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# High diagnosis rate for nonimmune hydrops fetalis with prenatal clinical exome from the Hydrops-Yielding Diagnostic Results of Prenatal Sequencing (HYDROPS) Study

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**PURPOSE:** Nonimmune hydrops fetalis (NIHF) presents as life-threatening fluid collections in multiple fetal compartments and can be caused by both genetic and non-genetic etiologies. We explored incremental diagnostic yield of testing with prenatal exome sequencing (ES) for NIHF following a negative standard NIHF workup.

**METHODS:** Participants enrolled into the Hydrops-Yielding Diagnostic Results of Prenatal Sequencing (HYDROPS) study met a strict definition of NIHF and had negative standard-of-care workup. Clinical trio ES from fetal samples and parental blood was performed at a CLIA-certified reference laboratory with clinical reports returned by geneticists and genetic counselors. Negative exomes were reanalyzed with information from subsequent ultrasounds and records.

**RESULTS:** Twenty-two fetal exomes reported 11 (50%) diagnostic results and five possible diagnoses (22.7%). Diagnosed cases comprised seven *de novo* dominant disorders, three recessive disorders, and one inherited dominant disorder including four Noonan syndromes (*PTPN11*, *RAF1*, *RIT1*, and *RRAS2*), three musculoskeletal disorders (*RYR1*, *AMER1*, and *BICD2*), two metabolic disorders (sialidosis and multiple sulfatase deficiency), one Kabuki syndrome, and one congenital anemia (*KLF1*).

**CONCLUSION:** The etiology of NIHF predicts postnatal prognosis and recurrence risk in future pregnancies. ES provides high incremental diagnostic yield for NIHF after standard-of-care testing and should be considered in the workup.

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## INTRODUCTION

Hydrops fetalis (HF) is abnormal fluid collections in multiple fetal compartments, such as skin edema, pulmonary effusions, or ascites observed during prenatal ultrasound. It affects at least 1.6 in 10,000 pregnancies and is associated with a high risk of fetal demise.[1] Multiple pathologic processes are implicated in HF including alloimmune hemolysis, infectious diseases, fetal anemia, cardiac and lymphatic malformations, metabolic derangements, and neurologic abnormalities.[2–6] HF resulting from Rh incompatibility has become rarer since the advent of routine Rho(D) immune globulin administration during at-risk pregnancies. Thereby, nonimmune HF (NIHF) represents the largest proportion of the epidemiologic burden. Many genetic causes of NIHF have clinical implications. During pregnancy, diagnosis guides clinical and familial decision-making by providing short- and long-term prognoses associated with genetic disorders including lifelong health concerns or targeted therapies, which can be initiated after birth. For future pregnancies, genetic diagnosis permits recurrence risk assessment and facilitates targeted prenatal genetic testing or preimplantation genetic testing for monogenic/single-gene defects (PGT-M).

Current clinical strategies for NIHF diagnosis include evaluations for alloimmune anemia, infectious etiologies, fetomaternal hemorrhage, and some genetic causes.[7] The recommended genetic workup includes chromosomal microarray to detect chromosomal copy-number variants (CNVs). Other testing may include biochemical testing[2,3] or gene panels for disorders known to be associated with hydrops, such as Noonan syndrome.[8] Modern genetic testing technologies permit large sequencing panels with some commercial panels simultaneously evaluating 130 genes associated with NIHF. As many rare diseases cause NIHF, these panels are far from comprehensive.[9] Exome sequencing (ES) involves broader genetic testing, evaluating the majority of disease genes with a single test. This is useful for many nonspecific clinical presentations of genetic disorders that present with overlapping and clinically indistinguishable phenotypes.[10] Studies applying prenatal ES to pregnancies with wide-ranging fetal presentations, including a limited number of hydropic fetuses, identified several novel gene associations for NIHF.[11–13] These studies were designed for disease gene discovery and prenatal phenotyping by enrolling cases with high likelihood of genetic etiologies such as consanguineous families.[14]

Multiple groups assessed the yield of prenatal ES for various indications. In one study of karyotypic normal fetuses with structural anomalies, ES identified genetic causes in 10% of cases.[15] In another study, ES identified diagnostic genetic variants in 8.5% of fetuses with structural anomalies which increased to 15.4% in fetuses with multisystem anomalies.[16] Another series demonstrated genetic diagnoses in 20.6% of fetuses with

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congenital anomalies.[17] By grouping multiple presentations into sequencing cohorts, these studies included few cases of NIHF.[16] However, in the PAGE study, 3/33 (9.0%) of fetuses with hydrops were diagnosed by ES. These pregnancies were enrolled at the time of hydrops diagnosis; therefore, this study provides an estimated yield of ES for NIHF before other clinical testing is performed to exclude common nongenetic etiologies. This likely underestimates the incremental diagnostic yield after negative standard-of-care evaluation. Recently, a series of 127 pregnancies reported a 29% diagnostic yield using a broader definition of NIHF, including cases with a single fetal compartment fluid collection[18] and a higher yield of 34% among the subset of 77 cases with two or more fetal fluid collections.

In our Hydrops-Yielding Diagnostic Results Of Prenatal Sequencing (HYDROPS) study, we present a prospective series of 22 pregnancies with NIHF based on *strict* phenotypic inclusion criteria of fluid collection in two or more fetal compartments (skin edema, ascites, pleural effusion, pericardial effusion) and a documented nondiagnostic workup based on Society for Maternal–Fetal Medicine (SMFM) guidelines for common infectious and immune etiologies, fetomaternal hemorrhage, and chromosomal disorders[7] to evaluate the incremental diagnostic yield of clinical trio ES for this indication.

## MATERIALS AND METHODS

We conducted a prospective study of sequentially referred pregnancies complicated by NIHF with cases referred from maternal–fetal medicine (MFM) practices across the country (14 states). The study was registered on ClinicalTrials.gov under identifier NCT03911531. Investigators at Thomas Jefferson University (TJU) enrolled participants from January 2019 to July 2020. Mother–father–fetus/neonate trios of NIHF pregnancies were recruited from multiple MFM divisions across the country after standard-of-care testing did not identify an etiology for the NIHF.

### Subject identification and enrollment

Participant screening ensured that study inclusion criteria were met. Inclusion criteria consisted of confirmation of hydrops fetalis after the first trimester and a complete negative workup for NIHF. We used *strict* phenotypic description for NIHF diagnosis including presence of at least two of the following: skin edema, ascites, pleural effusion or pericardial effusion. Increased nuchal translucency alone and isolated fluid collection in one fetal compartment (i.e., fetal ascites alone) did not satisfy the definition of NIHF. Neither polyhydramnios nor placentomegaly were considered criteria for NIHF diagnosis. All cases had documentation of negative standard workup including clinical testing for infection (parvovirus, cytomegalovirus, toxoplasmosis, and syphilis), alloimmune anemia, fetomaternal hemorrhage (Kleihauer–Betke test or middle cerebral artery Doppler), and chromosomal disorders (microarray). Exclusion criteria included abnormal karyotype, pathogenic or likely pathogenic findings on microarray, documented alloimmune anemia, infectious or fetomaternal hemorrhage as an etiology for hydrops, unobtainable parental DNA, hydrops diagnosed concomitantly with intrauterine fetal demise, donor egg or donor sperm utilized for conception, fetus or infant diagnosed with lysosomal storage disease, and parental age under 16 at the estimated date of delivery. Presence of other fetal anomalies was not an exclusion criterion. For two cases with variants of uncertain significance (VUS) on microarray, the array reports were reviewed by two clinical geneticists and a genetic counselor at time of enrollment and were determined not be suspected causes of NIHF.

A genetic counselor provided pretest counseling by telephone. Subjects provided written informed consent. Both parents provided blood samples for DNA isolation. Fetal samples were collected by MFM specialists at referring institutions through the workup already being performed as part of the standard-of-care and in one case cord blood was collected at delivery.

### Exome sequencing

Clinical ES was performed at the PerkinElmer® Genomics Laboratory on genomic DNA using the Agilent v6CREv2 targeted sequence capture method. Direct sequencing of amplified captured regions was performed using 2×100 bp reads on Illumina next-generation sequencing systems. Bases were deemed sufficiently covered at 20× and exons were considered fully covered if all coding bases plus three nucleotides of flanking sequence were covered at least 20×. Alignment to the human reference genome (hg19) was performed and variants were identified in the targeted regions. Variants were called at a minimum coverage of 8× and an alternate allele frequency of at least 20%. Single-nucleotide variants meeting internal quality assessment guidelines were confirmed by Sanger sequence analysis. CNV software (Biodiscovery™) detects deletions and duplications of at least three exons. Only CNVs related to the reported phenotype were returned. Primary data analysis was performed using Illumina DRAGEN Bio-IT Platform v.2.03. Secondary and tertiary data analysis was performed using PerkinElmer's internal ODIN v.1.01 software for single-nucleotide variants and Biodiscovery's NxClinical v.5.1 or Illumina DRAGEN Bio-IT Platform v.2.03 for CNVs and absence of heterozygosity. The analyzed regions of genes include coding exons and 10 bp of flanking intronic regions. Variants were evaluated by their reported frequency in the Genome Aggregation Database (gnomAD),[19] Human Gene Mutation Database (HGMD), and ClinVar.[20] Given the prevalence of the disease in the general population, variants with a population frequency greater than expected were considered "benign" variants. "Benign" and "likely benign" variants were not reported. Silent and intronic variants beyond +/−3 base pairs of splice junctions were not reported unless suspected to be pathogenic. Variants were classified in accordance with American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) variant classification guidelines.[21] Clinical reports were generated within the CLIA environment and signed by American Board of Medical Genetics and Genomics (ABMGG) certified laboratory directors.

### Variant return

Clinical ES reports were available within two to three weeks of sample receipt by the laboratory and returned to the family within days of result regardless of current pregnancy status (ongoing pregnancy, fetal demise, or live birth). ES reports were reviewed by a genetic counselor, a MFM geneticist, and a pediatric geneticist and grouped into categories based on diagnostic clarity. Reports were designated as "diagnosed" if a classified pathogenic or likely pathogenic genotype was identified for a disorder known to be associated with NIHF (one variant for dominant disorder and two variants in *trans* based on biparental inheritance for autosomal recessive disorders). Reports were designated as having a "possible diagnosis" if they included VUS in genes associated with NIHF or de novo variants in dominant disease genes not previously associated with NIHF but known severe syndromic presentations in infancy. The remaining "undiagnosed" reports were cases with no variants associated with NIHF or only a single inherited pathogenic variant in an autosomal recessive disease gene.

Clinical reports were returned to families by a genetic counselor. In cases with a suggested but nondefinitive diagnosis on initial ES analysis, additional clinical testing to evaluate uncertain variants, such as clinical enzymology or parental clinical laboratory testing (e.g., peripheral smears), was requested when available to help clarify variant classifications based on ACMG/AMP criteria. As exome analysis is phenotype driven, for cases without diagnoses on initial analysis, exome sequencing data were reanalyzed by the clinical laboratory with additional clinical information as it became available after further medical records review.

## RESULTS

### Sample demographics

The demographics of the 22 pregnancies and 44 parents presenting to MFM practices across 14 states are described in Table 1. Hydrops was identified between 15 weeks 3 days and 32 weeks 5 days gestational age (mean: 23.6 weeks; standard deviation: 5.2 weeks). Maternal age ranged from 19 to 37 years (mean: 29.6 years; standard deviation: 3.8 years) and paternal age ranged from 21 to 42 (mean: 31.2 years; standard

**Table 1.** Demographic characteristics.

| Family ID/ state | Maternal age/ethnicity | Paternal age/ethnicity | GA at diagnosis | Pregnancy outcome | Sample source | Prestudy genetic findings |
|---|---|---|---|---|---|---|
| NIHF1-001 KS | 27 years/Caucasian | 27 years/Hispanic | 15 weeks 3 days | IUFD at 23 weeks 0 days | Fetal cystic hygroma fluid | Microarray: normal male |
| NIHF1-002 TX | 30 years/Asian | 31 years/Asian | 31 weeks 6 days | Live born | Cord blood | Microarray: 35 kb VUS loss of 17q24 |
| NIHF1-003 MD | 24 years/Black, African American | 23 years/Black, African American | 19 weeks 0 days | Live born | Amniotic fluid | Microarray: normal male |
| NIHF1-004 VA | 30 years/Caucasian | 42 years/Caucasian | 19 weeks 6 days | IUFD at 21 weeks 5 days | Amniotic fluid | Microarray: normal male |
| NIHF1-005 OH | 19 years/Caucasian | 21 years/Caucasian | 20 weeks 4 days | Live born | Amniotic fluid | Microarray: normal male<br>Noonan panel: negative |
| NIHF1-006 OH | 29 years/Caucasian | 32 years/Caucasian | 23 weeks 3 days | Live born, death HOL 1 | Amniotic fluid | Microarray: normal male |
| NIHF1-007 ND | 30 years/Caucasian | 32 years/Caucasian | 29 weeks 3 days | Live born, death HOL 35 | Amniotic fluid | Microarray: normal female |
| NIHF1-008 AZ | 30 years/Caucasian | 39 years/Caucasian | 23 weeks 0 days | Live born, death HOL 1 | Amniotic fluid | Microarray: normal female |
| NIHF1-009 MD | 33 years/South Asian | 35 years/South Asian | 24 weeks 0 days | Termination at 26 weeks 2 days | Amniotic fluid | Microarray: normal male<br>Noonan panel: negative |
| NIHF1-010 PA | 30 years/Caucasian | 31 years/Caucasian | 32 weeks 1 day | Live born, death DOL 2 | Amniotic fluid | Microarray: normal female<br>Noonan panel: negative |
| NIHF1-011 SC | 27 years/Caucasian | 31 years/Caucasian | 18 weeks 4 days | Live born, death DOL 28 | Amniotic fluid | Microarray: normal female<br>Noonan panel: negative |
| NIHF1-012 TX | 28 years/Hispanic | 29 years/Hispanic | 20 weeks 3 days | Termination at 21 weeks 3 days | Amniotic fluid | Microarray: normal female |
| NIHF2-013 NC | 31 years/Caucasian | 33 years/Caucasian | 32 weeks 5 days | IUFD at 32 weeks 5 days | Amniotic fluid | Microarray: 222 kb VUS duplication at 12q13 |
| NIHF2-014 IL | 26 years/Caucasian | 23 years/Caucasian, Hispanic | 17 weeks 1 day | IUFD at 27 weeks 5 days | Products of conception | Microarray: normal male |
| NIHF2-015 MD | 28 years/Caucasian | 30 years/Asian | 29 weeks 6 days | IUFD at 32 weeks 2 days | Fetal quadriceps | Microarray: normal female |
| NIHF2-016 IL | 31 years/Hispanic | 29 years/Caucasian | 29 weeks 4 days | IUFD at 30 weeks 2 days | Placental tissue | Microarray: normal male |
| NIHF1-017 MI | 30 years/Caucasian | 29 years/Caucasian | 19 weeks 2 days | Live born | Amniotic fluid | Microarray: normal male |
| NIHF1-018 DE | 32 years/Caucasian | 32 years/Caucasian | 19 weeks 5 days | Termination at 20 weeks 5 days | Amniotic fluid | Microarray: normal female<br>Arthrogryposis panel: negative |
| NIHF2-019 NJ | 37 years/Caucasian | 38 years/Caucasian | 23 weeks 3 days | IUFD at 24 weeks 3 days | Amniotic fluid | Microarray: normal female |
| NIHF1-020 OH | 35 years/Caucasian | 36 years/Caucasian | 24 weeks 3 days | Live born, death HOL 1 | Amniotic fluid | Microarray: normal male |
| NIHF2-021 MD | 30 years/Hispanic | 29 years/Hispanic | 19 weeks 5 days | IUFD at 25 weeks 6 days | Amniotic fluid | Microarray: normal male |
| NIHF1-022 ND | 34 years/Caucasian | 34 years/Caucasian | 25 weeks 2 days | IUFD at 27 weeks 2 days | Amniotic fluid | Microarray: normal female |

*DOL* day of life, *GA* gestational age, *HOL* hour of life, *IUFD* intrauterine fetal demise, *NIHF1* DNA obtained during continuing pregnancy, *NIHF2* DNA obtained after fetal demise.

**Table 2.** Molecular results of prenatal ES in the HYDROPS study.

| Case ID | Diagnosis category | Disease (inheritance) | Gene/transcript | Coding change | Zygosity | Parental source | ACMG/ AMP class | Max population frequency[a] | Functional predictions[b] | Other evidence/ comment |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cases with diagnosis** | | | | | | | | | | |
| NIHF1-004 | Diagnosed | Spinal muscular atrophy, lower extremity-predominant 2A/ B (AD) | *BICD2*/ NM_001003800.1 | c.2081G>A/p.Arg694His | Het | Dn | L-Path | 0% | Deleterious | Variant at same AA reported for arthrogryposis multiplex; reclassified after additional phenotyping |
| | Unlikely | COL2A1-related disorder (AD) | *COL2A1*/ NM_001844.4 | c.3656A>G/p.Asp1219Gly | Het | Mat | VUS | 0.003% | Deleterious | Variant at same AA previously reported with spondyloepiphyseal dysplasia |
| | Unlikely | Omodysplasia (AD) | *FZD2*/ NM_001466.3 | c.407T>A/p.Leu136Gln | Het | Mat | VUS | 0% | Deleterious | |
| | Unlikely | Bruck syndrome (AD) | *PLOD2*/ NM_182943.2 | c.2038C>T/p.Arg680Ter | Het | Mat | L-Path | 0% | LOF | Previously reported in compound Het affected |
| NIHF1-006 | Diagnosed | Noonan syndrome (AD) | *PTPN11*/ NM_002834.3 | c.923A>G/p.Asn308Ser | Het | Mat | Path | 0% | Mixed | Multiple reported cases |
| NIHF1-007 | Diagnosed | Dyserythropoietic anemia (AD) | *KLF1*/ NM_006563.3 | c.973G>A/p.Glu325Lys | Het | Dn | Path | 0% | Deleterious | Multiple reported cases/published functional studies |
| NIHF1-008 | Diagnosed | Noonan syndrome (AD) | *RAF1*/ NM_002880.3 | c.779C>T/p.Thr260Ile | Het | Dn | L-Path | 0% | Deleterious | Reported with hypertrophic cardiomyopathy |
| NIHF1-009 | Diagnosed | Multiple sulfatase deficiency (AR) | *SUMF1*/ NM_182760.3 | c.691dup/p.Try231fs | Hom | Both | Path | 0.02% | LOF | Reported in compound Het affected case |
| | Unlikely | Mucopolysaccharidosis I (AR) | *IDUA*/ NM_000203.3 | c.757G>T/p.Gly253Cys | Het | Mat | VUS | 0.4% | Deleterious | Reported once in MPS I patient |
| | | | | c.806C>G/p.Ser269Cys | Het | Pat | VUS | 0.08% | Deleterious | |
| NIHF1-011 | Diagnosed | Sialidosis (AR) | *NEU1*/ NM_000434.3 | c.238C>A/p.Pro80Thr | Het | Mat | L-Path | 0% | Deleterious | Same AA previously reported; biochemically confirmed |
| | | | | c.1230_1234delinsGCCAAA/ p.Ser410fs | Het | Pat | L-Path | 0% | LOF | Case reclassified after biochemical studies |
| NIHF1-012 | Diagnosed | Noonan syndrome (AD) | *RIT1*/ NM_006912.5 | c.270G>C/p.Met90Ile | Het | Dn | Path | 0% | Mixed | Reported in Noonan syndrome, this variant and same AA |
| NIHF1-017 | Diagnosed | Kabuki syndrome (AD) | *KMT2D*/ NM_003482.3 | c.15239_15240del/p. Val5080fs | Het | Dn | Path | 0% | LOF | |
| NIHF1-020 | Diagnosed | Osteopathia striata (XLD) | *AMER1*/ NM_152424.3 | c.1000G>T/p.Glu334Ter | Hemi | Dn | Path | 0% | LOF | |
| NIHF2-021 | Diagnosed | Central core disease; Congenital Neuromuscular Disease (AR) | *RYR1*/ NM_000540.2 | c.11778 + 1G>T/splice variant | Hom | Both | Path | 0% | LOF | |
| NIHF1-022 | Diagnosed | Noonan syndrome (AD) | *RRAS2*/ NM_001177314.1 | c.110A>T/p.Gln37Leu | Het | Dn | Path | 0% | Deleterious/ published GOF effect | Reported in Noonan syndrome, this variant and same AA |

**Table 2** continued

| Case ID | Diagnosis category | Disease (inheritance) | Gene/transcript | Coding change | Zygosity | Parental source | ACMG/ AMP class | Max population frequency[a] | Functional predictions[b] | Other evidence/ comment |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cases with Possible Diagnosis** | | | | | | | | | | |
| NIHF1-001 | Possible | Dehydrated stomatocytosis and/or perinatal edema (AD); Lymphatic malformation (AR) | *PIEZO1*/ NM_001142864.2 | c.1495G>A/p.Val499Ile | Het | Pat | VUS | 0.15% | Tolerated | |
| | | | | c.6905G>A/p.Arg2303His | Het | Mat | VUS | 0.05% | Mixed | Reported in case of AD stomatocytosis |
| NIHF1-003 | Possible | Charcot–Marie–Tooth disease, type 2F (AD) | *HSPB1*/ NM_001540.3 | c.476_477del/p.Pro159fs | Het | Dn | L-Path | 0.003% | LOF | Reported in infant with progressive paralysis and mildly affected father[25] |
| NIHF2-014 | Possible | Mucopolysaccharidosis VII (AR) | *GUSB*/ NM_000181.3 | c.1084G>A/p.Asp362Asn | Het | Mat | VUS | 0.003% | Mixed | Previously reported in a case with MPS VII |
| | | | | c.1747G>A/p.Gly582Arg | Het | Pat | VUS | 0.002% | Deleterious | |
| NIHF2-016 | Possible | Adams–Oliver syndrome (AD) | *NOTCH1*/ NM_017617.3 | c.6913_6916del/p.Asn2305fs | Het | Dn | VUS | 0% | LOF | Reported after additional phenotyping |
| | Unlikely | Familial hypertrophic cardiomyopathy (AR) | *ALPK3*/ NM_020778.4 | c.3077delinsTCATT/p. Ser1026fs | Het | Pat | Path | 0% | LOF | |
| NIHF1-018 | Possible | Dehydrated stomatocytosis and/or perinatal edema (AD); Lymphatic malformation (AR) | *PIEZO1*/ NM_001142864.2 | c.4556A>C/p.Gln1519Pro | Het | Pat | VUS | 0.07% | Mixed | |
| | | | | c.5728G>A/p.Glu1910Lys | Het | Pat | VUS | 0.4% | Tolerated | |
| | | | | c.6328C>T/p.Arg2110Trp | Het | Mat | VUS | 0.002% | Mixed/ published GOF effect | Previously reported in 2 patients with AD stomatocytosis |
| **Cases without diagnostic results** | | | | | | | | | | |
| NIHF1-002 | Undiagnosed | No variants related to hydrops reported | | | | | | | | |
| NIHF1-005 | Undiagnosed | No variants related to hydrops reported | | | | | | | | |
| NIHF1-010 | Undiagnosed | No variants related to hydrops reported | | | | | | | | |
| NIHF2-013 | Undiagnosed | Mucopolysaccharidosis I (AR) | *IDUA*/ NM_000203.3 | c.208C>T/p.Gln70Ter | Het | Mat | Path | 0.2% | LOF | Reported in multiple affected Hom or compound Het cases |
| NIHF2-015 | Undiagnosed | No variants related to hydrops reported | | | | | | | | |
| NIHF2-019 | Undiagnosed | No variants related to hydrops reported | | | | | | | | |

*AA* amino acid, *ACMG/AMP* American College of Medical Genetics and Genomics/Association for Molecular Pathology, *AD* autosomal dominant, *AR* autosomal recessive, *dn* de novo, *ES* exome sequencing, *GOF* gain of function based on published experiment, *Hemi* hemizygous, *Het* heterozygous, *Hom* homozygous, *L-Path* likely pathogenic, *LOF* loss of function (truncation, frameshift, or splice), *Mat* maternal, *MPS* mucopolysaccharidosis, *Pat* paternal, *Path* pathogenic, *VUS* variant of uncertain significance, *XLD* X-linked dominant.

[a]Max subpopulation allele frequency in gnomAD database.

[b]Tolerated, deleterious, or mixed based on consensus of PolyPhen-2, MutationTaster, and SIFT.

deviation: 5.1 years). All pregnancies met *strict* ultrasound criteria for hydrops, which included fluid collection in two or more fetal compartments. Prior to enrollment, all participants had a documented negative infectious workup, immune workup, and screening for fetomaternal hemorrhage. Chromosomal microarray did not identify CNVs in 20 (91%) cases and reported small CNV VUS (222-kb duplication at 12q13 and 35-kb deletion at 17q24) in 2 (9%) cases where they were not thought to be contributory to NIHF. The proband samples included amniocytes (17 samples) where residual DNA was available from the clinical lab after previous microarray was performed, and one sample each for products of conception after pregnancy loss, placenta, cord blood, fetal quadriceps, and cystic hygroma fluid. In six trios, DNA was obtained after fetal demise, though in all cases, hydrops was present prior to fetal demise. Consistent with the literature, the survival rate in our series is very low.[1] Nine cases (41%) had intrauterine fetal demise (IUFD), of which three were enrolled prior to demise. Of the 13 pregnancies without IUFD, three families electively terminated prior to sequencing result return. Of the ten livebirths, six (60%) reported neonatal demise within the first month of life.

### Diagnosed cases

Molecular results from the ES are shown in Table 2. There were 11 cases (50%) where the results demonstrated a likely NIHF-associated genotype, which included eight cases with a diagnosis based on a pathogenic or likely pathogenic variant in a dominant gene (seven autosomal and one X-linked) and three with homozygous or compound heterozygous pathogenic or likely pathogenic variants in an autosomal recessive gene. Cases with a definitive diagnosis included four cases of Noonan syndrome (*PTPN11*, *RAF1*, *RIT1*, and *RRAS2*), three musculoskeletal disorders (*RYR1*-associated disease, *AMER1*-associated osteopathia striata, and *BICD2*-associated spinal muscular atrophy), two inborn errors of metabolism (sialidosis and multiple sulfatase deficiency), one case of Kabuki syndrome, and one congenital anemia (*KLF1*). The case with homozygous pathogenic variants in *SUMF1*, diagnostic for multiple sulfatase deficiency (MSD), also reported compound heterozygous VUS in *IDUA*, but these were thought less likely to contribute to the presentation given the other definitive diagnosis. In one case, a pathogenic Noonan syndrome variant was inherited from a previously undiagnosed mother. Subsequently, she was referred for cardiology screening and reported a normal echocardiogram, consistent with the broad range of phenotypic expressivity in this disorder.[8] Only one of these cases with a definitive diagnosis, Kabuki syndrome, survived past one month of life.

### Possible diagnoses

Five cases (22.7%) with possible diagnoses included three cases of VUS in genes known to cause NIHF and two cases with de novo variants in candidate genes related to severe childhood syndromes not previously observed with NIHF.

For the cases with VUS in known NIHF genes, two cases included compound heterozygous VUS in *PIEZO1*, a gene associated with autosomal dominant stomatocytosis with perinatal edema or autosomal recessive lymphatic dysplasia. In both cases, one of the reported variants (*PIEZO1* p.Arg2303His in NIHF-01 and *PIEZO1* p.Arg6328Trp in NIHF-18) was previously reported in the literature[22,23] as possibly being related to autosomal dominant stomatocytosis; therefore, we requested hematologic evaluations of the parents. Parents of one of the two cases provided peripheral smears, which had normal findings. For both these cases, the previously reported variants were identified in *trans* with a *PIEZO1* variant found in 0.07% to 0.4% of the normal population. One of these alleles contains two VUS in *cis*. The allele frequency of these variants makes them too rare to exclude potential roles in hydropic pregnancies. While *PIEZO1*-associated lymphatic malformations are a well-described cause of NIHF, there was not enough evidence to definitively diagnose this based on these variants. The third possible diagnosis case reported compound heterozygous VUS concerning for mucopolysaccharidosis (MPS) VII, another known cause of NIHF. One of these two variants was previously reported in an MPS VII patient[24] and the other was predicted to be deleterious. Due to fetal demise, samples were not available for definitive biochemical analysis to reclassify this case.

For the two candidate genes, one case included a de novo loss-of-function variant of *HSPB1* in the proband that was previously reported in an infant with severe progressive motor neuropathy after receiving a tetanus vaccination, while his mildly symptomatic father was also found to carry the variant.[25] Of note, routine tetanus vaccination was not performed in the pregnancy we report. Given the wide spectrum of clinical presentation, including infantile onset, this known pathogenic variant was considered a likely candidate for fetal akinesia and NIHF. The other candidate gene, *NOTCH1*, is associated with Adams–Oliver syndrome, a disorder known to have severe infantile presentations. This *de novo* variant was classified as a VUS and thus remains a possible diagnosis. Of the cases with possible diagnoses, only the one with the *HSPB1* variant survived past one month of life.

### Undiagnosed cases

The reported genotypes in six cases did not suggest a cause for NIHF and remain undiagnosed. In five cases, no variants were reported. In one case, a heterozygous pathogenic variant was reported in *IDUA* associated with an autosomal recessive MPS I which is not diagnostic without a second variant in *trans*. Nevertheless, a second variant may be undetected on exome (e.g., deep intronic variant). Due to fetal demise, biochemical testing was not performed, leaving this case undiagnosed.

### Previous testing

Other clinical tests appropriate for NIHF workup, but not considered standard-of-care, were not uniformly performed prior to enrollment. Based on the exome results, biochemical analysis for lysosomal storage disorders would have been informative for three of the cases (13.6%), but was not performed on any cases referred to this study.[2] Noonan sequencing panels were performed on four cases prior to enrollment and were negative. Two of these four cases received diagnostic exome results in our study (*SUMF1* and *NEU1*). Of the 18 cases not previously tested for Noonan syndrome, ES diagnosed 4 (22.2%) with Noonan syndrome. One case had a negative arthrogryposis panel prior to enrollment.

Parental expanded carrier screening (ECS) reported relevant results in one case where, at the time of hydrops diagnosis, a paternal sialidosis variant was reported, but the maternal variant was not reported. This highlights known limitations of ECS, which does not report VUS, and may have significant residual carrier risk after negative testing.

### Variant reanalysis and reclassification

In few cases, variant classifications were upgraded by the clinical laboratory after additional phenotyping. The variants for sialidosis were initially reported as VUS, but after biochemical confirmation were reclassified as likely pathogenic. In another case, a *BICD2* variant classified as VUS was upgraded to likely pathogenic after ultrasound evaluation suggested fetal

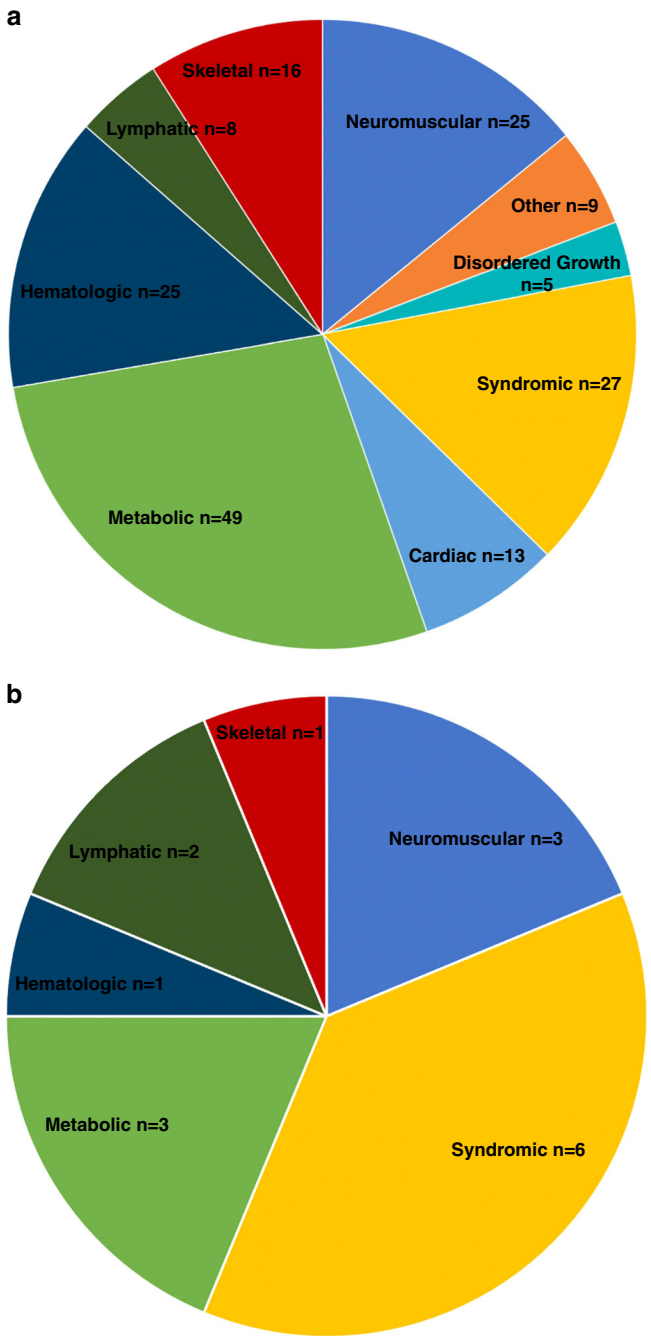


**Fig. 1 Distribution of categories of monogenic disorders implicated in NIHF.** (**a**) Categories of monogenic etiologies with strong and emerging evidence for causing nonimmune hydrops fetalis (NIHF) based on literature review adapted from Quinn et al.[9] This distribution shows the different types of single-gene disorders that can present with NIHF, but does not reflect the frequency of different etiologic causes given reporting bias in the literature. (**b**) Distributions of categories of monogenic etiologies identified in the HYDROPS study. This shows the distribution of categories of single-gene disorders identified in cases with likely and possible diagnoses in this study.

akinesia consistent with a neuromuscular diagnosis. The *NOTCH1* variant was not initially reported, but findings of cardiac abnormalities on subsequent fetal ultrasound allowed reanalysis. This variant, located near the terminus of the gene and possibly escaping nonsense-mediated decay, lacks sufficient evidence to apply ACMG/AMP loss-of-function criteria and remains a VUS.

### Categories of genetic etiologies

Our previous systematic review of monogenic causes of NIHF cataloged the evidence for genes reported in the literature associated with NIHF.[9] Using the same criteria, we now consider *RRAS2* to have strong evidence because of its association with Noonan syndrome though this is the first reported case of *RRAS2*-associated NIHF. *AMER1* and *HSPB1* have emerging molecular evidence because these are the first reported cases of NIHF associated with pathogenic variants in these genes. *NOTCH1* is a candidate gene for association with NIHF because the reported variant is considered a VUS. All other diagnosed cases reported variants in genes with multiple published reports of NIHF and strong evidence for causing NIHF.

In our previous categorization of genetic disorders that lead to NIHF, we could not assess the relative frequency of different causes due to reporting bias[9] (Fig. 1a). Based on our small series, the frequency of different causes is not uniformly distributed (Fig. 1b) as certain monogenic etiologies are more common. Larger cohorts are required to better assess this distribution.

## DISCUSSION

NIHF has many causes undetectable with current clinical workup.[9] In this HYDROPS study, cases met the strict SMFM definition of NIHF including multiple fetal compartment involvement and documented negative standard-of-care workup. While one third could have been diagnosed with narrower testing strategies including biochemical testing and a RASopathy panel, analysis of a greater number of genes with ES provided an incremental diagnostic yield of over 50% (standard error: 10.6%) of the initially undiagnosed cases and found an additional 22.7% (standard error: 8.9%) with possible diagnoses. The incremental diagnostic yield of ES in NIHF was higher in our study than the diagnostic yield in studies of congenital structural anomalies. A possible explanation for the higher diagnostic yield of ES in NIHF is that hydrops is a specific phenotype with many genetic etiologies and is more likely related to an identifiable genetic etiology than nonspecific malformations that may have nongenetic causes. As ES is phenotypically directed analysis, it is reasonable that our *strict* adherence to the phenotypic definition of NIHF in our study increased diagnostic utility of the testing. Additionally, the required completion of standard-of-care workup to exclude common nongenetic etiologies enriched for cases with genetic etiologies. Our study did not select for other factors that might enrich for genetic diagnoses such as familial recurrence, consanguinity, or associated syndromic anomalies. Five cases had negative panel based testing (four Noonan and one arthrogryposis) prior to enrollment, which could lead to underestimated diagnostic yield of exome by counting cases with previous nondiagnostic sequencing. These did not affect overall results since, on ES, two received diagnoses, one received a possible diagnosis, and two remained undiagnosed.

The recent publication by Sparks et al.[18] demonstrated a diagnostic yield of 29% in a study of fetal ES with fluid accumulation in one or more fetal compartments and a higher yield of 34% among their subgroup of 77 cases with fluid collections in two or more fetal compartments. Our study is unique since it was designed to assess the incremental diagnostic yield of ES over the current SMFM guidelines for NIHF. We defined NIHF based on the current guidelines of two or more fetal fluid collections compared with a broader definition

of a single compartment, such as an isolated increased nuchal translucency or isolated fetal ascites. We achieved this by only enrolling patients after a completely negative standard-of-care workup for NIHF was confirmed using the SMFM guidelines. While the previous study[18] also excluded cases with known etiologies, the specifics of previous testing were not discussed. Notably, fetomaternal hemorrhage, a known nongenetic etiology of NIHF, was not mentioned as an exclusion criterion in their study.[26] Another unique aspect of our study is that only trios were enrolled. All these reasons likely account for our trend toward a higher diagnostic rate. It is important to note that though the diagnostic yield appears high, our estimate is limited by small sample size and is consistent with previously published results. Our diagnostic rate of 11 of 22 cases is not statistically different than the rate of 26 of 77 cases reported by Sparks et al.[18] ($p = 0.165$, $Z$-test for two proportions).

Another potential source of the high diagnostic rate relates to the severity of presentations; clinicians and families might be more motivated to seek research testing in cases where future testing is limited due to impending fetal demise. Given our study's recruitment from multiple centers, it is unclear if our enrolled population represents a full spectrum of NIHF presentations or more severe presentations from each site.

Diagnoses identified through ES can affect clinical management for parents and the fetus. In one of our cases, a mother received a diagnosis of Noonan syndrome. Guidelines for screening individuals with Noonan syndrome for cardiomyopathies and cancers now guide her long-term clinical management.[27] Fetal diagnosis of a lysosomal storage disease may allow earlier initiation of enzyme replacement therapy after birth.[2]

The return and disclosure of clinical exome results require careful interpretation of the test report by clinicians experienced with molecular testing. This is especially true for pregnancies where results may affect continuation of pregnancy or future family planning decisions. In this study, half of the cases received a definitive diagnosis from ES, though some variants required additional confirmatory studies. For cases with possible diagnoses, some are likely to be truly related to disease and some may not be disease-causing. Involvement of clinical geneticists is important to determine appropriate follow-up testing to clarify uncertain results. Some of the most useful information provided by genetic diagnosis, especially given the high rate of poor clinical outcomes, is clarification of recurrence risk and options for early testing or PGT-M for future pregnancies. Recurrence risk is as low as around 1% for the cases associated with de novo dominant variants (7 autosomal and 1 X-linked) due to possibility of gonadal mosaicism, 25% for cases of biparental inheritance of autosomal recessive variants (4 cases), or as high as 50% as seen in the case of maternally inherited Noonan syndrome.

While the racial distribution in our study is close to the overall racial population distribution in the United States, the small size of the study led to skewing of minority representation. Since causes of NIHF are panethnic, often due to de novo variation, this does not affect the generalizability of our results. Given the rarity of NIHF, our study represents a large number of cases enriched for genetic diagnoses through the exclusion of more common etiologies. We were limited by lack of follow-up testing in several cases where additional biochemical or hematologic testing in the child or parents could allow variant reclassification. Especially given the high rates of pregnancy losses associated with NIHF, obtaining a postnatal sample was not always possible and parents grieving a pregnancy loss are not necessarily focused on further diagnostic studies. Nevertheless, prenatal ES for NIHF offers a high diagnostic yield for appropriately selected cases, provides clinically useful information, and should be considered in cases where the NIHF etiology remains undiagnosed after exclusion of common causes.

## DATA AVAILABILITY

All de-identified data supporting this study is available upon request from the authors.

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## AUTHOR CONTRIBUTIONS

Conceptualization: H.B.A., S.I.B. Data curation: M.M.M., E.C. Formal analysis: H.B.A., S.I.B. Investigation: H.B.A., M.M.M., S.M.R., K.S., R.L., M.H., E.C., S.I.B. Project administration: M. M.M., S.M.R., K.S., A.Q., B.V., B.F., E.C. Resources: C.H., B.F., R.L., M.H. Writing—original draft: H.B.A., S.I.B. Writing—review & editing: H.B.A., M.M.M., S.M.R., K.S., C.H., A.Q., B.V., B.F., R.L., M.H., E.C., S.I.B.

## ETHICS DECLARATION

TJU Hospital's Institutional Review Boards (IRB) approved ES protocols 18D.728 and 19D.700 for hydropic pregnancies when DNA was obtained during a continuing pregnancy or after fetal demise respectively. Subjects provided written informed consent approved by the TJU IRB.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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