Resequencing of VEGFR3 pathway genes implicate GJC2 and FLT4 in the formation of primary congenital chylothorax

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To the Editor,
Primary isolated congenital chylothorax (CCT) is a lymphatic disorder affecting 1 in 20,000 pregnancies. It is defined as pleural effusion with nonfasting triglycerides level of >110 mg/dl or 80% lymphocytes of its cells (Al-Tawil et al., 2000). CCT may develop already during the second trimester and its clinical course varies from mild pleural effusions to life-threatening fetal hydrops. Severe and progressive pleural effusions with consecutive fetal hydrops may impair fetal lung development with pulmonary hypoplasia and neonatal death in up to 40% of cases (Dorsi et al., 2018). Interestingly, the CCT phenotype presents only pre- and perinatally. Affected fetuses successfully treated with state-of-the-art pre- and perinatal management including intrauterine pleuroamniotic fluid drainage do not show long-term recurrence of pleural effusions or other major impairments related to their CCT (Resch et al., 2012).

Lately, deep genotyping and phenotyping have provided novel opportunities for the identification of drug targets and/or novel treatment strategies within the context of personalized medicine. In the field of lymphatic disorders, monogenic causes have been identified in a relatively large proportion of syndromic cases. For example, some of the monogenic disease genes associated with lymphatic disorders code for proteins involved in the RAS/MAPK signaling cascade (e.g., RAF1, RASA1, RIT1, and SOS1), a cascade accessible to treatment with mTOR and MEK inhibitors (Sevick-Muraca & King, 2014). Another important pathway that has been associated with monogenic lymphatic disorders is the VEGFR3 signaling cascade, which has been accounted for about 35% of familial and 8% of sporadic lymphedema (Mendola et al., 2013). The VEGFR3 pathway has been shown to be accessible to monoclonal antibody treatment and kinase inhibitors (Saif et al., 2016). Studies in mouse lymphatic endothelial cells suggest that the RAS/MAP signaling cascade is linked to the VEGFR3 signaling cascade through ets-mediated p300 recruitment and histone
acetylation of the mouse Vegfr3 gene (Ichise et al., 2012). The implication of VEGFR3 pathway genes in the formation of CCT might allow for new targeted treatment options. Therefore, we hypothesize VEGFR3 pathway genes to cause not only various forms of syndromic lymphatic disease but also primary isolated CCT. We chose especially those VEGFR3 pathway genes that have been reported for isolated lymphatic malformations without additional syndromic features of other organ systems, which are FLT4 (lymphatic malformation 1, MIM: 153100), VEGFC (lymphatic malformation 4, MIM: 615907), and GJC2 (lymphatic malformation 3, MIM: 613206). Moreover, CCT features have been associated with FLT4 and GJC2. Here, we identified two novel variants in two independent patients with isolated primary CCT. At this point, we are unable to be definitive regarding the pathogenicity of both variants, leaving them as variants of uncertain significance (VUS). Nevertheless, our observation suggests FLT4 and GJC2 as putative candidate genes for primary isolated CCT.

In detail, we used Molecular Inversion Probe (MIP) sequencing as a cost-efficient approach for targeted resequencing of three VEGFR3 pathway genes previously associated with lymphatic disease (FLT4, VEGFC, and GJC2) in a cohort of 31 isolated primary CCT patients and 44 of their unaffected parents. The study was approved by the Institutional Review Board of the University Hospital Bonn (Lfd. Nr.152/18) and informed consent was obtained prior to inclusion. DNA was extracted from blood or saliva samples. To cover all five protein-coding transcripts of FLT4, VEGFC, and GJC2 we designed 79 MIPs (Table S1). The final pooled MIP libraries were processed on a MiSeq sequencer (Illumina, San Diego, CA, USA) using 2 × 125 bp reads. Variants in patients and parents were filtered for QD (Quality by Depth) >10, coding position, LoF or non-synonymous SNV with gnomAD minor allele frequency of <0.0001, since all three genes have been associated with dominant lymphatic disorders. The remaining variants were controlled for individual read quality with >10 reads and visual quality control using the software IGV. Then, variants were filtered for conservation in vertebrate and deleterious prediction by at least three of four in silico prediction tools (SIFT, Polyphen2, Mutation Taster, and CADD). The remaining variants were validated by Sanger sequencing and segregation analysis was performed in all available family members. To acquire further CCT patients with variants in the VEGFR3 pathway genes FLT4, VEGFC, and GJC2, we submitted the genes in GeneMatcher (Sobreira et al., 2015).

Two variants, one in FLT4 and one in GJC2, passed all filter criteria. The first variant represents a novel heterozygous missense variant in GJC2 (c.T775C, p.Ser259Pro) in patient CHT31_501. The male patient CHT31_501 was delivered by cesarean section as the second child of a second gravida with a gestational age of 36 + 1 weeks and a birth weight of 3200 g. He was prenatally diagnosed with hydrodysmotosis of unknown cause and postnatally presented with bilateral CCT and hydrodysmotosis, respiratory failure of newborn, and neonatal cardiac failure. After birth, he was intubated and mechanically ventilated, his pleural effusions were treated successfully with bilateral placement of pleural drains. Sonographic examination of the brain, kidneys, and heart did not show any pathological findings. No infectious or any other cause for the hydrodysmotosis could be found.

The boy was given up for adoption and discharged in good general condition after 32 days. DNA of his biological parents and siblings is not available. The two older siblings of patient CHT31_501 were from a different father. The GJC2 variant (c.T775C, p.Ser259Pro) is not reported in gnomAD, its residue is conserved in vertebratea and the amino acid change is predicted to be deleterious by all four in-silico prediction tools, and is therefore fulfilling the ACMG criteria of VUS (PM2, PP3) (Table 1). GJC2 encodes for gap junction gamma-2 protein, a protein of the connexin families. Six connexin proteins form a connexon hemichannel and two connexons form a membrane spanning intercellular channel, the gap junction. GJC2 has been associated with autosomal recessive hypomyelinating leukodystrophy (MIM: 608804), autosomal recessive spastic paraplegia (MIM: 613206), and autosomal dominant lymphatic malformation and familial lymphedema (MIM: 613480). Carriers are reported to present with peripheral lymphedema with variable onset between childhood and adolescence. As GJC2 is involved in gap junction formation it has been hypothesized that disease variants in GJC2 impact the gap junctions’ function for pulsatile lymphatic flow (Ferrell et al., 2010). The amino acid change found in individual CHT31_501, p.Ser259Pro is located in the extracellular part of the highly conserved connexin domain (Figure 1a). This location is in direct proximity to p.Arg260Cys, which has been found causative for familial lymphedema (Ferrell et al., 2010) suggesting possible implication of the p.Ser259Pro change in the expression of CCT in our patient.

Second, we identified a novel heterozygous missense variant in FLT4 (c.G3827T, p.Gly1276Val) in patient CHT25_501. The male patient CHT25_501 was delivered by spontaneous vaginal delivery as the first child of a second gravida with a gestational age of 38 + 5 weeks and a birth weight of 3480 g. He was prenatally diagnosed with bilateral pleural effusions and received intrauterine pigtail catheter pleural drainage three times. The intrauterine recurrence of the pleural effusions without identification of a hemodynamic cause, rendered the diagnosis of isolated non-syndromic CCT most likely. Perinatally, the pigtail catheters were found in irregular extracorporal position. However, postnatal sonography showed remission of the pleural effusions with little or no effusions left. Mechanical ventilation was not necessary at any time. Postnatal sonographic examination of the brain, abdomen, and heart did not show any pathological findings. No infectious or any other metabolic cause for the pleural effusions could be identified. He was discharged home in good clinical condition after 9 days. Patient CHT25_501 inherited the FLT4 variant from his unaffected mother (CHT25_402) who had an abortion of a 13-week fetus (CHT25_502) with a severe congenital heart defect. DNA of this fetus is not available. The FLT4 variant (c.G3827T, p.Gly1276Val) is not reported in gnomAD, its residue is conserved in vertebrates and the amino acid change is predicted to be deleterious by all four in-silico prediction tools, and is therefore fulfilling the ACMG criteria of VUS (PM2, PP3) (Table 1). FLT4, also known as VEGFR3, encodes for vascular endothelial growth factor receptor 3 (VEGFR3, Uniprot: P35916), a tyrosin-protein kinase acting as cell-surface receptor for VEGFC and VEGFD. The protein consists of an extracellular region with multiple immune globulin domains, a transmembrane domain, and a
cytoplasmic region harboring the tyrosine kinase catalytic domain. FLT4 has been associated with various types of autosomal dominant inherited congenital heart defects (MIM: 618780), autosomal dominant juvenile capillary hemangioma (MIM: 602089), and autosomal dominant lymphatic malformation (MIM: 153100). The phenotypic spectrum in families with autosomal dominant FLT4 associated lymphatic malformations varies from asymptomatic individuals, early-onset peripheral lymphedema of different severity to congenital hydrothorax with hydrops fetalis, even within a single family with the same variant (Ferrell et al., 1998). The reported penetrance of hereditary lymphedema in families with FLT4 variants is 88% (Spiegel et al., 2006). Most of the variants reported to cause lymphatic malformations are located within the tyrosine kinase catalytic domain (pp. 845–1173) (Evans et al., 2003). However, the here identified FLT4 variant in family CHT25 resides within the cytoplasmic domain of the VEGFR3 receptor but not the tyrosine kinase catalytic domain (Figure 1b). The novelty of the here identified variant, and its deleterious prediction suggest its possible implication in the expression of CCT in patient CHT25_501. Reduced penetrance can explain the healthy appearance of the variant-carrying mother. In a targeted interview with the mother, questions concerning various symptoms of lymphatic malformations were answered in the negative, confirming her as an unaffected individual. This finding is in line with Ferrell et al.’s report on reduced penetrance and variable expressivity of lymphatic malformations caused by FLT4 variants. Whether the aborted fetus with a congenital heart defect also carried the variant remains elusive. The GeneMatcher submission did not yield any further CCT patients with variants in FLT4, VEGFC, or GJC2.

To summarize, our targeted sequencing approach of three VEGFR3 pathway genes identified two VUS in GJC2 and FLT4. Genetic variants in other sequences of the genome cannot be captured with the here applied method. While there is no functional data to support the identified variants in GJC2 and FLT4 as disease causing to this point; our a priori hypothesis for the implication of both genes in the formation of isolated CCT was based on the preexisting scientific literature describing CCT as an associated phenotypic feature for FLT4 associated lymphatic malformation 1 (MIM: 153100), and GJC2 associated lymphatic malformation 3 (MIM: 613206) (Ghalamkarpour et al., 2009; Munger et al., 2017). The identification of two novel variants, both conserved in vertebrates with the respective amino acid changes predicted to be deleterious by all in-silico prediction tools used, renders it possible that they are implicated in isolated primary CCT. To confirm our findings of the VEGFR3 pathway genes GJC2 and FLT4 as potentially novel disease genes for CCT, the identification of additional patients carrying potential disease-causing variants and functional studies are warranted. For example, RNA studies in newborn patients with acute CCT could assess the temporo-spatial expression of disease causing genes to further explore the

### Table 1: Molecular details and clinical features of individuals with variants in GJC2 and FLT4

| Family details | Family CHT 31 | Family CHT 25 |
|----------------|--------------|--------------|
| Individual CHT 31_501 | CHT 25_501 | CHT 25_502 |
| Zygosity | Heterozygous | Heterozygous | no DNA |
| Inheritance | N/A | Autosomal dominant | N/A |
| GeneVariant location | GJC2 (NM_020435) c.T775C, p.S259P | FLT4 (NM_182925) c.G3827T, p.G1276V |
| Variant consequence | Missense | Missense | N/A |
| ChromosomeDNA location | Chr1 (NC_00001.10) g.228346234T>C | Chr5 (NC_00005.9) g.180036034G>T |
| Exon | 2/2 | 29/30 | N/A |
| GnomAD MAF | Not reported | Not reported | N/A |
| Polyphen-2 | Probably damaging | Probably damaging | N/A |
| SIFT | Deleterious | Deleterious | N/A |
| Mutationtaster | Disease causing | Disease causing | N/A |
| CADD | 23.7 | 32 | N/A |
| ACMG criteria | VUS (PM2, PP3) | VUS (PM2, PP3) | N/A |
| Clinical features | | | |
| Ethnicity | Caucasian | Caucasian | |
| Gender | Male | Male | N/A |
| Age of onset | Congenital | Congenital | Congenital |
| Primary phenotype | Bilateral chylothorax with hydrops fetalis | Bilateral pleural effusions | Heart defect |
| Secondary phenotype | No | No | Nuchal edema |
| Prenatal intervention | No | Intrauterine pigtail catheter | ToP at 13 weeks of gestation |

Abbreviations: N/A, not applicable; ToP, termination of pregnancy; VUS, variant of uncertain significance.
pathomechanisms of CCT. Unfortunately, respective tissue is not available for the patients presented in this study. In this respect, the affected tissue is likely to be the lymphatic vessel system, which was not assessed by biopsies in the patients included in this study. Hence, analysis of possible disease variants would require testing of these variants in a vertebrate model system like zebrafish reporter lines, for example, Tg(batf3MIN:eGFP) (Frétaud et al., 2021), which were beyond the scope of the present study.

Overall, our study suggests GJC2 and FLT4 as putative candidate genes for primary isolated CCT and would represent a phenotype expansion to the previously reported CCT as part of a syndromic lymphatic disease. The implication of these VEGFR3 pathway genes in primary CCT might provide access to distinct druggable targets for monoclonal antibodies and kinase inhibitors. This would be a novel approach to the currently limited causal treatment options for CCT.

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CONFLICT OF INTEREST
All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS
Heiko Reutter and Andreas Müller initiated the study. Heiko Reutter acquired the respective funding and provided the laboratory resources. Sophia Schneider, Jeshurun C. Kalanithy, Annegret Geipel, Brigitte Strizek, Heiko Reutter, and Andreas Müller collected the patients with clinical information and DNA that build the basis for the genetic analysis of this study. Sophia Schneider, Ricarda Köllges, Jil D. Stegmann, Frederic Thieme, Alina C. Hilger, Lea Waffenschmidt, Julia Fazaal, Kerstin U. Ludwig, and Heiko Reutter planned and performed the MIP experiments. Sophia Schneider together with Ricarda Köllges, Jil D. Stegmann, Frederic Thieme, and Heiko Reutter evaluated the MIP data. Sophia Schneider, Jeshurun C. Kalanithy and Heiko Reutter designed the figure. Sophia Schneider and Heiko Reutter took the main lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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