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Fetal hydrops and the Incremental yield of Next generation sequencing over standard prenatal Diagnostic testing (FIND) study: prospective cohort study and meta-analysis

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CONTRIBUTION

What are the novel findings of this work?

This is a novel systematic review assessing the incremental yield of exome sequencing over chromosomal microarray analysis/ karyotyping in non-immune hydrops fetalis. An apparent incremental yield exome sequencing is demonstrated.

What are the clinical implications of this work?

Prenatal exome sequencing should be considered in prenatally diagnosed non-immune hydrops fetalis that is unexplained by standard genetic testing and either isolated or associated with additional fetal structural anomalies.

CONFLICT OF INTEREST

RYE and JL report grants from the Health Innovation Challenge Fund during the conduct of the PAGE study. DJM reports grants for travel expenses from Congenica to attend educational symposia during the conduct of the PAGE study. MEH reports grants from the Wellcome Trust and the UK Government Department of Health during the conduct of the study and personal fees from Congenica, outside of the submitted work. MDK is a member of Illumina's International Perinatal Advisory Group (but receives no payment for this) and is the Fetal Medicine Representative for the Central and South GLH. He is also the RCOG representative on the Joint Colleges Committee of Genomic and Genetic Medicine and the Royal College of Obstetricians and Gynaecologists Genomic Taskforce. ERM has received travel expenses, accommodation and consultant fees for participating in an Illumina International Advisory Group after completion of the PAGE study. MDK is funded through the Department of Health, Wellcome Trust and Health Innovation Challenge Fund (award number HICF-R7-396) for the PAGE and PAGE2 research studies complete August 2019. LSC was partially funded by the same group in relation to PAGE. RJW receives funding from Illumina and NIH for research. MN has been funded from Ultragenyx and the NIH for research relevant to included cases. All other authors declare no competing interests.

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Abstract

OBJECTIVES: Determine the incremental yield of next generation sequencing (predominantly exome sequencing (ES)) over quantitative fluorescence-polymerase chain reaction (QF-PCR) and chromosome microarray analysis (CMA)/karyotyping in; (i) all cases of prenatally diagnosed non-immune hydrops fetalis (NIHF); (ii) isolated NIHF; (iii) NIHF associated with additional structural anomalies and; (iv) NIHF according to severity (i.e., two cavities versus three or more cavities affected).

METHODS: A prospective cohort study (from an extended group of the Prenatal Assessment of Genomes and Exomes (PAGE) study) of n=28 cases of prenatally diagnosed NIHF undergoing trio ES following a negative QFPCR and CMA/karyotype was combined with a systematic review of the literature. Electronic searches of relevant citations from MEDLINE, EMBASE and CINAHL and clinicaltrials.gov (January 2000 – October 2020) databases was performed. Studies included were those with: (i) ≥ n=2 cases of NIHF undergoing sequencing; (ii) testing initiated based on prenatal ultrasound-based phenotype and; (iii) a negative CMA/karyotype. PROSPERO Registration No. CRD42020221427.

RESULTS: The PAGE cohort study noted the additional diagnostic yield of ES was 25.0% (n=7/28) for all NIHF, 21.4% (n=3/14) for isolated NIHF and 28.6% (n=4/14) for non-isolated NIHF. From the meta-analysis, the pooled incremental yields from n=21 studies (n=306 cases) were 29% (95% CI 24-34%, I^2=0%, p<0.00001) in all NIHF, 24% (95% CI 16-33%, I^2=0%, p<0.00001) in isolated NIHF and; 38% (95% CI 28%-48%, I^2=6%, p<0.00001) in NIHF associated with additional anomalies. In the latter, congenital limb contractures were the most prevalent additional structural anomaly at 17.3% (n=19/110). Incremental yield did not differ significantly based upon hydrops severity. The commonest genetic disorders identified were RASopathies in 30.3% (n=27/89), most commonly due to *PTPN11* variants in 44.4% (n=12) and the predominant inheritance pattern was autosomal dominant in monoallelic disease genes 57.3% (n=51/89), of which most were *de novo* 86.3% (n=44).

CONCLUSIONS: Use of prenatal next generation sequencing in both isolated and non-isolated NIHF should be considered in developing clinical pathways. Given the wide range of potential syndromic diagnoses and heterogeneity in prenatal phenotypes of NIHF, exome or whole genome sequencing may prove to be a more appropriate testing approach than a targeted gene panel testing strategy.

Keywords

exome sequencing; fetus; hydrops; prenatal diagnosis; next generation sequencing; nonimmune hydrops fetalis

INTRODUCTION

Nonimmune hydrops fetalis (NIHF) is traditionally defined as fluid accumulation in two or more fetal body cavities (in cases not secondary to maternal red cell alloimmunization).[1] It affects up to 1 in 1700 pregnancies, with associated high risks of perinatal morbidity and mortality.[2] Excluding infection, fetal structural anomalies (FSAs) and complications of twin pregnancies, aneuploidy may explain a quarter of cases, with chromosome microarray (CMA) demonstrating a further abnormality of copy number variants (CNVs) in 6-14%.[3,4] Despite this, the definitive diagnostic yield of CMA over standard G-banding karyotype is moderate and following exclusion of the aforementioned causes up to 50% of NIHF remains unexplained, with a significant proportion thought to be secondary to single gene variants.[5] Over 170 genes have been identified as being associated with NIHF and until the recent revolution of next generation sequencing (NGS), testing for such conditions has relied upon targeted gene testing and enzyme assays.[3,6] Single gene causes of NIHF are associated with significant risks of perinatal death or neurodevelopmental sequalae.[2] Establishing a diagnostic aetiology prenatally is a vital step in facilitating informed decision making (for both parents and clinicians), considering options such as termination of pregnancy, planning neonatal care and addressing recurrence risks.[2] The latter could theoretically be mitigated using novel technologies such as preimplantation genetic testing.[7] While individual case cohort studies have assessed the diagnostic yield of exome sequencing (or an alternative sequencing approach) over Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR) and CMA or karyotype in NIHF, they are heterogenous in relation to populations assessed and genetic platforms used.[3] There is a need to integrate existing data on single gene disorders underlying NIHF given this heterogeneity. Hence, the aims of this study were to evaluate the incremental diagnostic yield of prenatal exome sequencing (ES) (or an alternative sequencing technology) in; (i) all NIHF; (ii) isolated NIHF; (iii) NIHF associated with fetal structural anomalies (FSAs) and; (iv) NIHF according to severity (i.e., two cavities versus three or more cavities affected).

METHODS

Extended Prenatal Assessment of Genomes and Exomes (PAGE) study Cohort

This included prospectively identified cases of prenatally confirmed NIHF from an extended cohort of the Prenatal Assessment of Genomes and Exomes (PAGE) Study.[8] For the purposes of the FIND study, we defined NIHF as ultrasonographically prenatally confirmed pathological fluid accumulations in ≥two fetal cavities, where cases with aneuploidy, congenital infection, alloimmunization or and twin-twin transfusion syndrome had been excluded.[1,2] The final PAGE cohort included n=850 fetuses (published cohort n=596) with trio ES performed in instances when an ultrasound-confirmed FSA was detected.[8] Such cases were recruited between October 2014 and May 2018 across 34 fetal medicine

centres in England and Scotland, with ES performed centrally at the Wellcome Trust Sanger Institute.[8] PAGE eligibility criteria included: (i) prenatal detection of a FSA after 11-weeks' gestation; (ii) availability of proband and parental DNA and; (iii) negative QF-PCR and CMA or karyotype testing. The PAGE study methodology has been published previously and utilized a standard ES approach with variant interpretation based on a targeted virtual 1628 gene panel for developmental disorders.[8,9] Phenotypes of all cases were classified using Human Phenotype Ontology (HPO) terms,[10] and those defined as Hydrops Fetalis HP:0001789 were selected and further analysed to determine if the criteria for NIHF for the purposes of the FIND study were met. Cases were further classified into 'isolated' and 'associated with additional FSAs' using the HPO approach to coding additional anomalies. Fetal phenotypes were described by fetal medicine specialists/sonographers and documented principally on Viewpoint® Version 5.6.16 (GE Healthcare). Variants were classified in accordance with the American College of Medical Genetics and Genomics (ACMG) guidelines as agreed by a clinical review panel and incidental findings (IFs) were not reported.[11] Pathogenic and likely pathogenic variants explaining the fetal phenotype were confirmed using Sanger sequencing and results returned to parents after the end of pregnancy. Ethical approval was obtained from the Research Ethics Committees at the West Midlands – South Birmingham (ref: 13/WM/1219) and the Harrow - REC reference number 01/0095. Local Research and Development offices subsequently approved the study at each participating organisation.

Systematic review and meta-analysis

Information sources—This review was performed in a standardized fashion in line with recommended methods for systematic reviews and PRISMA guidance and was prospectively registered [PROSPERO No. CRD42020221427].[12,13] The following databases were searched electronically for relevant citations, from January 2000 (ES was not an available technology prior to this) until October 2020: MEDLINE, EMBASE, CINAHL and clinicaltrials.gov. The search strategy consisted of relevant Medical Subject Headings (MeSH) terms, keywords and word variants for 'exome sequencing', 'fetus' and 'abnormality' were used with alternative terms encompassing 'genome sequencing', 'exome', fetal', 'prenatal', 'antenatal', 'defect' and 'anomaly'. Bibliographies of relevant articles were searched manually and experts in prenatal genomics were also contacted to identify further relevant studies. The search strategy is available from the corresponding author on request.

Study selection—The inclusion criteria for study selection were any prospective or retrospective cohort studies or case series which: (i) included two or more cases of NIHF undergoing ES (or an alternative sequencing strategy such as gene panels); (ii) initiated testing based on prenatal ultrasound-based phenotype; (iii) had a negative CMA/karyotype result and; (iv) results of genetic testing were known. Where ES was initiated postnatally, such cases were included if testing was based upon the prenatal phenotype and instances where sequential Sanger sequencing was utilised were also included. When studies were not specific to NIHF exclusively, data regarding such cases were extracted from the paper or via author request. All study abstracts were screened by two reviewers (F.M. and M.D.K.) and full manuscripts were subsequently reviewed when further information was required.

Data extraction and quality assessment—Both reviewers independently extracted data on study characteristics and outcome data using a proforma. Data extracted from studies, when obtainable, included: ultrasound phenotype, sequencing approach, reported variants, source of fetal DNA, turnaround time, fetal outcome, maternal age and gestational age at testing. Quality assessment was performed using modified Standards for Reporting of Diagnostic Accuracy (STARD) criteria.[14]. Criteria deemed most important to optimise accuracy were: (i) trio analysis; (ii) use of ACMG criteria for variant interpretation; (iii) Sanger sequencing validation and; (iv) description of the prenatal phenotype.

Data analysis—Descriptive tables were produced detailing study characteristics and outcomes. The incremental diagnostic yield, or risk difference, with 95% CI, of ES (or alternative sequencing strategy) over QF-PCR and CMA or karyotyping was calculated for each study and as a pooled value for: (i) all NIHF; (ii) isolated NIHF; (iii) NIHF associated with additional structural anomalies and; (iv) NIHF according to severity. Where reported, pooled values for variants of uncertain significance (VUS) and IFs was also determined. Risk differences from each study were pooled using a random effects model throughout to estimate incremental yield by a previously published method which facilitated calculation with adjustment for 'zero' values from negative QF-PCR and CMA or karyotype testing.[9,15] Results were displayed in Forest Plots with corresponding 95% confidence intervals (CIs). Heterogeneity was assessed graphically within the forest plot and statistically using Higgins' I^2. Publication bias was assessed graphically using funnel plots. Statistical analysis was performed using RevMan version 5.3.4 (Review Manager, The Cochrane Collaboration, Copenhagen, Denmark) statistical software.

RESULTS

Extended PAGE cohort

Of the 850 cases of prenatal structural anomaly which underwent ES, there were n=28 (3.3%) cases that met the definition for NIHF. Of these 50% (n=14) were apparently isolated and 50% (n=14) were associated with additional FSAs. In the majority of cases (96.4%; n=27) the original genetic test was CMA, with the remainder being karyotype with most proband DNA originating from cultured amniocytes (50%; n=14). The diagnostic yield of ES overall in all NIHF was 25.0% (n=7/28) and was 21.4% (n=3/14) and 28.6% (n=4/14) in isolated NIHF and NIHF associated with additional FSA respectively. Where additional anomalies associated with pathogenic variants were present, there were most commonly congenital limb contractures due to arthrogryposis multiplex congenita (HP0002804) 75% (n=3/4). In instances where no pathogenic variant was obtained, the commonest additional anomalies were cardiac, genitourinary and thoracic in nature (each 50.0% (n=5/10)). One case of Noonan syndrome was initially not detected as pathogenic as it was filtered out of the bioinformatic pipeline due to inheritance from an apparently unaffected parent. Subsequently the pipeline was adjusted so that such variants were not filtered out even if inherited. The incidence of VUS was 7.1% (n=2/28). Pathogenic variants and VUS are described and outlined in supplementary tables S1 and S2.

Systematic review and meta-analysis

Where a study was suitable for inclusion but data were incomplete, the corresponding authors were contacted to request further data (n=5), regarding fetal phenotype, of which two responded and provided full datasets.[16,17] One of these, the study from Columbia University Medical Centre, New York provided an extended dataset from the paper by Petrovski, *et al.* 2019.[16] In addition, to the extended PAGE Study cohort[8], there were a further n=20 studies which met the inclusion criteria as demonstrated in Figure 1.[2,8, 16-34] Table 1 highlights the characteristics of included studies and Figure 2 shows the overall quality assessment.

Systematic review outcomes

In total n=21 studies were included with a total of n=306 NIHF cases. Where stated (n=217), there were n=107 (49.3%) cases of apparently isolated NIHF (on prenatal detailed ultrasound) and n=110 (50.7%) cases associated with additional FSAs. The mean maternal age and gestation at testing was 30.9 (+/−3.5 SD) years and (21.9 +/−5.4 SD) weeks, respectively. Fetal DNA was obtained in the majority of cases via amniocentesis; 50.6% (n=121/239) with the initial test prior to ES performed; CMA; 84.0% (n=257) and the remainder G-banding karyotype. Where documented (n=12 studies), the median turnaround time for ES was 40 (range 7-140) days. Pregnancy outcome was available for (32.4%, 99/306 of cases (termination of pregnancy; n=79 (30.9%); in-utero demise; n=57 (22.3%) livebirth and; n=21 (8.1%) neonatal death). When reported, the pooled incremental yield for VUS and IFs was 19% (95% CI 6-22%, I^2=62%, p=0.003) and 4% (95% CI −1-9%, I^2=0%, p=0.09), respectively. Pathogenic variants and VUS are outlined in supplementary tables S1 and S2.

Systematic review pathogenic variants

The apparent incremental yields with ES (or an alternative sequencing strategy) in (i) all NIHF, (ii) isolated NIHF and (iii) NIHF associated with additional anomalies are demonstrated in Forest plots (Figures 3a-c) and were 29% (95% CI 24-34%, I^2=0%, p<0.00001), 24% (95% CI 16-33%, I^2=0%, p<0.00001) and, 38% (95% CI 28%-48%, I^2=6%, p<0.00001) respectively. The corresponding funnel plots are displayed in supplementary figures S1-2. The commonest additional anomalies in the presence of pathogenic variants were those affecting the upper and/or lower limbs due to congenital contractures (HP:0002803); 17.3% (n=19/110). Where the NIHF phenotype was described, the incremental yield of pathogenic variants was not significantly greater where the hydrops was more severe (two cavities versus three or more cavities affected); 34% (95% CI 23-45%, I^2=0%, p<0.00001) and 30% (95% CI 19-40%, I^2=0%, p=0.003) respectively p=0.26. Where pathogenic variants were documented (n=89) (supplementary table 1) the commonest genetic disorders were (i) RASopathies 30.3% (n=27), primarily due to *PTPN11* variants 44.4% (n=12/27); (ii) musculoskeletal disorders 14.6% (n=13), primarily due to *RYR1* variants 46.2% (n=6/13) and; (iii) inborn errors of metabolism 12.4% (n=11), primarily due to *GUSB* variants 54.5% (n=6/11) The predominant inheritance pattern was autosomal dominant in monoallelic disease genes 57.3% (n=51), of which most were *de novo* 86.3% (n=44). Where the type of ES performed was stated [Table 1] (n=20 studies), the overall

incremental yield did not differ significantly dependent on whether a panel or whole exome approach was used; 26% (95% CI 16-36%, I^2=0%, p<0.00001) and 27% (95% CI 19-36%, I^2=25%, p<0.00001) respectively.

DISCUSSION

This systematic review demonstrates substantial incremental yield with NGS (principally ES) over QF-PCR and CMA or karyotyping of 29% in cases of prenatally diagnosed NIHF. This yield was higher among cases with additional FSAs, but severity of NIHF did not demonstrate a significant difference in the incremental yield. In the majority of instances pathogenic variants were *de novo* in autosomal dominant disease genes, predominantly in those causative of RASopathies.

The findings of the final PAGE cohort and systematic review were broadly concordant, with a lower yield in the cohort study, which may be explained by the smaller case number as well as the unselected approach to case selection. The dominance of RASopathies and of *de novo* variants in autosomal dominant disease genes is expected and not mutually exclusive.[2] Incremental yield was higher in instances where additional FSAs were present, predominantly so in cases of congenital arthrogryposis, which is intuitive as contractures are a common musculoskeletal phenotype of higher diagnostic yield with sequencing. Again this was unsurprising as contractures are seen commonly in the highest yielding musculoskeletal phenotype group.[35] In contrast, isolated NIHF was seen commonly within the RASopathies; 47.8% (n=11/23). This is in keeping with the variable phenotype reported in the RASopathies and supports the use of prenatal ES in cases of isolated NIHF.[36] There is phenotypic variability in cases with known RASopathy pathogenic variants, as well as in cases with pathogenic variants in other types of genetic diseases. This supports the use of ES or WGS, rather than a targeted or stepwise approach, in the investigation of NIHF.[37] One must always respect the role of QF PCR or conventional karyotyping in NIHF, given the high incidence of aneuploidy.[38] However, given the limited additional yield of CMA compared to karyotype and the ability of WGS to detect structural variants, it may be reasonable in the future as clinical and technical application of NGS technology includes validated CNV detection, to consider this as the second line test after QF-PCR or conventional karyotype.[5] The list of novel causative genes in NIHF is constantly expanding, and with time the yield with prenatal NGS will likely improve as more genes are discovered and out understanding of the prenatal phenotype develops.[2,37] This is supported by the high number of class III variants (VUS) identified within candidate genes from this study, high-lighted by the largest series in this study.[2] Re-analysis and potential re-classification of VUS is currently underway for the PAGE cohort which may increase the diagnostic yield.

Due to the relatively high yield evident in isolated NIHF from this study (and individual papers in the literature) it was decided to include NIHF (from March 2021) as an indication for inclusion in the R21 pathway of the National Health Service (NHS) England National Genomic Test Directory for Rare and Inherited Disease.[36,39] This (R21) pathway is a nationally (England presently) commissioned rapid prenatal ES service for fetuses with multiple, multisystem, major and selected isolated FSAs which is performed by two Genomic Laboratory Hubs in line with a set protocol.[40] Inclusion of hydrops fetalis has been

discussed as an inclusion phenotype and adopted in April 2021. Furthermore, the on-going Fetal Oedema and Lymphatic Disorder (FOLD) study is presently ongoing in the UK.[41]

Our study based its selection criteria upon the routine definition of what constitutes NIHF.[1] It has been proposed that this definition be expanded to include pathological fluid accumulation in one or more fetal body cavity, inclusive of a large nuchal translucency (NT)[>3.5 mm] or cystic hygroma.[2] This is being further explored but appears a reasonable argument given the large variability in NIHF phenotypes as well as their complex evolution and sometimes resolution seen in causative syndromes such as the RASopathies and is supportive by our finding that the mere presence of NIHF as opposed to its severity influence diagnostic yield with ES.[2,42] Prenatal ES performed at the time of an isolated increased NT or pleural effusion for instance may be the only snapshot to obtaining a prenatal diagnosis and is indicative of the nature of evolving and resolving NIHF phenotypes. There are a need for studies which track the evolution of the phenotype and respective diagnostic yields with NGS. Despite this, prenatal ES offered in cases of isolated elevated NT appears to offer a modest increase in diagnostic yield over CMA at around 5-7%.[2,42-44] It would appear to not just be the mere presence of the increased NT but its severity (≥5mm), persistence and association with additional anomalies that influence diagnostic yield with NGS.[2,37,44]

The strength of this systematic review lies in its novelty in concept, the robust methodology utilized as well as collaboration between experts of some of largest contemporary series in this area.[2,8,16,17] The relatively small number of cases (n=306) represents the largest reviews of prenatal NIHF cases and did not appear to impact upon heterogeneity. Due to absence from the literature, no included studies used a WGS approach, hence the difference in yield between WGS and ES could not be assessed. This is likely to change in the coming years and will likely prove more beneficial due to its all-in-one ability to detect most chromosomal and genetic differences.[7,39]

In conclusion, the use of prenatal NGS in both isolated NIHF and NIHF associated with additional FSAs should be considered in developing clinical pathways. Given the vastness of syndromic categories and heterogeneity in prenatal phenotypes of NIHF, a whole exome or genome sequencing approach in combination with accurate prenatal phenotyping is likely a more appropriate tool than a targeted or stepwise single gene testing strategy in achieving an optimum diagnostic yield. The current definition of NIHF in assessing yield appears appropriate, although further studies assessing expansion of this definition are required to support this.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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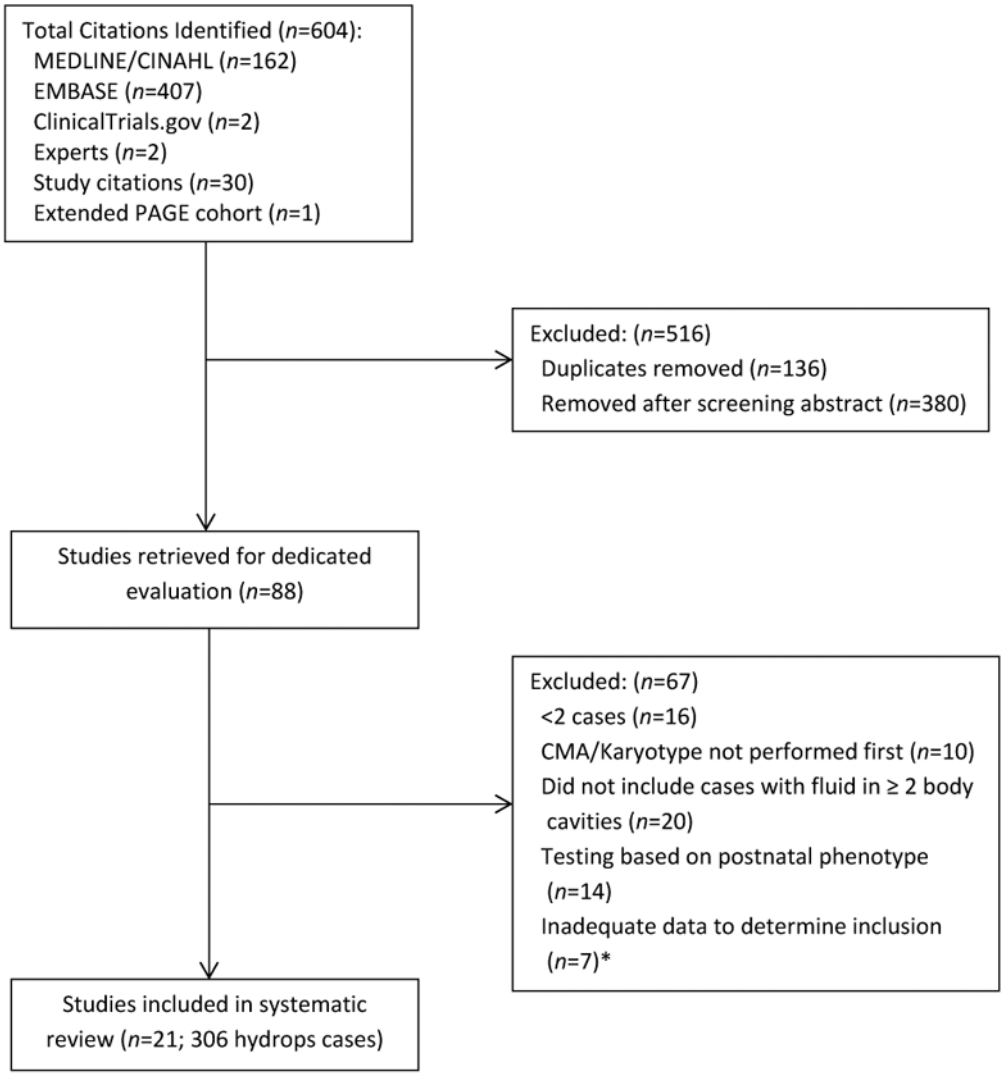

Figure 1 –.

Flowchart demonstrating included studies *Corresponding author contacted to request additional information

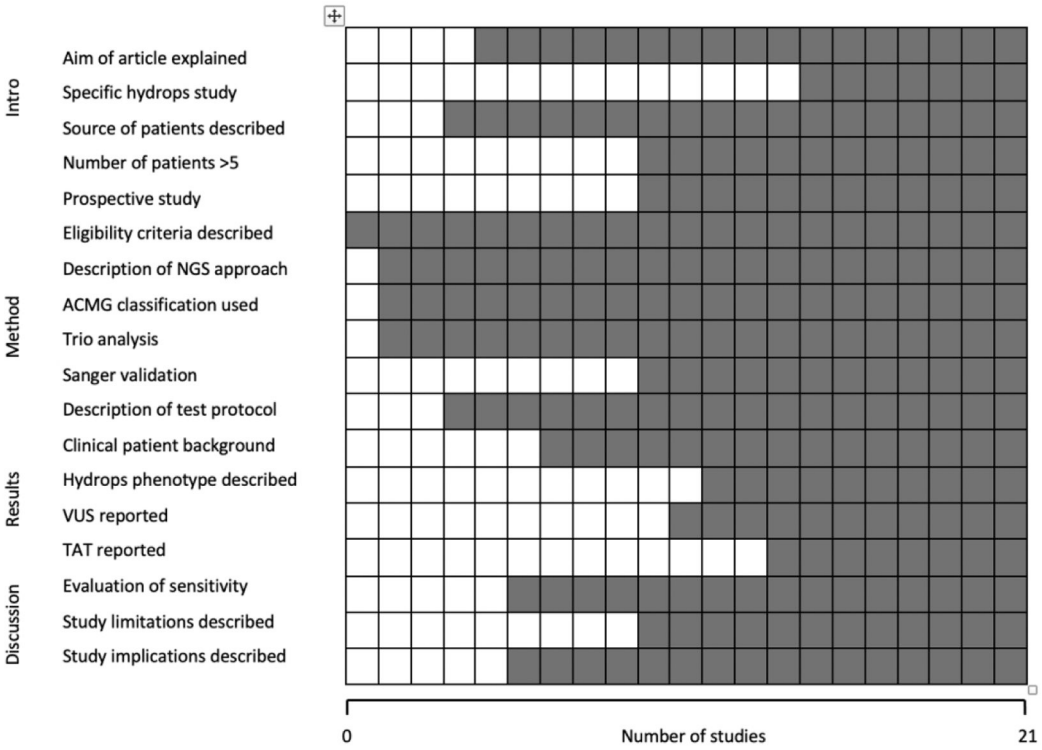

Figure 2 –.

Quality assessment of 21 studies included in systematic review, using modified Standards for Reporting of Diagnostic Accuracy criteria. ACMG, American College of Medical Genetics and Genomics; NGS, next-generation Sequencing; TAT, turnaround time, VUS, variants of uncertain significance. ☐ No ■ Yes

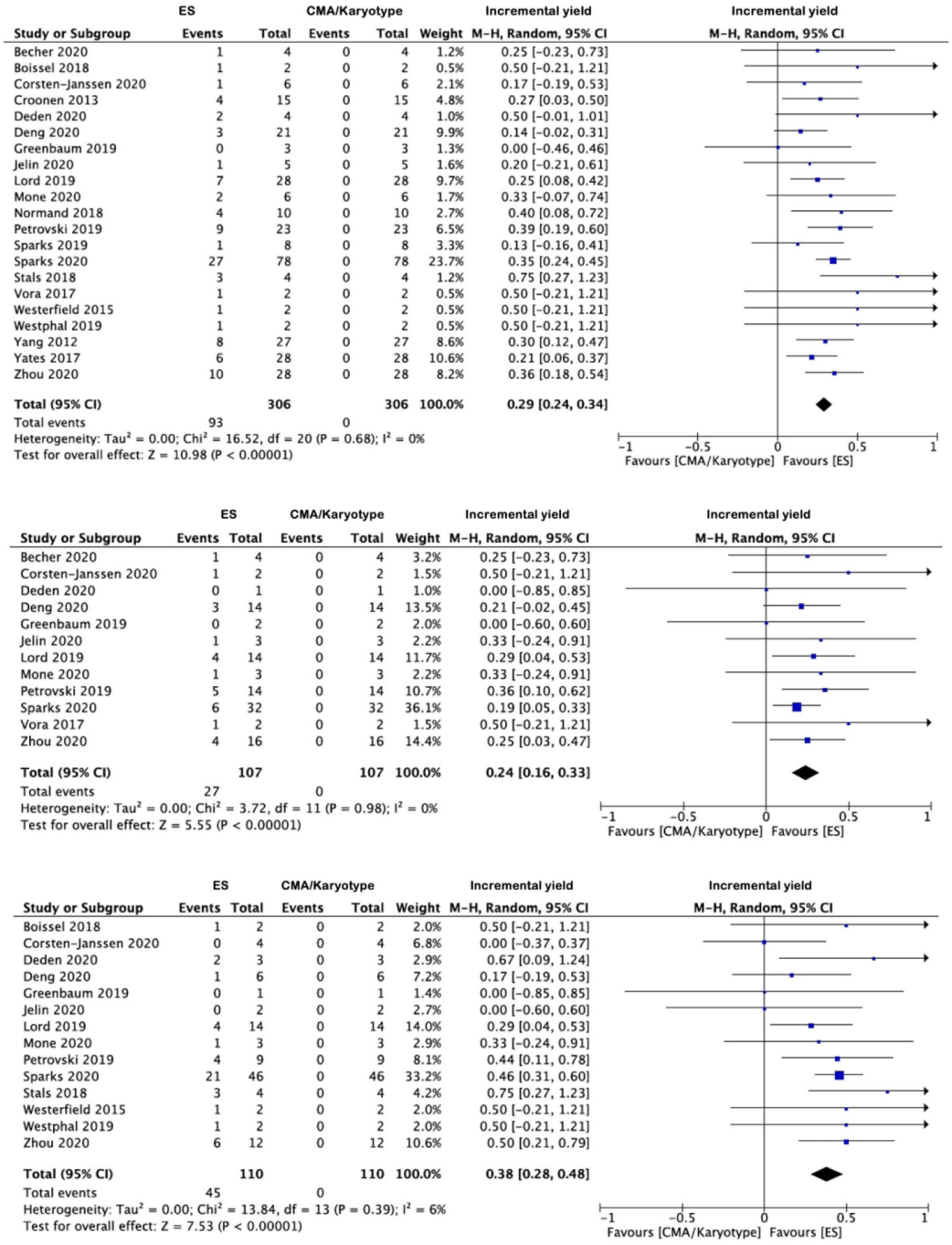

Figure 3 -

Forest plots showing incremental yield of exome sequencing (or an alternative sequencing strategy) over chromosomal microarray analysis/karyotyping in fetuses with prenatally detected non-immune hydrops fetalis (NIHF), overall (a) and in those with isolated NIHF (b) and NIHF with additional fetal structural anomalies (c). Only first author of each study is given. Refers to cases with a normal CMA result. CMA = chromosome microarray; M–H = Mantel–Haenszel.

Table 1-

Characteristics of included studies [CE, clinical exome; FSA, fetal structural anomaly, NIHF, nonimmune hydrops fetalis; N/S, not-stated; WES, whole exome sequencing *coverage not stated]

Study	Next Generation Sequencing Approach	Number of NIHF cases		
		All NIHF	Isolated NIHF	NIHF and additional FSAs
Becher, *et al.*[26]	WES Trio 103 × coverage Roche SeqCap EZ MedExome Plus capture + Illumina NextSeq 500	4	4	0
Boissel *et al.*[18]	WES Trio 110 × coverage Agilent capture + Illumina HiSeq 2000 or 2500	2	0	2
Corsten-Janssen, *et al.*[32]	WES Trio 20 × coverage Agilent capture + Illumina NextSeq500	6	2	4
Croonen, *et al.*[33]*	Clinical Exome; Noonan Panel Illustra amplification. Sequencer not stated	15	N/S	N/S
Denden, *et al.*[27]	WES Trio 200-300 × coverage Agilent capture + Illumina NextSeq500	4	1	3
Deng, *et al.*[19]	WES Trio 120 × coverage Agilent capture + Illumina HiSeq XTen or Novaseq 6000	21	14	6
Jelin, *et al.*[20]	WES Trio depth of coverage <10 removed Agilent capture + Illumina Hi-Seq 2500	5	3	2
Greenbaum, *et al.*[28]	WES Trio 100 × coverage Capture kit unknown + Illumina sequencing	3	2	1
Lord *et al.*[8]	Trio WES Panel 1628 genes Agilent capture + Illumina Hi-Seq 2500 98.3% of the bait regions covered at a minimum depth of 5 ×	28	14	14
Mone, *et al.*[34]	Trio WES Panel 1628 genes Agilent capture + Illumina Hi-Seq 2500 98.3% of the bait regions covered at a minimum depth of 5 ×	6	3	3
Normand *et al.*[21]	WES Trio Coverage 150 × Roche NimbleGen capture Illumina Genome Analyzer IIx platform/HiSeq 2000	10	N/S	N/S
Petrovski *et al.*[16]	WES Trio Nimblegen SeqCap EZ capture + Illumina Hiseq 2500. Average read coverage 89.3 reads Bioinformatic signatures	23	14	9
Sparks, et al. 2019[29]*	WES × 1 Clinical exome × 7 Details not specified	8	N/S	N/S
Sparks 2, et al. 2020[2]*	WES Trio llumina HiSeq 2500 or Illumina NovaSeq 6000	78	32	46
Stals *et al.*[23]	WES Parents only 80 × coverage Agilent capture + Illumina HiSeq 2500 or NextSeq500. Only include het rare (MAF<0.001) variants in same gene in both parents	4	0	4
Vora *et al.*[22]*	CE and WES Trio Illumina Hi-Seq 2500	2	2	0
Westerfield, *et al.*[30]	WES Trio 130 × coverage Roche NimbleGen capture + Illumina Genome Analyzer IIx or HiSeq 2000	2	0	2
Westphal *et al.*[24]	WES Trio 20,000 genes 150 × coverage	2	0	2
Yang, *et al.*[31]*	Clinical exome; Lymphoedema panel Oligo 6.1 PCR amplification + ABI. PRISM 3000 DNA sequencer	27	N/S	N/S
Yates *et al.* [25]	WES Trio 140 × coverage Agilent capture + Illumina HiSeq 2000 or 2500	2 8	N/S	N/S

Study	Next Generation Sequencing Approach	Number of NIHF cases		
		All NIHF	Isolated NIHF	NIHF and additional FSAs
Zhou, et al.[17]*	WES Trio in recurrent NIHF Agilent capture + Illumina HiSeq X Ten	28	16	12