Lymphoscintigraphic abnormalities associated with Milroy disease and Lymphedema-Distichiasis syndrome

Running Title: Lymphoscintigraphic abnormalities in MD and LDS

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Abstract *(250 words max)*

Background: Primary lymphedema is genetically heterogeneous. Two of the most common forms of primary lymphedema are Milroy disease (MD) and Lymphedema-Distichiasis Syndrome (LDS). This study aims to look further into the pathogenesis of the two conditions by analyzing the lymphoscintigram images from affected individuals to ascertain if it is a useful diagnostic tool.

Methods and results: The lymphoscintigrams of patients with Milroy disease and Lymphedema-Distichiasis Syndrome were analyzed, comparing the images and transport parameters of the two genotypes against a control population. Lymphoscintigrams were available for 12 MD and 16 LDS patients (all genetically proven diagnoses).

Eight of the twelve (67%) lymph scans performed on patients with MD demonstrated little or no uptake from the initial lymphatics and poor visualization of the inguinal lymph nodes. These changes were consistent with a "functional aplasia" i.e. the lymphatic vessels were present but appear to be ineffective in absorbing the interstitial fluid into the lymphatic system. In patients with LDS the lymphoscintigraphic appearances were different. In 12 of the 16 scans (75%), the lymph scans were highly suggestive of lymphatic collector reflux.

Quantification revealed a significantly reduced uptake of tracer within the inguinal lymph nodes and a higher residual activity in the feet at 2 hours in MD compared to LDS and compared to controls.

Conclusion: Lymphoscintigraphic imaging and quantification can be characteristic in specific genetic forms of primary lymphedema and may be useful as an additional tool for in-depth

phenotyping, leading to a more accurate diagnosis and providing insight into the underlying mechanism of disease.

Keywords

Milroy disease, Lymphedema-distichiasis syndrome, lymphoscintigram, lymphedema, reflux, functional aplasia.

Condensed abstract

Two of the most common genetic forms of primary lymphedema are Milroy disease (MD) and Lymphedema-Distichiasis Syndrome (LDS). This study aims to analyze the lymphoscintigrams from affected individuals to look at the pathogenesis of the two conditions and to ascertain if this investigation provides useful diagnostic and mechanistic features. We conclude that lymphoscintigraphic imaging and quantification can be characteristic in these two specific genetic forms of primary lymphedema and may be useful as an additional tool for in-depth phenotyping, leading to a more accurate diagnosis and providing insight into the underlying mechanism of disease.

Introduction

The primary function of the lymphatic vasculature is to absorb the protein-rich fluid that is filtered from blood capillaries into the interstitial spaces. This fluid is absorbed in the first instance by the initial lymphatic vessels and then transported via larger collecting lymphatic vessels to the thoracic duct via lymph nodes, and ultimately to the cardiovascular circulation. Collecting lymphatic vessels possess valves that prevent lymphatic backflow when the smooth muscle cells in the walls of the vessels systematically contract to pump the lymph proximally.[1]

Primary lymphedema is caused by a developmental defect in the lymphatic system and leads to chronic swelling, which most often occurs in the lower limbs. Two of the most frequent genetic forms of primary lymphedema are Milroy disease (MD; OMIM 153100) and Lymphedema-Distichiasis syndrome (LDS; OMIM 153400). Both are of autosomal dominant inheritance and causative genes have been identified for each condition.

MD is caused by pathogenic variants in the *FLT4 (VEGFR3*) gene on chromosome 5, which codes for vascular endothelial growth factor receptor 3.[2, 3] All pathogenic variants to date have been located in the tyrosine kinase domain.[4, 5] Patients usually present with lower limb swelling at birth, most often the dorsum of the feet (Figure 1a). The swelling is almost always distal to the knee. Common features include marked pedal edema, deep interphalangeal creases of the toes and small dysplastic nails (Figure 1b). In many patients, the lower limb veins are of large calibre indicating venous involvement.[6] One third of affected males are born with, or will develop, hydroceles.[7] The swelling of the dorsum of the feet can sometimes be seen *in utero* on antenatal ultrasound examination.

Until recently, Milroy disease was thought to be due to aplasia of the initial lymphatic vessels. Mice with a heterozygous, inactivating *Vegfr3* mutation and swelling of the limbs are lacking subcutaneous lymphatic vessels.[8] However, analysis of skin biopsies from patients with *FLT4 (VEGFR3)* mutations showed that dermal lymphatic vessels were not absent but that their density was reduced by 51–61% in the foot and 26–33% in the forearm.[6] This study demonstrated that the lymphatic vessels in the feet of patients with Milroy disease have profound functional failure as lymphatic filling was reduced by 86-91% compared to controls. Despite a reduction of the number of lymphatics in the forearm, there was no reduction in function. Furthermore, nine out of the ten patients studied exhibited saphenous vein reflux. The presence of venous reflux indicates that *FLT4 (VEGFR3)* has a role in venous development as well as lymphatic development.[6]

LDS is caused by heterozygous, loss of function, pathogenic variants in the forkhead box transcription factor C2 gene *(FOXC2*).[9, 10] Phenotypic features of LDS include late onset swelling of the lower limbs (Figure 1c) typically around puberty but can be as late as the 5th decade. Other features include distichiasis (aberrant eyelashes growing from the meibomian glands in the inner eyelids) (Figure 1d), a high incidence of varicose veins, ptosis, congenital heart disease and cleft palate.[11]

FOXC2 is expressed in lymphatic and venous valves. Defective valves and abnormal recruitment of smooth muscle cells around lymphatics have been demonstrated. Valve failure is thought to give rise to lymph and venous reflux in the erect posture.[12]

Early diagnosis of both conditions is important for appropriate management. Access to diagnostic genetic testing may not be available in many lymphedema clinics. However, the

majority of radiology departments should be able to undertake lymphoscintigraphy. Lymphoscintigraphy has been established as a gold-standard investigation for lymphedema.[13, 14] A representative image of the lymph drainage pathways may be produced using a gamma camera following an interdigital injection (e.g. in the web space between the toes) of a radiolabelled tracer (e.g. colloidal albumin labelled with Technetium-99m) (Figure 2). If applying a standardized protocol, reproducible imaging can be obtained[15] and it is recommended that compression stockings be removed before injection and all subsequent imaging.[16] Lymphoscintigraphy has predominantly been used as a qualitative measure, evaluating the appearance and number of lymph nodes in the axilla or groin, but quantitative measures can also be given[17, 18] and can prove to be a more sensitive approach to the diagnosis[19]. Here we describe the lymphoscintigraphy findings in patients with pathogenic variants in either the *VEGFR3* or *FOXC2* gene.

Materials and Methods

Design

A retrospective analysis of records of patients seen in the specialist Primary and Pediatric Lymphedema clinic at St George's University Hospitals NHS Foundation Trust was carried out. Using the Primary Lymphedema Register, all patients with pathogenic variants in *FLT4* (*VEGFR3)* (Milroy disease (MD)) and *FOXC2* (Lymphedema-Distichiasis Syndrome (LDS)) were identified. All genetic testing was performed in the DNA laboratory at the SW Thames Regional Genetics Service. Those that had undergone lymphoscintigraphy were identified and their scans evaluated.

Participants (affected individuals)

A total of 169 patients were identified, 104 patients had a pathogenic variant in *FLT4 (VEGFR3)* (NM_182925) and 65 had a pathogenic variant in *FOXC2* (NM_005251). Patients who did not have lymphoscintigrams were excluded (n=140). One lymphoscintigram was excluded, as there were no quantification values. Twelve patients with MD and 16 patients with LDS were molecularly proven and had had lymphoscintigraphy with quantification and were, therefore, included in this study.

Control participants

Twenty-two suitable participants were included as a comparison control group on the basis that both lower limbs were clinically and lymphoscintigraphically normal. The lymphoscintigram scans were identified from the Radiology Department, St George's NHS Foundation Trust.

Lymphoscintigraphy

Lower limb lymphoscintigraphy was performed according to standard local procedure. ^{99m}Tc-Nanocoll (0.2 ml, 25 MBq; GE Healthcare, Little Chalfont, Buckinghamshire, UK) was injected subcutaneously into the web space between the first and second metatarsophalangeal joints of the toes of each foot. Imaging was performed using a single head Argus Epic gamma camera (MIC Ltd, Fleet, Hampshire, UK; 128 x 128 matrix, low-energy general purpose collimator). Participants were lying supine for all examinations. No exercise was performed.

Images were taken at 15 minutes and 2 hours post-injection to demonstrate the lymphatic drainage pathways and uptake of the tracer into the inguinal lymph nodes (Figure 2). All lymphoscintigrams were reviewed by clinicians with expertise in reading lymphoscintigraphy

(PSM, SM, KG, SDH). Scans were reported independently by at least 2 clinicians who were blinded to the diagnosis/genotype.

Abnormal morphological features were identified as:

(i) **reduced regional node and lymphatic collector imaging (delay),** including functional aplasia (no uptake within lymphatic collectors and nodes) or functional hypoplasia (reduced activity within lymphatic collectors and nodes) at 2 hours

(ii) **imaged popliteal nodes,** indicating lymph diversion via the sub-fascial (deep muscle) system; and

(iii) **dermal backflow** (rerouting through the skin).

These outcome measures were considered the most discriminating and in line with recently published criteria.[20]

Quantification of uptake of tracer within a region of interest over the ilio-inguinal lymph nodes, and activity overlying the foot depot at two hours post injection, was calculated. Lymphatic transport was determined by calculating the percentage of tracer, relative to amount injected, accumulating in regional lymph nodes after 2 hours. Uptake of more than 8% within the ilio-inguinal nodes was considered normal, but uptake of less than 8% was considered indicative of lymphatic impairment. Thresholds were obtained from normative patient data, which are used in lymph scan assessment at St George's Hospital and elsewhere.[20] Quantification values of the tracer retention rate and tracer uptake in the lymph nodes (left and right) were compared across the three groups (MD, LDS and controls) and tested for significance using a non-parametric Kruskal-Wallis test. To measure any difference between the affected participant groups, LDS

and MD, the retention rate as well as lymph node uptake were tested for significance using the non-parametric Mann-Whitney U test. All statistical tests were performed using the SPSS v25 software package (IBM).

Results

Twelve scans from patients with pathogenic variants in *FLT4 (VEGFR3), 9 (*75%) female, 3 (25%) male and 16 scans from patients with pathogenic variants in *FOXC2*, 8 (50%) female, 8 (50%) male, were analyzed (Table 1). The mean age of the patients at the time of their scan was 30 (±SD 16.0) years for MD and 27 (±SD 14.9) years for LDS. Available control group scan data, based on 22 individuals, 13 (59%) female and 9 (41%) male, with a mean age of 43 (±SD 14.5) years (at the time of the scan), was used as a comparison group (Table 1).

Lymphoscintigraphy images for Milroy Disease

Eight of 12 scans for the individuals with Milroy disease clearly demonstrated minimal or no uptake of tracer into the lymphatic collectors or inguinal lymph nodes at 2 hours and a high level of tracer retention in the feet (i.e. a pattern of "functional aplasia", interpreted as initial lymphatic vessels present but not working) (Table 2).

Four of the lymphoscintigrams were atypical (Table 3). Two of the scans (MD8 and MD9) demonstrated unilateral abnormalities. In both, the right lower limb was characteristic of Milroy disease with functional aplasia, but in MD8 there was normal lymphatic drainage in the left lower limb with clearly visualized inguinal lymph nodes. MD9 showed some dermal backflow of tracer around the left ankle. In MD10, the retention and uptake figures are indicative of Milroy disease, but the scan is atypical as a tortuous appearance of the lymphatic channels is observed with bilateral dermal backflow, suggestive of superficial rerouting of tracer. A faint popliteal

lymph node was seen in the calf area, suggesting a degree of re-routing of tracer via the deep lymphatic system. MD11 showed a level of uptake, which is unusual for Milroy disease. Additionally, popliteal lymph nodes were seen in the calf and thigh area of the right leg.

Lymphoscintigraphy images for LDS

The lymphoscintigrams for the individuals with LDS showed a very different picture from those of MD and from normal. Twelve of the 16 lymphoscintigrams demonstrated very similar features that were considered to be characteristic of LDS (Table 4); namely, widened and tortuous lymphatic channels with an accumulation of tracer activity in the skin and subcutis of the distal portion of the lower limbs creating an outline of the lower leg ('profiling of the leg'). There often appeared to be increased uptake of tracer within the inguinal lymph nodes, which appeared increased in number, and the deeper popliteal nodes were clearly visualized (Table 4, e.g. knee area of LDS17 and LDS20:1).

Four of the lymphoscintigrams were less characteristic for LDS (Table 5). LDS21:1 demonstrated unilateral changes, uptake on the left was within the normal range. On the right, the changes were consistent with functional hypoplasia, with no profiling of the limb. The scan for LDS21:2 was more comparable to the functional aplasia seen in the MD cases with high retention values in the feet and very little uptake of tracer in the legs or groins at 2hrs. There was a subtle profiling, indicating dermal backflow, in the right limb. LDS18:2 there was evidence of dermal backflow above the left ankle but insufficient to create profiling of the leg. LDS22 showed scrotal dermal backflow, probably indicating reflux of tracer, in addition to popliteal node uptake on the right and dermal backflow/rerouting in the thighs and legs.

Lymph transport quantification

1) Normal controls: Lymphoscintigraphy had previously been performed on unaffected individuals for comparison (for another study). Twenty-two participants had normal imaging and quantification of both lower limbs (44 limbs). The mean retention rate at the injection site of both legs was nearly identical with a mean of 71.1% in the right leg and 70.7% in the left leg (Table 1). Similarly, the mean nodal uptake of the right leg and left leg was reported 17.3% and 16.3% respectively.

2) MD: Of the 24 limbs scanned in patients with MD, 18 had more than 93% of tracer retained at the site of the injection at 2 hours (Tables 2 and 3), with a median value of 95.6% in right leg and 88.5% in left leg (Table 1). 18 of the 24 limbs showed nodal uptake figures of <0.2% in the groin at 2hrs (Tables 2 and 3) with a median value of 0.1% uptake for both legs (Table 1). Statistical analysis using the non-parametric Kruskal Wallis test for the quantification figures (foot retention and nodal uptake) for all MD cases (including atypical scans in Table 2) was significantly different from those of the LDS and control groups (Table 1).

3) LDS: In terms of quantification, the LDS scans showed more variability than those for MD. The injection depot site retention figures ranged from 52% (which is considered normal) to 98% (which indicates impaired uptake) with a mean of 82.0% retention in the right leg and 82.6% in the left leg (Table 1). Of the 32 limbs scanned, 13 limbs showed a normal uptake from the depot in the foot (i.e. retention figures of <80%). The quantification figures for uptake of tracer in the groin area after 2 hrs was also variable and ranged from 0% (which indicates impaired uptake) to 32.1% (which is considered high and might suggest coexistent venous disease) with mean values of 4.5% and 7.0% in the right and left legs respectively (Table 1).

The difference between the injection depot site retention (in percentage) and the lymph node tracer uptake (in percentage) in the right and left leg at 2 hours in MD, LDS and controls is evident (Figure 3).

Additional tests for significance specifically between MD and LDS were performed, including all scans (Supplementary Table 1) and including only the typical scans of both groups (Supplementary Table 2) using the non-parametric Mann-Whitney U test. This confirmed a statistically significant difference of the mean retention at depot right leg and mean inguinal nodal uptake right leg between all patients with MD and LDS, the left leg also showed a difference although it did not reach statistical significance. A statistical difference between the two groups was reached for both lower limbs if only the 'typical' scans were considered (Supplementary Table 2).

Discussion

Milroy disease (MD) and Lymphedema Distichiasis (LDS) are two of the most frequent genetic causes of primary lymphedema. There are often marked differences between MD, LDS and other types of primary lymphedema in their clinical presentation. One of the problems with making a clinical diagnosis only, is that many clinicians will diagnose every type of primary lymphedema as Milroy disease. The identification of causal genes for primary lymphedema allows for genetic testing and a specific diagnosis according to genotype can be made. Genetic testing may not always be available and, if tested, variants of unknown significance in these genes may be identified making molecular diagnosis difficult. The clinical assessment of the patient is essential for making an accurate diagnosis, targeting the genetic test and interpretation of the result. This

study investigated if lymphoscintigraphy in primary lymphedema can provide characteristic features that are diagnostically specific and therefore helpful in phenotyping and genotyping.

In a normal lymphoscintigram using a bipedal depot injection (Figure 2) the radioactive isotope is taken up symmetrically from the feet, and the main lymphatic tracts and inguinal lymph nodes are clearly visualized two hours after injection. Milroy disease typically demonstrated a 'functional aplasia' whereby tracer did not appear to be absorbed by initial lymphatics and consequently little tracer drainage was seen in the legs with little or no uptake (and therefore no visualization) in ilio-inguinal lymph glands and a high level of retention at the depot at 2 hours post-injection. Given that previous studies in humans have revealed the histological presence of dermal initial lymphatic vessels,[6] the interpretation is that the lymph vessels, although present, are not working, hence the term 'functional aplasia'. The analysis of tracer retention at the injection depot and lymph node uptake was found to be useful in supporting the image findings with statistically significant differences between MD, LDS and controls. MD demonstrated a high injection site retention of tracer and no, or little, drainage to, or accumulation within, the ilio-inguinal nodes.

In contrast, in LDS, the lymph scan images were clearly different and demonstrated what was interpreted as collector vessel reflux. This was because typical scans showed superficial rerouting of the lymph drainage, with the tracer imaged within the skin and subcutis of the legs (dermal backflow) so giving an appearance of the legs seen in profile. Furthermore, the fact that tracer accumulation within the skin was usually highest in the vicinity of the lower leg and ankle supported a mechanism of reflux. The idea of lymph reflux as the mechanism for LDS is not without precedent. Mutations in *FOXC2* are known to interfere with lymphatic valve development[1] and reflux was suggested in traditional radio contrast lymphography.[21] Despite the

reflux and inefficient drainage, the bulk of imaged nodes could be profound. This was interpreted as indicating the hyperplasia of ilio-inguinal nodes as seen on traditional lymphography.[21] There could be some retention of the tracer at the injection depot and a reduction of the uptake in the groin, but it was nowhere near as striking as in MD. Previous studies in mice and humans with *FOXC2* mutations have demonstrated that both the lymphatics and veins have small, dysplastic and leaky valves with abnormal recruitment of smooth muscle.[1] The finding that lymph drainage in LDS is influenced by orthostasis (effects of gravity) also support the mechanism of reflux.[12]

There was some inevitable variability in the lymphoscintigraphy results for both genotypes as demonstrated in Tables 3 and 5. This is independent of the type of mutation, with intra-familial variation. These differences are difficult to explain but may depend on other factors, for example, the age of the patient at the time of the scan, or damage following episodes of cellulitis.

This report demonstrates a significant difference in the quantification between the two conditions reflecting differing underlying mechanisms (Table 1). In Milroy disease, there is limited absorption of interstitial fluid into the initial lymphatic vessels and, in the main, very poor transport and uptake in the ilio-inguinal nodes. In LDS, the lymphatic vessels are able to absorb interstitial fluid and there are measurable levels of transport of tracer to the ilio-inguinal lymph glands but substantial amounts of lymphatic backflow down the leg, due to defective lymphatic valves unable to oppose the gravitational forces working against lymphatic flow. The popliteal nodes are frequently visualized. Tracer administered around the lateral foot and ankle, as given for Sentinel Lymph Node (SLN) identification, may drain via the deep route, but tracer administered into the web spaces, under normal circumstances, follows an epifascial route. Popliteal node visualization after subcutaneous foot web space injection is an important sign of abnormal lymphatic function in patients with clinical lymphedema of the lower extremities.[22]

The visualization of popliteal lymph nodes on imaging, therefore indicates that reflux also occurs into the deeper drainage systems.

In conclusion, lymphoscintigraphic imaging and quantification can be characteristic in both Milroy disease and Lymphedema-Distichiasis syndrome and so be useful as an additional tool for in-depth phenotyping, leading to a more accurate diagnosis and providing insight into the underlying mechanism.

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Author Disclosure Statement

The authors declare no conflict of interest and have no competing financial interests.

Ethical Approval

Ethical approval for this study was obtained from the South West London Research Ethics Committee (REC Ref: 05/Q0803/257).

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Figures

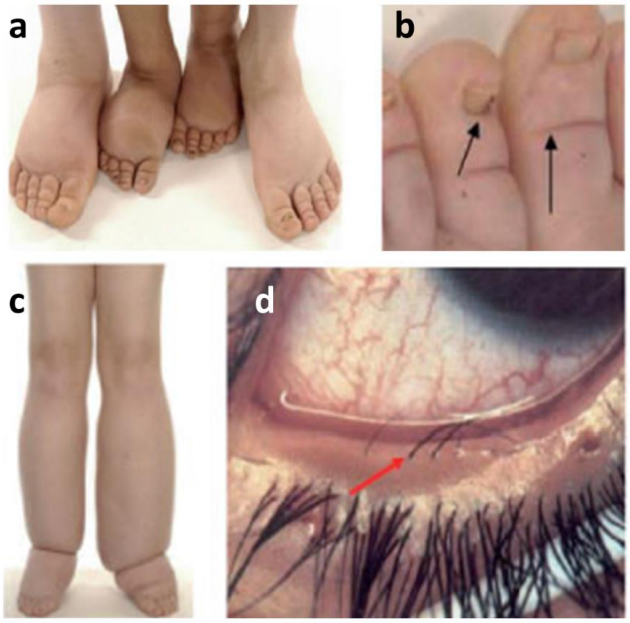


Figure 1. Phenotypic features observed in Milroy disease and Lymphedema-Distichiasis syndrome. (a) Dorsal pedal edema and (b) deep toe creases and small dysplastic nails in Milroy disease (arrows). (c) Bilateral lower limb edema and (d) distichiasis (arrow) seen in LDS.

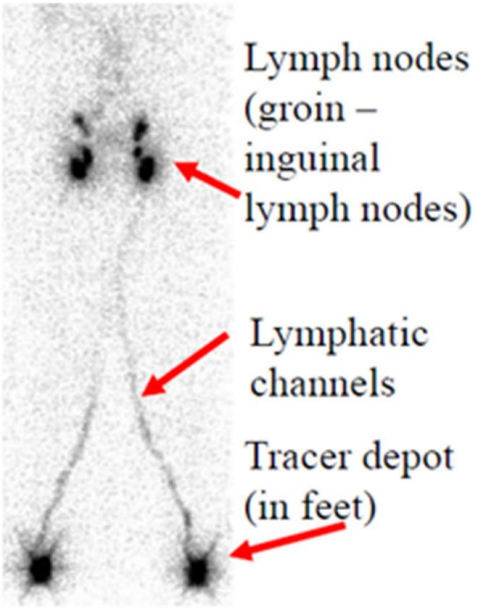


Figure 2. Lower limb lymphoscintigraphy in an unaffected subject showing symmetrical migration of radionuclide (Technetium-99) through discrete lymph vessels up into the inguinal lymph nodes 2 hours after injection.

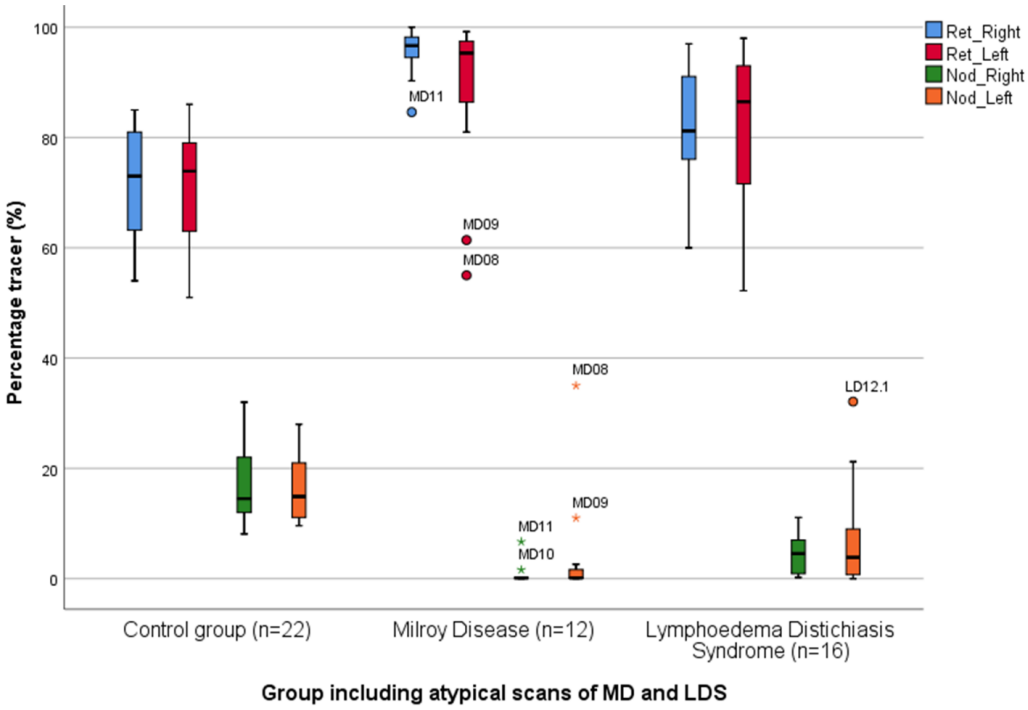
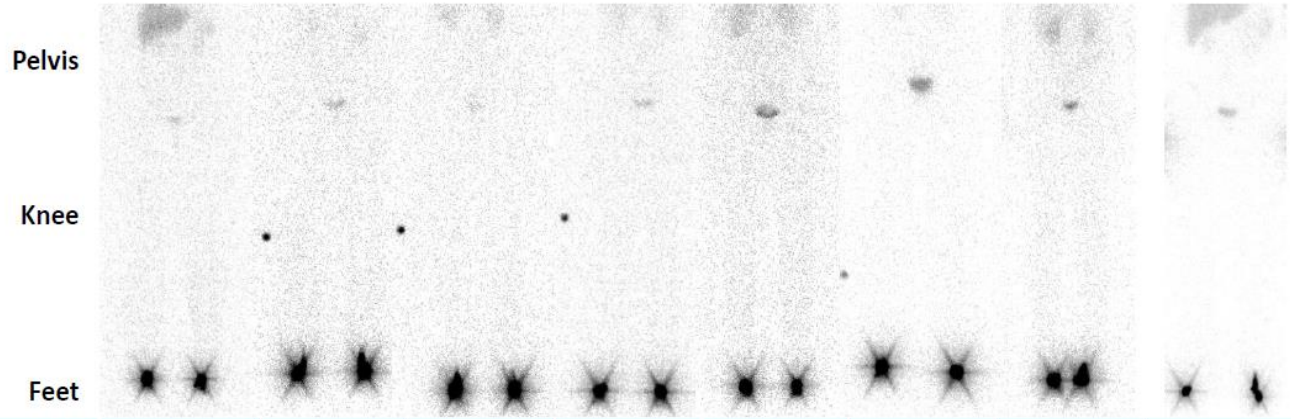


Figure 3. Boxplot showing the percentage retention rate and the lymph node uptake in the right and left leg at 2 hours of the control group, Milroy disease and Lymphedema-Distichiasis syndrome. Outliers are identified by patient ID number. Ret_Right, retention rate right leg; Ret_Left, retention rate left leg; Nod_Right, lymph node uptake right leg; Nod_Left, lymph node uptake left leg.

Table 1. Demographic data. Number of males and females (%) within each group, mean age (±SD) per group and total dataset. Mean retention rate (±SD) of contrast agent at 2 hours per leg, mean inguinal nodal uptake (±SD) at 2 hours per leg was compared across the three groups and tested for significance using the non-parametric Kruskal Wallis test. LDS, Lymphedema-Distichiasis syndrome; MD, Milroy disease; SD, standard deviation.

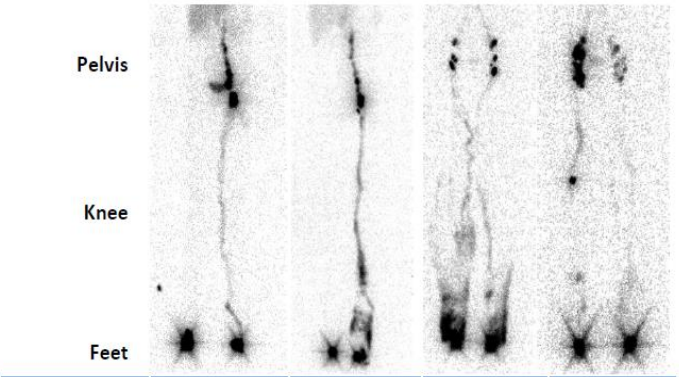
Demographics	**Normal n=22 (44%)**	**LDS n=16 (32%)**	**MD n=12 (24%)**	**Total n=50 (100%)**
Male	13 (59%)	8 (50%)	3 (25%)	24 (48%)
Female	9 (40.9%)	8 (50%)	9 (75%)	26 (52%)
Mean age at scan (±SD)	43 ±14.5	27 ±14.9	30 ±16.0	35 ±16.5
				P-value
Mean retention at depot right leg (±SD)	71.1% (±9.8)	82.0% (±10.6)	95.6% (±4.4)	<0.001
Mean retention at depot left leg (±SD)	70.7% (±9.7)	82.6% (±13.4)	88.5% (±15.0)	<0.001
Mean inguinal nodal uptake right leg (±SD)	17.3% (±7.5)	4.5% (±3.4)	0.8% (±1.9)	<0.001
Mean inguinal nodal uptake left leg (±SD)	16.3% (±5.7)	7.0% (±9.0)	4.2% (±10.2)	<0.001

Table 2. Lymphoscintigraphy imaging of typical Milroy disease (MD). All lower limb scans are given as anterior view. Quantification figures 2hrs post injection are given as % of tracer retention in right and left foot and tracer uptake in the ilio-inguinal nodes. Genetic variant and predicted protein change are also shown. Patients MD2:1 and MD2:2 are related. The black dot in MD2:1, MD2:2, MD3 and MD5 is the orientation marker.



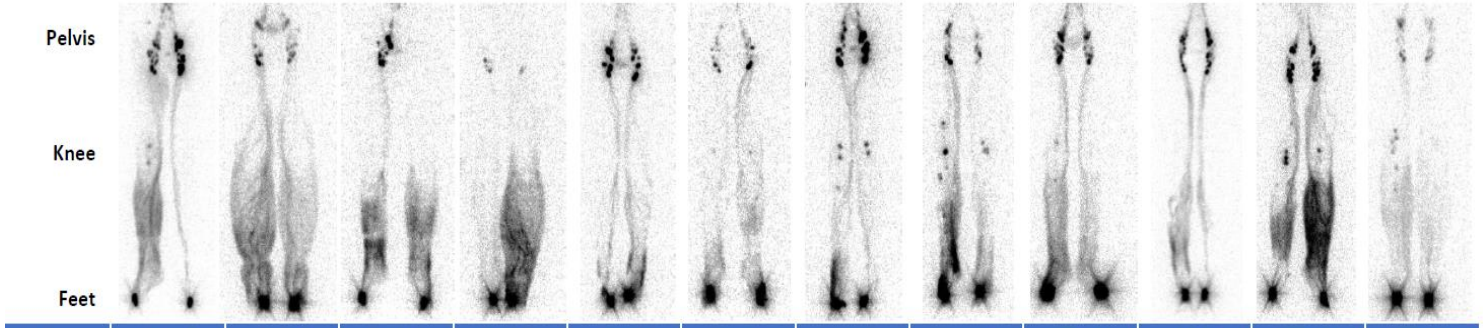
Patient	MD 1		MD 2:1		MD 2:2		MD 3		MD 4		MD 5		MD 6		MD 7	
Lower Limb	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Tracer retention in foot	94.0	81.0	97.7	99.2	98.4	97.1	100.0	94.6	95.7	93.4	95.0	96.0	96.3	97.7	98.0	98.0
Uptake in ilio-inguinal nodes after 2 hrs	0	0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0	0	0.1	0.1	0	0
VEGFR3 Variant	c.3071G>A		c.3097T>G		c.3097T>G		c.3122G>C		c.3157A>T		c.3169G>C		c.3410C>T		c.[2680_2682delATG (+) 3433C>T]	
Predicted protein change	p.Gly1024Glu		p.Cys1033Gly		p.Cys1033Gly		p. Arg1041Pro		p.Ile1053Phe		p.Gly1057Arg		p.Pro1137Leu		p.M894del + p.Arg1145Cys	
Gender	F		F		F		M		F		F		F		M	
Age at time of scan	53		55		28		36		21		10		7		32	

Table 3. Lymphoscintigraphy imaging of atypical Milroy disease (MD). All lower limb scans are given as anterior view. Quantification figures 2hrs post injection are given as percentage of tracer retention in right and left foot and tracer uptake in the ilio-inguinal nodes. Genetic variant and predicted protein change are also shown. Orientation marker in MD8 indicating the right-hand side of the scan. However black dots seen in the right and left calf area of MD10, and right calf and thigh of MD11 are popliteal nodes.



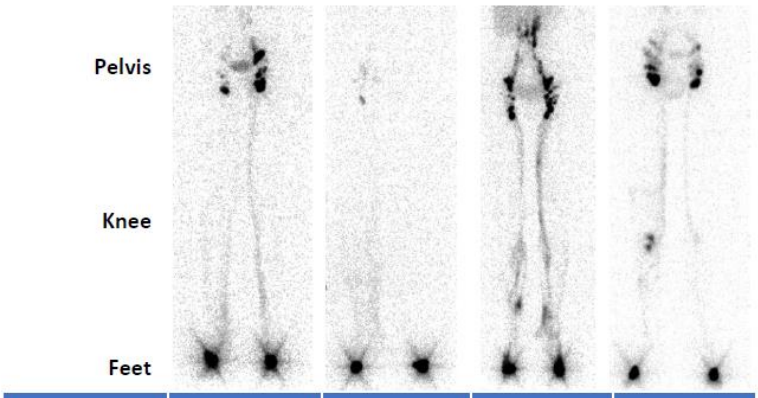
Patient	MD 8		MD 9		MD 10		MD 11	
Lower Limb	Right	Left	Right	Left	Right	Left	Right	Left
Tracer retention in foot	97.0	55.0	99.7	61.4	90.3	91.8	84.6	97.2
Uptake in ilio-inguinal nodes after 2 hrs	0.05	35.0	0.1	11	1.6	2.6	6.7	0.7
VEGFR3 Variant	c.3121C>T		c.3122G>C		c.3122G>A		c.3323_3325 delTCT	
Predicted protein change	p.Arg1041Trp		p.Arg1041Pro		p.Arg1041Pro		p.Phe1108del	
Gender	F		F		F		M	
Age at time of scan	9		39		39		35	

Table 4. Lymphoscintigraphy imaging of typical Lymphedema-Distichiasis Syndrome (LDS). All lower limb scans are given as anterior view. Quantification figures 2hrs post injection are given as percentage of tracer retention in right and left foot and tracer uptake in the ilio-inguinal nodes. Genetic variant and predicted protein change are also shown. Patients LDS12:1 and LDS12:2 are related, so are LDS15:1 and LDS15:2 and LDS20:1 and LDS20:2. LDS18:1 is related to LDS18:2 in Table 5.



Patient	LDS 12:1		LDS 12:2		LDS 13		LDS 14		LDS 15:1		LDS 15:2		LDS 16		LDS 17		LDS 18:1		LDS 19		LDS 20:1		LDS 20:2	
Lower Limb	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Tracer retention in foot	60.0	52.2	79.0	88.0	69.4	84.7	88.0	70.0	81.3	85.0	94.1	91.0	81.1	69.8	76.6	96.7	84.2	96.0	75.5	73.2	69.1	67.0	95.2	94.0
Uptake in ilio-inguinal nodes after 2 hrs	5.5	32.1	2.0	1.0	5.9	0.2	0.4	0.3	9.0	5.5	0.8	2.2	6.2	21.2	3.6	0.7	3.5	1.6	7.2	14.9	7.8	7.1	1.0	0.7
FOXC2 Variant	c.223T>G		c.223T>G		c.256C>T		c.361C>T		c.374C>T		c.374C>T		c.595dupC		c.595dupC		c.666dupG		c.710_713 dupTGCA		c.818dupA		c.818dupA	
Predicted protein change	p.Tyr75Asp		p.Tyr75Asp		p.Gln86X		p.Arg121Cys		p.Ser125Leu		p.Ser125Leu		p.His199Pro fs*264		p.His199Pro fs*264		p.Pro223Ala fs*240		p.Gln238His fs*226		p.Asn274Glu fs*189		p.Asn274Glu fs*189	
Gender	M		M		M		F		F		F		M		F		F		F		M		F	
Age at time of scan	12		37		16		29		13		45		14		24		10		29		13		34	

Table 5. Lymphoscintigraphy imaging of atypical Lymphedema distichiasis syndrome (LDS). All lower limb scans are given as anterior view. Quantification figures 2hrs post injection are given as percentage of tracer retention in right and left foot and tracer uptake in the ilio-inguinal nodes. Genetic variant and predicted protein change are also shown. Patients LDS21:1 and LDS21:2 are related. Patients LDS18:2 is related to patient LDS18:1 in Table 4.



Patient	LDS 21:1		LDS 21:2		LDS 18:2		LDS 22	
Lower Limb	Right	Left	Right	Left	Right	Left	Right	Left
Tracer retention in foot	96.0	92.0	97.0	98.0	86.0	73.4	79.5	91.6
Uptake in ilio-inguinal nodes after 2 hrs	0.7	8.0	0.2	0	6.8	10.0	11.1	6.2
FOXC2 Variant	c.134delC		c.134delC		c.666dupG		c.712C>T	
Predicted protein change	p.Pro45Arg fs*27		p.Pro45Arg fs*27		p.Pro223Ala fs*240		p.Gln238X	
Gender	M		M		F		M	
Age at time of scan	15		53		40		53	

Supplementary tables

Supplementary Table 1. Mann-Whitney U test testing all LDS and MD quantification values from lymphoscintigraphy for significant difference (LDS, lymphedema distichiasis syndrome; MD Milroy disease).

Measurement	LDS (n=16)	MD (n=12)	P-value
Mean retention at depot right leg (±SD)	82.0% (±10.6)	95.6% (±4.4)	<0.001
Mean retention at depot left leg (±SD)	82.6% (±13.4)	88.5% (±15.0)	0.078
Mean inguinal nodal uptake right leg (±SD)	4.5% (±3.4)	0.8% (±1.9)	<0.001
Mean inguinal nodal uptake left leg (±SD)	7.0% (±9.0)	4.2% (±10.2)	0.025

Supplementary Table 2. Mann-Whitney U test testing for significance between the typical LDS and MD quantification values from lymphoscintigraphy (LDS, lymphedema distichiasis syndrome; MD, Milroy disease).

Measurement	LDS (n=12)	MD (n=8)	P-value
Mean retention at depot right leg (±SD)	79.5% (±10.3)	96.9% (±1.9)	<0.001
Mean retention at depot left leg (±SD)	80.6% (±14)	94.6% (±5.8)	<0.001
Mean inguinal nodal uptake right leg (±SD)	4.4% (±2.9)	0.8% (±0.1)	<0.001
Mean inguinal nodal uptake left leg (±SD)	7.3% (±10.2)	0.8% (±0.1)	<0.001