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GENETIC VARIANTS IN THE TRANSCRIPTION FACTOR NETWORK: THE IMPACT OF *FOXC1*, *NOTCH1*, *RORC*, *FOXC2* AND *SOX18* IN PRIMARY LYMPHEDEMA

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ABSTRACT

Lymphedema is a chronic inflammatory disease caused by defective lymphatic system function or development that proceeds in abnormal fluid drainage and edema of the peripheral tissues. This retrospective study evaluates the potential role of transcription factors in the development of primary lymphedema. We analyzed 408 Italian primary lymphedema patients for pathogenic variants in genes related to the transcription factor network. Eighteen likely pathogenic genetic variants were identified in 5 genes - FOXC1, FOXC2, NOTCH1, RORC, *and* SOX18 - *most of which have not been previously reported in the literature. Although majority of these genetic variants resulted in premature termination of the proteins, a single missense variant was identified in* FOXC2 *and analyzed in this study using molecular modeling approaches to eval-uate its structural changes. These results sug-gested possible deleterious effects of the ana-lyzed variant and supported its potential pathogenicity. Our findings provide additional evidence that associates variants in transcription factors to the onset of lymphedema and support the addition of the candidate genes* FOXC1, NOTCH1, *and* RORC *in primary lymphedema diagnostic practice.*

Keywords: Primary lymphedema; Tran-

scription factors; Regulatory networks; Molecular modeling; *FOXC2*

INTRODUCTION

Lymphedema is a chronic condition characterized by abnormal fluid buildup in peripheral tissues. This pathology arises from dysfunctions of the lymphatic system, a network of vessels responsible for draining interstitial fluid, transporting dietary fats, and supporting immune function (1,2). Lymphedema occurs when the amount of interstitial fluid in peripheral tissues overload the functional reserve of the lymphatic system, finally resulting in fluid stagnation and accumulation (2,3). The impaired lymph circulation causes edema mainly in lower and upper extremities. Progression of lymphedema is divided into 4 clinical stages (0-3). Stage 0 is non-visible without swelling, where the patient suffers only from heaviness and aching after physical activity, while stage 3 is characterized by the presence of dermal metaplasia and lymphostatic elephantiasis (3). At present, lymphedema does not have a definitive cure. Treatments are very limited and symptomatic, and lymphedema can progress rapidly and result in heaviness, aching, infections (3-5), and reduced mobility (6). While the causes can be broadly categorized as primary (genetic) or secondary (acquired), the underlying mechanisms often converge on disruptions of lymphatic function. Primary lymphedema is hereditary, primarily caused by defects in genes involved in lymphatic development and function. Secondary lymphedema occurs during life, after the lymphatic system is damaged due to surgeries, infections, or other diseases (2,3,5). Primary lymphedema affects 1 in 100,000 individuals, while secondary is more common and affects 1 in 1,000 people (7).

Recent research has highlighted the critical role of transcription factor networks in directing lymphatic vessel formation and function (*Fig. 1*) (8,9). These networks act as master regulators, orchestrating the expression of genes essential for lymphatic development through the action of specialized proteins called transcription factors (TFs). These proteins bind to specific DNA sequences in gene promoters or enhancers, acting as molecular switches that enable selected gene transcription (9-11). Mutations in key genes within TFs networks have been linked to various forms of lymphedema and offer valuable insights into the disease's pathogenesis (12-16). The major players in the TFs network include the SOX18/COUP-TFII/PROX1 axis (*Fig. 1*). Transcription factor SOX-18 (SOX18) and COUP transcription facotr 2 (COUP-TFII) work in tandem to activate Prospero homeobox protein 1 (PROX1), a master regulator that directly controls the expression of numerous genes required for lymphatic vessel development (8,9,17). Additionally, Forkhead box protein C1 (FOXC1) and Forkhead box protein C2 (FOXC2), belonging to the Forkhead box protein family, contribute by influencing lymphatic vessel growth and maintaining proper vessel structure (10,18).

This study aims to identify novel genetic variants in genes within the TFs network and discuss their potential association with lymphedema onset. Elucidating the link between specific genetic variations and the development of lymphedema could improve diagnosis and risk stratification, and guide novel therapeutic strategies.

MATERIALS AND METHODS

Selection of Subjects and Sample Processing

In this study, we retrospectively investigated genetic and clinical data collected from 408 Italian patients with primary lymphedema. Patients were not distinguished between syndromic and non-syndromic lymphedema, and only the lymphatic phenotype was assessed. The same cohort has been studied previously by Dundar et al. (19). Before genetic testing, all participants received genetic counseling, where we gathered detailed information on their personal and family medical history. We explained the implications of genetic testing and obtained written informed consent from each participant, following the ethical principles outlined in the Declaration of Helsinki. This study was approved by the Ethical

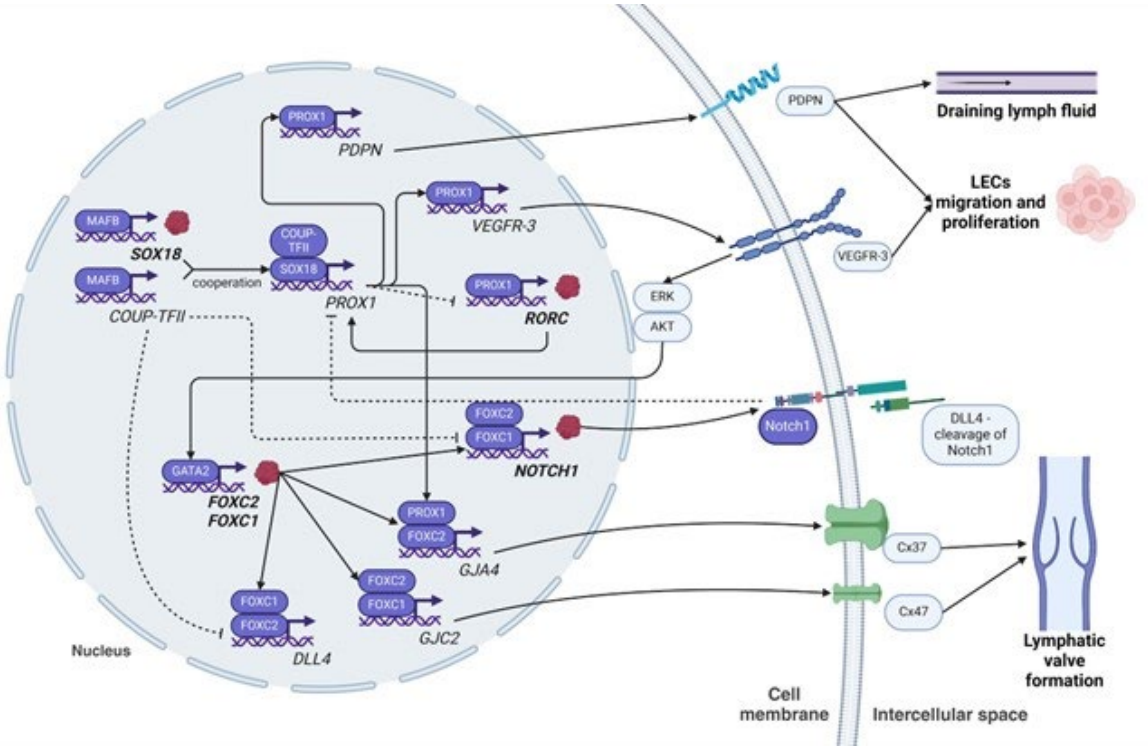

Fig. 1. *Schematic view of the lymphatic endothelial cell and key transcription factors acting in lymphatic system development and function (highlighted in dark blue bubble frames). Solid lines represent enhanced interaction, while dashed lines represent inhibition of transcription. Created with* ***https://www.biorender.com/****.*

Committee of Azienda Sanitaria dell'Alto Adige (Italy) under Approval No. 132-2020. We proceeded with DNA extraction, using either saliva or peripheral blood samples. To isolate genomic DNA, we employed a commercially available kit (SaMag Blood DNA Extraction Kit, Sacace Biotechnologies, Como, Italy) following the manufacturer's established protocol.

Selection of the Genes and Sequencing

This study focused on genes involved in the lymphatic TFs networks, specifically *FOXC1, FOXC2, NOTCH1, RORC* and *SOX18.* More information regarding gene-pathway correlation and difference between candidate and diagnostic genes can be found in Bonetti et al. (20). Analysis involved target capture using custom oligonucleotide-based panels (Illumina Nextera Rapid Capture Custom Assay and Twist Custom Panel EF Workflow) and was followed by sequencing of 150 bp paired-end reads on an Illumina MiSeq sequencer, in accordance with the manufacturer's protocols. Details regarding primer sequences, PCR reaction and sequencing conditions are available upon request.

Bioinformatic Analyses and Variant Classification

Raw data from sequencing were obtained in the form of Fastq files. These files were then mapped and aligned to a reference genome using BWA software (https://bio-bwa.sourceforge.net/). To enhance data quality and facilitate variant calling, duplicate reads were removed using a combination of SAMBAMBA (https://lomereiter.github.io/sambamba/) and GATK's MarkDuplicates tool (https://gatk.broadinstitute.org/hc/en-us). Subsequently,

the BAM alignment files were further optimized with GATK's RealignerTargetCreator and IndelRealigner (https://gatk.broadinstitute.org/hc/en-us). To assess the prevalence of identified variants in the general population, minor allele frequencies were retrieved from the Genome Aggregation Database (GnomAD) (https://gnomad.broadinstitute.org/). *In silico* predictions of the potential functional impact of these nucleotide variations were performed using VarSome (https://landing.varsome.com/varsome). Finally, the variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines and an internal algorithm developed by Cristofoli et al. (21) into five categories: pathogenic, likely pathogenic, variant of uncertain significance, likely benign, or benign. We report only pathogenic and likely pathogenic variants. Detailed descriptions of the bioinformatics workflow can be found in Donato et al. (22).

In silico prediction of the effect of identified missense variant

To visualize the impact of mutations on the three-dimensional structure of FOXC2 protein, we employed protein structure modeling techniques. The crystal structure of FOXC2 DNA binding domain in complexed with DBE2 DNA (Protein Data Bank ID: 6AKO) was used for modeling (23). The mutations were introduced into the modeled structures using the mutagenesis tool in PyMOL (https://www.pymol.org/). Furthermore, the SIFT and PolyPhen scores were retrieved using the Ensembl Variant Effect Predictor (VEP) (https://www.ensembl.org/info/docs/tools/vep/index.html), and the predicted changes in protein stability upon mutation were assessed using the mCSM web server (https://biosig.lab.uq.edu.au/mcsm/stability). 6AKO, which contains the 3D coordinates of the FOXC2-DBD/DNA complex, was subjected to Molecular Dynamics (MD) simulations, performed by Gromacs (https://manual.gromacs.org/current/download.html). AMBER99 was chosen as the force field, which contains parametrizations for both nucleotides and amino acids. The complex was placed in a triclinic box with a minimum spacing of 1.2 nm. Solvation was done using TIP3P water molecules, and the system was neutralized with Mg^{2+}/Cl^{-}. Energy minimization was performed via the gradient descent algorithm, with step size of 0.01, maximum force threshold of 1000.0 kJ/mol/nm, for 25000 steps. Consequently, two equilibration steps of position-restrained molecular dynamics in the NVT and NPT ensembles were performed, 100 ps each. The V-rescale thermostat and the Parrinello-Rahman barostat were used, with reference temperature of 300K and pressure of 1 bar, respectively. Finally, a molecular dynamics production run of 100 ns, followed by 2 additional replicas of 50 ns, with a 2-fs integration step, were performed for each of the variants simulated.

RESULTS

Identifying variants with possible pathogenic impact on lymphedema patients

This retrospective study aimed to identify in a cohort of lymphedema patients genetic variants within five TFs genes, *FOXC1, FOXC2, NOTCH1, RORC,* and *SOX18,* which are crucial for lymphatic system function and development. We identified 18 likely pathogenic variants in 18 unrelated patients. Clinical characteristics of the probands are reported in *Table 1.* The study probands had an average age of 50±20, ranging from 10 to 89 years old. Most patients (33%) developed lymphedema symptoms as adults. Participants mainly experienced edema in one or both lower limbs.

Our analysis identified four likely pathogenic variants within candidate genes associated with lymphedema: two in *FOXC1*, and one each in *NOTCH1* and *RORC* (detailed in *Table 2*). The remaining 14 variants were found in two diagnostic genes: *FOXC2* and *SOX18* (*Table 3*). The majority of reported genetic variants were nonsense, resulting in early termination of the protein. Importantly, these variants have not been reported previously in public databases or the published

TABLE 1
Clinical features of probands in which genetic variants were identified

Characteristic		Case Subjects (n=18)
Age	Mean (±SD) Median	50 ± 20 53
Sex	Females/Males	13/5 (72 %/28 %)
Period of onset	Congenital Childhood (1-10) Youth (11-17) Adult (>18) Unknown	1 (6 %) 3 (17 %) 2 (11 %) 6 (33 %) 6 (33 %)
Age of onset	Mean (±SD) Median Unknown	23 ± 18 21 7
Familiarity	Sporadic Familiar Unknown	12 (67 %) 4 (22 %) 2 (11 %)
Location	Lower limb Lower limbs Unknown	5 (23 %) 8 (36 %) 9 (41 %)

TABLE 2
Genetic variants of candidate genes (*FOXC1*, *NOTCH1* and *RORC*)

Gene	Nucleotide change	SNP ID	Amino acid change	ACMG Classification	Frequency in GnomAD
FOXC1	NM_001453:c.78C>A	NA	p.(Tyr26Ter)	LP	0
FOXC1	NM_001453:c.75C>A	NA	p.(Tyr25Ter)	LP	0.00000184
NOTCH1	NM_017617:c.2940T>A	rs213335 0399	p.(Cys980Ter)	LP	NA
RORC	NM_005060:c.73C>T	NA	p.(Gln25Ter)	LP	NA

There is no lymphedema reference available for any of these variants; NA: Not Available; LP: Likely Pathogenic

TABLE 3
Genetic variants of diagnostic genes (*SOX18* and *FOXC2*)

Gene	Nucleotide change	SNP ID	Amino acid change	ACMG Classification	Frequency in GnomAD	Lymphedema reference
SOX18	NM_018419:c.39_42del	rs1270867434	p.(Arg17Alafs Ter72)	LP	0.0000127	NA
SOX18	NM_018419:c.526 G>T	NA	p.(Gly176Ter)	LP	0	NA
FOXC2	NM_005251:c.284 T>A	NA	p.(Leu95Ter)	LP	NA	NA
FOXC2	NM_005251:c.544 del	NA	p.(His182Thrf sTer19)	LP	NA	NA
FOXC2	NM_005251:c.785 _786del	NA	p.(Ala262Glyf sTer200)	LP	NA	NA
FOXC2	NM_005251:c.374 C>T	rs121909106	p.(Ser125Leu)	LP	6.195e-7	(12,13,24-28)
FOXC2	NM_005251:c.532 G>T	NA	p.(Glu178Ter)	LP	NA	NA
FOXC2	NM_005251:c.134 7del	NA	p.(Asn449Lys fsTer23)	LP	NA	NA
FOXC2	NM_005251:c.342 del	NA	p.(Gly115Alaf sTer86)	LP	NA	NA
FOXC2	NM_005251:c.764 C>A	NA	p.(Ser255Ter)	LP	0	NA
FOXC2	NM_005251:c.970 del	rs1974229664	p.(Ala325Prof sTer12)	LP	0.00000357	NA
FOXC2	NM_005251:c.106 1del	NA	p.(Leu354Arg fsTer16)	LP	NA	NA
FOXC2	NM_005251:c.459 C>G	NA	p.(Tyr153Ter)	LP	NA	NA
FOXC2	NM_005251:c.101 7C>A	NA	p.(Tyr339Ter)	LP	0	NA

NA: Not Available; LP: Likely Pathogenic

literature, except for one variant in the *FOXC2* gene (rs121909106), which a missense variant that replaces serine (Ser) at amino acid position 125 of the FOXC2 DNA binding domain with leucine (Leu).

In silico analysis of the missense variant in FOXC2

To understand how the missense variant in the *FOXC2* gene may impact pathogenicity in lymphedema patients, a structural analysis was conducted based on computational predictions. The missense variant in *FOXC2* gene, altering Ser with Leu at position 125, was modeled *in silico,* and the protein-DNA complex was subjected to molecular dynamics (MD) simulations. The variant is located in the H3 helix of the FOXC2 DNA binding domain and participates in the interaction with the core DNA sequence GTAAACA (*Fig. 2*). The H3 helix contains four amino aicd residues that participate in direct interactions with the DNA, namely: asparagine 118 (Asn118), arginine 121 (Arg121), histidine 122 (His122), and Ser125, one of which includes

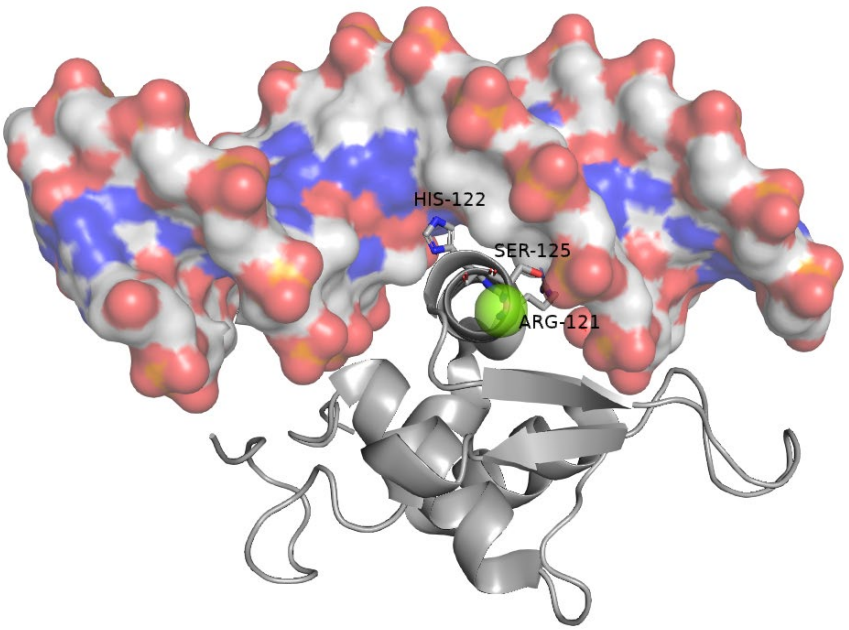

Fig. 2. *FOXC2 DNA binding domain (shown in grey) interacting with the major groove of DNA (shown as surface representation). The light green sphere represents the magnesium ion found in the crystal structure, while the red sphere represents a water molecule potentially mediating the interaction between arginine121 and DNA. The three important amino acids of the H3 helix (Arg121, His122, and Ser125) are shown as sticks.*

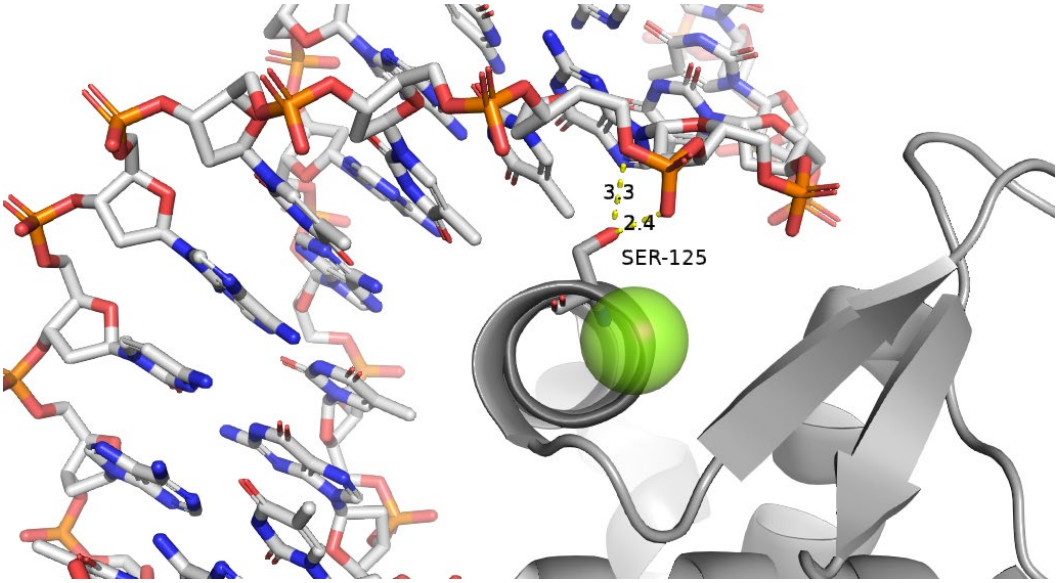

Fig. 3. *Centered view of the H3 Helix and Ser125 interaction with the phosphate backbone of thymine11. Protein is shown as grey cartoon, DNA residues are shown in light grey, with orange representing the phosphate backbone; the magnesium ion, found in the crystal structure bound to the end of the H3 helix and forming ionic interaction with Asp128 (not shown), is shown in light green. Hydrogen bonds are shown as yellow dashed lines, and each hydrogen bond distance in ångströms is shown.*

TABLE 4
In silico predictions of missense variant identified in the *FOXC2* gene (NM_005251)

Gene	Amino Acid Change	SIFT	PolyPhen	Predicted Stability Change	Stability Effect	Mutation taster
FOXC2	p.(Ser125Leu)	0	1	-0.186 kcal/mol	Destabilizing	DC

DC: Disease Causing

our variant Ser125Leu (*Fig. 2*). Specifically, Ser125 interacts with the phosphate backbone of T11' through hydrogen bonds (*Fig. 3*). According to electrophoretic mobility shift assays (EMSA) results from Chen et al. (23), the Ser125Leu variant showed the greatest reduction in DNA-binding affinity. However, Differential Scanning Fluorimetry (DSF) revealed a slight right shift of the melting temperature (T_m) when compared to the wildtype, indicating a greater thermal stability for the mutant complex [as seen in Fig. 8C from Chen et al. (23)]. We investigated the Ser125Leu variant using molecular modeling and molecular dynamics and found that a continuous hydrogen bond between serine at 125 and the phosphate backbone was present in the whildtype but absent in the mutant simulation (Supplementary Videos V1, and V2). Furthermore, *in silico* predictions show a slight destabilizing effect on the stability of the resulting protein (*Table 4*). We therefore suggest that the Ser125Leu mutantion, which introduces a hydrophobic amino acid to the binding site, could disrupt hydrogen bond formation with the phosphate backbone, probably leading to reduced binding affinity between the H3 helix of the FOXC2 DNA-binding domain and DNA.

DISCUSSION

Defects in the lymphatic system may lead to impaired function and result in diseases such as lymphedema (10,29). This study investigated the presence of pathogenic or likely pathogenic variants in genes correlated to the TFs networks (8,9,20). TFs regulate the transcription of genes expressed in lymphatic cells (*Fig. 1*) (8,10). Their mutations have been associated with syndromic or non-syndromic lymphedema (12-16,30), highlighting the significant role the TFs play in lymphatic development and function.

Our study reports four likely pathogenic variants in the candidate genes *FOXC1, NOTCH1,* and *RORC*, and 14 variants in the diagnostic genes *FOXC2* and *SOX18*. Most of the identified variants were nonsense or frameshift mutations, causing the premature termination of resulting proteins and possibly leading to loss of function. The FOXC2 Ser125Leu variant, on the other hand, has been experimentally shown to reduce the binding affinity of the protein to the DNA (23). In this study, our computational analyses, including molecular dynamics simulations, further revealed that the mutant may disrupt the formation of hydrogen bonds essential for intraction with DNA.

FOXC1 (Forkhead box protein C1), a member of the FOX protein family, is a crucial TF involved in various developmental processes, particularly in the eye, heart, kidney, and lymphatic system. *FOXC1* (OMIM: *601090) is expressed predominantly during development and regulates gene expression by binding to specific DNA sequences known as forkhead box motifs (31). In the lymphatic system, FOXC1 contributes to lymphatic vessel formation and maintenance. It regulates the expression of genes essential for lymphatic endothelial cell differentiation and proliferation, such as those in MAPK/ERK signaling (32). Additionally, FOXC1 cooperates with

FOXC2 in regulating lymphatic valve formation (*Fig. 1*) (18). Mice models with specific deletion of *Foxc1* in LECs show increased LEC proliferation, abnormal vessel morphology, and defective sprouting of LECs from blood veins (32,33), emphasizing the role of *FOXC1* in the development of lymphatic vessels. Mutations in this gene have not been correlated to lymphatic malformations in OMIM. However, we identified two likely pathogenic nonsense mutations in lymphedema patients, providing further evidence for its implications in lymphedema onset.

NOTCH1 (OMIM: *190198) encodes a transmembrane receptor called Neurogenic locus notch homolog protein 1 (NOTCH1). This protein is vital for cell-cell signaling and is involved in various developmental processes, including forming lymphatic valves and vessels (34). NOTCH1 contains EGF-like domains crucial for membrane binding (35). NOTCH signaling is a complex intercellular process that involves the interaction of NOTCH1 with ligands expressed by neighboring cells. When NOTCH1 binds to its ligand, such as DLL4 (36,37), it undergoes proteolytic cleavage, releasing the intracellular domain of the protein. The domain then enters the nucleus and regulates the expression of target genes, such as *COUP-TFII* and *PROX1* (*Fig. 1*) (38). NOTCH1 regulates the sprouting of lymphatic vessels from blood vessels, ensures that lymphatic vessels are connected correctly to blood vessels and tissues (34,39,40), and regulates valve formation (41). Mutations in the gene can disrupt NOTCH signaling, impairing lymphatics formation and function, as shown *in vitro* (41,42). Mice models lacking the functional *Notch1* gene exhibited abnormal blood vessels and artery morphology (43) and defective vascular development (44). LEC-specific *Notch1* deleterious mice show increased proliferation and sprouting of LECs (40), overgrowth of lymphatics continued with failure of lymphatic vessel separation from a cardinal vein (42), and defective valve formation (41). These findings support the regulative role of *NOTCH1* in lymphatic development. Michelini et al. connected seven rare missense variants in the *NOTCH1* gene to the lymphedema predisposition (15). This study identified one likely pathogenic nonsense mutation (rs2133350399).

RORC (also known as *RORG* or *RORγ*; OMIM: *602943) is a gene encoding transcriptional regulator of the nuclear receptor family (NR1 subfamily). RORC (RAR-related orphan receptor C) exists in two isoforms and functions as an activator, developmental protein, DNA binder, and receptor. ROR receptors bind to specific DNA regions called ROR response elements (RORE) through two zinc finger domains (45). The C-terminal AF-2 motif facilitates interactions with diverse cofactors crucial for accurate transcriptional regulation. The cofactors differ based on the tissue, developmental stage, and promoter type. (45-50). RORC plays a vital role in various biological processes, including immune response, lipid, steroid, and glucose metabolism, and circadian rhythm (45,51-55). However, its specific molecular mechanism in these processes is currently unknown. RORC reacts with other TFs to regulate lymphatic vessel formation and maintenance. It regulates the expression of *PROX1* and supports its nuclear localization. RORC interacts with PROX1 via the AF2 domain, mutually modulating their transcriptional activity. In a feedback loop, PROX1 acts as a repressor for the expression of *RORC*, while RORC enhances the level of *PROX1* expression (*Fig. 1*) (56). *In vitro* and *in vivo* studies showed that RORC contributes to the development of the lymphatic system, mainly the organogenesis of lymph nodes, Peyer's patches (45,48,57,58), and the differentiation of innate lymphoid cells 3 (ILC3) that are associated with the growth of lymphatic capillaries around lymph nodes (59-61). Pathogenic mutations in this gene have been linked to lymphedema in literature. Babu et al. and Babu & Nutman reported the role of *RORC* in filariasis lymphedema (62,63). Ferrel et al. (2008) observed three single nucleotide variants in *RORC* (64) and Michelini et al. reported two heterozygous variants in primary lymphedema patients that were negative to variants in other lymphedema-associated genes (16). Our study identified one novel likely pathogenic heterozygous vari-

ant (NM_005060:c.73C>T) in the *RORC* gene.

SOX18 (OMIM: *601618) encodes a crucial transcription factor belonging to the SRY-related HMG-box (SOX) family. SOX18 is composed of a highly conserved HMG-box domain needed for DNA binding and a transcription activation domain (TAD) that enables binding to other TFs (65). To bind the DNA, SOX18 recognizes specific sequences located in the promoters of targeted genes – Sox DNA-binding motifs (66). This protein is involved in the development and cell specialization. As it is mainly expressed in developing LECs, *SOX18* is essential for initiating LEC specification, lymphatic vessel sprouting, and maturation (11,67-69). The expression of *SOX18* is activated via MAPK/ERK signaling (67,70). Then, SOX18 cooperates with COUP-TFII and activates the transcription of the master regulator – *PROX1* (*Fig. 1*) (11,71), ensuring the development of LECs from BECs (72,73). Homozygous mice (*Sox18-/-*) show blocked LECs differentiation from the cardinal veins, abnormal lymphatic vessel morphology, and skin edema, with few cases of postnatal lethality (11,68). Mutations in this gene can lead to lymphatic malformations and lymphedema. *SOX18* mutations are linked to Hypotrichosis-lymphedema-telangiectasia syndrome (OMIM: *607823 – AR; *137940 – AD), which is characterized by lower limb lymphedema, absent facial hair, and thin skin (14,74,75).

In this study, we found two likely pathogenic variants in primary lymphedema patients supporting the significant role of SOX18 in lymphatic system function and lymphedema onset. FOXC2 is a transcription factor belonging to the forkhead box (FOX) family. It binds to specific DNA regions known as forkhead box motifs via the DNA-binding Forkhead domain, ensuring its interaction with chromatin and nuclear localization (23,76). *FOXC2* (OMIM: *602402) is necessary for various tissues development and function, including the lymphatic system (77,78). It modulates the MAPK/ERK signalling pathway as well as other genes involved in lymphangiogenesis, LECs differentiation and valve formation, such as *PROX1, NFATc1* and connexins (18,30,32,79-83). Heterozygous mice with a targeted disruption of *Foxc2* exhibit lymphatic vessel and lymph node hyperplasia, retrograde lymph flow, and incomplete lymphatic valves. Affected mice display the lymphedema phenotype (84). On the other hand, homozygous mice experience severe morphological defects in lymphatics, such as increased LECs proliferation, enlarged lymphatic nodes and vessels, defects in the sprouting of LECs from veins (32,33,84), and degeneration of the lymphatic valves (79,80,85). Defects in the *FOXC2* gene are connected dominantly to the lymphedema-distichiasis in OMIM (*153400), which manifests lymphedema in the lower extremities. The role of *FOXC2* in lymphedema is well-established and reviewed in the literature, linking many variants to the development of lymphedema (12,13,86-90). We identified 12 likely pathogenic variants in this gene. Most of them were frameshift or nonsense. One variant was missense, altering serine at position 125, located in the Fork-head domain. Our variant substituting serine for leucine (rs1219-09106) has been described previously (*Table 3*) as causative for lymphedema-distichiasis. Additionally, molecular dynamics simulations showed a loss of hydrogen bonding capability between the H3 helix of the FOXC2 DNA binding domain, possibly leading to a decrease in binding affinity.

Limitations of the study

Our research reviews the potential role of TFs in lymphedema. However, this study has a few limitations. Firstly, by applying an NGS panel composed of 99 genes, we could overlook other substantial genes associated with the TFs network and with the onset of primary lymphedema. Secondly, we omitted the functional studies that could show the impact of identified variants on lymphatics *in vitro* and *in vivo*. We estimated the pathogenicity of variants only with *in silico* predictors and molecular modeling. Further studies are thus necessary to validate the functional impact of these variants and elucidate their role in disease development. Lastly, we were unable

to perform segregation analysis to confirm the inheritance patterns of the identified variants due to limited family data. Despite the limitations, we analyzed a large cohort of Italian lymphedema patients and identified 18 variants in TF genes potentially associated with lymphedema onset.

CONCLUSION

This study investigated the potential association of TFs to the development of lymphedema. We revealed the presence of 17 novel variants in the genes *FOXC1*, *NOTCH1*, *RORC*, *FOXC2* and *SOX18*, while one genetic variant found in *FOXC2* was linked previously to the onset of lymphedema in the literature. Most of the identified variants resulted in premature termination of the proteins, possibly leading to the loss of their function. Molecular modeling analysis of one missense variant in *FOXC2* evaluated its structural changes and suggested its potential deleterious effects and pathogenicity. Our findings provide further evidence that correlates genetic variants in TFs to lymphedema development, supporting the addition of *FOXC1*, *NOTCH1,* and *RORC* in primary lymphedema diagnostic practice.

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CONFLICT OF INTEREST

All affiliations of the authors with private companies have been declared to make clear the position regarding the interests of these companies. The authors are affiliated with private companies for which there could be a possible conflict of interest. The authors of this article are reported to be patent inventors.

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