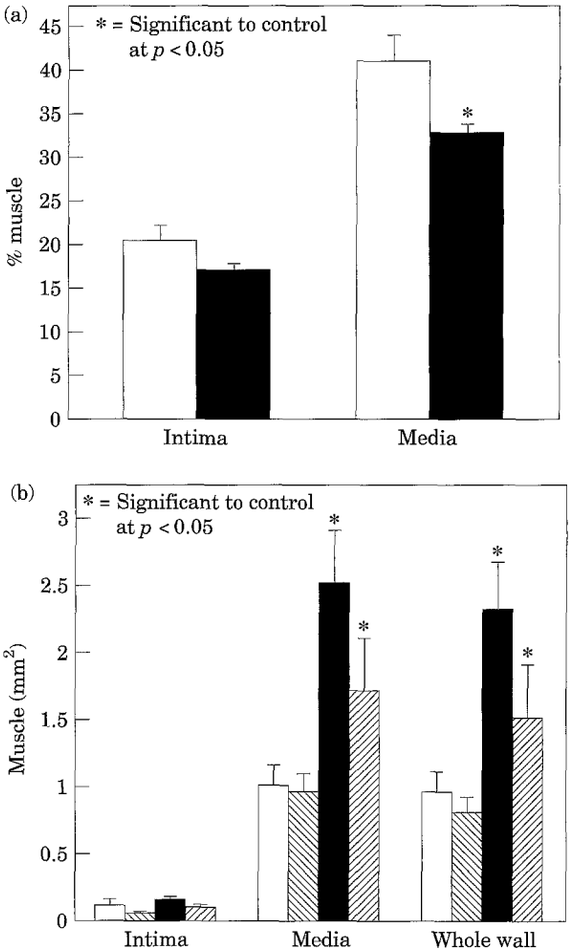

Fig. 3. (a) % muscle on control and varicose veins. (□) control; (■) varicose. (b) total muscle content in control and varicose veins. (□) control proximally; (▨) control distally; (■) varicose proximally; (▨) varicose distally.

Discussion

The present study has shown that varicose saphenous veins have greater cross-sectional areas of intima, media, whole wall and lumen than control veins. The differences affect both proximal and distal regions but are not accompanied by changes in wall thicknesses. These findings suggest that in varicosis the dilation which occurs is not accompanied by a generalised wall thinning. Any thinning which occurs is compensated by thickening elsewhere and this is effected, in part at least, by the production of new wall material.

These findings support others from our laboratories (unpublished observations) which have shown by combined light and electron microscopy, that there are significant alterations of wall morphology at proximal and distal sites, and in different tunics, of varicose long saphenous veins. Varicose saphenous veins were larger with a greater cross-sectional areas (and hence volumes) of tunica intima and tunica media.

The lumen area of a vessel depends not only on transmural pressure and tension generated in the vessel wall[3] but also on any increase in wall area due to hypertrophy/hyperplasia of constituent elements such as muscle to counteract the distending force. The increased lumen area found here suggests that this equilibrium has been disrupted in varicosis.

No significant differences in intima, media or whole wall thicknesses between varicose and control veins were observed. However, an increased variation in varicose vein wall thickness was noticed, possibly reflecting different stages of the varicose process. Within a varicose vein, regions of thinning (blowouts) were often compensated by areas of thickening, whereas other regions were normal and unaffected by fibrosis. Thickness variation also explains why no overall difference in mean wall thickness was found. Blowouts consisted of thinned out collagen (the muscle cells had disappeared completely), lined only by endothelial and subendothelial tissue and appeared to represent the end stage of varicosis.

No evidence of site variation was found in control veins but varicose veins had significantly larger areas of tunica media and whole wall in proximal regions. In part, this was due to signficantly larger amounts of collagen, elastin and muscle in these regions. No variation was seen in intimal area or its constituents between proximal and distal regions. Since no fluctuation along the length of the normal long saphenous vein was found, variation along the varicosed saphenous vein appears to be a reflection of varicosis itself and could be one reason for past discrepancies as to the composition of varicose vein wall, since results obtained would depend on the region of vein sampled.

When compared to equivalent regions in controls, varicose saphenous veins contained significantly higher amounts of collagen in the whole wall, media and intima both proximally and distally. This agrees with previous findings.[6,14,25,26] Proximally, significantly higher relative amounts of collagen were seen in the media, intima and whole wall and distally higher percentages were seen in the media when compared to controls. These findings show that varicosis is related to increased production of collagenous tissue which is in excess of (disproportionate to) the increase in intima, media and wall area.

Our findings question the wisdom of assessing collagen and elastin concentration as an index of content. Varicose saphenous veins in this study possessed more massive walls and so it is possible that decreases in their collagen and elastin concentration could disguise increases in their content. The conflicting findings in the literature of increases, no changes and decreases in concentrations must be treated warily because all could be accompanied by increases in absolute amounts.

Histologically, collagen fibres were seen to be invading and breaking up regular muscle layers of the media in varicosis. Scattered elastic fibres were also visible in the media, lying between disrupted muscle layers. Fibrosis was particularly evident in the inner longitudinal and circular muscle layers of the media. Areas of fibrous infiltration were not uniformly distributed and in some sections involved only part of the vein wall. Even when the vein wall was grossly varicose, some normal areas with regular muscle bundle formation could be seen.

The separation and disruption of muscle cells caused by increased collagenous material may lead to diminished tone and vein wall dilation, as "effective contraction cannot occur unless individual cells are in communication with each other".[6]

Others report abnormalities in the structure and types of collagen fibrils, suggesting that this is a factor in wall fibrosis. Various types of collagen (I–V) have been identified in normal and varicose veins. It has been proposed that varicose and potentially varicose veins contain more of collagen types I and III than normal veins.[14] As yet, however, the functional significance of different collagen types found in vein walls is not fully understood but it may prove to be relevant in understanding the causation of varicose veins.

There was comparatively more smooth muscle in the media of control *vs.* varicose veins, though no difference was seen in intimal muscle content and

there was a higher level of smooth muscle in varicose veins overall. Consequently, the pathological abnormality in varicosis may not be a deficiency of muscle, but the inability of muscle to provide the necessary tone in vessel walls due to the break up of its regular structure by fibrous tissue. Rose and Ahmed[6] suggested that fibrous tissue invades muscle layers, disturbing their regular cellular pattern. In areas most affected by fibrosis the regular pattern of collagen fibres is also disturbed, as is the supporting elastic tissue. Differences in collagen fibril diameter between proximal and distal sites (Weaver *et al.* 1994, unpublished data) suggest that veins in the thigh are under greater stress than in the calf. This is consistent with our finding that wall areas are greater proximally and with independent observations that more collagen and elastin are found proximally.[21] Psaila and Melhuish[13] found that the breaking strength and breaking energy needed to disrupt the vein wall were greater in the thigh than at the knee.

Whilst muscle hypertrophy in varicose vein walls with disorganization of connective tissue support, has been suggested[11,15,16] others in contrast, have reported muscle loss with replacement by fibrous tissue.[6,26] This investigation reports increased absolute amounts of muscle in varicosis but a decreased proportional amount when compared to control veins. It may be that the large proportional increase in collagenous tissue, causing dense fibrosis, contributes to break up of muscle layers.

Although the results suggest a primary defect in wall structure, it is not known whether muscle cells separate because of an intrinsic muscle abnormality (which is followed by collagen infiltration) or abnormal collagen fibre production. According to Rose and Ahmed[6], smooth muscle cells can transform into connective tissue cells, secreting collagen. The stimulus for fibrous infiltration is unknown. Genetic factors may be involved and an individual could inherit a wall weakness due to abnormal muscle cells or abnormal collagen fibres.

The limitations affecting this study concerned mainly availability of control tissue obtained from femoral-popliteal bypass operations. Such vein samples came from patients with ischaemic limbs and it is uncertain what effect this may have had on vein structure. Several of the control (and varicose) samples came from patients over 50 years and age-linked changes begin between 50 and 60 years.[27] The variation in the collagen content of controls, depending on their age, could be the reason for discrepancies between workers claiming increases or decreases in the collagen content of varicose veins. Future studies should look at the wall composition of both dilated and non-dilated portions of varicose veins in comparison to controls, effect of duration of varicosis and age/sex matched samples.

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Accepted 14 August 1995