# *MET* somatic activating mutations are responsible for lymphovenous malformation and can be identified using cell-free DNA next generation sequencing liquid biopsy

Maria Palmieri, MS,[a] Laura Di Sarno, MS,[a] Andrea Tommasi, MD,[a,b] Aurora Currò, MD,[a,b] Gabriella Doddato, MS,[a] Margherita Baldassarri, MD,[a] Elisa Frullanti, PhD,[a] Annarita Giliberti, MS,[a] Chiara Fallerini, PhD,[a] Aldo Arzini, MD,[c] Annamaria Pinto, MD,[b] Massimo Vaghi, MD,[c,d] *and* Alessandra Renieri, MD, PhD,[a,b] *Siena and Crema, Italy*

## ABSTRACT

**Objective:** Germline mutations of either the endothelial cell-specific tyrosine kinase receptor TIE2 or the glomulin (*GLMN*) gene are responsible for rare inherited venous malformations. Both genes affect the hepatocyte growth factor receptor c-Met, inducing vascular smooth muscle cell migration. Germline mutations of hepatocyte growth factor are responsible for lymphatic malformations, leading to lymphedema. The molecular alteration leading to the abnormal mixed vascular anomaly defined as lymphovenous malformation has remained unknown.

**Methods:** A group of 4 patients with lymphovenous malformations were selected. Plasma was obtained from both peripheral and efferent vein samples at the vascular malformation site for cell-free DNA extraction. When possible, we analyzed tissue biopsy samples from the vascular lesion.

**Results:** We have demonstrated that in all four patients, an activating *MET* mutation was present. In three of the four patients, the same pathogenic activating mutation, T1010I, was identified. The mutation was found at the tissue level for the patient with tissue samples available, confirming its causative role in the lymphovenous malformations.

**Conclusions:** In the present study, we have demonstrated that cell-free DNA next generation sequencing liquid biopsy is able to identify the *MET* mutations in affected tissues. Although a wider cohort of patients is necessary to confirm its causative role in lymphovenous malformations, these data suggest that lymphovenous malformations could result from postzygotic somatic mutations in genes that are key regulators of lymphatic development. The noninvasiveness of the method avoids any risk of bleeding and can be easily performed in children. We are confident that the present pioneering results have provided a viable alternative in the future for lymphovenous malformation diagnosis, allowing for subsequent therapy tailored to the genetic defect. (J Vasc Surg: Venous and Lym Dis 2020;■:1-5.)

**Keywords:** cfDNA; Liquid biopsy; Lymphovenous malformation; *MET* mutation; Noninvasive technique

Among the venous anomalies, the two most represented subclasses are venous malformations (VMs) at ~95% and glomuvenous malformation at 5%.[1] Low-flow VMs result from an error in vascular morphogenesis. The TIE2 receptor tyrosine kinase was the first gene to be associated with inherited VM development. This receptor is located on chromosome 9p21 and is specific for endothelial cells.[2-5] TIE2, through action in ANGPT1 (angiopoietin 1), upregulates hepatocyte growth factor (HGF), leading to the abnormal growth of veins. Thus, mice overexpressing Angpt1 in the skin will develop more, larger, and more highly branched vessels.[6]

Loss of function mutations in the glomulin (*GLMN*) gene are responsible for the more rare hereditary glomuvenous malformations. They are mostly situated on the extremities and implicate the skin and subcutis but seldom the mucosa. Unlike other vein malformations, these multifocal, frequently hyperkeratotic, injuries will be painful on palpation and cannot be completely flattened by compression. The mode of inheritance is paradominant, with a second somatic alteration inducing the pathogenicity. Glomulin interacts with the HGF receptor c-Met, inducing vascular smooth muscle cell migration and angiogenesis.[7]

A common feature to all blood and lymphatic vessels is the presence of endothelial cells as the luminal cell layer. Dysfunction of the *LYVE1* and *VEGFR3* genes or *VEGF-C, VEGF-D, PROX1*, *NRP2*, and *ANGPT2* involved in lymphangiogenesis and/or lymphatic vessels could be potential candidates for lymphatic malformations.[1]

From the Medical Genetics, University of Siena,[a] and the Genetica Medica, Azienda Ospedaliera Universitaria Senese,[b] Siena; and the Chirurgia Vascolare, Ospedale Maggiore di Crema, Largo Ugo Dossena,[c] and the Radiologia interventistica, Ospedale Maggiore di Crema,[d] Crema.

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Correspondence: Alessandra Renieri, MD, PhD, Medical Genetics, University of Siena, Policlinico "Santa Maria alle Scotte", Viale Bracci, 2, Siena 53100, Italy (e-mail: alessandra.renieri@unisi.it).

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HGF, and its receptor c-MET, previously unrecognized lymphedema genes, are attractive candidate genes for lymphedema.[8] HGF-mediated activation of cMet has promoted angiogenesis and lymphangiogenesis in cell models and in vivo.[9] Somatic-activating *MET* mutations have been identified in some cancers, including non–small-cell lung cancer and germline mutations in renal carcinoma.[10]

The mutations occur within the juxtamembrane region (T1010I and D1028N) and are known to be activating mutations identified rarely in lung cancer. Mutations occurring at *MET* exon 14, even if they do not produce juxtamembrane-missing variants, might be responsible for such neoplasms and, therefore, could be considered as exon 14 driver mutations, rather than as a polymorphism.[11] Human c-Met mutations can be studied in the nematode *Caenorhabditis elegans*, in which induction of the activating mutation p.T1010I will induce abnormal vulval development, vulval hyperplasia, and lower fecundity.[12]

Next generation sequencing (NGS)-liquid biopsy (LB) using cell-free DNA (cfDNA) is a novel approach that enables the early detection and monitoring of cancer.[13-15] The use of NGS-LB overcomes the space-time limit of tissue biopsies and represents a new possibility for diagnosing and monitoring vascular malformations due to somatic mosaicism.

Mosaic somatic mutations occur after the first zygotic divisions and exclusively affect specific cell lines. Thus, the cell lines harboring the variant will generate mutated tissues and the unmutated cell lines will develop normal tissues. Therefore, some anatomic areas will generate the disease and others will remain normal. Using NGS-LB, we identified a somatic activating mutation in p.T1010I and p.D1028N of the *MET* gene, not previously associated with VMs, although already known to be very well involved in the distrusted signalling pathway of both inherited VMs and glomuvenous malformations. The present study complied with institutional guidelines, and the ethics committees of the Azienda Ospedaliera Universitaria Senese, Siena, approved the present study (project name, GeVaMa_2015, v.4_21-02-2020).

## METHODS

**Patient enrollment and sample collection.** At the Medical Genetics Unit of the Azienda Ospedaliera Universitaria Senese, Siena, Italy, we enrolled four patients with VM in the present study. In the context of the genetic counseling, informed consent, clinical data, genealogic trees, and family history were collected for each patient. For patient 3, the Division of Vascular Surgery, Ospedale Maggiore, Crema, collected blood samples from the efferent vein at the lesion site and different formalin-fixed paraffin-embedded (FFPE) tissue samples were stored until use.

### ARTICLE HIGHLIGHTS

- **Type of Research**: Multicenter, prospective study
- **Key Findings**: Cell-free DNA next generation sequencing liquid biopsy was able to identify somatic mutations of the *MET* gene in all lymphovenous malformations investigated. The mutations were also confirmed at the tissue level when tissue samples were available.
- **Take Home Message**: Liquid biopsy applied to vascular malformations represents an innovative and effective approach capable of overcoming the limitations of tissue biopsy.

**Obtaining cfDNA from plasma.** For each patient, 10 mL of blood was collected in cfDNA BCT tubes (Streck, La Vista, Neb). The plasma was stored at −80°C until use. cfDNA extraction was performed from 4 mL of plasma using the MagMAX cell-free Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, Waltham, Mass). The cfDNA quantity was assessed using the Qubit dsDNA High Sensitivity test kit and a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, Calif). The quality was assessed using the Agilent High Sensitivity DNA kit (Agilent Technologies, Palo Alto, Calif) and an Agilent 2100 Bioanalyzer (Agilent Technologies).

**NGS sequencing of cfDNA.** The cfDNA library was set up using the Oncomine Pan-Cancer Cell-Free Assay gene panel (ThermoFisher Scientific), and sequencing was performed using the Ion Proton sequencer (Life Technologies, Carlsbad, Calif). The gene panel was designed to identify single nucleotide variants, insertions and deletions, gene fusions, and copy number variations to a detection limit of 0.05%. The Ion Reporter Server System (ThermoFisher Scientific) was used to perform variant analysis. The same panel was used to sequence genomic DNA (gDNA) from patient 3.

**Hematoxylin and eosin staining.** For patient 3, tissue samples had been taken from the right upper limb, FFPE, serially cut (10-μm sections), and rehydrated with 100% xylene and 100%, 95%, and 70% ethanol before immersion in water. The slices were then stained with hematoxylin and eosin and dehydrated.

**gDNA extraction from tissues.** For patient 3, gDNA was extracted from FFPE tissue samples using the MagCore Genomic DNA FFPE One-Step Kit for the MagCore System (Diatech Pharmacogenetics SRL, Ancona, Italy) following the manufacturer's instructions. gDNA was quantified using the Qubit Fluorometer and Qubit dsDNA High Sensitivity Assay (Life Technologies, Carlsbad, Calif).

## RESULTS

Patient 1 was a 26-year-old woman with angiomatous lesions on her forehead, perinasal and perioral regions, both hands, abdominal region, and left lower limb. Laser therapy on both upper and lower limbs was performed, with partial benefit for the hands. However, the laser therapy had caused ulcers in the lower limbs. A venous echocardiogram-color Doppler examination of the lower limbs showed mild ectasia of the great saphenous vein and insufficiency of the perforating veins, in addition to ectatic veins on the foot. The patient also reported skin fragility of the external auditory canal. The *MET* c.3029C>T p. (The1010Ile) mutation was detected with a variant allele frequency (VAF) of 0.32% from the peripheral vein using NGS-LB analysis.

Patient 2 was a 34-year-old woman with congenital angiomas of the left upper limb and left side of the chest. The patient reported congenital hypertrophy of her left upper limb, mainly at her hand and third finger. A previous spinal magnetic resonance imaging examination had revealed the presence of multiple dorsal neuromas. The patient also reported two lipomas, previous obesity, and troncular neuropathy of both median nerves. NGS-LB analysis detected the same pathogenic c.3029C>T p.(The1010Ile) mutation in the *MET* gene at a VAF of 0.97%.

Patient 3 was a 36-year-old woman with telangiectasias of the right hand. The first vascular surgery had been performed when she was 2 years old, with subsequent further interventions and progressive loss of function of the limb. Computed tomography scans showed a paravertebral mixed capillary lymphatic VM that extended along the right anterolateral chest wall and forearm. The malformation affected both the cutaneous and subcutaneous and the muscle tissues. *MET* mutation c. 3082G>A; p.(Asp1028Asn) was detected using NGS-LB analysis, with a VAF of 0.11%. For patient 3, we were also able to perform an analysis of the available tissue samples, which confirmed the existence of the mutation at the malformation site with a VAF of 0.011%, confirming its likely causative role in the lymphovenous malformations.

Patient 4 was a 61-year-old man with congenital angiodysplasia and right lower limb elephantiasis that extended up to the lumbar region. The lesion had increased in extent during adolescence, when the initial episode of lymphangitis had been reported. The patient also had intestinal lymphangiomatosis with episodes of gastrointestinal bleeding. Patient 4 also had features of Klippel-Trenaunay syndrome. *PIK3CA* c.1357G>A p.(Glu453Lys) and *MET* c.3029C>T, p.(The1010Ile) mutations were detected using NGS-LB analysis with a VAF of 0.36% and 0.09%, respectively. The clinical features and molecular results obtained for all 4 patients are shown in Fig and Table.

## DISCUSSION

MET activation occurs through the binding of receptor tyrosine kinases and its ligand, HGF. This binding leads to activation of the downstream PI3K/mTOR (phosphoinositide 3-kinase/mammalian target of rapamycin), STAT (signal transducer and activator of transcription), and MAPK (mitogen-activated protein kinase) pathways. The erroneous Met/HGF regulation has been studied and demonstrated for various cancers. It leads to oncogenesis by increasing cell proliferation and survival, epithelial–mesenchymal transition, tumor invasion, and tumor angiogenesis.[16]

Several gene mutations, such as amplification, copy number variations, and dysregulation of pathways, can lead to *MET* signaling defects. Although rare, several *MET* mutations have been observed in the kinase domain, rather than in the juxtamembrane or extracellular domain, for several tumors.[17]

It is also essential to consider the alternative splicing that occurs in *MET* exon 14. Several alterations affecting exon 14 and adjacent intronic regions will induce splicing alterations, which will lead to different protein isoforms, including the isoform with exon 14 skipping.[11] Some of these aberrant RNA transcripts can, thus, modulate disease onset and progression.

The p.T1010I mutation is located in the juxtamembrane domain and causes greater cellular proliferation and less cell adhesion. Furthermore, when the domain has mutated, it will show an increase in cellular polarity and a more disordered cellular pattern and cytoarchitecture.

Previous evidence has shown the p.T1010I alteration in patients with cancer, in particular, in patients with thyroid cancer, and might reflect a *MET* exon 14 alteration rather than a polymorphism owing to its oncogenic characteristics.[17] The suspicion of a polymorphism was raised by a family history of papillary renal cell carcinoma in which the germline alteration did not segregate with the disease.[18]

The prevalence of the MET p.T1010I mutation in the population overall is 0.07% according to the Exome Aggregation Consortium and 1.1% in the European population. All 4 of our analyzed patients with a lymphovenous malformation had this mutation. Definitely, a larger cohort of patients is needed to establish with certainty the frequency of the identified variant among patients with lymphovenous malformations.

Exon 14 skipping mutations prolong *MET* oncogenic activity by inhibition of Met receptor degradation. This type of oncogenic event could have clinical effects and could provide therapeutic options for several cancer types. In mammary epithelium, the presence of p.T1010I resulted in the formation of cellular colonies, cell migration, and invasion in vitro and tumor growth and invasion in vivo.[19] Mahjoubi et al[20] reported a case of lung

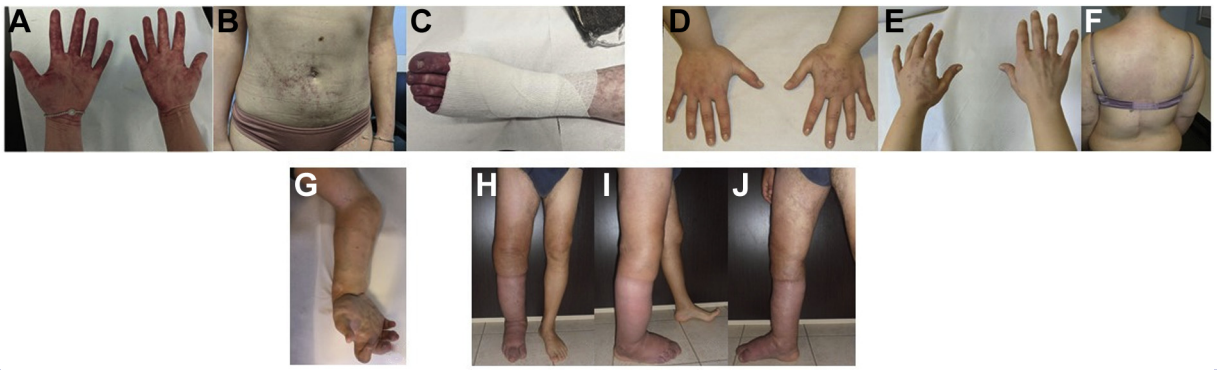


**Fig.** Clinical features of the patients. Patient 1: angiomatosis in the hands **(A)**, abdomen **(B)**, and left lower limb **(C)**. Patient 2: diffuse venous and lymphatic malformations **(D-F)**. Patient 3: lymphatic malformation of the right forearm **(G)**. Patient 4: right lower limb angio-osteohypertrophy in Klippel-Trenaunay syndrome with prominent lymphatic involvement **(H-J)**.

adenocarcinoma with a *MET* exon 14 donor splice site mutation (D1028N) that had been treated with efficacy using crizotinib. The *MET* exon 14 skipping alteration is a pharmacological target of Met inhibitors, such as crizotinib, cabozantinib, and capmatinib, and/or another potent Met inhibitor, such as foretinib. Their use has resulted in dose-dependent inhibition of growth in c-MET–amplified cells, with concomitant induction of apoptosis. Thus, the use of Met inhibitors could be a new treatment approach for patients with cancer.[11,21]

At present, vascular malformations are treated by surgical removal or sclerotherapy. The latter involves the use of alcohol or toxic agents capable of destroying aberrant blood vessels. However, depending on the anatomic location of the malformation, both treatments can be inadequate, with regrowth occurring often. Thus, different treatment options are needed.

Given the invasiveness of performing a tissue biopsy and the inaccessibility of some vascular sites, we evaluated the efficacy of NGS-LB for detecting cfDNA fragments released by the malformation into the bloodstream. We compared the blood from the efferent venous sample with that from the peripheral venous sample. We found that the cfDNA was enriched with mutated fragments released from the cells in the vascular lesion, in line with a causative role of the identified mutation.

Therefore, this procedure allows for the detection of even low-grade mosaicism. cfDNA analysis revealed pathogenic variants in exon 14 of the *MET* gene. The tissue biopsy analysis, performed for patient 3, validated the alteration at the malformation site, confirming its likely causative role. The broad and variable phenotypic spectrum is related to the different allele frequency of the MET variant in each single. Furthermore, it is possible that the MET mutation is not the only mutation causing the disease. Also, it is likely that the concomitant presence of other mutations with different degrees of expression would contribute to and could explain the phenotypic variability.

**Table.** Patient clinical features and molecular findings

| Pt. No. | Code No. | Gender | Age, years | Phenotype | Peripheral vein NGS-LB finding | Efferent vein NGS-LB finding |
|---|---|---|---|---|---|---|
| 1 | 5410/19 | Female | 26 | Diffuse extratroncular venous anomalies | *MET* [p.(T1010I)], 0.32% | NA |
| 2 | 5543/19 | Female | 34 | Diffuse disseminated venous cutaneous malformations associated with hypertrophy of fatty tissues | *MET* [p.(T1010I)], 0.97% | NA |
| 3 | 5461/19 | Female | 36 | Venous lymphatic malformation of upper right limb | NA | *MET* [p.(D1028N)], 0.11% |
| 4 | 278/20 | Male | 61 | Venous lymphatic malformations and Klippel-Trenaunay syndrome | *MET* [p.(T1010I)], 0.09% | NA |

*NA*, Not applicable; *NGS-LB*, next generation sequencing liquid biopsy; *Pt. No.*, patient number.

## CONCLUSIONS

From these data, we believe that the use of NGS-LB with blood samples from the efferent vein of the vascular malformation will allow for the identification of somatic mosaic mutations that have very low frequency and are the basis of the vascular phenotype. This approach overcomes the difficulty of tissue biopsy and enables the transition to precision medicine.

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## AUTHOR CONTRIBUTIONS

Conception and design: MV, AR
Analysis and interpretation: MP, LDS, EF, AP, MV, AR
Data collection: MP, LDS, AT, AC, GD, MB, EF, AG, CF, AA, AP, MV, AR
Writing the article: MP, LDS, AT, AC, GD, MB, EF, AG, CF, AP, MV, AR
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## REFERENCES

1. Uebelhoer M, Boon LM, Vikkula M. Vascular anomalies: from genetics toward models for therapeutic trials. Cold Spring Harb Perspect Med 2012;2:a009688.
2. Vikkula M, Boon LM, Carraway KL III, Calvert JT, Diamonti AJ, Goumnerov B, et al. Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. Cell 1996;87:1181-90.
3. Wouters V, Limaye N, Uebelhoer M, Irrthum A, Boon LM, Mulliken JB, et al. Hereditary cutaneomucosal venous malformations are caused by TIE2 mutations with widely variable hyper-phosphorylating effects. Eur J Hum Genet 2010;18:414-20.
4. Brahami N, Subramaniam S, Al-Ddafari MS, Elkaim C, Harmand PO, Sari BE, et al. Facial cutaneo-mucosal venous malformations can develop independently of mutation of TEK gene but may be associated with excessive expression of Src and p-Src. J Negat Results Biomed 2017;16:9.
5. Natynki M, Kangas J, Miinalainen I, Sormunen R, Pietila R, Soblet J, et al. Common and specific effects of TIE2 mutations causing venous malformations. Hum Mol Genet 2015;24:6374-89.
6. Suri C, McClain J, Thurston G, McDonald DM, Zhou H, Oldmixon EH, et al. Increased vascularization in mice overexpressing angiopoietin-1. Science 1998;282:468-71.
7. Brouillard P, Vikkula M. Vascular malformations: localized defects in vascular morphogenesis. Clin Genet 2003;63:340-51.
8. Finegold DN, Schacht V, Kimak MA, Lawrence EC, Foeldi E, Karlsson JM, et al. HGF and MET mutations in primary and secondary lymphedema. Lymphat Res Biol 2008;6:65-8.
9. Nakagaw T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 2010;328:62-7.
10. Giordano S, Maffe A, Williams TA, Artigiani S, Gual P, Bardelli A, et al. Different point mutations in the Met oncogene elicit distinct biological properties. FASEB J 2000;14:399-406.
11. Pilotto S, Gkountakos A, Carbognin L, Scarpa A, Tortora G, Bria E. MET exon 14 juxtamembrane splicing mutations: clinical and therapeutical perspectives for cancer therapy. Ann Transl Med 2017;5:2.
12. Siddiqui SS, Loganathan S, Krishnaswamy S, Faoro L, Jagadeeswaran R, Salgia RC. elegans as a model organism for in vivo screening in cancer: effects of human c-Met in lung cancer affect C. elegans vulva phenotypes. Cancer Biol Ther 2008;7:856-63.
13. Palmieri M, Baldassarri M, Fava F, Fabbiani A, Gelli E, Tita R, et al. Two point-NGS analysis of cancer genes in cell free-DNA of metastatic cancer patients. Cancer Med 2019;9:2052-61.
14. Crowley E, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. Nat Rev Clin Oncol 2013;10:472-84.
15. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. N Engl J Med 2018;379:1754-65.
16. Sylvester PW. Targeting met mediated epithelial-mesenchymal transition in the treatment of breast cancer. Clin Transl Med 2014;3:30.
17. Ma PC, Kijima T, Maulik G, Fox EA, Sattler M, Griffin JD, et al. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res 2003;63:6272-81.
18. Schmidt L, Junker K, Nakaigawa N, Kinjerski T, Weirich G, et al. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene 1999;18:2343-50.
19. Liu S, Meric-Bernstam F, Parinyanitikul N, Wang B, Eterovic AK, Zheng X, et al. Functional consequence of the MET-T1010I polymorphism in breast cancer. Oncotarget 2015;6:2604-14.
20. Mahjoubi L, Gazzah A, Besse B, Lacroix L, Soria JC. A never-smoker lung adenocarcinoma patient with a MET exon 14 mutation (D1028N) and a rapid partial response after crizotinib. Invest New Drugs 2016;34:397-8.
21. Sohn SH, Kim B, Sul HJ, Choi BY, Kim HS, Zang DY. Foretinib inhibits cancer stemness and gastric cancer cell proliferation by decreasing CD44 and c-MET signaling. Onco Targets Ther 2020;13:1027-35.

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