Valvular Heart Disease

Mutations in FOXC2 Are Strongly Associated With Primary Valve Failure in Veins of the Lower Limb

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Background—Mutations in the FOXC2 gene cause lymphedema distichiasis, an inherited primary lymphedema in which a significant number of patients have varicose veins. Because lymphedema distichiasis is believed to be caused by lymphatic valve failure (reflux), and FOXC2 is highly expressed on venous valves in mouse embryos, we tested the hypothesis that FOXC2 mutations may be linked to venous valve failure and reflux.

Methods and Results—The venous system of the leg was investigated with Duplex ultrasound. Pathological reflux was recorded by color Duplex ultrasound in all 18 participants with a FOXC2 mutation, including 3 without lymphedema. Every participant with a mutation in FOXC2 showed reflux in the great saphenous vein (n=18), compared with only 1 of 12 referents (including 10 family members; P<0.0001, Fisher exact test). Deep vein reflux was recorded in 14 of 18 participants.

Conclusions—FOXC2 is the first gene in which mutations have been strongly associated with primary venous valve failure in both the superficial and deep veins in the lower limb. This gene appears to be important for the normal development and maintenance of venous and lymphatic valves. (Circulation. 2007;115:1912-1920.)

Key Words: valves ▪ genes ▪ patients ▪ ultrasonics ▪ veins ▪ lymphedema

Valves in the veins of the leg become incompetent as a consequence of structural failure, vein wall dilatation, or deep vein damage, i.e., deep vein thrombosis. Consequently, venous reflux (retrograde flow) occurs during limb dependency, leading to sustained periods of distal high venous pressure not relieved by ambulatory exercise. Valvular failure and elevated venous pressure can cause varicose veins, edema, lipodermatosclerosis, and ulceration. These secondary effects develop in 9% of men and 7% of women in the general population between 18 and 64 years of age, with a higher prevalence in subjects between 55 and 64 years of age (25% of men, 12% of women). The prevalence of varicose veins in the general population increases in those over 35 years of age, ranging from 30% with minor varicosities (often unreported) to 6% with severe symptoms. The treatment of venous disease accounts for ~2% of national healthcare resources in the United Kingdom.

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The cause of primary venous valve failure remains unknown. A genetic predisposition to varicose veins has been proposed for many years, and venous function in twins, measured by impedance plethysmography, indicates a strong genetic influence. Individuals are more likely to be affected by varicose veins when parents and siblings have varicose veins. A study of 134 families reported that the risk of developing varicose veins was 90% when both parents were affected, whereas the risk was only 20% for individuals who had unaffected parents. The specific genes that are involved in both venous function and primary venous valve failure remain to be determined. A recent study in a twin cohort indicated linkage of varicose veins to candidate marker D16S520 on chromosome 16. This region of chromosome 16 contains genes coding for, among others, FOXC2, FOXL1, FOXF1, and IRF8. The results of these studies show that further work on possible target genes for venous disease is warranted.

FOXC2 encodes a regulatory transcription factor and is situated on chromosome 16q24.3. FOXC2 is implicated in both lymphatic and vascular development, but its exact role is unknown. In animal models, foxc2 is expressed in developing mesenchyme cells that later develop into connective tissue, blood vessels, and lymphatic vessels. At a later stage of development, foxc2 is expressed on both the...
endothelial and smooth muscle cells of developing blood vessels\textsuperscript{13} and on the venous and lymphatic valve leaflets.\textsuperscript{16,17} Homozygous null mice (foxc2\textsuperscript{−/−}) die during development or immediately after birth and show abnormal remodeling that produces nonfunctioning blood vessels.\textsuperscript{18} FOXC2 is highly expressed on venous valves of adult wild-type mice, which suggests this gene is important for their development and/or maintenance (T. Petrova, PhD, unpublished data, 2006).

Heterozygous mutations in FOXC2 in humans cause lymphedema distichiasis syndrome (LDS), a dominantly inherited primary lymphedema.\textsuperscript{10,11,19} Although the specific mutation in FOXC2 differs between families, the mutations are rare, and those identified in the forkhead domain produce a protein that lacks DNA binding capability.\textsuperscript{21} Affected patients commonly have bilateral lymphedema of the lower legs and feet and aberrant eyelashes (distichiasis) arising from abnormally differentiated meibomian glands.\textsuperscript{20} The lymphedema usually develops at or after puberty, whereas the distichiasis can be present at birth. Lymphoscintigraphy demonstrates lymph reflux in the large lymphatic vessels of the leg,\textsuperscript{20} and nearly half of lymphedema distichiasis patients have visible varicose veins.\textsuperscript{20}

The various clues from human lymphedema distichiasis and animal mutation models, as well as the common embryological origin of lymphatics and veins,\textsuperscript{22–24} lead to the hypothesis that mutations in FOXC2 affect the development of both lymphatic vessels and veins, particularly the function of the valves. If the hypothesis is true, primary venous valve failure should be more frequent in patients with FOXC2 mutations. The aim of the present study was to determine whether the prevalence of venous reflux in lower limb veins is greater in patients with FOXC2 mutations than in referents. Participants from families with confirmed FOXC2 mutations were examined by conventional color Doppler duplex ultrasound of lower-limb veins. The primary outcome measure was the prevalence of nonsegmental venous valve reflux. Family members with FOXC2 mutations (with or without lymphedema) were compared with family members without FOXC2 mutations. Venous duplex ultrasound requires calf compression to augment proximal venous flux before release of compression allows retrograde flow to test for valve competency. To assess whether the strength of compression influenced the prevalence of venous reflux, we devised a novel method for determining the magnitude of the augmented proximal flux. In addition, we developed a new method for measuring whether FOXC2 mutations alter the magnitude of any evoked reflux. The new methodologies permit an objective measure of the effect of the distal compression and any evoked reflux in venous duplex examinations.

**Methods**

**Participants**

Two groups were examined, the FOXC2 mutation group (the “FOXC2 group”) and a reference group. The FOXC2 group comprised 18 participants (8 males, 10 females) from 7 families with known heterozygous FOXC2 mutations.\textsuperscript{10,20} Four families had deletions in the FOXC2 gene, 2 families had insertions, and 1 family had a nonfunctioning missense mutation. Fifteen had lymphedema distichiasis (lymphedema of the lower legs and distichiasis). Three had FOXC2 mutations with associated distichiasis but no lymphedema. These mutation carriers were from the same families as those with lymphedema; 2 had insertions, and 1 had a deletion in the FOXC2 gene. The reference group consisted of 12 participants (4 males, 8 females) with no FOXC2 mutation and no evidence of lymphedema or distichiasis. Ten were family members of participants in the FOXC2 group.

**Unilateral Examination With Color Duplex Ultrasound**

The first 13 participants were studied bilaterally (8 from the FOXC2 group, 5 from the reference group). Because results obtained from each leg were not significantly different, and time constraints made the completion of studies (including other lymphatic studies) difficult within 1 day, the remaining participants were examined unilaterally, with the more swollen of the legs used for study. For the present report, the unilateral results are presented for each individual examined. In the 3 participants with a FOXC2 mutation but no lymphedema, the left leg was examined in 2 participants and the right leg in 1 participant. In total, 10 left legs and 8 right legs were studied in the FOXC2 group. For unilateral examination in the reference group, 7 left and 5 right legs were studied.

To facilitate systematic mapping of valve function in the lower limb, the leg veins were classified into 3 groups, which were further subdivided into a total of 9 segments (Figure 1). The segments in group 1 consisted of the great saphenous vein in the upper and lower thigh and the lesser saphenous vein in the calf (the superficial veins). Group 2 consisted of the proximal and distal femoral vein and the proximal and distal popliteal vein (the deep veins). Group 3 comprised the superficial-deep communications, namely, the saphenofemoral junction in the groin and saphenopopliteal junction behind the knee. Each of the 9 segments of vein (upper and lower great saphenous vein; lesser saphenous vein; proximal and distal femoral vein; proximal and distal popliteal vein; saphenofemoral junction; and saphenopopliteal junction) was studied with an Acuson 128 ultrasound machine (Acuson, Stevenage, United Kingdom) at 7 MHz in a temperature-controlled room. The thigh veins were examined with the participant standing, whereas the calf veins were examined in the sitting position, with the legs dependent. The calf communication veins were not assessed. Each vein segment was imaged both in cross section and longitudinally. Doppler shift was used to measure the red cell velocity and direction of flow. When the vessels were imaged in cross section, the direction of the Doppler shift was represented by a color scale over the blood vessels (red=flow in proximal direction, blue=flow in distal, retrograde direction). In longitudinal images, the numerical velocity of red blood cells within each segment was plotted against time (Figure 2). A transient increase in proximal flux was generated by manually squeezing the limb distal to the segment under examination. On releasing the distal compression, significant amounts of blood pass distally (pathological reflux) if the valve proximal to the site of examination does not close properly. For assessment of the calf veins, the foot was compressed; for assessment of the thigh veins, the calf was compressed.

**Measurement of Pathological Reflux on the Nominal Scale**

Measurements of flux and reflux were performed from prints of the Doppler waveform at a later time point and blinded for the group of origin. Significant pathological reflux was defined by the following criteria: (1) reflux detected qualitatively in the cross-sectional color Duplex image; and (2) in the longitudinal image, peak reflux velocity and duration of reflux were measured from the Doppler waveform. The most common criteria for pathological reflux were used, namely,
peak reflux velocity >10 cm/s and duration of reflux >0.5 seconds after release of distal compression.25,26 Pathological reflux was only diagnosed if both of the above criteria were fulfilled. Pathological reflux was categorized, therefore, as either "present" or "absent" for each venous segment (the statistical "nominal scale"). Measurements were not always possible for every segment of vein in all participants because of variations in venous anatomy27 and occasional technical limitations (ie, poor definition of the deep veins). Results are stated as a number or percentage of the measurements that were successfully obtained.

Quantitative Measurement of the Magnitude of Augmented Proximal Flux and Reflux (Interval Scale)
The increase in proximal flux caused by a distal compression is influenced by individual variations in limb volume, venous anatomy, and the strength of compression. To test whether distal compression caused a similar challenge to the valves, ie, a similar degree of proximal flux augmentation in the 2 groups, the area under the proximal flux curves (in arbitrary units) was compared for each segment of vein in the FOXC2 and reference groups. Because the evoked proximal flux varied over a wide range, reflux severity was normalized by expressing it as a ratio of the evoked proximal flux in the same segment of vein. Therefore, the area under the reflux curve (AUC_reflux) was divided by the area under the proximal flux curve (AUC_proximal flux), ie, AUC_reflux / AUC_proximal flux for each segment of vein to determine (1) the magnitude of both proximal flux augmentation in response to distal compression and (2) the severity of reflux for every unit of proximal flux produced. This approach thus provided quantitative information on the statistical "interval scale."

Statistical Analysis
The prevalence of venous reflux in the vein segments of the reference group and the FOXC2 group was compared with the Fisher exact test for nominal scale results. For interval scale results, differences in proximal flux were tested with the Student unpaired t test; ratios of the average reflux/average proximal flux per participant were compared with the Mann-Whitney U test; and linear regression analysis was used to compare slopes (Figure 3). Differences were considered significant when P<0.05. Means are followed by SDs.

Ethics Committee Approval
Ethics approval was obtained from both Wandsworth and St Thomas’ Local Research Ethics Committees (protocol numbers 03.0015 and EC03/139, respectively). Each participant gave informed written consent.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Demographic Group Information
FOXC2 Group
Mean age was 40.2±14.2 years (mean±SD, limits 20 to 61 years). Average height was 1.70±0.10 m; average weight
was 71.0±14.3 kg; body mass index averaged 25.0±3.6 kg/m²; and 1 participant was a smoker.

Reference Group
Mean age was 44.3±13.8 years (limits, 23 to 66 years). Average height was 1.70±0.10 m, weight was 70.3±13.6 kg, body mass index was 24.9±3.6 kg/m², and 2 participants were smokers.

Magnitude of Proximal Flux Augmentation
Proximal flux augmentation caused by distal compression was not statistically different in the group with FOXC2 mutations (area under the proximal flux curve 24.9±38.6, mean±SD; n=18) versus the reference group (18.2±10.8; n=12, including 10 related to members of the FOXC2 group; P=0.24, Student unpaired t test). This was also true if each vein segment group was compared separately (Table 1). This indicated that the presence of edema did not compromise the effect of the distal compression on proximal flux augmentation (Figure 3). Furthermore, there was no difference between FOXC2 participants with and without lymphedema.

Unilateral Findings Representative of Both Legs
In the first 13 participants examined bilaterally, only 3 segments (<5% of measurements) were different between the legs (specifically, the popliteal vein in 2 FOXC2 participants and the femoral vein in 1 FOXC2 participant). All referents showed identical results between legs. Further results were obtained unilaterally for a total of 18 participants in the FOXC2 group and 12 referents.

Significant Venous Valve Failure Was Uncommon in Referents
Table 2 and Figure 4 show the prevalence of reflux in the reference and FOXC2 groups for each vein segment. Reflux was detected in only 4 (3.8%) of a total of 105 recordings in referents. Even when present, reflux occurred only in 1 of the 9 segments of vein in the referents affected.

Venous Reflux Was Always Present in Participants With Mutations in FOXC2
In contrast to reference results, pathological reflux was observed in all 18 participants with a FOXC2 mutation. The
percentage of segments showing reflux in a given individual varied between 25% and 100%, averaging 63.0±21.2%. Some veins were more prone to reflux than others. The prevalence of reflux in the great saphenous vein was 100% (18/18; Figures 4 and 5). Reflux was observed in 45 (96%) of 47 recordings taken from superficial veins. In addition to superficial vein reflux, 78% of the FOXC2 group had recordable deep vein reflux; 9 had extensive deep venous reflux (all deep veins refluxing, 4 participants; all deep veins in the thigh refluxing, 5 participants); 4 exhibited no deep vein reflux; and 5 exhibited reflux in isolated segments of the deep veins in addition to reflux in the saphenofemoral junction. Reflux was observed in 23 (64%) of 47 recordings taken from superficial-deep communications and 31 (46%) of 67 recordings taken from deep veins.

All but 1 of the vein segments in the FOXC2 group (superficial, deep, and superficial-deep communications) had a higher prevalence of reflux than in the corresponding segment of the reference group (P<0.0001 to 0.05, Fisher exact test). In only the distal popliteal vein did the prevalence of reflux not reach significance (P=0.06). Table 2 shows the comparisons for individual vein segments.

**Magnitude of Reflux**

In the veins showing reflux in the FOXC2 group, the average reflux/average proximal flux ratio (the amount of reflux per

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**TABLE 1. Magnitude of Proximal Flux Augmentation**

<table>
<thead>
<tr>
<th>Vein Group and Segment</th>
<th>Reference Group (Max N=12)</th>
<th>FOXC2 Group (Max N=18)</th>
<th>P (Student Unpaired t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great saphenous (proximal)</td>
<td>18.3±7.8</td>
<td>26.6±35.5</td>
<td>0.50</td>
</tr>
<tr>
<td>Great saphenous (distal)</td>
<td>23.1±9.7</td>
<td>17.0±7.1</td>
<td>0.22</td>
</tr>
<tr>
<td>Lesser saphenous</td>
<td>11.2±6.4</td>
<td>8.2±5.4</td>
<td>0.36</td>
</tr>
<tr>
<td>Superficial/deep communications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saphenofemoral</td>
<td>10.5±2.4</td>
<td>18.1±10.9</td>
<td>0.15</td>
</tr>
<tr>
<td>Saphenopopliteal</td>
<td>8.5±4.2</td>
<td>31.2±33.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Deep</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral (proximal)</td>
<td>32.5±9.9</td>
<td>35.1±17.5</td>
<td>0.42</td>
</tr>
<tr>
<td>Femoral (distal)</td>
<td>33.3±7.3</td>
<td>24.7±16.2</td>
<td>0.36</td>
</tr>
<tr>
<td>Popliteal (proximal)</td>
<td>15.1±6.0</td>
<td>17.1±12.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Popliteal (distal)</td>
<td>11.6±3.1</td>
<td>8.5±6.7</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Max indicates maximum. Values are mean±SD, in arbitrary units.
unit of proximal flux) in the superficial veins, 2.1±0.8, was almost 4 times that in the deep veins (0.6±0.2), as shown by linear regression analysis (Figure 3; \( P=0.0007 \)). Both were much greater than the reflux/proximal flux ratio in referents (0.10±0.11; \( P<0.0001 \), Mann-Whitney \( U \) test). There was no difference in the size of the evoked proximal flux between the superficial and deep vein segments in the FOXC2 group (area under the curve 18.9±23.9 and 30.2±52.5; \( P=0.32 \), unpaired \( t \) test).

**FOXC2 Mutations Caused Venous Valve Failure Irrespective of the Presence of Lymphedema**

Three members of the FOXC2 group did not have lymphedema but had mutations in FOXC2 (mutation carriers). This allowed examination of the effects of FOXC2 mutations independent of the presence of lymphedema. The mutation carriers showed a similar pattern of reflux to the 15 participants with lymphedema distichiasis (Table 3). Reflux was observed in 16 (64%) of 25 recordings from mutation carriers. This prevalence of reflux was almost identical to that in the LDS participants, in whom reflux was present in 83 (66%) of 125 recordings. Of particular note is that deep venous reflux was recorded in all FOXC2 mutation carriers.

**Discussion**

The present study is the first, to the best of our knowledge, that identifies a link between a specific gene, FOXC2, and

**Table 2. No. of Vein Segments Showing Pathological Reflux**

<table>
<thead>
<tr>
<th>Vein Group and Segment</th>
<th>Reference Group (Max ( N=12 ), n/N (%))</th>
<th>FOXC2 Group (Max ( N=18 ), n/N (%))</th>
<th>( P ) (Fisher Exact Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Superficial</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great saphenous (proximal)</td>
<td>1/12 (8.3)</td>
<td>18/18 (100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Great saphenous (distal)</td>
<td>1/12 (8.3)</td>
<td>15/15 (100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lesser saphenous</td>
<td>0/12 (0)</td>
<td>12/14 (85.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Superficial/deep communications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saphenofemoral</td>
<td>1/12 (8.3)</td>
<td>15/18 (83.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Saphenopopliteal</td>
<td>0/10 (0)</td>
<td>8/18 (44.4)</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>Deep</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral (proximal)</td>
<td>1/12 (8.3)</td>
<td>10/18 (55.6)</td>
<td>0.018</td>
</tr>
<tr>
<td>Femoral (distal)</td>
<td>0/11 (0)</td>
<td>6/16 (37.5)</td>
<td>0.05</td>
</tr>
<tr>
<td>Popliteal (proximal)</td>
<td>0/12 (0)</td>
<td>9/15 (60.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Popliteal (distal)</td>
<td>0/12 (0)</td>
<td>4/18 (33.3)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Max indicates maximum. Values are no. of measurements showing reflux/No. of participants in whom measurements were technically successful (%).

**Figure 4.** Percentage of recordings that showed reflux in each segment of vein. A, Reference group; B, FOXC2 group.
primary venous valve failure in both deep and superficial veins. Mutations in the \textit{FOXC2} gene were strongly associated with venous reflux in every participant. The great saphenous vein was always incompetent in the presence of a \textit{FOXC2} mutation. A significant proportion of the \textit{FOXC2} group also had reflux in the deep veins and superficial-deep venous communications.

Color Duplex ultrasound is the best technique available for the noninvasive examination of venous function.\textsuperscript{25} Venous valves are difficult to image clearly with ultrasound. Venous valves ensure that venous blood flows centrally. Flow in the opposite direction (reflux) is used as a surrogate for valve failure. A small degree of reflux may occur in the presence of a functioning valve,\textsuperscript{26} so valve failure is only considered to be present when there is >0.5 seconds of venous reflux with peak velocity >10 cm/s.\textsuperscript{28,29} Significant reflux has been shown to occur at single sites in 32% of clinically normal humans with no evidence of venous disease.\textsuperscript{30} Significant reflux at a single site was recorded in a similar percentage of the reference group (33%) in the present study but with no reflux in the neighboring vein segments. Segmental or single-site reflux is likely to be hemodynamically unimportant.

Venous duplex ultrasound is an operator-dependent method. In particular, the distal compression provocation and its effect on augmenting proximal venous flow is dependent on leg girth, venous anatomy, and amount of pressure applied. To investigate whether the presence of edema influenced the primary outcome measure, namely, the presence or absence of venous reflux, a new method was devised to measure the magnitude of the proximal flux augmentation by measuring the area under the proximal flux curve. The magnitude of proximal flux augmentation varied over a wide range (Figure 3) but was not influenced by the edema. Furthermore, by calculating the ratio of the area under the curve for reflux as a degree of the area under the proximal flux curve, the magnitude of the reflux normalized for the distal compression effects can be calculated. It is suggested that this additional but simple methodology brings greater objectivity and reliability to venous duplex examinations.

The central feature of venous incompetence is the defective function of the semilunar venous valves. Incompetent veins have an altered wall structure, with abnormal expression of collagen,\textsuperscript{31} loss of endothelium,\textsuperscript{32,33} abnormal migration of smooth muscle cells,\textsuperscript{33} and a reduction in the number of valves per unit length of vein.\textsuperscript{34} It is not known which of these pathological changes happens first. Structural alterations in the vein wall could increase susceptibility to dilation during exposure to increased venous pressure, leading to valve failure, reflux, and possibly valve regression.\textsuperscript{1} Alternatively, if the valve number is reduced in a given segment of vein, the fluid pressure acting on each valve is increased, leading to valve failure and triggering pathological changes in the vein wall.\textsuperscript{34}

The valves in the superficial veins were always affected by the \textit{FOXC2} mutation. Primary varicose veins in the general

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**TABLE 3. Breakdown of \textit{FOXC2} Mutation Results Into Those Participants With Lymphedema and Mutation Carriers Without Detectable Lymphedema**

<table>
<thead>
<tr>
<th>Vein Group and Segment</th>
<th>Lymphedema (N=15)</th>
<th>Distichiasis (N=15)</th>
<th>Mutation Carriers (N=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Great saphenous (proximal)</td>
<td>15/15 (100)</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Great saphenous (distal)</td>
<td>12/12 (100)</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Lesser saphenous</td>
<td>12/13 (92.3)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>Superficial/deep communications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saphenofemoral</td>
<td>12/15 (80.0)</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Saphenopopliteal</td>
<td>8/15 (53.3)</td>
<td>0/3 (0)</td>
<td></td>
</tr>
<tr>
<td>Deep</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral (proximal)</td>
<td>8/15 (53.3)</td>
<td>2/3 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Femoral (distal)</td>
<td>4/13 (30.8)</td>
<td>2/3 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Popliteal (proximal)</td>
<td>6/12 (50.0)</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Popliteal (distal)</td>
<td>6/15 (40.0)</td>
<td>0/3 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Values are no. showing reflux/No. of participants in whom measurements were technically possible (%).
population usually result from superficial vein incompetence. On the other hand, deep venous reflux is functionally more relevant than superficial venous reflux and was present in the deep veins in 78% of FOXC2 participants. Half of the FOXC2 group had complete or nearly complete deep venous failure. This is the first time both superficial and deep venous incompetence has been associated with mutations in a specific gene, namely, FOXC2.

The magnitude of reflux was greater in refluxing superficial veins than in refluxing deep veins. The vein walls and valves of the superficial veins are more prone to structural failure than the deep veins, probably because the deep veins are better supported by the muscle and fascia, whereas superficial veins are only surrounded by loose connective tissue and fat. This is in keeping with the reasoning that superficial veins fail before deep veins during the progression of venous disease.30

Little is known about the genes involved in valve development and maintenance. The expression pattern of FOXC2 in the veins of adults has not been studied in detail, although it is known to be expressed on the endothelium and smooth muscle cells of blood vessels13 and on the venous valves in mouse embryos.16 Because venous valve failure was recorded in all participants with a mutation in FOXC2, including those without clinical signs of lymphedema (carriers), it appears likely that FOXC2 may have a role in venous valve function and therefore varicose veins.

The main embryological lymph sacs, from which the major lymphatic vessels sprout, arise from the venous system during development.24,35 Both lymphatics and veins contain valves to ensure unidirectional flow. Lymphoscintigraphy performed in patients with LDS (FOXC2 mutations) demonstrates “dermal backflow” of tracer away from the normal deep lymphatic routes into small skin vessels. The dermal backflow seen during dependency, and in this particular lymphedema, is believed to be the result of lymph reflux,20 analogous to the venous reflux demonstrated in the present study. Therefore, not only do the lymphatics and veins have a common embryological origin, but they also share a common failure of their valves associated with mutations in FOXC2. This interpretation is supported by the recent observation that FOXC2 inactivation in transgenic mice leads to an absence of valves in lymphatic vessels.16

As a consequence of the present study, color Doppler ultrasound now forms part of the routine assessment of suspected LDS cases at our institution. After the termination of recruitment for the present study, 8 more patients were diagnosed with LDS. All 8 showed the presence of venous valve failure in a similar pattern to that seen in the present study; 7 had superficial venous reflux, with 1 other having previously had venous surgery; 5 of 8 showed deep venous reflux. The present results indicate that the swelling in LDS may be of mixed lymphovenous origin, which may have implications for future treatment. Although conventional venous surgery approaches are contraindicated because of the increased risk of infection and possible damage to the already dysfunctional lymphatic system, the newer selective endovenous ablation treatments may prove helpful in reducing excessive fluid filtration and swelling.36

These results indicate that functioning FOXC2 is required for normal venous function, and more specifically for valve development and/or maintenance in humans. FOXC2 plays an important role in the development of both lymphatic and venous systems, with mutations causing lymphatic and venous abnormalities. The pathways and mechanisms by which FOXC2 acts on valve development and maintenance require further elucidation. Venous disease is a common condition, and further studies are now warranted to determine whether FOXC2 abnormalities play a role in the development of primary varicose veins in the general population.

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Disclosures

Professor Mortimer is on the editorial/advisory board for Lymphatic Research and Biology and Lymphology. Professors Mortimer and Levick and Dr Stanton are investigators on a Wellcome Trust program grant examining breast cancer-related lymphoedema. Professor Jeffery holds funding from the London IDEAS Genetic Knowledge Park. The remaining authors report no conflicts.

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**CLINICAL PERSPECTIVE**

The veins in the lower leg require competent valves to ensure proximal flow of blood toward the heart, against gravity. A failure of the valves results in venous reflux (retrograde flow) and sustained periods of high venous pressure, which can lead to varicose veins, edema, lipodermatosclerosis, and ulceration. The cause of primary venous valve failure is unknown. A genetic predisposition to varicose veins has been proposed, but the genes responsible for valve development and function remain unidentified. Mutations in the FOXC2 gene cause lymphedema distichiasis, an inherited form of primary lymphedema in which ≃50% of affected individuals have varicose veins. Because FOXC2 is highly expressed on venous valves in mouse embryos, we tested the hypothesis that FOXC2 mutations are linked to venous valve failure and reflux by investigating affected individuals with venous duplex ultrasound. Every one of 18 participants with a FOXC2 mutation showed pathological reflux in their great saphenous veins, and deep venous reflux was observed in 14. Thus, FOXC2 is the first gene in which mutations have been strongly associated with primary venous valve failure in both superficial and deep veins. Because lymph reflux has also been consistently observed on lymphoscintigraphy in humans with FOXC2 mutations, it would appear highly likely that FOXC2 is important for the normal development and maintenance of both venous and lymphatic valves. The pathways and mechanisms by which FOXC2 acts on valve development need further elucidation, but these results could lead to interventions capable of influencing valve growth and repair.
Mutations in FOXC2 Are Strongly Associated With Primary Valve Failure in Veins of the Lower Limb
Russell H. Mellor, Glen Brice, Anthony W.B. Stanton, Jane French, Alberto Smith, Steve Jeffery, J. Rodney Levick, Kevin G. Burnand and Peter S. Mortimer

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