**Original research**

**Biallelic ANGPT2 loss-of-function causes severe early-onset non-immune hydrops fetalis**

Marie F. Smeland, Pascal Brouillard, Trine Prescott, Laurence M Boon, Bodil Hvingel, Cecile V Nordbakken, Mona Nystad, Øystein L. Holla, Miikka Vikkula

**ABSTRACT**

**Background** Hydrops fetalis, a pathological fluid accumulation in two or more body compartments, is aetiologically heterogeneous. We investigated a consanguineous family with recurrent pregnancy loss due to severe early-onset non-immune hydrops fetalis.

**Methods and results** Whole exome sequencing in four fetuses with hydrops fetalis revealed that they were homozygous for the angiopoietin-2 (ANGPT2) variant Chr8 (GRCh37/Hg19): 6385085T>C, NM_001147.2:c.557A>G. The substitution introduces a cryptic, exonic splice site predicted to result in loss of 10 nucleotides with subsequent shift in reading frame, leading to a premature stop codon. RNA analysis in the heterozygous parents demonstrated loss of detectable mutant allele, indicative of loss-of-function via nonsense-mediated mRNA decay. Serum ANGPT2 levels were reduced in the parents. In a pregnancy with a healthy, heterozygous child, transiently increased fetal nuchal translucency was noted.

**Conclusion** Pathogenic heterozygous ANGPT2 missense variants were recently shown to cause autosomal dominant primary lymphoedema. ANGPT2 is a ligand of the TIE1-TIE2 (tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 and 2) pathway. It is critical to the formation and remodelling of blood and lymphatic vessels and is involved in vessel maintenance. ANGPT2 knockout mice die from generalised lymphatic dysfunction. We show here that a homoygous pathogenic variant causes loss-of-function and results in severe early-onset hydrops fetalis. This is the first report of an autosomal recessive ANGPT2-related disorder in humans.

**INTRODUCTION**

Hydrops fetalis is a pathological accumulation of fluid in two or more body compartments. This aetiologically heterogeneous condition is caused by increased interstitial fluid production or decreased lymphatic return, and may result in increased nuchal translucency, lymphatic malformations (e.g. cystic hygroma), subcutaneous fluid accumulation, pleural/pericardial effusion, ascites and placentation thickening.

Historically, red cell allo-immunisation was the major cause of hydrops fetalis. Today, non-immune hydrops fetalis (NIHF; Mendelian Inheritance in Man, MIM: 236730) constitutes approximately 90% of cases. The estimated prevalence of hydrops fetalis ranges from 1:1500 to 1:4000 births. Early onset and aetiology are prognostic predictors in NIHF. Presentation in the first trimester increases the likelihood of a fetal aneuploidy. In a prenatal series of more than 12,000 pregnancies, almost half of 66 NIHF cases were attributed to a chromosomal abnormality. Aneuploidy conferred an extremely poor prognosis. In liveborn infants with NIHF, neonatal mortality is as high as 60%.

Bellini et al. presented a classification scheme for NIHF based on underlying mechanism, including chromosomal imbalance, infection, placental abnormality, twin-to-twin transfusion, cardiovascular disorders, syndromes and lymphatic dysplasia. In an update, they classified 19.8% of cases as idiopathic. In a recent review including only cases meeting the strict criteria for NIHF, the aetiology was identified in 86.3%. The most prevalent aetiologies in the study by Laterre et al. were chromosomal imbalance (33 of 102; 32.4%), lymphatic dysplasia (14 of 102; 13.7%) and cardiovascular disorders (10 of 102; 9.8%). The authors also developed a flow chart outlining a systematic diagnostic approach that emphasises the importance of next-generation sequencing (NGS).

In a series of 127 cases of prenatally diagnosed NIHF, which were aetiologically unresolved by standard analyses, a genetic cause was detected in 29% by whole exome sequencing (WES). A comprehensive systematic review of monogenic causes of NIHF identified 517 candidate genes, including 177 for which evidence was strong or emerging. Of these 177, 5.1% were lymphatic-related genes. Autosomal recessive inheritance was the most frequent (61.8%) when aetiology was known, although all Mendelian modes of inheritance were represented.

NIHF due to lymphatic abnormalities is a form of generalised lymphatic dysplasia, a subgroup of primary lymphoedema. In Genomics England’s PanelApp, evidence of an association between pathogenic variants in 40 genes and primary lymphoedema is strong (‘green’ category genes; panel version: 2.12, 13 March 2021). For fetal hydrops (panel version 1.25, 13 March 2021), the evidence for causation is strong for 56 genes. The two panels overlap with 19 genes.

Subsequent to the original publication of primary lymphoedema involving the vascular endothelial growth factor 3 gene (VEGFR3, encoded by the FLT4 gene), the central role of VEGFR3 in
signalling was identified. Genes more recently added to the pathway include ADAMTSL3, FBXL7 and ARAF. New pathways involving lymphangiogenesis and remodelling have also been identified and involve genes such as PIEZO1, EPHB4, CALCR and GDF2.

In 2020, heterozygous missense mutations in angiopoietin-2 (ANGPT2) were reported to cause primary lymphoedema with reduced penetrance and variable expressivity in five unrelated families, implicating another endothelial growth factor receptor pathway, the ANGPT2–TIE2 tyrosine kinase pathway.

Here we report a consanguineous couple with at least four fetuses with severe early-onset NIHF and homozygosity for a loss-of-function (LOF) variant in ANGPT2. This work underscores the pivotal role of ANGPT2 in lymphatic development and adds to the growing list of genes associated with NIHF.

### PATIENTS AND METHODS

#### Methods

**Nucleic acid extraction**

DNA was extracted from EDTA blood with EZ1 DSP DNA Blood Kit using an EZ1 Advanced XL (QIAGEN) from amniocytes (for WES) and chorion villus biopsies (for SNP array analysis) using QiaSymphony (QIAGEN) or QIAamp DNA Micro Kit (QIAGEN). For quantitative fluorescent (QF)-PCR analysis of amniocytes and chorion villus biopsies, rapid DNA extraction was performed using InstaGene Matrix Chelex 100 (Bio-Rad, California, USA).

RNA was extracted from whole blood using TRIzol reagent (Invitrogen, California, USA) and retrotranscribed with moloney murine leukaemia virus RT (ThermoFisher, Massachusetts, USA). For RT-PCR, primers were chosen in exons distant from those carrying the pathogenic variant NM_001147.2: c.557A>G and

#### Table 1  Clinical information of the members of a family with ANGPT2 c.557A>G c.557_566del

| Individual/pregnancy | III-3 | III-4 | III-5 | III-7 | III-8 | III-9 | II-1 | II-2 | II-6 |
|----------------------|-------|-------|-------|-------|-------|-------|------|------|------|
| **Phenotype**        | Healthy girl | IUFD, week 24+2 | IUFD, week 27+3 | TOP, week 14 | Miscarriage, week 14 | Healthy boy | Healthy father | Healthy mother | Maternal aunt |
| **Sex**              | Female | Male | Female | Male | Male | Male | Female | Female | Female |
| **Sample/analysis**  | Blood | Amnion | CVS | Amnion | Fibroblasts | Umbilical cord | Blood | Blood | Blood |
| **Analysis**         | WES | WES | WES | WES | WES | Sanger | WES | WES | Sanger, WES |
| **Genotype**         | Het | Hom | Hom | Hom | Hom | Het | Het | Het | WTTW |
| **Outcome**          | Healthy. | Severe, progressive hydrops, IUFD week 24. | Severe, progressive hydrops, IUFD week 27. | Early detected hydrops, TOP week 14. | Early detected severe hydrops, spontaneous abortion week 14. | Healthy. | Healthy. | Healthy. | ‘Oedema’ in childhood, lower extremity lymphoedema. |
| **Early US, including NT** | Week 11+2 normal, no measurement of NT. | Week 11 normal, no measurement of NT. | Week 12+3 NT 4–5, cystic hygroma subcutaneous fluid. | Week 12+4 cystic hygroma, subcutaneous fluid. | Week 11+0 NT 3.7, generalised subcutaneous fluid, cystic hygroma. | Week 12+4 NT 4.5 | – | – | – |
| **Later US**         | Week 18 normal. | Week 19 normal. (LMP). | Week 16 hydrothorax, subcutaneous fluid, cystic hygroma. | Week 14+4 cystic hygroma and hydrothorax. | Week 12+1 NT 6–7mm. | Week 14+2 NT normal. | – | – | – |
| **PM**               | NA | Yes | Yes | NA | NA | NA | – | – | – |
| **Cardiac**          | NA | Tetralogy of Fallot | Normal at PM | NA | NA | NA | – | – | – |
| **Skeletal/growth**  | NA | 2-week discrepancy EDD from LMP and from US. | Short long bones, bell-shaped thorax (due to hydrothorax/ hypoplastic lungs. | NA | NA | NA | – | – | – |
| **IHC in lymphatic endothelium** | NA | No observed increase in number of lymphatic vessels | No observed increase in number of lymphatic vessels | NA | NA | NA | – | – | – |
| **Placenta**         | NA | Hydropic placenta. Thick (3 cm), oedematous. Weight 360g. | Hydropic placenta. Thick (2.9 cm). Weight 440g. Abnormal vascular architecture/ proliferation of vessels. | NA | NA | NA | – | – | – |

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CVS, chorionic villus biopsy; EDD, estimated due date; Het, heterozygous; Hom, homozygous; IHC, immunohistochemistry; IUFD, intrauterine fetal death; LMP, last menstrual period; NA, not assessed/not applicable; NT, nuchal translucency; PM, postmortem examination; TOP, termination of pregnancy; US, ultrasound; WES, whole exome sequencing; WT, wild-type.
the benign marker variant NM_001147.2:c.882G>A (sequences available on request). RNA was also isolated from cultured fibroblasts derived from a paternal skin biopsy.

ELISA measurements of ANGPT2

ANGPT2 protein levels were measured in blood-EDTA plasma using the Human Angiopoietin-2 Quantikine ELISA Kit (R&D Systems, Minnesota, USA), according to the manufacturer’s protocol.

Whole exome sequencing

Nextera Rapid Capture Exome sample prep and enrichment (Illumina, California, USA) were carried out according to the manufacturer’s instructions. Libraries were sequenced 2×150 bp on a NextSeq500 (Illumina). Bcl2fastq2 conversion software (Illumina) was used for de-multiplexing. Reads were aligned to reference genome GRCh37/hg19 using Burrows-Wheeler Aligner, BWA. Picard was used to remove PCR duplicates. The Genome Analysis Toolkit was applied for base quality score recalibration, insertion and deletion (INDEL) realignment, and single nucleotide variant and INDEL discovery. Sequence variants were annotated with Annovar. Regions of homozygosity were detected using BCFtools/Samtools. Filtus software was used for bioinformatic filtering of variants. Integrative Genomics Viewer was used for NGS data visualisation. Splice site predictions were performed using SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder through Alamut Visual software (Interactive Biosoftware, Rouen, France).

Samples from both parents, four affected fetuses and one clinically unaffected child were exome-sequenced. ANGPT2 was analysed by NGS in a blood sample from the maternal aunt (II-6) as a control of her Sanger sequencing result.

Sanger sequencing

DNA from samples II-6 (including a control sample) and III-9 were Sanger-sequenced on an ABI 3130xl using standard protocols to determine ANGPT2 genotype. For the other samples, Sanger sequencing was used to verify ANGPT2 results from NGS.

QF–PCR (III-4, III-5 and III-7)

QF-PCR/rapid aneuploidy diagnostics test was performed with the Elucigene QST*Rplus2 Kit (Elucigene Diagnostics, Manchester, England). Fragments were analysed using capillary electrophoresis on ABI 3500xl Genetic Analyzer (Applied Biosystems, California, USA) with accompanying GeneMapper software according to the manufacturer’s protocols.

Standard G-banding/karyotyping of amniocytes and parental blood samples (III-4, II-1 and II-2)

G-banding of metaphase chromosomes was performed on amniocytes from fetus III-4 and sodium-heparin peripheral blood samples from the parents (II-1 and II-2) according to standard protocols.

SNP array analysis (III-4, III-5 and III-7)

Molecular karyotyping of DNA extracted from amniocytes and chorion villi was performed using SNP array analysis (CytoScan HD; Affymetrix, California, USA). Filtering of data from SNP array analysis was performed using ChAS (Chromosome Analysis Suite) software (Affymetrix) and Cartagenia Bench Lab CNV (Agilent Technologies, California, USA). Positions refer to Genome Reference Consortium Human Genome Build 37 (GRCh37) (hg19). The following were the filter settings for analysis: 400 kb and 30 markers for loss, 90 markers for gains; 5 Mb and 50 SNP markers for regions of homozygosity.

Lymphohistochemical staining in postmortem samples (III-4 and III-5)

Lymphohistochemical staining in skin/subcutaneous tissue from the neck and lung was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections with marker D2-40 (podo- planin) (Roche) for lymphatic endothelium.

Placental examination (III-4 and III-5)

Placental FFPE tissue was stained with H&E according to the manufacturer’s protocol to visualise the microanatomy of the placenta (nuclear components, cytoplasmic collagen and elastic fibres, muscle fibres and red blood cells).
RESULTS

Clinical summary

The clinical and molecular findings are summarised in table 1 and figure 1.

The healthy mother (II-1) and father (II-2) are second cousins once removed and originally from central Asia. She has polycystic ovary syndrome. Both parents have normal haemoglobin profiles and neither harbours an alpha-globin deletion. Maternal parvovirus serum IgG in the fifth pregnancy was indicative of previous infection. Screening for maternal erythrocyte and platelet antibodies was negative. Standard karyotyping (G-band analysis) of the parents was normal. There is no clinical suspicion of lymphoedema in either parent, although their prenatal and neonatal histories are not available.

The mother presented at age 26 years in the second trimester of her fourth pregnancy (III-4) due to fetal hydrops. Fetal ultrasound at 11+5 weeks had been reported as normal, although nuchal translucency was not assessed. Routine ultrasound at 19+5 weeks revealed a male fetus (III-4) with severe hydrops. Additional findings included gestational age by ultrasound at 17+4 weeks, no evidence of fetal anaemia based on cerebral artery Doppler, and negative rapid testing for trisomy 13, 18 and 21 and cytogenetic karyotyping in amniocytes. Maternal serology and PCR analysis of amniotic fluid detected no evidence of a viral infection. At 20 weeks, fluid accumulation was increased and tetralogy of Fallot was noted. Intrauterine death occurred at 26+3 weeks.

The mother’s first pregnancy ended in an early miscarriage (III-1).

The couple has a healthy daughter (III-2) born following a normal pregnancy in which an ultrasound at 11+2 weeks was reported as normal, although nuchal translucency was not assessed. She is now school-aged and there is no suspicion of oedema anamnestically.

The third pregnancy resulted in a miscarriage (III-3) at 9 weeks.

In the fifth pregnancy, severe hydrops including a large nuchal cyst was detected at 12 weeks in the female fetus (III-5). Results of rapid testing for trisomy 13, 18 and 21 and chromosomal microarray on DNA extracted from a chorionic villus biopsy were normal. The hydrops progressed and the couple was informed of a poor prognosis, including the risk of maternal mirror syndrome. Fetal demise occurred at 27 weeks (figure 2). Autopsy revealed severe hydrops with hydrothorax, hypoplastic lungs, a bell-shaped thorax, short long bones and a hydropic placenta.

The sixth pregnancy ended in a termination (III-6) after detection of a blighted ovum at 10+4 weeks.

The seventh pregnancy was terminated (III-7) at 15 weeks after detection of severe subcutaneous oedema and a cystic hygroma at 12+4 weeks. Rapid aneuploidy testing was normal. Chromosomal microarray was normal and demonstrated homozygosity stretches in common with fetuses III-4 and III-5, in keeping with parental consanguinity.

The eighth pregnancy ended in a miscarriage (III-8) at 14–15 weeks following detection of increased nuchal translucency (week 11+0, 3.7 mm, >99th centile) and severe generalised subcutaneous fluid accumulation.

In the ninth pregnancy (III-9), ultrasound at 12+2 gestational weeks showed increased nuchal translucency (4.5 mm, >99th centile) with a narrow brim of subcutaneous fluid extending to the upper back. However, at 14+2 weeks no abnormal fluid accumulation was seen. Pregnancy proceeded uneventfully and a healthy male infant was born at term. He is now 12 months old and there has been no suspicion of oedema at any time.

Clinical assessment of additional family members

Both parents have several siblings, but only the maternal 30-year-old sister (II-6) has been available for investigation. She reported childhood ‘oedemas’, but her paediatric record is unavailable. In young adulthood, she presented with intermittent swelling of the ankles and feet, especially on the left. Lymphoscintigraphy of her lower extremities failed to visualise the lymphatic vessels or the inguinal nodes on the left. In the right leg, delayed clearance and several foci of increased uptake were interpreted as evidence of abnormal lymphatic vessels morphology.

Figure 2 Photograph of a fetus (III-5) with intrauterine death at 27 gestational weeks. Note the severe subcutaneous oedemas, deformed facial features and large cystic hygroma of the neck. Created by the authors.
**Whole exome sequencing**

WES revealed rare, coding or splice site variants compatible with autosomal recessive inheritance in seven genes not previously been associated with hydrops fetalis (table 2). These were submitted to GeneMatcher. The only potentially relevant match was for ANGPT2, a case series with autosomal dominant lymphoedema, which was then unpublished. Subsequently, in a new pregnancy with an affected fetus (III-8), exome sequencing excluded all candidate genes except ANGPT2. The detected variant in our family, (GRCh37/Hg19): 8:6385085T>C, NM_001147.2:c.557A>G p.(Asp186Gly) in exon 3, was identified in the homozygous state in all investigated hydropic fetuses and in the heterozygous state in the unaffected parents (figure 1 and table 1). The ANGPT2 variant was absent from gnomAD and predicted activation of a cryptic 5' splice site between c.556 and c.557, leading to removal of 10 bp from the 3' end of exon 3 and introduction of a premature termination codon: r.557_566del p.(Asp186Valdelfs*3).

Individual II-6 did not carry ANGPT2 c.557A>G. No pathogenic or likely pathogenic variants causative of monogenic lymphoedema were detected in the 40 genes subsequently analysed (PanelApp’s ‘green’ genes, primary lymphoedema; V.2.2, 6 June 2021).

**Sanger sequencing**

The unaffected brother (III-9) was heterozygous for NM_001147.2:c.557A>G, whereas the maternal aunt (II-6) did not carry the variant (a control sample was also analysed by Sanger sequencing and by WES).

**mRNA analyses**

mRNA from the heterozygous parents showed only the wild-type (WT) allele at position c.557 (figure 3). Loss of the allele carrying the likely pathogenic variant c.557A>G was confirmed by genotyping a second variant NM_001147.2:c.882G>A p.(Thr294=) in exon 5, heterozygously present in both parents’ DNA. Only the G-allele was observed in mRNA from fibroblasts and lymphocytes. Thus, both parents expressed only a single WT ANGPT2 allele, in keeping with degradation of the mutant allele with a premature termination codon by nonsense-mediated mRNA decay.

To confirm that ANGPT2 expression was reduced, plasma concentrations were measured by ELISA. The heterozygous father’s plasma concentration was reduced (672.2 pg/mL) compared with non-pregnant controls; however, it was approximately half of the mean level (18 900 pg/mL) in pregnant controls (table 3).

| Individual | Gender | Mutation | ANGPT2 plasma concentration (pg/mL) |
|------------|--------|----------|------------------------------------|
| Control 1  | Female | –        | 1690                               |
| Control 2  | Female | –        | 1197                               |
| Control 3  | Male   | –        | 2354                               |
| Control 4  | Female | –        | 1729                               |
| Control 5  | Female | –        | 1419                               |
| Control 6  | Male   | –        | 2297                               |
| Control 7  | Male   | –        | 1479                               |
| Control 8  | Female | –        | 2844                               |
| Control 9  | Female | –        | 4751                               |
| LE-623-10* Female | c.1475G>A | 1247 |
| II-1       | Male   | c.557A>G | 672                                |
| II-2       | Female | c.557A>G | 82941                              |

*Patient previously published. **The mother (II-6) was pregnant at the time of sampling. ANGPT2, angiopoietin-2.
Lymphohistochemical staining and placental examination
No obvious lymphatic abnormalities were noted, possibly due to substantial autolysis, which was present in tissue specimens from the affected fetuses. Placentas from pregnancies III-4 and III-5 were thick and oedematous (table 1). Abnormal maturation of chorionic villi was present in III-5.

DISCUSSION
Two of the authors recently reported heterozygous pathogenic sequence variants in ANGPT2 as a cause of dominantly inherited primary lymphoedema.26 Here, we report a homozygous variant, NM_001147.2:c.557A>G.557_S66del, in a consanguineous family with recurrent, severe, early-onset hydrops fetalis. The unaffected parents and their two unaffected children are heterozygous and the four affected fetuses homozygous for this LOF variant.

ANGPT2 c.557A>G activates a cryptic splice site and prompts nonsense-mediated mRNA decay, NMD/LOF
Rather than having an effect via an amino acid substitution, the variant activates a cryptic RNA splice site, predicted to remove 10bp from the coding sequence, leading to a premature stop codon after the third of nine exons, (p.Asp186Valfs*3). Nonsense-mediated mRNA decay results in destruction of this mutant transcript. As confirmed in the unaffected parents, the loss of one allele is reflected by a halving of the expected amount of ANGPT2 plasma protein. ANGPT2 levels in the unaffected heterozygous children have not been assayed as their samples were unavailable. In the homozygous affected fetuses, there was likely a complete absence of ANGPT2 with resultant impaired lymphatic development and severe, lethal hydrops fetalis. Lymphatic dysplasia was not histologically evident in the skin and lung specimens from two affected fetuses. However, at the time of microscopy, autolysis was in process (data not shown). Taken together with earlier reports in humans and the murine model, we nonetheless believe that lymphatic dysplasia is the likely underlying cause of ANGPT2-related fetal hydrops.

Angpt2 knockout mice have disturbed angiogenetic remodelling
The murine knockout mimics the severe phenotype described in this family. In the vascular system, ANGPT2 is considered an antagonist of ANGPT1-induced TIE1-TIE2 (tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 and 2) signalling, while it seems to play a comparatively prominent activating role in lymphatic vasculature. Angpt2 knockout mice have normal embryonal vascular development, but disrupted postnatal angiogenic remodelling.24 In addition, they exhibit major lymphatic vessel defects and generalised lymphatic dysfunction, including chylous ascites. Large lymphatic vessels are replaced by a ragged mesh, and small vessels exhibit abnormal patterning.25 The lymphatic phenotype, but not the blood vascular remodelling defects appearing later in development, can be rescued by expressing Angpt1 instead of Angpt2 in the Angpt2 locus, showing an activating role of Angpt2 in lymphatics, and in contrast an antagonistic role in blood vessels.26 ANGPT2 blocking antibodies also affect the formation of button-type lymphatic endothelial cell (LEC) junctions, adherence junction stability or lymphatic valve formation and maturation of collecting lymphatic vessels in mouse embryos.26 In contrast to the fetuses in our family who died before 27 weeks of gestation, mice homozygous for deletion of Angpt2 were born at normal frequencies, but die from severe chylous ascites and other lymphatic defects by 2 weeks postnatally.26 Rare mice that survived to adulthood had severe ascites and lymph stasis.26

Heterozygous ANGPT2 LOF mutations cause mild congenital lymphoedema
In the original report of seven affected individuals from five families with heterozygous ANGPT2 mutations, the disorder was characterised by mild lymphoedema in early life, with resolution in three individuals during the first months after birth or adolescence and reduced penetrance in two unaffected mutation carriers.23 A heterozygous whole-gene deletion was identified in a 1.5-year-old boy followed for lower leg oedemas first noted at age 8–9 weeks.24 Three reported amino acid substitutions (Asn304Lys, Cys435Ser and Arg492Gln) resulted in LOF together with partially inhibited secretion of the WT allele in vitro, which may have a partial dominant-negative effect on ANGPT2 signalling.24 Variable effects on ANGPT2 are further exemplified by a fifth mutation (Thr299Met) that leads to gain-of-function, inducing increased lymphatic proliferation when expressed in the mouse ear.24 The associated clinical phenotype was progressive, hypothesised to be due to hyperplasia. However, genotype-phenotype correlations are tentative due to the low number of cases.24 These results indicate an optimal window of activity for ANGPT2, from which deviation upwards or downwards may significantly hamper development of lymphatic vasculature.

Is there a lymphoedema phenotype in c.557A>G heterozygotes?
Heterozygosity for c.557A>G causes haploinsufficiency, and we aimed to delineate the phenotype of the heterozygotes in our family. In individual III-9, significantly increased nuchal translucency was detected on ultrasound at week 12 but was completely resolved 2 weeks later. Pregnancy was monitored closely, and there were no other signs of oedema in the fetus or the mother. The healthy newborn boy was not oedematous, and no oedemas developed by age 12 months. Heterozygosity for the pathogenic sequence variant might explain his transiently and significantly increased nuchal translucency; no other likely cause was found. Indeed, one of the individuals reported by Leppänen et al.24 also had increased nuchal translucency early in pregnancy. In our family, there is no evidence of primary lymphoedema in the heterozygous child nor in the parents, although pregnancy and neonatal records were not available for all. Thus, postnatal transient lymphoedema cannot be excluded. Furthermore, genetic predisposition may play a role when secondary lymphoedema develops in other contexts, for example, after surgery for breast cancer.34 35 Cellulitis is a well-recognised complication of primary lymphoedema.36 Whether or not clinically unaffected heterozygotes for a pathogenic ANGPT2 variant are at increased risk of secondary lymphoedema and subsequently for cellulitis is not known.

What is the cause of lymphoedema in individual II-6?
Lymphoscintigraphy of individual II-6 revealed lower extremity lymphoedema, abnormal morphology of lymphatic vessels in the right leg and absence of detectable lymphatic vessels in the left leg. Sanger sequencing of two separate blood samples and targeted NGS failed to identify the likely pathogenic variant in ANGPT2 or any likely pathogenic variant in primary lymphoedema genes. Thus, the cause of her lymphoedema remains unknown.

Phenotype in the affected fetuses
This family demonstrates uniquely the natural course of complete LOF of ANGPT2 in humans. With one exception, the affected
pregnancies were not terminated, but monitored until fetal demise. All three affected fetuses, for which a nuchal translucency measurement in the first trimester was available (III-5, III-7 and III-8), showed increased values, approaching what is seen in nuchal lymphatic malformations (cystic hygromas). Progression of hydrops was gradual from increased nuchal translucency to cystic hygroma, extensive subcutaneous fluid accumulation, hydrothorax, and lastly ascites, with intrauterine death occurring between weeks 14 and 27. The significantly, but transiently, increased nuchal translucency in a heterozygous unaffected fetus (III-9) underscores the importance of repeated ultrasounds in the context of increased nuchal translucency of unknown cause. We noted no specific feature that distinguishes ANGPT2-related hydrops from other forms of NIHF.

Additional phenotypic features in the homozygous fetuses
Fetus III-4 had tetralogy of Fallot and fetus III-5 had a structurally normal heart. Two fetuses (III-7 and III-8) died too early to allow thorough assessment of cardiac anatomy. In a large cohort of individuals with tetralogy of Fallot, there was no increased burden of rare variants in ANGPT2 (K Devriendt, MD, personal communication, December 2020). Thus, it is unclear whether congenital heart malformations are a feature of the homozygous ANGPT2 phenotype. WES of fetus III-4 did not reveal any other pathogenic or likely pathogenic variants in genes known to be associated with tetralogy of Fallot.

Fetus III-5 revealed short long bones radiographically. This could have been due to general growth retardation via placental dysfunction or more specifically related to vascular/lymphatic abnormalities.

Fetus III-4 was probably growth retarded given the 2-week discrepancy between estimated due date based on last menstrual period versus sonographic estimation of gestational age. The length of the long bones was not commented on autopsy.

ANGPT2 bialelic variants: a rare cause of hydrops fetalis
Biallelic LOF variants are likely a rare cause of recessive lymphatic-related hydrops fetalis. We are not aware of any other biallelic ANGPT2 LOF variants in other cohorts of fetal hydrops to date, and no other match for ANGPT2 was identified via GeneMatcher. The condition seems to be very severe, perhaps even universally lethal. Other cases may remain undiagnosed due to early fetal demise in the absence of molecular genetic investigations, as suggested by Quinn et al. In the same signalling pathway as ANGPT2, other genes have known phenotypes related to inherited monol- lelic or somatic variants. Somatic and constitutional variants in the TIE2-encoding gene TEK cause venous malformations via a gain-of-function mechanism, while constitutional LOF variants may underlie primary congenital glaucoma. ANGPT1 variants are reported in primary congenital glaucoma and hereditary angio-oedema. Homozygous LOF of ANGPT1 and TEK is expected to be embryonically lethal in humans, as in mice, due to defective blood vessel formation, but not expected to cause fetal hydrops.

WES in the diagnostic work-up of NIHF
The Society for Maternal-Fetal Medicine’s guidelines for the diagnostic work-up of NIHF from 2015 does not address testing for monogenic disorders. However, given the rapid identification of new single gene disorders, WES is recommended as the preferred sequencing approach for NIHF. The likelihood of identifying new disease genes via single cases is high given the severity and decreased viability of the phenotype, as in this family.

CONCLUSION
In conclusion, we report the first family with severe, early-onset hydrops fetalis caused by a homozygous LOF variant in ANGPT2. Our findings are in accordance with the previous identification of heterozygous missense variants causing autosomal dominant primary lymphoedema and with the crucial role of ANGPT2 in lymphatic development demonstrated by the murine knockout model. Biallelic ANGPT2 LOF is yet another of the many monogenic causes of NIHF. Subsequent studies may be able to confirm our suspicion of an underlying generalised lymphatic dysplasia. Heterozygous carriers of LOF variants may manifest signs of lymphatic insufficiency, such as dependent lymphoedema or transiently increased fetal nuchal translucency in early pregnancy.

Correction notice This article has been corrected since it was published online first. The gene name in the title has been italicised.

Twitter Miikka Vikkula @MiikkaVikkula

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Contributors All authors of this manuscript fulfill the criteria for authorship. PB and OLH performed molecular studies. MFS, BH, LMB and CVN conducted clinical investigations and collected patient samples. PB, MFS, TP, OLH, MN, CVN and MV planned the study. PB, MFS, TP, OLH, MN, BH and MV wrote the manuscript. All authors revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript. OLH is responsible for the overall content as guarantor.

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Competing interests PB has received support from Belgian Fonds de la Recherche Scientifique for attending meetings and/or travel.

Patient consent for publication Parental/guardian consent obtained.

Ethics approval The parents provided informed, written consent on behalf of themselves, their children and fetuses, as did the maternal aunt for inclusion in the study and for publication of clinical information, results of laboratory investigations and photographs, in accordance with IRB approval from the University of Louvain, Brussels, Belgium (B403201629786). Initial investigations were performed in a diagnostic setting.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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ORCID iDs Marie F Smeland http://orcid.org/0000-0002-1015-6338 Pascal Brouillard http://orcid.org/0000-0001-9548-8229 Laurence M Boon http://orcid.org/0000-0001-8273-3328 Mona Nystad http://orcid.org/0000-0002-0555-6951 Øystein L Holla http://orcid.org/0000-0002-7697-857X Miikka Vikkula http://orcid.org/0000-0002-6236-338X

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