

Light sheet microscopy-based 3-dimensional histopathology of the lymphatic vasculature in Emberger syndrome

Lichtblattemikroskopie-basierte 3-dimensionale Histopathologie des Lymphgefäßsystems beim Emberger-Syndrom

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Key words

light sheet microscopy, histopathology, lymphovascular diseases, lymphoedema

Schlüsselwörter

Lichtblattemikroskopie, Histopathologie, Lymphovaskuläre Erkrankungen, Lymphödem

Bibliography

DOI <https://doi.org/10.1055/a-1191-8380>

Phlebologie 2020; 49: 242–248

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ISSN 0939-978X

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ABSTRACT

Introduction Lymphovascular diseases represent a heterogeneous group of inherited and sporadic disorders and refer to a range of possible underlying pathologies and pathogenesis.

Emberger Syndrome, an inherited form of lymphedema, is characterized by bilateral lower limb lymphedema, however, upper limbs do not show any signs of swelling.

To identify disease-associated histopathological alterations in patients with Emberger Syndrome and to elucidate potential histological differences between the lymphatic vasculature of upper and lower limbs, a detailed knowledge on the 3-dimensional tissue and vessel architecture is essential. However, the current gold standard in 2-dimensional histology provides only very limited spatial information.

Material and methods To elucidate the underlying vascular pathology in Emberger Syndrome on the cellular level, we applied the 3-dimensional visualization and analysis approach VIPAR (volume information-based histopathological analysis

by 3D reconstruction and data extraction) to entire whole-mount immunofluorescence-stained human tissue samples. VIPAR is a light sheet microscopy-based imaging technique, which allows 3-dimensional reconstruction of entire tissue biopsies followed by automated and semi-automated analysis of vascular parameters in 3-dimensional space.

Results Using VIPAR we could show that in Emberger Syndrome the dermal lymphatic vasculature is intact and non-disrupted.

However, lower limbs showed an hypoplastic lymphatic vasculature with absence of lymphatic valves in pre-collecting and collecting vessels. In contrast to the lower limbs, the lymphatic vasculature of the upper limbs showed no morphological alterations of lymphatic vessels and lymphatic valves compared to healthy controls.

Discussion Based on the 3-dimensional histopathological analysis we were able to perform a detailed phenotyping of lymphatic vessels in the upper and lower limb in Emberger Syndrome and to identify the underlying vascular pathology. In addition, we could show vascular alteration between the upper and lower limbs indicating a vascular heterogeneity of dermal lymph vessels causing the lower limb lymphedema.

ZUSAMMENFASSUNG

Einleitung Lymphovaskuläre Erkrankungen stellen eine heterogene Gruppe von Erkrankungen dar, die sowohl genetische als auch nicht-genetische Ursachen haben und in der zugrunde liegenden Pathologie und Pathogenese sowie in der Symptomatik sehr variieren können. Innerhalb der Gruppe der angeborenen lymphovaskulären Erkrankungen stellt das Emberger-Syndrom eine Ursache für bilaterale Beinlymphödem dar, die obere Extremität ist hingegen in der Regel nicht betroffen.

Um die erkrankungsassoziierten histopathologischen Veränderungen im Vergleich zum Gesunden und histologische Unterschiede zwischen der oberen und unteren Extremität zu identifizieren und neue Einblicke in die Pathogenese zu erhalten, ist eine detaillierte Kenntnis der 3-dimensionalen Gewebe- und Gefäßarchitektur essenziell. Dies ist jedoch mittels des aktuellen histologischen Goldstandards, der 2-dimensionalen Histologie, nur sehr limitiert möglich.

Material und Methoden Um die zugrunde liegende Pathologie auf zellulärer Ebene zu identifizieren, nutzten wir das

3-dimensionale Visualisierungs- und Analyseverfahren VIPAR (volume information-based histopathological analysis by 3D reconstruction and data extraction). Hierbei handelt es sich um eine Lichtblattmikroskopie-basierte optische Schnittbildgebung von kompletten, intakten Immunofluoreszenzgefärbten Gewebebiopsien. Im Anschluss an eine 3-dimensionale Rekonstruktion der optischen Schnittbilder erfolgt eine automatische bzw. semiautomatische Analyse verschiedener Gefäßparameter im 3-dimensionalen Raum.

Ergebnisse Es sollten Hautgewebeproben beim Emberger-Syndrom analysiert werden, um das vorwiegende Auftreten eines bilateralen Beinlymphödems bei regelhaftem Fehlen eines Armlymphödems zu untersuchen.

Mittels des VIPAR-Ansatzes wurden hierzu Gewebebiopsien der oberen und unteren Extremität bei Patienten mit Emberger-Syndrom untersucht. Es konnte bei den untersuchten Hautbiopsien gezeigt werden, dass das dermale lymphatische

Gefäßnetzwerk der oberen und unteren Extremität intakt und somit nicht disruptiert war. Im Vergleich zur unteren Extremität, die jeweils ein hypoplastisches Gefäßnetzwerk sowie ein Fehlen der Lymphgefäßklappen in den lymphatischen Präkollektoren und Kollektoren aufwies, zeigten sich in der oberen Extremität keine numerischen oder morphologischen Unterschiede der Gefäßklappen im Vergleich zu einer Kontrollprobe.

Fazit Durch die 3-dimensionale histopathologische Untersuchung war es möglich die Lymphgefäße der oberen und unteren Extremität beim Emberger-Syndrom detailliert zu beschreiben und die ursächliche vaskuläre Pathologie zu identifizieren. Ferner zeigten sich bei den untersuchten Proben anatomische Unterschiede zwischen oberer und unterer Extremität, die auf eine vaskuläre Heterogenität der dermalen Lymphgefäße hindeuten und somit die regelhafte Limitation des Lymphödems auf die untere Extremität erklären können.

Introduction

Besides inherited lymphatic vessel malformations, lymphovascular diseases include various forms of lymphoedema and represent a heterogeneous group of disorders with respect to the pathogenesis and underlying pathological changes in the tissues. The underlying cause may be genetically induced malformations of the lymphatic system, injuries, radiation, surgical procedures or infections [1, 2].

Emberger syndrome (ES), also known as deafness-lymphoedema-leukaemia syndrome, is one such rare genetic disorder. It is caused by a heterozygous mutation in the GATA2 gene that shows autosomal dominant inheritance with variable penetrance and expressivity. In addition to the characteristic onset of lower limb lymphoedema in the early years of life and sensorineural hearing loss, haematological abnormalities (acute myeloid leukaemia, cytopenia) and immunodeficiency have been described for ES [3–6].

Although the genetic cause is well-known and animal models have shown the significance of the GATA2 zinc-finger transcription factor in the development and function of the lymphatic vessels and valves, the alterations that the mutation causes in human lymphatic vessels have not been conclusively elucidated [3, 7–9]. It is also unclear why only the lower limbs are affected in ES, while the upper limbs usually show no signs of lymphoedema [3, 10, 11].

This lack of understanding can be attributed to the inadequacies of imaging techniques, which fail to provide a sufficient information on the vascular architecture at the cellular level and in a three-dimensional (3D) context [12].

Macroscopic imaging provides information on the lymph flow and possible reflux across a defective valve, but does not allow any conclusions to be made at the cellular level. On the other hand, microscopic techniques, such as that used for routine two-dimen-

sional (2D) histology, offer cellular resolution and information at cell level. However, the necessary detailed understanding of the spatial architecture is hardly possible on the basis of 2D histological sections alone, with the result that the complex vascular network and tissue architecture cannot be demonstrated and analysed adequately [12].

The aim of our study was to determine the underlying vascular pathology in ES and identify possible reasons for the characteristic absence of lymphoedema in the upper limbs. For this purpose, we used a mesoscopic imaging technique and performed whole-mount immunofluorescence staining of intact tissue biopsies for lymphatic endothelial cells followed by optical sectioning using a light sheet microscope. The obtained image stacks were then visualised and evaluated in 2D and 3D using VIPAR (volume information-based histopathological analysis by 3D reconstruction and data extraction).

Materials and methods

Antibodies

We used the following antibodies: rabbit anti-human PROX1 (102-PA32, polyclonal, Relia Tech), mouse anti-human PDPN (clone D2–40, monoclonal, Dako), Alexa dye-conjugated secondary antibodies (LifeTechnologies).

Immunofluorescent staining of the skin biopsies

Skin biopsies were fixed for four hours in 4% PFA/PBS at 4 °C and subsequently permeabilised (0.5% Triton X-100/PBS). The antibody staining (diluted in PermBlock) was performed after blocking in PermBlock (1% BSA, 0.5% Tween 20 in PBS). Samples were washed three times with PBS-T (0.1% Tween 20/PBS) after each staining step.

Light sheet microscopy

Once the whole samples had been immunofluorescence-stained, the tissue biopsies were optically cleared and visualized with a LaVision Ultramicroscope II (LaVision BioTec) at various magnifications. We performed the 3D reconstruction and analysis of the images with VIPAR [12].

Optical clearance of the skin biopsies

Immunofluorescent-stained skin biopsies were embedded in 1 % low-melting-point agarose and dehydrated in an ascending alcohol series (50 %, 70 %, 95 %, >99.0 %, >99.0 % methanol, each step lasting 30 minutes). The dehydrated samples were then incubated twice in benzyl alcohol/benzyl benzoate (BABB, ratio 1:2 (v/v)) for four hours each time and optically cleared. The optically cleared samples were stored in BABB [12, 13].

Ethics

Participants in the study were recruited through the Genetics and Lymphovascular Medicine Clinic, St. George's Hospital, London, United Kingdom (the study was approved by the South West London Research Ethics Committee, London, United Kingdom, REC ref: 05/Q0803/257). Subjects gave their informed consent to the study.

Results

The VIPAR-based approach to light sheet microscopy allows the optical cross-sectional imaging and 3D reconstruction of the lymphatic vascular anatomy in the skin

After immunofluorescent staining of the entire tissue biopsy, the samples were dehydrated in an ascending alcohol series and optically cleared with benzyl alcohol/benzyl benzoate (► Fig. 1A). The immunofluorescence-stained and optically cleared whole-mount preparations were then optically sectioned in their entirety using a LaVision UltraMicroscope II and the resulting image stacks consisting of 3000–6000 optical sections (spaced at 1–2 µm distance in the z section plane) were used for digital 3D reconstruction and analysis with VIPAR (► Fig. 1B, C).

To demonstrate the lymphatics in the tissue samples, the lymphatic vessels were stained for the surface protein podoplanin (PDPN) and the lymphatic endothelial cell nuclei for the transcription factor prospero homeobox protein 1 (PROX1) [12, 15]. This specific staining allowed the unequivocal identification of the lymphatic vessels in the biopsy samples (► Fig. 1D, E).

With the 3D reconstruction of the lymphatic vessels in the whole-mount biopsies, we were able to visualise the vascular architecture in both healthy and diseased tissue samples. In this way, we were able to demonstrate both lymphatic capillaries/sinusoids as well as precollectors and collectors. Lymphatic valves could also be clearly identified from the high PROX1 expression at the site of vascular bifurcations (► Fig. 1D, E, yellow arrows).

Demonstrating the 3D anatomy of the lymphatic vessels in tissue samples with this procedure offers a rapid and simple means of interpreting the vascular architecture and any pathological changes. However, the great complexity of a spatial vascular network makes it somewhat difficult to analyse and quantify vessel parameters in 3 dimensions. For this reason, we integrated automated segmentation and data extraction procedures into VIPAR to enable us to describe and quantify the lymphatic system in detail and in an unbiased manner. In this way, we were able not only to compare samples from different anatomical sites, but also to distinguish between patient samples and controls, so that it was possible to perform a detailed phenotyping of the tissue sample and thus any vascular pathology that may be present.

The 3D histopathological analysis of the lymphatic vessels in the lower limb in patients with Emberger syndrome shows a continuous hypoplastic lymphatic system as well as the absence of lymphatic valves

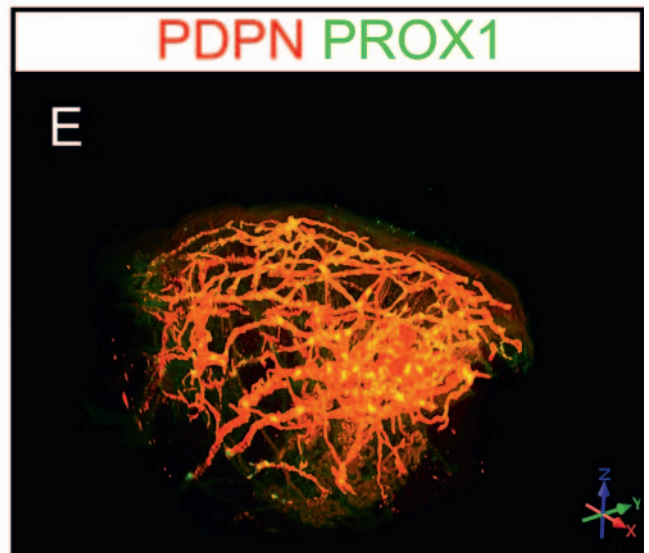
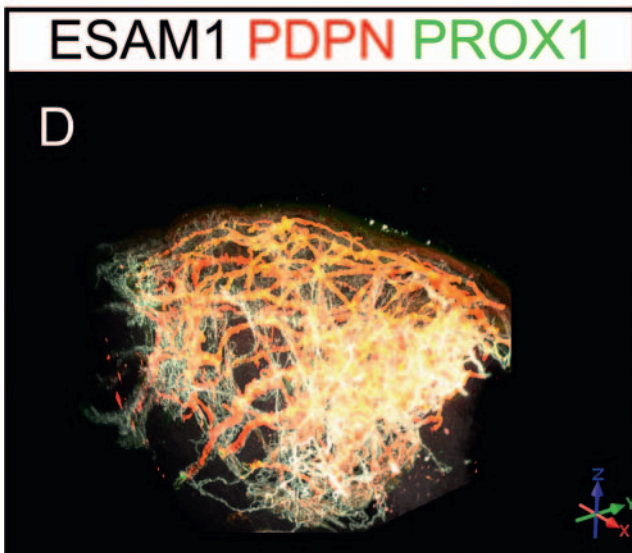
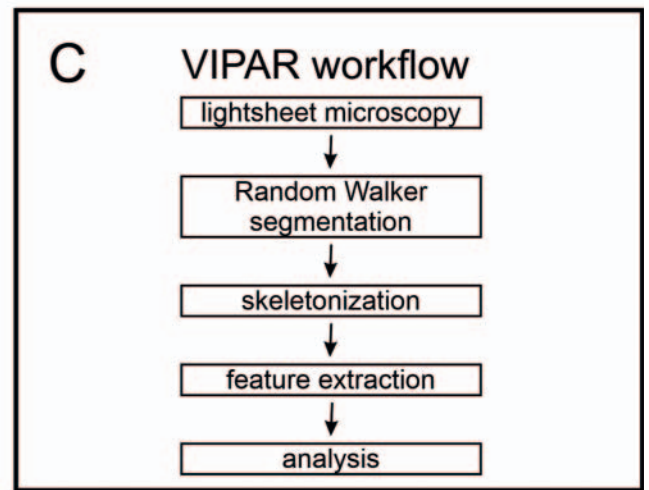
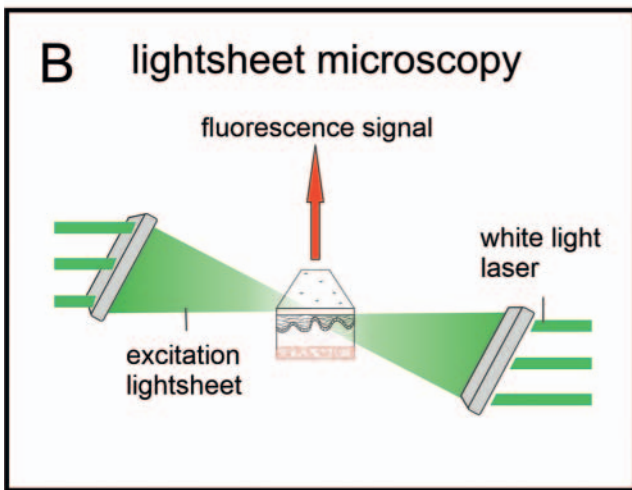
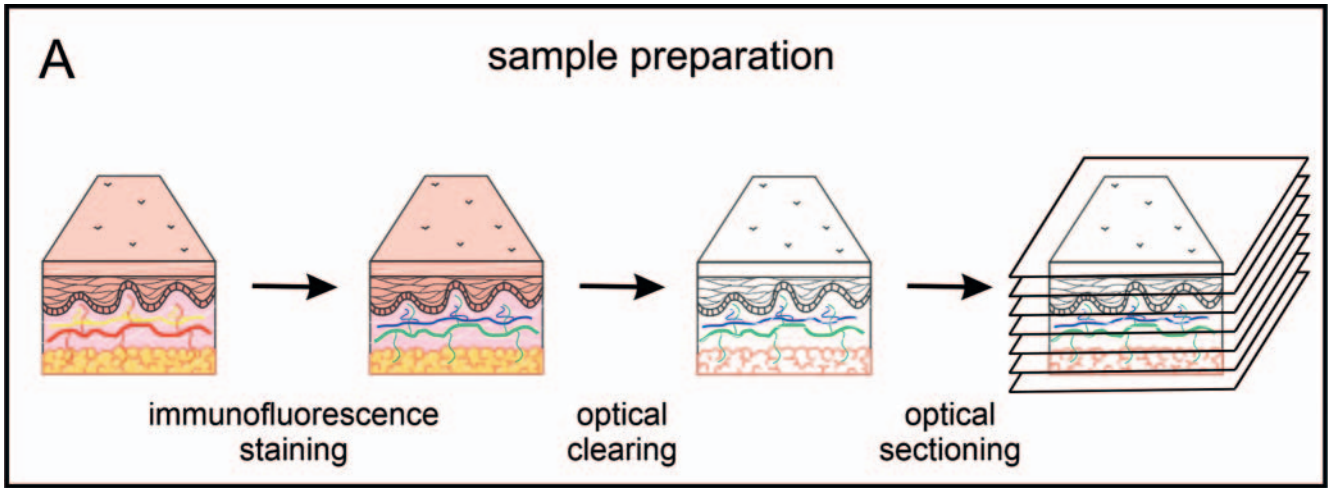
In order to identify the underlying pathological changes to the vessels in ES using VIPAR, we examined the skin biopsies from two patients with ES and compared them with healthy control samples. As these patients had severe lymphoedema of the lower limbs, but clinical examination had not shown any evidence of lymphoedema of the upper limbs, the first step was to use VIPAR to examine the legs more closely.

The 3D visualisation of the tissue samples from the legs of patients with ES showed a continuous undisrupted lymphatic network (► Fig. 2A). In comparison with control samples, however, the lower limbs showed a hypoplastic lymphatic system with vessels of reduced diameter (reduced by 13 % and 17 %) and a smaller ratio of lymph vessel volume to tissue volume (reduced by 24 % and 29 %). There was also less vascular branching. A clear differentiation between collectors, precollectors and capillaries/sinusoids was difficult, if not impossible (► Fig. 2A).

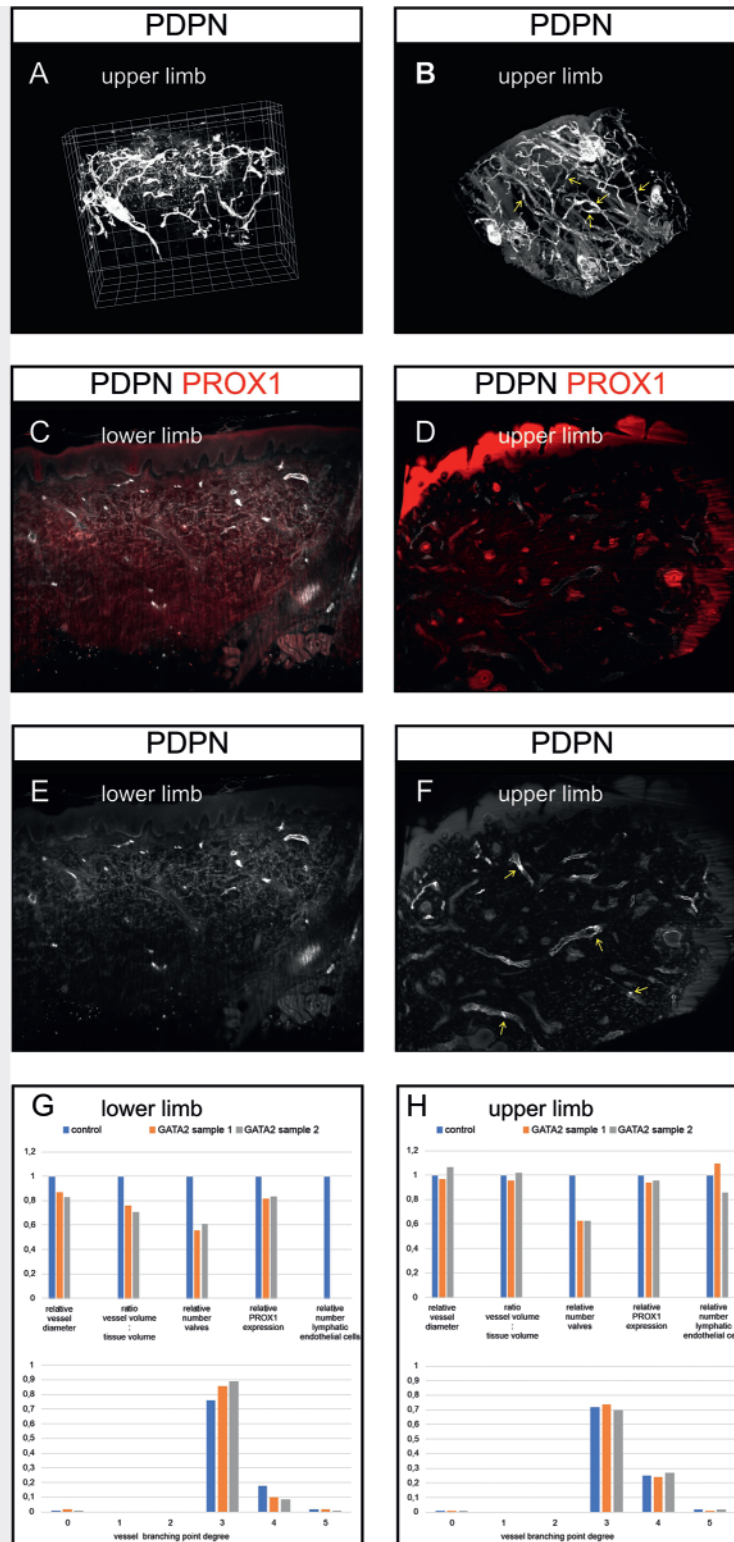
As GATA2 has an essential role in the transcription of multiple genes that are involved in valve morphogenesis and is thus important for the morphology and function of the lymphatic valves [4, 7–9, 14], we looked more closely at these valves.

Our analysis revealed the absence of valves in the lymphatic systems of the patients with ES (► Fig. 2A, C, E), but they could be clearly identified in the control samples on the basis of both morphological and molecular characteristics (not shown) [12]. In order to further characterise the changes and identify possible molecular mechanisms, we performed immunofluorescence-staining for the lymphatic transcription factor PROX1, which is also regulated by GATA2 and whose expression is essential for valve morphogenesis [4, 7].

The comparative analysis of PROX1 expression in the two patients with ES showed that, compared to the control samples, the relative expression of the transcription factor was reduced by 44 % and 39 % respectively in all lymphatic endothelial cells. This was also associated with an 18 % and 16 % reduction in the endothelial cells themselves (► Fig. 2G).



► **Fig. 1** Sample preparation, analysis workflow and 3D reconstruction of human tissue biopsies with VIPAR. **A** Whole-mount human tissue biopsies were immunofluorescence-stained dye, dehydrated and optically cleared in benzyl alcohol/benzyl benzoate. **B** Optically cleared samples were optically sectioned using a light sheet microscope and then **C** visualised and analysed with the VIPAR workflow. **D**, **E** 3-dimensional reconstructions of the dermal lymphatic vessels allowed the demonstration of the lymphatic architecture, as well as the identification of lymphatic valves (identified by high PROX1 expression).



► **Fig. 2** Light sheet microscopy-based 3-dimensional reconstruction and analysis of the dermal lymphatic vessels allows us to identify morphological differences in the vascular anatomy of the upper and lower limbs in patients with Emberger syndrome. **A–H** Whole-mount immunofluorescence-stained tissue biopsies of the lower and upper limbs from patients with Emberger syndrome were visualized and analysed with VIPAR. The lymphatic vessels were stained with antibodies to the surface marker podoplanin (PDPN) and to the transcription factor PROX1. For each limb, 3D reconstructions are presented **A, B** as well as individual representative 2-dimensional optical planes **C–F**. Lymph valves (identified from the high PROX1 expression) are indicated by the yellow arrows. In comparison with the upper limbs, the lower limbs show a clearly hypoplastic lymphatic system with an absence of valves. **G, H** Vascular 3D parameters were quantified with VIPAR and compared with control tissue samples.

Dermal lymphatic vessels in the upper limbs in patients with Emberger syndrome do not show any morphological differences or a reduction in lymph valves

Skin biopsies from the upper limbs were then examined with the method described previously. The 3D reconstruction (► Fig. 2B) demonstrated that, compared with healthy controls, the lymphatics in patients with ES showed no significant changes in architecture or morphology.

In contrast to the findings in the lower limbs, lymphatic valves were identified on the basis of the morphology and condensation of PROX1-positive nuclei (► Fig. 2B, D, E); these valves were present in numbers similar to those found in the control samples (► Fig. 2H). To determine whether the presence of these valves was also associated with an unchanged relative PROX1 expression compared to the controls, we performed an expression analysis by means of immunofluorescent staining. In doing so, we identified a 37% reduction in the relative expression of the transcription factor. The number of lymphatic endothelial cells did not show any relevant differences (6% reduction and 4% increase respectively). No other molecular or morphological abnormalities were detected (► Fig. 2H).

Discussion

In this study, we used the novel 3D histopathology technology VIPAR to reveal the underlying vascular pathology in the lymphatic vessels of two patients with ES. By comparing the findings with healthy controls, we were able not only to visualise the changes in 3D, but also to quantify these changes in three dimensions. The automated and semi-automated analytical algorithms integrated into VIPAR offer a rapid and unbiased quantification, which allows us to determine, for example, the vascular volume, the diameter and length of the vessels and the extent of branching.

Based on the 3D reconstruction of the tissue samples taken from the lower limbs, we could demonstrate that even though patients with ES had a continuous, i. e. undisrupted, lymphatic system, it was clearly hypoplastic (► Fig. 2A). The structural vascular changes in ES described in the 3D reconstruction were confirmed by the 3D analysis of the vessel parameters. This showed that the lymphatic vessels of the lower limbs in patients with ES have a smaller total volume related to the tissue volume and a smaller diameter. In addition, the analysis of branching confirmed that fewer branches are present in ES patients, although the vessels do not become fragmented (► Fig. 2G).

We also identified an absence of valves in the lower-limb lymphatic vessels, which made it difficult to distinguish between the various type of lymphatic vessel (capillaries/sinusoids, precollectors and collectors) (► Fig. 2A, C, E). Consideration of further 3D vascular parameters suggests an altered maturation of the lymphatic vessels, which needs to be addressed in more detail in future studies.

In order to understand whether the pathogenic germline mutation present in the patients' GATA2 gene, which affects protein expression, was responsible for the hypoplastic lymphatic system and the absence of valves, we analysed the expression of

the lymphatic transcription factor PROX1. Compared with healthy controls, PROX1 expression in the two patients with ES was reduced by 44% and 39% respectively. Subsequent quantification of the lymphatic endothelial cell nuclei showed reductions of 18% and 16% (► Fig. 2G).

As GATA2 regulates the expression of PROX1 via binding in the PROX1 promoter region and is essential for the morphogenesis of valves in the lymphatic vessels [4], the presence of the pathogenic mutation in GATA2 may be regarded as a possible cause of the hypoplastic lymphatic system and the absence of valves.

Furthermore, the absence of lymphatic valves indicates altered flow patterns in the vessels, which in turn may affect the expression of multiple genes and maturation of the vessels [7–9].

The changes in the vessels that we have described may explain the development of lymphoedema in the lower limbs seen in ES.

As we felt it important to determine why only the lower limbs are usually affected by lymphoedema in patients with ES [3, 11], we also carried out the analysis described above on tissue samples from the upper limbs.

Here we found that the lymphatic vessels in the examined skin biopsies from the upper limbs showed no significant differences with respect to volume, branching or vessel diameter. The vessels were connected to a continuous network and showed no vascular fragmentation. In addition, the number of lymphatic valves present was similar to the number found in the healthy controls (► Fig. 2B, D, F, H).

In this context, an expression analysis of the lymphatic endothelial cell nuclei for PROX1 was also carried out. There was a clear reduction in the relative expression despite the presence of morphologically unremarkable valves in the vessels (► Fig. 2H).

An overall assessment of our results indicates a vascular heterogeneity of the dermal lymphatic vessels in the upper and lower limbs of patients with ES. The vascular network shows morphological differences between the upper and lower limbs that explain why lymphoedema characteristically develops in the legs, but spares the arms.

This may indicate that the signalling pathway within the lymphatic endothelial cells differs between the upper and lower limbs or that other genes are able to compensate for the altered GATA2 signalling pathway and the reduction of PROX1 expression depending on the anatomical site.

To allow further interpretations of these findings, future studies should include additional parameters (e. g. gravitation, flow rate) that could also have an effect on the vascular anatomy of the upper and lower limbs and may thus explain the phenotype.

Conclusion

Due to the novel imaging technique which allows 3-dimensional histopathological analysis of entire tissue biopsies, we were able to gain new insights into the vascular pathology of Emberger syndrome and collect evidence indicating a vascular heterogeneity in the dermal lymphatic vessels. In future studies, the concept of vascular heterogeneity should be investigated in a larger cohort as well as in other inherited diseases with lymphoedema affecting both the lower and the upper limbs. Given the possibility of future

novel therapeutic interventions, these findings may be key to developing curative treatment methods for patients with Emberger syndrome.

Financial support

The study was supported by the Lymphatic Malformation Institute, USA, and the German Society of Lymphology (Research prize, 2019). The author is a member of the Berlin Institute of Health-Charité Clinician Scientist Program, which is financed by Charité-Universitätsmedizin Berlin and the Berlin Institute of Health.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgement

I would like to thank Nils R. Hansmeier and Sascha Ulferts (Charité-Universitätsmedizin Berlin) for their help in preparing and analysing the samples. My thanks also to Dominik Drees of the Computer Science Department at Münster University for his support in the data analysis.

References

- [1] Mortimer PS, Rockson SG. New developments in clinical aspects of lymphatic disease. *J Clin Invest* 2014; 124 (3): 915–921
- [2] Schulte-Merker S, Sabine A, Petrova TV. Lymphatic vascular morphogenesis in development, physiology, and disease. *J Cell Biol* 2011; 193 (4): 607–618
- [3] Ostergaard P, Simpson MA, Connell FC. Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome). *Nat Genet* 2011; 42 (10): 929–931
- [4] Kazenwadel J, Secker GA, Liu YJ. Loss-of-function germline GATA2 mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature. *Blood* 2012; 119 (5): 1283–1291
- [5] Donadieu J, Lamant M, Fieschi C. Natural history of GATA2 deficiency in a survey of 79 French and Belgian patients. *Haematologica* 2018; 103 (8): 1278–1287
- [6] Brambila-Tapia AJL, García-Ortiz JE, Brouillard P. GATA2 Null Mutation Associated With Incomplete Penetrance in a Family With Emberger Syndrome. *Hematology* 2017; 22 (8): 467–471
- [7] Kazenwadel J, Betterma KL, Chong CE. GATA2 is required for lymphatic vessel valve development and maintenance. *J Clin Invest* 2015; 125 (8): 2979–2994
- [8] Mahamud MR, Geng X, Ho YC. GATA2 controls lymphatic endothelial cell junctional integrity and lymphovenous valve morphogenesis through miR-126. *Development* 2019; 146 (21): dev184218
- [9] Cha B, Geng X, Mahamud MR. Complementary Wnt Sources Regulate Lymphatic Vascular Development via PROX1-Dependent Wnt/ β -Catenin Signaling. *Cell Rep* 2018; 25 (3): 571–584.e5
- [10] Greene AK, Grant FD, Slavin SA. Lower-extremity lymphedema and elevated body-mass index. *N Engl J Med* 2012; 366 (22): 2136–2137
- [11] Connell FC, Gordon K, Brice G. The classification and diagnostic algorithm for primary lymphatic dysplasia: an update from 2010 to include molecular findings. *Clin Genet* 2013; 84 (4): 303–314
- [12] Hägerling R, Drees D, Scherzinger A. VIPAR, a quantitative approach to 3D histopathology applied to lymphatic malformations. *JCI Insight* 2017; 2 (16): e93424
- [13] Hägerling R, Pollmann C, Andreas M. A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J* 2013; 32 (5): 629–644
- [14] Pollmann C, Hägerling R, Kiefer F. Visualization of lymphatic vessel development, growth, and function. *Adv Anat Embryol Cell Biol* 2014; 214: 167–186
- [15] Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 1999; 98 (6): 769–778