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Original Article

Pathogenic variants in *HGF* give rise to childhood-to-late onset primary lymphoedema by loss of function

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Abstract

Developmental and functional defects in the lymphatic system are responsible for primary lymphoedema (PL). PL is a chronic debilitating disease caused by increased accumulation of interstitial fluid, predisposing to inflammation, infections and fibrosis. There is no cure, only symptomatic treatment is available. Thirty-two genes or loci have been linked to PL, and another 22 are suggested, including *Hepatocyte Growth Factor* (*HGF*). We searched for *HGF* variants in 770 index patients from the Brussels PL cohort. We identified ten variants predicted to cause *HGF* loss-of-function (six nonsense, two frameshifts, and two splice-site changes; 1.3% of our cohort), and 14 missense variants predicted to be pathogenic in 17 families (2.21%). We studied co-segregation within families, mRNA stability for non-sense variants, and *in vitro* functional effects of the missense variants. Analyses of the mRNA of patient cells revealed degradation of the nonsense mutant allele. Reduced protein secretion was detected for nine of the 14 missense variants expressed in COS-7 cells. Stimulation of lymphatic endothelial cells with these 14 HGF variant proteins resulted in decreased activation of the downstream targets AKT and ERK1/2 for three of them. Clinically, HGF-associated PL was diverse, but predominantly bilateral in the lower limbs with onset varying from early childhood to adulthood. Finally, aggregation study in a second independent cohort underscored that rare likely pathogenic variants in *HGF* explain about 2% of PL. Therefore, HGF signalling seems crucial for lymphatic development and/or maintenance in human beings and *HGF* should be included in diagnostic genetic screens for PL.

Keywords: primary lymphoedema; hepatocyte growth factor; whole-exome sequencing; lymphatic system

Introduction

The lymphatic system is a major regulator of tissue homeostasis, and defects can lead to lymphoedema (MIM: 153100). This may be due to developmental problems and/or damage to lymphatic vessels and/or lymph nodes [1]. Lymphoedema is characterized by accumulation of protein-rich interstitial fluid, especially in extremities, causing chronic swelling. It is often accompanied by accumulation of fat and cutaneous fibrosis.

Existing treatments focus on alleviating symptoms by repetitive manual lymphatic drainages, daily use of compressive garments, and surgery in selected cases. However, relapse of the disease and infections are a common problem [2]. There is currently no cure. This is why it is necessary to develop new therapeutic approaches, which requires a better understanding of the underlying pathophysiological mechanisms.

Lymphoedema is classified as secondary when it is due to an external cause, such as an infection or invasive therapy that causes damage to the lymphatic system. On the other hand, when no such cause can be identified, it is classified as primary. Primary lymphoedema (PL) can be fully developed at birth or appear later in life, e.g. during puberty or adulthood [3]. PL can be hereditary, and genetic causes have been identified, particularly

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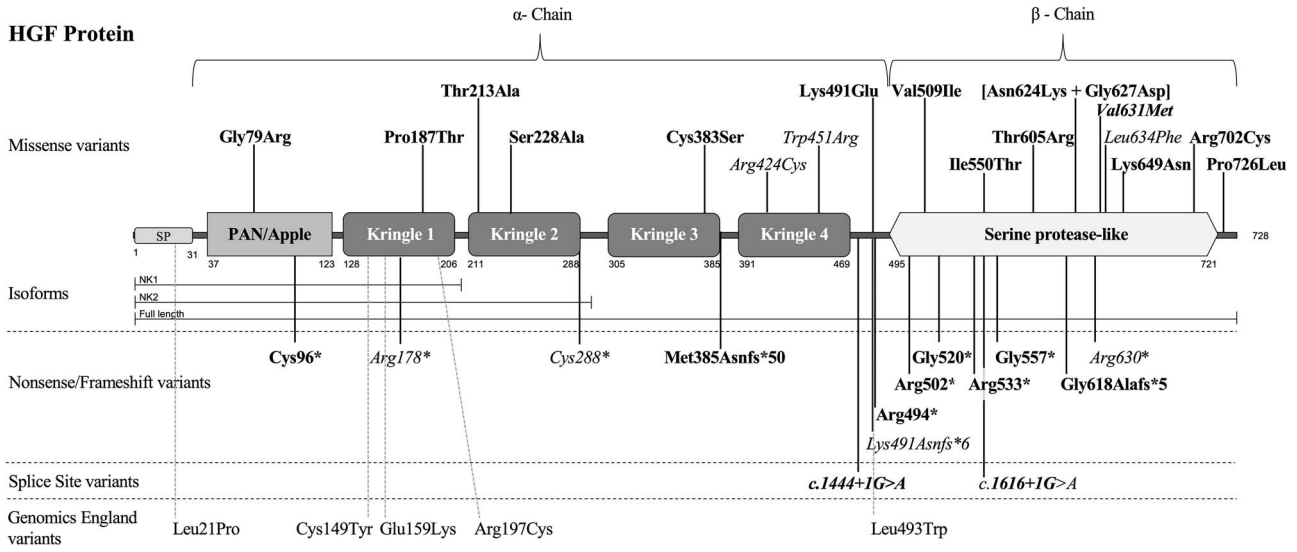

Figure 1. Schematic representation of HGF with position of variants detected in lymphoedema patients. HGF includes an N-terminal PAN/apple domain, four kringle domains and a serine protease-like domain. Domain positions given in amino acid numbers. Two shorter isoforms (NK1 & NK2) shown. The variants, organized by type, have an allele frequency < 0.0014 in GnomAD and have not been detected in more than three distinct pathologies in our internal database. Variants from this study, in bold; published variants [5, 6], in italics. One variant in our Brussels PL cohort and also reported, bold italics. SP, signal peptide. Nucleotide positions according to NG_016274.2 (NM_000601.6) and amino acids to Uniprot P14210. Variants between [ ] are allelic.

for congenital forms [1]. As of today, PL has been formally linked to 27 genes [1, 4]. In addition, five chromosomal loci and an additional 22 potential PL genes have been suggested by association or identification of rare and unvalidated variants [1, 4]. Many of the mutated and validated PL genes result in dysfunction of the vascular endothelial growth factor C (VEGFC)/vascular endothelial growth factor receptor 3 (VEGFR3) and/or Angiopoietin (ANGPT)/TIE signalling axes, which are considered as central pathways regulating lymphatic development [1].

The *Hepatocyte Growth Factor* (*HGF*) and *Hepatocyte Growth Factor Receptor* (formally named as the MET Proto-Oncogene, *MET*) are among the genes in which variants have been suggested to be associated with PL in two small case series [5, 6]. HGF is a 728 amino acid-long protein composed of a signal peptide, an alpha and a beta chain (Fig. 1) [7]. It is produced and secreted by cells of mesenchymal origin, including endothelial cells and fibroblasts. Pro-HGF, the inactive form, undergoes maturation, including an extracellular cleavage between Arg494 and Val495 by a urokinase-type plasminogen activator, thrombin, type II transmembrane enzyme matriptase or hepsin, to become active [8, 9]. The two chains are subsequently joined by a cysteine bond between Cys487 and Cys604 [7].

During embryonic development of vertebrates, HGF and MET have been detected in different tissues undergoing morphogenesis, including liver, placenta, and muscles [10]. The MET receptor is strongly expressed in lymphatic endothelial cells (LECs) of the lymphatic vessels [11]. In zebrafish, the knockdown of *met* showed decreased liver size due to disrupted HGF-MET signalling [12]. HGF-MET signalling is also involved in tissue repair, and the proliferation and migration of lymphatic endothelial cells [11]. This signalling is proposed to involve the activation of a downstream target in common with VEGFC-VEGFR3 signalling [13]. When HGF binds to the MET receptor, the ligand-receptor complex undergoes oligomerization, leading to activation of downstream targets such as PI3K-AKT and RAS-MAPK [1, 13]. Subcutaneous or transgenic administration of HGF in mice induced the formation of lymphatic vessels [11]. Therefore, HGF is considered as a good candidate gene for primary lymphoedema, and we investigated the relationship between HGF variants and PL in a cohort of 770 index patients.

Results

Ten HGF loss-of-function variants

The Brussels PL cohort of 770 PL index patients was explored for rare likely pathogenic *HGF* variants. The Genome Aggregation Database (GnomAD) pLI score of *HGF* is 1, indicating that *HGF* is intolerant of loss-of-function (LoF) variants. We detected six nonsense, two frameshifts and two splice-site variants in ten index patients (five female and five male), corresponding to 1.3% of the cohort (Fig. 1, Table 1, Supplementary Fig. 1A). They have not been reported in any disease previously, and only one (*c.1504C>T*; p.Arg502*) was reported once in GnomAD v3. No likely pathogenic variant was detected in the other known PL genes for these patients. Co-segregation analysis of these variants in available family members unravelled five additional affected individuals (four females, one male), as well as five unaffected carriers (one female, four males) (Supplementary Fig. 1A).

Stability of HGF mRNA with LoF variants

We obtained lymphocytic RNA from variant-carrying patients LE-169-1 (*c.1153dup*; p.Met385Asnfs*50), LE-535-10 (*c.1504C>T*; p.Arg502*) and LE-1181-10 (*c.1597C>T*; p.Arg533*) to assess *HGF* mRNA stability. The mutant allele was significantly less abundant than the wild-type allele in RT-PCR amplicons from all three patients, demonstrating nonsense-mediated mRNA decay (NMD) (Fig. 2).

HGF missense variants

The Z-score for *HGF* missense variants in GnomAD is 1.85, indicating that fewer amino acid substitutions are observed than expected, suggesting negative selection against such changes. In total, fourteen rare missense variants were identified in

Table 1. Individuals from the ten families with pathogenic LoF HGF variants.

Patient	Gender	HGVS DNA	HGVS Protein	Occurrence GnomAD v3	Age at onset (years)	Lymphoedema Phenotype	Associated clinical signs	ACMG classification
LE-291-10	F	*c.288_289delinsAA*	p.Cys96*	0	38	Bilateral legs (whole)	Hyperkeratosis	Pathogenic
LE-169-1	M	*c.1153dup*	p.Met385Asn*fs*50	0	No PL	Asymptomatic	None	Pathogenic
LE-169-10	F	*c.1153dup*	p.Met385Asn*fs*50	0	12	Bilateral legs (whole) and arms	None	
LE-641-1	M	*c.1444+1G>A*	p.?	0	No PL	Asymptomatic	None	Pathogenic
LE-641-10	F	*c.1444+1G>A*	p.?	0	6	Right leg (distal)	Hyperkeratosis	
LE-1189-2	F	*c.1480C>T*	p.Arg494*	0	No PL	Asymptomatic	None	
LE-1189-10	F	*c.1480C>T*	p.Arg494*	0	16	Bilateral legs and mild left hand	None	Pathogenic
LE-1189-12	M	*c.1480C>T*	p.Arg494*	0	No PL	Asymptomatic	None	
LE-1189-100	M	*c.1480C>T*	p.Arg494*	0	4	Left leg and right hand	None	
LE-1189-101	F	*c.1480C>T*	p.Arg494*	0	8	Bilateral legs and arms	None	
LE-535-10	M	*c.1504C>T*	p.Arg502*	1	7	Bilateral legs (whole) and arms	Hyperkeratosis	Pathogenic
LE-535-11	M	*c.1504C>T*	p.Arg502*	1	No PL	Asymptomatic	None	
LE-754-10	F	*c.1558G>T*	p.Gly520*	0	15	Bilateral legs (whole)	Hyperkeratosis	Pathogenic
LE-1181-10	M	*c.1597C>T*	p.Arg533*	0	35	Right leg (whole)	None	Pathogenic
LE-1260-10	F	*c.1616+1G>A*	p.?	0	16	Bilateral legs and arms	None	Pathogenic
LE-1260-11	F	*c.1616+1G>A*	p.?	0	5	Bilateral legs	None	
LE-563-10	M	*c.1669G>T*	p.Gly557*	0	14	Bilateral legs (whole) and arms	Hyperkeratosis, frequent cellulitis	Pathogenic
LE-563-100	F	*c.1669G>T*	p.Gly557*	0	12	Bilateral legs (whole)	None	
LE-563-101	F	*c.1669G>T*	p.Gly557*	0	12	Bilateral legs (whole)	Frequent cellulitis	
LE-602-10	M	*c.1853del*	p.Gly618Ala*fs*5	0	25	Bilateral legs (distal)	Hyperkeratosis	Pathogenic

No variants were detected for the other known PL genes. Colour alternated per family. -, not applicable; bold patient code, index case; HGVS, Human Genome Variation Society; PL, lymphoedema; distal, PL below the knee; whole, PL along the whole lower limb. Variant classifications based on the guidelines of American College of Medical Genetics and Genomics (ACMG).

seventeen families (Fig. 1, Table 2, Supplementary Fig. 1B). These variants were predicted as pathogenic by at least five different software programs out of 20 used. The Ser228Ala is shared by two families, and Val631Met by three. LE-47-11 carries two allelic *HGF* missense variants [Asn624Lys+Gly627Asp] inherited from his asymptomatic father. Co-segregation analyses for all likely pathogenic missense variants identified two additional affected carriers (LE-47-12 and LE-804-2) and eleven unaffected carriers (Supplementary Fig. 1B). In total, we had thirty individuals carrying *HGF* missense variants, twelve males and eighteen females. (Table 2).

To assess the effects of the missense variants on the predicted 3D structure of HGF, we used AlphaFold2.3 software [14]. However, the variants at positions Gly79, Pro187, Thr213, Ser228, Cys383, Lys491, Ile550, Thr605, [Asn624+Gly627], Lys649, Arg702 and Pro726 were in unfolded regions for which predictions were not possible. The variants at positions Val509 and Val631 did not predict any structural changes. We also used AlphaMissense as an additional tool to predict pathogenicity and four variants in the Kringle 1 domain were predicted likely pathogenic (Table 2 and Supplementary Table 1) [15].

Genetic burden analysis

In order to determine the relevance of LoF and missense *HGF* variants in lymphoedema susceptibility, a genetic burden analysis was performed by comparing the frequency of rare variants in whole exome sequencing (WES) data of the Brussels PL cohort (n = 765) with that of other pathologies (n = 1438). In total, 22/765 lymphoedema cases (2.87%) carried a rare *HGF* variant compared to 6/1438 controls (0.42%). This burden was statistically highly significant (P-value < 0.0001). Similarly, in the Genomics England's 100 000 Genomes project, a Whole Genome Sequencing project (The National Genomic Research Library v5.1, Genomics England [16]) five out of 156 individuals with PL (3.21%) had a rare (GnomAD < 0.001, CADD>15) *HGF* variant compared to 95 out of 15 998 controls (0.59%) (P-value = 0.0009, Fig. 1 & Supplementary Table 1).

Effect of HGF missense variants on HGF secretion and maturation

We assessed the functional impact of HGF missense variants from the Brussels PL cohort *in vitro*. Upon overexpression in COS-7 cells, all pro-HGFs with a missense variant were detected in cell lysates with no statistically significant change in expression level compared to the wild-type HGF (Fig. 3A and B).

The amount of HGF produced by COS7 cells was then quantified by ELISA (n = 3) (Fig. 3C). No HGF secretion was detected in the negative control (transfected with an empty vector). Four of the missense variants had HGF levels close to wild-type. Gly79Arg was ~50% more secreted, while Cys383Ser was barely detectable. Pro187Thr, Thr213Ala, Ser228Ala, Val509Ile, Ile550Thr, [Asn624Lys+Gly627Asp], Arg702Cys and Pro726Leu showed a 30% to 50% reduction in secretion.

We subsequently analysed the processing of HGF variants in the conditioned medium of COS-7 cells. In pooled supernatants from three experiments, the α and β-chains of HGF were clearly visible around 60 kDa and 35 kDa, respectively, as well as variable levels of pro-HGF around 100 kDa (Supplementary Fig. 2). HGF processing was normal for all except Gly79Arg, Leu491Glu and Val631Met, as some of the mutant HGF remained as unprocessed

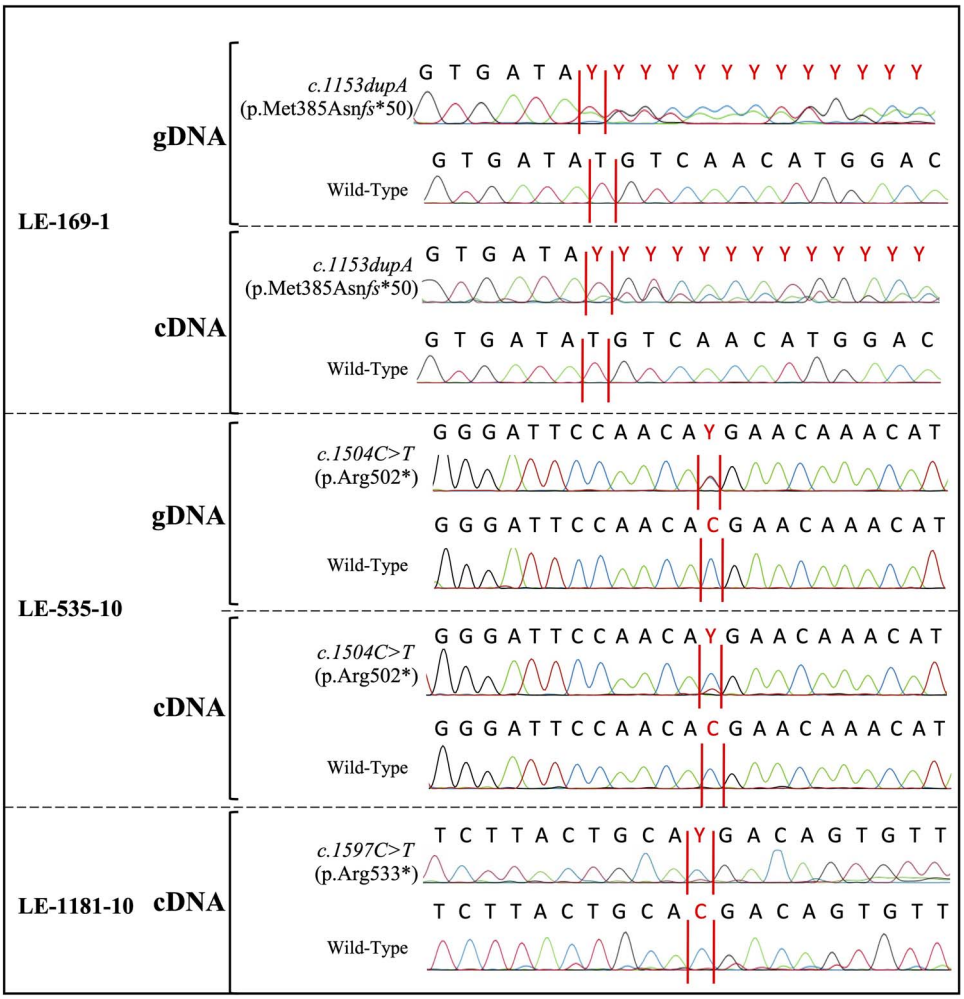

Figure 2. RNAs with premature *HGF* stop codons are degraded by nonsense-mediated mRNA decay. Electropherograms with genomic (g)DNA and lymphocytic/lymphoblastic cDNA sequences around the variants in heterozygous patients and unaffected control individuals (wild-type). Mutated nucleotide shown between vertical red lines and with "Y". Frameshift-induced double sequence also marked with "Y". Heterozygous mutant alleles are well detected in gDNA, hardly visible on cDNA. gDNA not available for sanger sequencing for LE-1181-10.

pro-HGF. In addition, the β-chain, which normally appeared as a doublet of bands, appeared as a single upper band for the Lys649Asn variant, which is predicted to create a new N-glycosylation site.

Effect of missense variants on HGF activity

To study whether MET receptor tyrosine kinase phosphorylation would be affected by HGF, we used conditioned media produced by COS-7 cells to treat LECs for 10 min and characterized the cell lysates using phospho-tyrosine kinase receptor arrays. We used this option as no specific phospho-MET antibody was sensitive enough to detect the level of phosphorylation of endogenous MET in LECs. We found that the treatment with wild-type HGF only induced phosphorylation of MET, compared with the negative control (Supplementary Fig. 3A). Phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) and insulin-like growth factor receptor-1 (IGFR1) remained unchanged compared with the negative control, and VEGFR3 was not stimulated. To ascertain that we used cells that had retained their LEC-phenotype and expressed VEGFR3, we also stimulated them with VEGFC (2179-VC-025, R&D Systems) and detected robust phosphorylation of the VEGFR3 receptor (Supplementary Fig. 3A). VEGFC also induced phosphorylation of the insulin receptor and of VEGFR2. Phosphorylation of IGFR1 did not differ between the control condition and VEGFC.

To better understand the intracellular signalling pathways that might be affected by our variants, we also used a phospho-kinase array. Out of 43 intracellular kinases tested, HGF stimulation increased phosphorylation of AKT-Ser473, eNOS, ERK1/2, GSK3-α/β, WNK1, and PRAS40 (Supplementary Fig. 3B). We then examined by immunoblotting the effects of *HGF* missense variants on phospho-proteins for which antibodies existed, including AKT (Ser473), eNOS (Ser1177), ERK1/2, GSK3- α/β, PRAS40 (Thr246) and WNK1 (Fig. 4, Supplementary Fig. 4). Three out of 14 missense variants showed significantly decreased phosphorylation of AKT (Cys383Ser, Lys649Asn and Arg702Cys), two decreased phosphorylation of ERK1/2 (Cys383Ser and Arg702Cys); and one also decreased phosphorylation of GSK3β (Cys383Ser), suggesting reduced activity of these HGF variants (Fig. 4, Supplementary Fig. 4). None of the missense variants showed a significant change in phosphorylation of eNOS, PRAS40 or WNK1 compared with wild-type (Supplementary Fig. 4E–G).

As some variants appeared to be overexpressed relative to WT, we also investigated whether the short term (10 min) exposure to increased doses of wild-type HGF might lead to variations in the activation of downstream targets in LECs. However, different

Table 2. Individuals from the 17 families with an HGF missense variant.

Patient	Gender	HGVS DNA & Protein	Occurrences GnomAD v3	Age at onset (years)	Variants in AD PL genes (consensus > =3; GnomAD < 100)	Lymphoedema Phenotype	Associated clinical signs	ACMG/AlphaMissense classification
LE-345-2	F	c.235G>A p.Gly79Arg	1	No PL	None	Asymptomatic	None	VUS/Likely Benign
LE-345-10	M	c.235G>A p.Gly79Arg	1	Congenital	None	Unilateral leg (left) + genital	Capillary malformation, scoliosis	
LE-153-10	F	c.559C>A p.Pro187Thr	0	Congenital	None	Unilateral leg (right) + genital	None	VUS/Likely pathogenic
LE-658-2	F	c.637A>G p.Thr213Ala	0	No PL	PIEZO1 (p.Phe533Leu) GnomAD: -	Asymptomatic	None	Likely Benign/Likely Benign
LE-658-10	F	c.637A>G p.Thr213Ala	0	In utero	PIEZO1 (p.Tyr1763*) GnomAD: 10	-	Hydrops Fetalis	
LE-658-11	M	None	-	In utero	PIEZO1 (p.Tyr1763*) GnomAD: 10	-	Hydrops Fetalis	-
LE-363-10	F	c.682T>G p.Ser228Ala	45	12	None	Bilateral leg + genital	Yellow/upturned nails, Papillomatosis	VUS/Ambiguous
LE-885-10	F	c.682T>G p.Ser228Ala	45	Unknown	None	Lymphoedema	None	VUS/Ambiguous
LE-907-10	F	c.1147T>A p.Cys383Ser	0	21	None	Bilateral leg	Obesity, hyperkeratosis	Pathogenic/Likely Pathogenic
LE-804-2	F	c.1471A>G p.Lys491Glu	0	29	None	Bilateral leg	None	Likely Benign/Ambiguous
LE-804-10	F	c.1471A>G p.Lys491Glu	0	17	None	Bilateral leg	Cerebral Glucose Transporter Deficiency	
LE-313-10	M	c.1525G>A p.Val509Ile	0	44	None	Bilateral leg	None	VUS/Likely Benign
LE-740-10	F	c.1649T>C p.Ile550Thr	0	13	ITGA9 (p.Gly344Glu) GnomAD: 19 FOXC2 (p.Leu124Phe) GnomAD: -	Bilateral leg	None	VUS/Ambiguous
LE-102-2	F	c.1814C>G p.Thr605Arg	2	No PL	None	Asymptomatic	None	Likely Benign/Likely Benign
LE-102-10	M	c.1814C>G p.Thr605Arg	2	Congenital	FLT4 (p.Gly1131Ser) GnomAD: -	Bilateral leg (Milroy's phenotype)	None	
LE-47-1	M	c.1872C>A p.Asn624Lys c.1880G>A p.Gly627Asp	4 4	No PL	None	Asymptomatic	None	N624K VUS/Likely Benign G627D VUS/Ambiguous
LE-47-11	M	c.1872C>A p.Asn624Lys c.1880G>A p.Gly627Asp	4 4	Congenital	None	Milroy's phenotype	None	
LE-47-12	F	c.1872C>A p.Asn624Lys c.1880G>A p.Gly627Asp	4 4	In utero	None	Unknown	Unknown	
LE-126-1	M	c.1891G>A p.Val631Met	160	No PL	None	Asymptomatic	None	Benign/Likely Benign
LE-126-10	F	c.1891G>A p.Val631Met	160	15	GATA2 (p.Arg396Trp) GnomAD: -	Bilateral leg	Emberger syndrome	
LE-857-1	M	c.1891G>A p.Val631Met	160	No PL	None	Asymptomatic	None	Benign/Likely Benign
LE-857-10	F	c.1891G>A p.Val631Met	160	Congenital	PIEZO1 (p.Thr1317Pro) GnomAD: -	Bilateral leg and arm + facial oedema	Hydrops Fetalis	
LE-977-10	F	c.1891G>A p.Val631Met	160	15	ADAMTS3 (c.2260+1G>A) GnomAD: 5	Bilateral leg	None	Benign/Likely Benign
LE-977-100	F	c.1891G>A p.Val631Met	160	No PL	None	Asymptomatic	Obesity	
LE-197-1	M	c.1947G>T p.Lys649Asn	0	No PL	None	Asymptomatic	None	VUS/Likely Benign
LE-197-10	M	c.1947G>T p.Lys649Asn	0	10	PIEZO1(c.7049+2_7049+3delTG) GnomAD: -	Unilateral leg (left)	None	
LE-795-1	M	c.2104C>T p.Arg702Cys	0	No PL	None	Asymptomatic	None	Likely Pathogenic/Likely Pathogenic
LE-795-10	M	c.2104C>T p.Arg702Cys	0	No PL	None	Asymptomatic	None	
LE-795-12	F	c.2104C>T p.Arg702Cys	0	6	MET (p.Glu927Lys) GnomAD: -	Unilateral leg (left)	None	
LE-93-1	M	c.2177C>T p.Pro726Leu	0	No PL	SHANK3 (p.Arg1478Cys) GnomAD:1 PIEZO1(p.Arg908Trp) GnomAD:21	Asymptomatic	None	VUS/Likely Benign
LE-93-10	F	c.2177C>T p.Pro726Leu	0	Congenital	SHANK3 (p.Arg1478Cys) GnomAD:1 PIEZO1(p.Arg908Trp) GnomAD:21	Unilateral leg (left)	Distichiasis	

-, not applicable; underlined patient code, same variant in different pedigrees; italic LE-code, two variants in same individual; bold code, index case; HGVS, Human Genome Variation Society; PL, lymphoedema. Variant classifications were done based on the guidelines of the American College of Medical Genetics and Genomics (ACMG) and using AlphaMissense, respectively. VUS, Variant of Uncertain Significance.

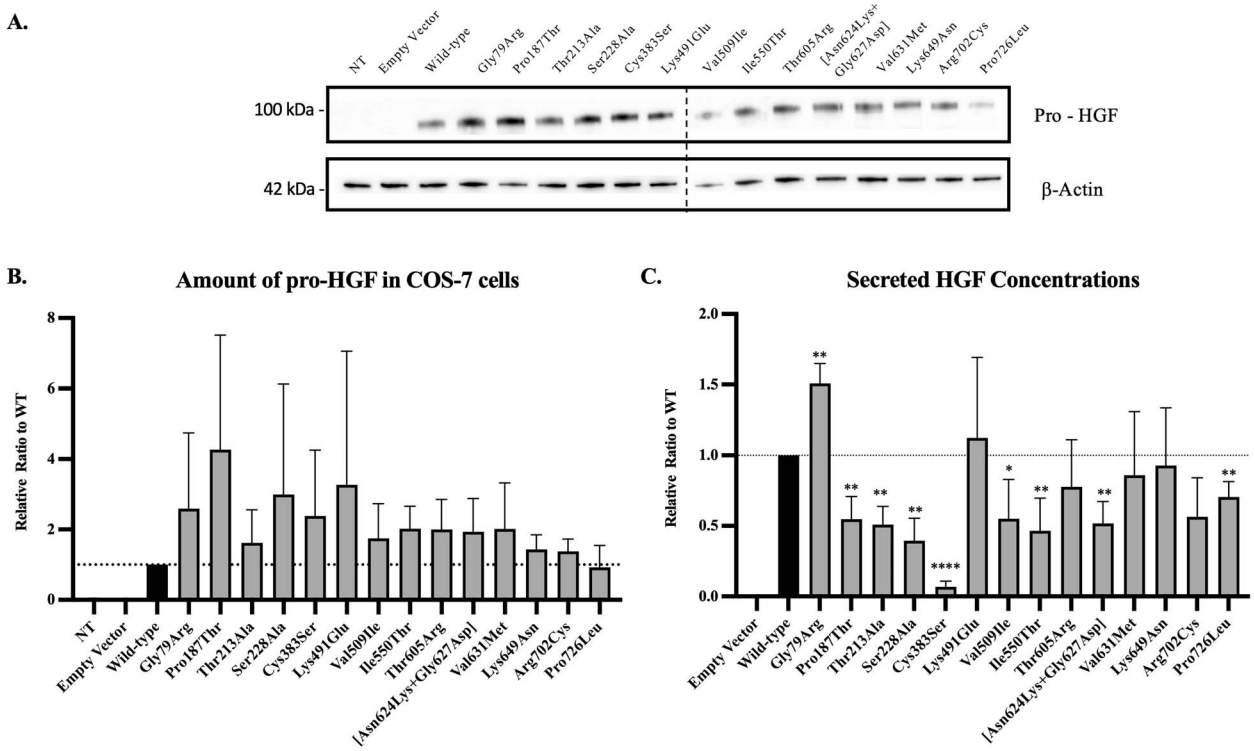

Figure 3. Effect of variants on HGF expression and secretion in COS-7 cells. Cells were transiently transfected with an empty vector or plasmids for wild-type and variant human *HGF*. NT, non-transfected. Standard deviations indicated with bars. (A) Representative western blot of cell lysates revealed expression of pro-HGF (~100 kDa) and β-actin (~42 kDa) as loading control. (B) Quantification of pro-HGF in western blots of cell lysates (n = 3). Fold-change concentrations relative to wild-type. (C) ELISA quantification of HGF in the supernatants (n = 3). Fold-change concentrations relative to wild-type. Significant differences with wild-type (evaluated using a two-tailed Wilcoxon test) indicated with * for P-value ≤ 0.05; ** for P-value ≤ 0.01. Two gels were used to load the samples, separated by a dashed line.

concentrations of HGF did not induce statistically significant alterations (Supplementary Fig. 5). On the bases of these results, it seems that the Cys383Ser, Lys649Asn and Arg702Cys variants have reduced HGF activity, whereas this is not the case for the other eleven variants (Supplementary Fig. 6). For nine of them, however, secretion or processing is abnormal.

Screening for likely pathogenic variants in other known PL genes

The 17 index patients with missense variants were also screened for other known autosomal dominant or recessive genes responsible for PL. We found a few VUSs in 9 of them (Table 2). These included a heterozygous *PIEZO1* variant in four patients (including one with a *SHANK3* VUS), an *ADAMTS3*, *FLT4*, *GATA2* or *MET* VUS in one patient each, as well as *ITGA9* and *FOXC2* VUS in one patient.

Phenotype-genotype correlation

To assess whether the severity of PL correlates with the type of *HGF* variant, we examined the clinical data of patients with *HGF* LoF variants and the three missense variants with reduced HGF activity (Cys383Ser, Lys649Asn and Arg702Cys). All ten index patients with an *HGF* LoF variant had bilateral lower limb lymphoedema, with the exception of LE-1189-100, LE-641-10 and LE-1181-10, who had unilateral lower limb PL. Six of the patients with bilateral lower limb PL also had upper limb lymphoedema (Table 1, Supplementary Fig. 1A). In all patients with *HGF* LoF variant, the onset of lymphoedema was late, between 4 and 38 years of age (Table 1). In these ten families, we observed four asymptomatic males out of nine male carriers of the variant, as well as one unaffected female out of 11 female carriers, reflecting reduced penetrance (90.9% for females and 55.6% for males; 75% overall).

The three index cases with missense variants displaying reduced HGF activity had bilateral (n = 2) or unilateral (n = 1) lower limb PL without upper limb involvement. Overall penetrance was 50% and patients had late-onset lymphoedema. There was no difference between men and women in the severity of the clinical phenotype for these three variants.

Lymphoscintigraphy for LE-291-10, a patient with a premature stop codon (Cys96*), showed lack of lymph node labelling on the affected right limb and no lymph drainage (Fig. 5). Lymphoscintigraphy for LE-535-10 (Arg502*) showed no drainage on either lower limb and no visible inguinal or iliac lymph nodes with distal dermal back flow. Lymphoscintigraphy for LE 197-10 (Lys649Asn) showed an absence of lymph node labelling for the affected left limb with subcutaneous diffusion of the radiotracer into the lower limb. To compare HGF-induced lymphoedema with others, we also compared lymphoscintigraphy from a patient with Milroy's disease due to a VEGFR3 variant (Asn1042Ile). The patient had no visible drainage or lymph nodes, like our patient LE-535-10.

Discussion

This study shows that HGF is an important genetic factor predisposing to primary lymphoedema. Screening of 770 index PL patients within the Brussels PL cohort identified 27 patients with 24 distinct rare heterozygous *HGF* variants. Genetic burden analysis in two independent cohorts underscored that *HGF* variants increase susceptibility to lymphoedema.

Ten LoF variants resulted in haploinsufficiency, and this is probably also the case for the Cys383Ser substitution, which is

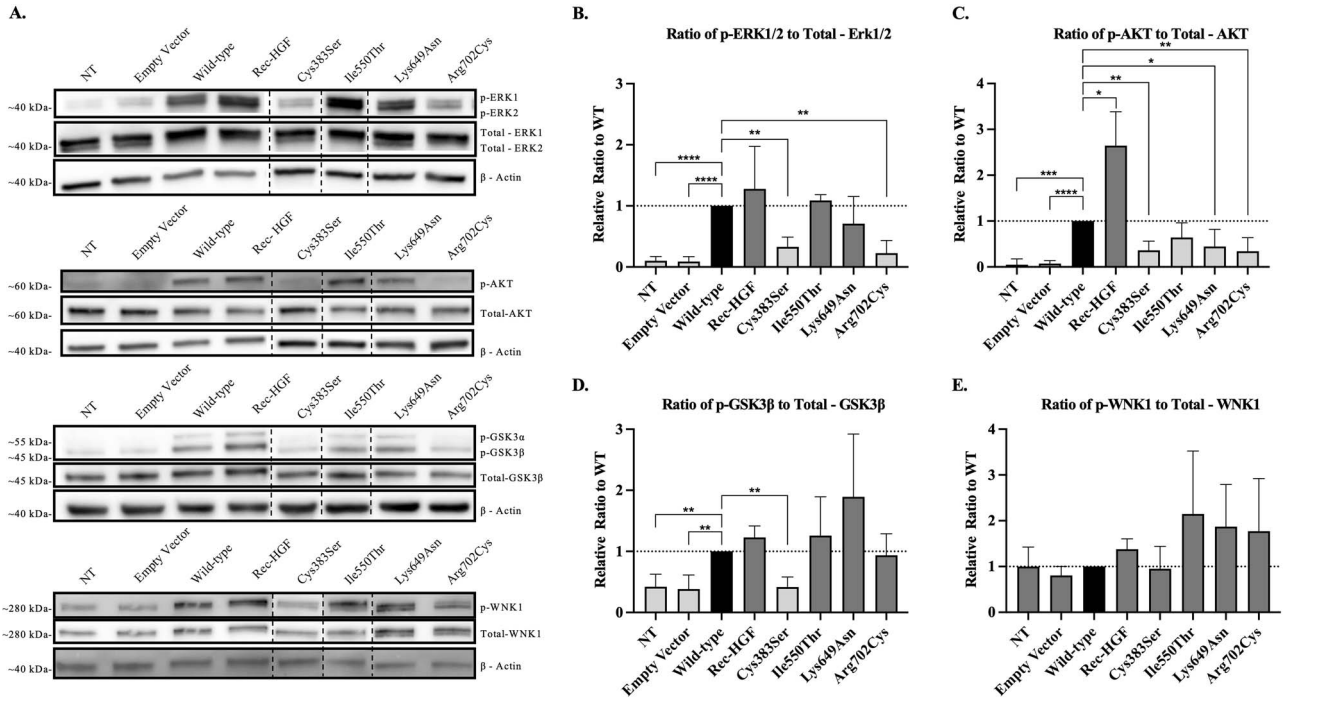

Figure 4. Effect of HGF mutants on phosphorylation of intracellular signalling molecules in LECs. Stimulation of LECs with same amount of recombinant wild-type HGF (rec-HGF) or HGF-containing conditioned medium from COS-7 cells expressing an empty vector, wild-type HGF, or one of four selected mutants. NT, non-treated. (A) Representative western blots of stimulated LEC cell lysates with levels of p-ERK1/2 (p44/p42), total ERK1/2, p-AKT (Ser473), total AKT, p-GSK3-α/β (Ser9), total GSK3-β, p-WNK1 (Thr60), total-WNK1 and β-actin. (B-E) quantification of the corresponding western blots (n = 3) relative to wild-type. Significant differences with wild-type (evaluated using a two-tailed Wilcoxon test) indicated with * for P-value ≤ 0.05; ** for P-value ≤ 0.01. Wild-type indicated with black bar; decreased level of phosphorylation is indicated in light grey bars. Gels were cropped to show only 4 missense variants. See the entire series and whole blots of variants in Supplementary Fig. 4.

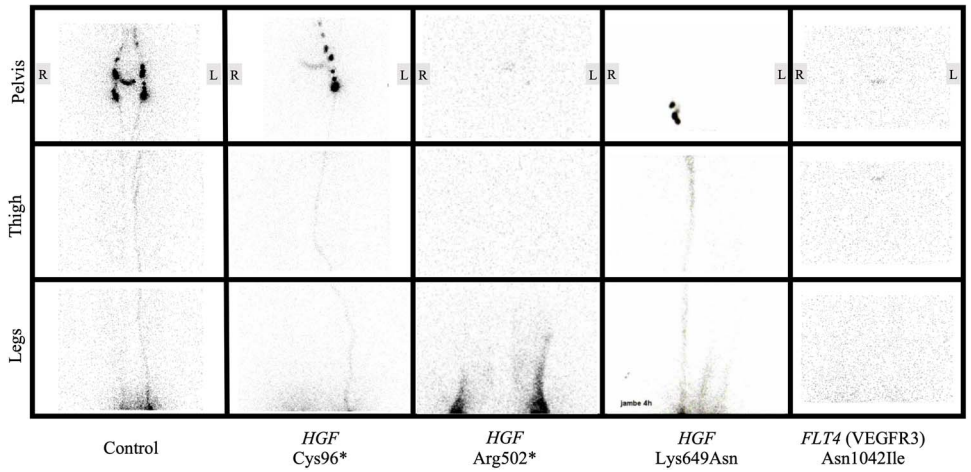

Figure 5. Altered lymphatic drainage in lower limbs of HGF patients compared to a VEGFR3-associated Milroy disease. Images 4 h after injection. Control: Drainage without delay in both legs, number of lymph nodes in pelvis normal. *HGF* stop codon Cys96* (LE-291-10): No drainage on right lower limb, altered drainage on left, with insufficient number of pelvic lymph nodes stained. *HGF* stop codon Arg502* (LE-535-10): No drainage on lower limbs and no lymph nodes, with distal dermal back flow. *HGF* missense variant Lys649Asn (LE-197-10): No drainage on lower limbs, subcutaneous diffusion of the radiotracer in left leg. *FLT4* (VEGFR3) Asn1042Ile: Patient with PL showing no drainage and no visible pelvic lymph nodes.

secreted in very low amounts and has minimal signalling activity (Supplementary Fig. 6). Cys383 is involved in a disulphide bond with Cys305. Consequently, this variant likely destabilizes the folding of the protein, preventing its secretion.

The Arg702Cys substitution may also belong to the LoF category, as the level of secretion is only 56% of that of the wild-type, and with an amount of this HGF variant equivalent to the wild-type, ERK1/2 activation is reduced by 77% and AKT activation by 87% compared with the wild-type (Supplementary Fig. 6). Overall, this mutant HGF would thus have an activity close to 0% on HGF signalling. Amino acid 702 is located close to Cys697, predicted in Uniprot to be involved in a disulphide bond with Cys669. The creation of a new cysteine in this region provides a new binding partner for Cys669, which may lead to incorrect folding of the C-terminus of HGF. The key role of the cysteine residues in stabilising the structure is reinforced by the fact that there are only three cysteine residues among the 60 last amino acids of HGF (Cys669, Cys679, Cys697), and they

are all involved in disulphide bonds (Cys679 being in pair with Cys612).

In total, the twelve pathogenic *HGF* variants had a penetrance of only about 70%, since 29.2% of patients were asymptomatic pathogenic variant-carriers. Phenotypes ranged from four-limb lymphoedema in most carriers of LoF *HGF* variants to a single leg-PL below the knee in one of the "reduced-function HGF substitutions". The four similar modifications (Arg178*, Cys288*, Lys491Asn*fs**6, and Arg630*) reported previously probably behave in the same way [5, 6]. Four-limb lymphoedema has so far only been associated with variants in the Gap junction gamma-2 (*GJC2*) gene and Turner syndrome [17]. Patients with *GJC2* variants were reported to have four-limb lymphoedema occurring between birth and the age of 40 [18, 19]. This is similar to our HGF data with variants causing four-limb lymphoedema and with age at onset ranging from 4 to 38 years.

The third substitution (Lys649Asn) with greatly reduced AKT activation, 29% reduced ERK activation and a fully glycosylated beta chain, could also be a pathogenic variant, although the other intracellular targets did not show any difference (Supplementary Fig. 6). This variant in fact introduces a third N-glycosylation site into the beta chain (in addition to Asn566 and Asn653) which appears to be efficiently recognized. Previous research has demonstrated the importance of glycosylation in MET function. Hypo-glycosylation of MET resulted in reduced activation of AKT and ERK1/2 without affecting HGF-MET binding activity [20]. However, the full implications of glycosylation on the HGF-MET interaction remain to be elucidated, particularly with respect to hyper-glycosylation.

The other eleven rare substitutions predicted to be pathogenic did not show conclusive evidence of being LoF variants. The Gly79Arg was 51% more secreted, but its specific activation of the downstream targets was comparable to that of the wild-type (Supplementary Fig. 6). Of the remaining missense variants, seven were 30% to 50% less secreted than wild-type, but without having a significant impact on downstream signalling in the *in vitro* experiments, while the remaining three (Lys491Glu, Thr605Arg and Val631Met) showed neither reduced secretion nor reduced downstream signalling. Therefore, these eleven variants are probably rare polymorphisms that may predispose to PL but may require other genetic variants to have an impact on lymphatic physiology *in vivo*. In some diseases, it has already been shown that variants in a single gene may not be sufficient to predispose to a disease but require another gene [21, 22]. Such a digenic or oligogenic mechanism could be at play with these "weak" HGF variants.

We identified bioinformatically predicted likely pathogenic variants in other known PL genes in four of these eleven patients (LE-102-10, LE-197-10, LE-658-10 and LE-977-10) and other VUSs in known PL genes for 4 of the remaining 7 patients. Among the likely pathogenic variants in other known PL genes, patient LE-102-10 carries a variant in the tyrosine kinase domain of VEGFR3, known to be responsible for Milroy's disease [23]. Thus, the HGF Thr605Arg substitution, which behaves like wild-type HGF in our functional assays, is likely a non-pathogenic polymorphism. Two patients had an HGF Val631Met substitution, which also behaves like wild-type HGF in our functional assays, in association with another variant. Patient LE-126-10 with evidence of Emberger syndrome (MIM: 614038), had a de novo *GATA2* variant at the same amino acid position that has been associated with lymphoedema due to Emberger syndrome [24], suggesting that the GATA2 variant is the most likely cause of the PL. The other patient, LE-977-10, had a predicted splice-site change in *ADAMTS3*. The patient's sister, who had the *HGF* missense variant, but no splice-site change in *ADAMTS3*, did not have lymphoedema. However, *ADAMTS3*-induced PL is recessive [25]. Thus, the cause for PL in this family remains elusive. Finally, patient LE-658-10 had a nonimmune hydrops fetalis and a paternally inherited nonsense variant in *PIEZO1* as well as an HGF Thr213Ala substitution. Her mother (LE-658-2) had the same HGF variant without PL, while her brother had the same PIEZO1 variant without the HGF substitution and yet with fetal hydrops. PIEZO1 variants have been associated with both recessive (MIM: 616843) [26, 27] and dominant nonimmune fetal hydrops (MIM: 194380) [28], so the genetic cause in this family might be linked to PIEZO1. Overall, these results suggest that some HGF missense variants are rare polymorphisms.

One patient (LE-93-10) with the HGF missense variant (Pro726Leu) had lymphoedema distichiasis (MIM: 153400), i.e. the presence of an aberrant second row of eyelashes [29]. Distichiasis is generally associated with *FOXC2* variants [30]. However, we did not identify any *FOXC2* point mutation nor a copy number change in the exome sequencing data, and the HGF variant behaved in the same way as wild-type HGF in the downstream signalling, although it was less secreted. The cause therefore likely lies elsewhere.

In addition to the full length HGF, two short naturally occurring human isoforms of HGF result from alternative splicing: NK1 and NK2 (Fig. 1). NK1 consists solely of the N-terminal PAN domain and the first Kringle domain. It has been defined as a MET agonist. In contrast, NK2 (truncated after Kringle 2 domain) is a partial antagonist [31]. Our results demonstrated that NMD degrades *HGF* transcripts with a premature stop codon. The fact that these variants responsible for haploinsufficiency are distributed throughout the protein and cause a similar phenotype in the patients argues in favour of their impact on the full-length HGF rather than on one of the NK1-HGF and NK2-HGF variants.

HGF-deficiency has been generated in mice by global deletion of the gene. Homozygous mutant embryos showed a severe phenotype with placental abnormalities and prenatal lethality [32]. Heterozygous mutant mice developed well and appeared normal. This difference between human heterozygotes with a phenotype and normal-looking mice probably reflects physiological differences and the influence of other genetic (laboratory mice are usually inbred lines, whereas human beings are extremely heterogeneous) and environmental factors. Therefore, tissue-specific *HGF* knock-out models seem necessary to explore a lymphatic-specific phenotype in mice. In addition, somatic second-hit mutations are also a mechanism by which LoF variants can become locally null, leaving cells without functional alleles [33–35]. However, confirmation of this phenomenon is hampered by the fact that surgery or biopsies are rarely performed for PL.

The serine/threonine kinase AKT has been shown to play a central role in cell signalling, first in angiogenesis and subsequently in lymphangiogenesis [36–38]. We showed that AKT and its targets were the most sensitive to variants in HGF. Therefore, the PI3K/AKT signalling in lymphangiogenesis seems essential not only for VEGF family signalling, but also for HGF. The phospho-kinase array suggested that eNOS, GSK3-α/β, PRAS40 and WNK1 were also phosphorylated upon treatment with wild-type HGF. However, none of the identified variants showed a significant change in phosphorylation levels compared to wild-type, with the exception of Cys383Ser-HGF for GSK3-β phosphorylation. These proteins may be targets of HGF-MET signalling, but other regulators of these proteins may compensate for the reduction in phosphorylation due to HGF variants.

In LECs, treatment with wild-type HGF resulted in phosphorylation of MET exclusively without altering phosphorylation of VEGFR2 and IGFR1. Although it has been suggested that HGF has an indirect mechanism of action through VEGFR3, this hypothesis is controversial [39]. Our results showed no phosphorylation of VEGFR3 upon treatment with wild-type HGF, as suggested by another report [11].

In conclusion, we showed that premature nonsense-generating variants of *HGF* cause late-onset familial primary lymphoedema by haploinsufficiency, which can affect all four limbs, with incomplete penetrance. Three HGF missense variants also result in significant loss of HGF function and reduced activation of its targets, impacting the central PI3K/AKT and ERK1/2 signalling pathways in LECs. Two of these variants were "cysteine mutants". Aggregation tests suggested that about 2% of primary lymphedema is due to likely pathogenic variants in *HGF*. It would be interesting to study the HGF missense variants further *in vitro* (e.g. with longer exposure of LECs to the HGF variants to study cellular behaviour and signalling) and *in vivo*, to elucidate the exact pathophysiological mechanisms by which they affect lymphangiogenesis. *HGF* should in any case be included in diagnostic tests for primary lymphoedema, although the pathogenicity of variants causing amino acid substitutions is difficult to classify without functional validation.

Materials and methods

Sample collection

Informed consents of the patients and their family members were obtained according to the regulations approved by the Ethical Committee of the Medical Faculty at the University of Louvain (Brussels, Belgium) under reference B403201629786 and the respective committees of our clinical collaborators. Peripheral blood samples were collected. DNAs from the patients were extracted using Wizard Genomic kit (Promega), and concentrations were measured on a Nanodrop 8000 (Thermo Scientific). Most of the primary lymphoedema patients of the Brussels PL cohort were of European ethnicity, but we also had patients with other ethnic backgrounds.

Sequencing

Sequencing of 5 index patients was performed using an Ion Personal Genome Machine (PGM) or an Ion Proton (Thermo Fisher Scientific), as described by Brouillard *et al* in 2021 [40]. The targeted sequencing panel was designed using www.ampliseq.com, and included known PL genes and some candidate genes, such as *HGF*. Whole exome sequencing was performed for the other 765 samples at Macrogen-Europe on Illumina HiSeq or NovaSeq machines, using either Agilent SureSelect (v6 or v7) or TWIST Exome (Twist Bioscience) capture kits. Reads were aligned against human reference genome hg38 to generate "*.bam*" files subjected to variant calling with the Genome Analysis Toolkit (GATK, Broad Institute). Data were imported in Highlander (http://sites.uclouvain.be/highlander/), a software developed in the Brussels laboratory to annotate, visualise and filter variants using multiple tools.

Variant calling and filtering

Filtering for possible disease-causing variants in *HGF* and the known PL genes (n = 27) were done using the following filtering criteria. The variants should be predicted to be damaging by at least five prediction algorithms out of twenty used (computed as *consensus prediction*). These were: BayesDel, Combined Annotation Dependent Depletion, ClinPred, Deogen, Functional Analysis through Hidden Markov Models (FATHMM), FATHMM-XF, Likelihood Ratio Test, Lists2, Mendelian Clinically Applicable Pathogenicity, MetaSVM, Mutation Taster, Mutation Assessor, Missense Variant Pathogenicity, Polyphen2, PrimateA1, Provean, Rare Exome Variant Ensemble Learner, SNPEff, Sorting Intolerant from Tolerant, and Variant Effect Scoring Tool. Variants should be read with a minimum of ten reads at the interrogated position and the allele frequency should be less than or equal to 0.0014 in the Genome Aggregation Database v3 (GnomAD, https://gnomad.broadinstitute.org) (the estimated prevalence of PL in the population is ∼1.4‰ [41] the variant should not be detected in more than three distinct pathologies in our internal database (>3000 WES datasets). Nucleotide positions are given according to NG_016274.2 (NM_000601.6).

Co-segregation analyses

WES or Sanger sequencing was used to assess variant co-segregation in families. For the latter, primers were designed to amplify 250–500 bp fragments around the variants (sequences available on request). The chromatograms were visualised using CLC Main Workbench (Qiagen).

Genetic burden analysis

Non-synonymous variants, insertion/deletions, and splice-site variants across the *HGF* gene were retrieved in the Brussels PL cohort of index patients, as well as in an index cohort of individuals with other pathologies from our Highlander database, serving as a control population. Variants were considered as potentially pathogenic if they had a frequency of less than or equal to 0.001 in GnomAD and were predicted deleterious by at least five software programs out of 20 used. Fisher's exact test was used for statistical analysis.

Replication cohort

For replication of the genetic burden analysis, whole genome sequencing data from the Genomics England 100 000 Genomes Project Rare Diseases program (The National Genomic Research Library v5.1, Genomics England [16]) was used. A primary lymphoedema cohort of 156 probands were identified with the "normalised specific disease" label: "Meige", "Milroy", or "Primary lymphoedema", without a molecular diagnosis. A control group of 15 598 individuals without a diagnosed condition and unrelated to each other and/or the lymphoedema cases were selected. Only canonical transcripts were considered and rare variants (gnomAD MAX_AF < 0.001) with functional impact of moderate (missense) or worse (stop gain, frameshifts, splice acceptor/donor) and a CADD>15 were filtered for. The REGENIE workflow was implemented for burden testing adjusting for age, sex and three ancestry defined principal components [42].

RT-PCR analyses

mRNA extraction was performed from blood using the TriPure Isolation reagent (Roche) or using NucleoSpin RNA II kit (ref 740 955, Machey-Nagel) after ultracentrifugation with Guanidine Isothiocyanate (GITC) (50 990, Sigma-Aldrich) and Cesium Chloride (CsCI) (540-5507UB, Merc), according to the protocol described by the provider. cDNA was synthesised using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), using random hexamers. Newly synthesised cDNAs and fresh dilutions of primers were used for PCR amplification of *HGF* fragments with a TAQ polymerase (Qiagen). Primers were designed to amplify fragments spanning over at least one exon-intron junction before and after

the exon containing the variant studied (sequences available on request).

Expression of recombinant HGF

COS-7 cells were maintained in DMEM supplemented with 10% FBS. The pCMV3-HGF expression vector containing full-length untagged human *HGF* cDNA (HG10463-UT, Sino Biological Inc.) was used as template. Mutations were generated using the QuickChange Site-Directed Mutagenesis method (Agilent). Primers were designed using QuickChange Primer Design (https://www.agilent.com/store/primerDesignProgram.jsp) (sequences available on request). Mutagenesis was initiated with 10 ng of vector DNA and the addition of 3 μl DMSO in a total reaction volume of 50 μl. Amplification consisted of 16 cycles (30 s denaturation at 95°C, 1 min annealing at 59°C, 9 min elongation at 68°C) and a final 9 min elongation at 67°C. The amplified products were digested using *DpnI* and transformed into JM109 competent bacteria. The entire *HGF* coding sequence of selected clones was verified by Sanger sequencing. An empty vector was also generated by removing the *HGF* using *KpnI*.

To produce recombinant HGF proteins, plasmids (3 μg) were transfected in 80–95% confluent COS-7 cells using JetPEI (Polyplus transfection), in 6-well plates. Six hours after transfection, the medium was changed to DMEM without phenol red (Thermofisher), 1% FBS and 1 mM pyruvate. Conditioned media were collected after 3 days (centrifuged at 400 g for 5 min, a pellet of cells removed and preserved in aliquots of 2 ml at −80°C), and cells were lysed during 30 min at 4°C with a buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 20 mM EDTA and supplemented with Complete Mini protease inhibitor cocktail tablets (Roche) and PhosSTOP (Roche). Cell lysates were sonicated and centrifuged at 15 000 g for 20 min. The Human HGF Quantikine ELISA Kit (R&D systems) was used to measure HGF concentrations in conditioned media. Samples were diluted 500× before ELISA performed according to the manufacturer's protocol.

HGF stimulation of LECs

To analyse the effects of HGF missense variants, lymphatic endothelial cells (LECs) (CC-2812, Lonza) were cultured with EGM-2MV medium until 90% confluency was reached in T-25 flasks. Cells were serum-starved overnight with FBS-free EGM-2MV medium before treatment. 50 or 10 ng/ml of HGF, 50 ng/ml of VEGFC (2179-VC-025, R&D Systems) or different dosages of recombinant HGF (294-HG, R&D systems) was diluted in serum-free EGM-2MV medium with 0.1% BSA. Subsequently, these were diluted in the medium of the empty vector-transfected COS-7 cells to normalise the volumes to the lowest concentration HGF variant sample. LECs were stimulated with each HGF preparation for a short duration (10 min), then lysed with the same protocol as for COS7 cells.

Tyrosine kinase receptor and intracellular kinase phosphorylation array

LEC stimulation with HGF (10 ng/ml) or VEGFC (50 ng/ml) was performed as described above. After 10 min of stimulation, LECs were lysed and explored for protein phosphorylation using the Human Phospho-RTK Array and Human Phospho-Kinase Array (R&D systems), according to the kit recommendations. The different spot intensities were evaluated by densitometric analysis using the ImageJ software (http://imagej.nih.gov/ij/).

Western blotting

Total protein concentrations of cell lysates were quantified using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were separated on SDS/PAGE, transferred onto PVDF membranes (Merck Millipore) and immunoblotted (overnight, in milk) with the following antibodies: AKT (9272, Cell Signaling), phospho-AKT-Ser473 (9271, Cell Signaling), eNOS (AF950, R&D Systems), phospho-eNOS-Ser1177 (C9C3) (9570, Cell Signaling), GSK-3α/β (AF2157, R&D Systems), phospho-GSK-3α/β-Ser21/Ser9 (AF1590, R&D Systems), HGF (AF-294-NA, R&D systems), p44/42 MAPK (ERK1/2) (9102S, Cell Signaling), phospho-p44/42 MAPK (ERK1/2)-Thr202/Tyr204 (9101, Cell Signaling), WNK1 (4979, Cell Signaling), phospho-WNK1-Thr60 (MAB4720, R&D Systems), PRAS40 (2610, Cell Signaling), phospho-PRAS40-Thr246 (D4D2) (13 175, Cell Signaling), α-Actinin (6487, Cell Signaling) and β-actin (A5441, Sigma Aldrich). The blots were subsequently washed and probed with appropriate HRP-conjugated secondary antibodies (anti-goat IgG, P0449, Dako; anti-mouse IgG, A5278, Sigma Aldrich; or anti-rabbit IgG, P0448, Dako). Bands were detected with enhanced chemiluminescence reagent (Perkin Elmer) and Supersignal West Femto Maximum Sensitivity substrate (Thermo Fisher Scientific). Images of the membranes were taken by Fusion Solo S (Vilber) and images were transferred to Image J software (http://imagej.nih.gov/ij/) to quantify band intensities. Results were transferred to PRISM software (https://www.graphpad.com/) to perform statistical tests.

Lymphoscintigraphy

Lymphoscintigraphy is a standardized, minimally invasive and reproducible procedure allowing morphological and functional exploration of the lymphatic circulation [43, 44]. It is performed after subcutaneous injection in the first inter-digital space of a small quantity of 99 m Tc-nano-colloid, which travels in the lymphatic circulation, releasing gamma radiation detected by a gamma camera [44]. Lymphoscintigraphy allows lymph node impregnation at the root of the limb, the presence of deep lymph nodes and signs of stasis. We assessed corresponding drainage activity at 45 min and 4 h for each patient.

Supplementary data

Supplementary data is available at *HMG Journal* online.

Conflict of interest statement: There are no competing financial interests concerning this work.

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