

ORIGINAL ARTICLE

5 Identification of candidate variants in 6 *NEB*, *OFD1*, and *SLCO2A1* in a product of 7 conception from a family with recurrent 8 miscarriages

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11 ABSTRACT

12 **Background:** Recurrent fetal loss can arise from a wide range of genetic abnormalities, many of which remain
13 undetected in the absence of comprehensive molecular testing. Trio-based clinical exome sequencing enables
14 the systematic identification of candidate mutations and variants of uncertain significance (VUS) that underlie
15 fetal loss and supports accurate diagnosis and informed genetic counselling for affected families.

16 **Methods:** Genomic DNA was extracted from fetal tissue and peripheral blood leukocytes of the couple, sib-
17 lings, and grandparents. Trio-clinical exome sequencing was performed, followed by Sanger sequencing to
18 confirm candidate variants. The variants were evaluated for pathogenicity and the pattern of inheritance.

19 **Results:** Trio-clinical exome sequencing identified two homozygous nonsynonymous variants in the *NEB*
20 gene, one in exon 6 (NM_001164507.2: c.367C>T; p.Arg123Cys) and another in exon 133 (NM_001164507.2:
21 c.20279A>G; p.Asp6760Gly) in the proband. In addition, a hemizygous nonsynonymous variant in exon 10 of
22 the X-linked *OFD1* gene (NM_003611.3: c.1035G>T; p.Lys345Asn) and a homozygous nonsynonymous variant
23 in exon 8 of the *SLCO2A1* gene (NM_005630.3: c.1105G>T; p.Gly369Cys) were observed in the proband. The
24 *NEB* and *SLCO2A1* variants were heterozygous in the couple, and only the mother was heterozygous for the
25 *OFD1* variant.

26 **Conclusion:** This study identified two candidate *NEB* gene variants (classified as VUS) as the most likely candi-
27 date contributors to recurrent fetal loss. The findings provide the basis for informed genetic counselling and
28 reproductive risk assessment.

29 **Keywords:** Clinical exome sequencing, fetal tissue, *NEB*, recurrent pregnancy loss, variants of uncertain signif-
30 icance, consanguinity.

31 Introduction

32 Recurrent pregnancy loss (RPL), defined as two or more
33 consecutive pregnancy losses, is one of the most common
34 complications encountered during pregnancy, with fetal
35 anomalies representing a significant contributing factor.
36 Approximately 70% of human conceptions fail to
37 achieve viability, and nearly 50% of pregnancies end in
38 miscarriage before the clinical recognition of a missed
39 period or the detection of embryonic cardiac activity (1).
40 Preimplantation genetic diagnosis (PGD) for aneuploidy
41 screening may be considered in couples with unexplained
42 RPL (2,3). Genetic analysis of fetal tissue from couples

43 experiencing more than two miscarriages can elucidate
44 the underlying etiology and support genetic counselling

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45	to inform prognosis for subsequent pregnancies (2).	106
46	Genetic factors, particularly chromosomal abnormalities,	107
47	are among the most common identifiable causes of	
48	RPL, although a significant proportion of cases remain	
49	unexplained or are attributable to non-genetic causes	
50	(1). Nongenetic factors include advanced maternal	
51	age, alcohol consumption, cigarette smoking, drug use,	
52	heavy caffeine intake, chronic maternal diseases, uterine	
53	malformations, previous induced abortions, structural	
54	reproductive tract abnormalities, and prior miscarriages	
55	(4).	
56	Pregnant individuals with a history of genetic disorders	
57	can be tested for single-gene disorders, chromosomal	
58	abnormalities, multifactorial or complex disorders, and	
59	teratogenic conditions to enable early diagnosis and	
60	intervention. Prenatal genetic defects can be detected	
61	through techniques such as karyotyping, chromosomal	
62	microarray analysis (CMA), and whole-exome	
63	sequencing (WES) of fetal DNA. Next-generation	
64	sequencing (NGS) has emerged as an invaluable tool,	
65	enabling not only the identification of novel disease-	
66	associated genes but also the clinical diagnosis of	
67	previously unexplained pathologies, thereby improving	
68	prenatal diagnostic accuracy (5).	
69	WES is increasingly applied in the evaluation of RPL,	
70	although its diagnostic yield remains relatively low in	
71	unselected cases (~2%-5%), particularly in the absence	
72	of structural abnormalities. However, the yield improves	
73	in carefully selected cases with suspected monogenic	
74	etiologies. In contrast, prenatal exome sequencing	
75	has demonstrated higher diagnostic utility in fetuses	
76	with structural anomalies, with an additional yield of	
77	approximately 30%-36% following negative CMA (6).	
78	In cases of recurrent fetal anomalies, the diagnostic yield	
79	may reach up to ~40%, highlighting the importance of	
80	phenotype-driven testing (7).	
81	Genetic variations in Nebulin (<i>NEB</i>), Oral-Facial-Digital	
82	Syndrome Type 1 (<i>OFDI</i>), and Solute Carrier Organic	
83	Anion Transporter Family Member 2A1 (<i>SLCO2A1</i>)	
84	have been associated with fetal anomalies and recurrent	
85	spontaneous miscarriages. Variants in <i>NEB</i> lead to	
86	muscle weakness and nemaline myopathy (8). The <i>NEB</i>	
87	gene (OMIM#161650), located on chromosome 2q23.3,	
88	causes nemaline myopathy 2 (OMIM#256030), an	
89	autosomal recessive skeletal muscle disorder. Variants	
90	in <i>OFDI</i> (OMIM#300170), located on chromosome	
91	Xp22.2, are responsible for Joubert syndrome 10	
92	(OMIM#300804) and oral-facial-digital syndrome	
93	type 1 (<i>OFDI</i> ; OMIM#311200). Joubert syndrome	
94	is characterized by abnormal hindbrain development,	
95	hypotonia, cerebellar ataxia, dysregulated breathing,	
96	and developmental delay. In contrast, oral-facial-digital	
97	syndrome presents with craniofacial, oral, and digital	
98	malformations and follows an X-linked dominant	
99	inheritance pattern with male lethality (9). Variants in	
100	<i>SLCO2A1</i> (OMIM#601460), located on chromosome	
101	3q22.1, cause primary hypertrophic osteoarthropathy	
102	2— <i>PHOAR2</i> (OMIM#614441) in the autosomal	
103	recessive form, while heterozygous variants result in the	
104	autosomal dominant form of <i>PHOAR2</i> (OMIM#167100).	
105	<i>PHOAR2</i> is a rare disorder characterized by digital	
	clubbing, pachydermia, and periostosis, with more severe	106
	manifestations observed in males (10,11).	107
	This study reports the identification of homozygous and	108
	hemizygous variants in three genes (<i>NEB</i> , <i>SLCO2A1</i> ,	109
	and <i>OFDI</i> , respectively) in a fetus from a couple with	110
	a history of multiple miscarriages, culminating in	111
	intrauterine fetal demise (IUFD).	112
	Materials and Methods	113
	Ethical considerations and informed consent	114
	The Institutional Ethics Committee of Madras Medical	115
	College (MMC) approved the study (Approval No:	116
	34042021; Dated: 07.04.2021), and written informed	117
	consent was obtained from all participants.	118
	Clinical history of the family	119
	A 28-year-old female with a history of multiple	120
	miscarriages resulting in IUFD was referred to the	121
	Department of Genetics, Dr ALM Post-Graduate	122
	Institute of Basic Medical Sciences, University of	123
	Madras, for diagnosis and genetic counseling. The	124
	couple was related by a consanguineous marriage	125
	(first cousins), and the mother had a poor obstetric	126
	history. During the first pregnancy, the fetus was	127
	male (confirmed by fetal/perinatal autopsy) at 13-	128
	14 weeks gestational age (by LMP), presenting with	129
	cardiac anomalies and bradycardia on ultrasound scan.	130
	Fetal autopsy revealed facial dysmorphism including	131
	hypertelorism (inner intercanthal distance 0.5 cm), flat	132
	nose with anteverted nares, long philtrum, elongated	133
	neck, and cardiomegaly with dilated ascending aorta	134
	and aortic arch with four systemic branches. The lung	135
	parenchyma was completely autolyzed and could not be	136
	assessed. Histopathological examination of the placenta	137
	revealed diffuse villitis, subchorionic hemorrhage, and	138
	microinfarctions; the pathologist noted that placental	139
	infection had probably contributed to IUFD. Skeletal	140
	muscle histopathology was not possible due to autolytic	141
	changes at 13-14 weeks gestational age. Chromosomal	142
	aneuploidy screening of the fetal tissue was performed	143
	using the KaryoLite BoBs assay, and no aneuploidy	144
	was detected in any chromosome, including X/Y. In the	145
	second pregnancy, an antenatal scan identified a cardiac	146
	anomaly, resulting in IUFD at 14 weeks of gestation.	147
	Cytogenetic analysis revealed normal karyotypes for	148
	the couple. Fetal tissue from the third pregnancy, along	149
	with peripheral blood samples from the couple, their	150
	siblings (B1 and B2: paternal siblings of the father; B3:	151
	maternal sibling of the mother), and the couple's parents	152
	(maternal grandmother, maternal grandfather, paternal	153
	grandmother, paternal grandfather), was collected for	154
	further genetic analysis.	155
	Pregnancy timeline	156
	Pregnancy 1: 13-14 weeks gestation (by LMP); male	157
	fetus (confirmed by autopsy); cardiac anomalies and	158
	bradycardia on ultrasound; fetal autopsy performed	159
	(facial dysmorphism, cardiomegaly with dilated	160
	ascending aorta; lung parenchyma autolysed; skeletal	161

muscle histopathology not possible due to autolysis); placental histopathology showed villitis, subchorionic haemorrhage, and microinfarctions; chromosomal aneuploidy screening (KaryoLite BoBs) on fetal tissue was normal, including X/Y; outcome: IUFD.

Pregnancy 2: 14 weeks gestation; cardiac anomaly identified on antenatal ultrasound; parental cytogenetic (karyotype) analysis was normal for both partners; no fetal autopsy or fetal genetic testing performed; outcome: IUFD.

Pregnancy 3 (proband): Evaluated at 12-13 weeks of gestation (by ultrasound crown-rump length; CRL 62 mm, corresponding to 12 weeks 4 days); non-immune hydrops fetalis (generalized edema, pleural effusion, ascites) with absent cardiac activity on ultrasound; early gestational age and autolytic changes precluded detailed autopsy and skeletal muscle histopathology; no dedicated aneuploidy assay (e.g., KaryoLite BoBs) was performed on this pregnancy; this fetus was the proband, which underwent trio-clinical exome sequencing (with the parents), maternal cell contamination (MCC) testing, and Sanger confirmation of candidate variants

DNA extraction

Genomic DNA was extracted from fetal tissue and whole blood samples using a modified phenol–chloroform–isoamyl alcohol (P:C:I) method (12). The concentration and purity of the extracted DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, DE, USA). DNA integrity was assessed by electrophoresis on a 0.8% agarose gel using a Mupid Gel Electrophoresis System (Takara, Japan) and visualized with a UV Gel Doc system (Syngene, G: Box Chemi-XX6).

Trio-clinical exome sequencing

Targeted sequencing was performed using a custom-designed clinical exome capture kit covering approximately 6,670 disease-associated genes. Genomic DNA was extracted from peripheral blood samples and subjected to selective enrichment of protein-coding regions, followed by library preparation. Sequencing was performed on the Illumina platform, generating high-quality data with an average sequencing depth of 241X, an average on-target depth of 129.3X, and >92% of target bases covered at ≥30X. MCC testing was performed separately on the DNA extracted from the fetal tissue sample at MedGenome Labs Ltd., Bangalore and was confirmed to be negative. As per the Pre-Natal Diagnostic Techniques (Regulation and Prevention of Misuse) Rules, 1994, the laboratory does not determine fetal sex from sequencing data; fetal sex was inferred from the hemizygous state of the X-linked *OFDI* variant in the proband. Raw reads were aligned to the human reference genome (GRCh38.p13) using the Sentieon aligner, following GATK best practices. Post-alignment processing included duplicate removal, base quality score recalibration, and indel realignment (13,14).

Variant calling and copy number detection

Variants were called using the Sentieon Haplotype Caller, generating an initial set of single nucleotide variants (SNVs) and small insertions/deletions (indels). Copy number variants (CNVs) were detected from the targeted sequencing data using ExomeDepth (v1.1.10), which identifies rare CNVs by comparing read depths to a matched aggregate reference dataset. The coding region coverage of *NEB* was 90.60%, and *SLCO2A1* was 100.00% (clinical exome report). The CNV detection sensitivity of the assay is 70%-75% for deletions/duplications greater than 10 bp; no large CNVs were identified in *NEB* or *SLCO2A1*. However, approximately 9.4% of the *NEB* coding region was not covered by the assay, and deep intronic variants, repeat expansions, or structural variants below the detection threshold may not have been identified.

Variant filtering and annotation

A stepwise variant filtering workflow was employed. All 6,670 genes in the clinical exome panel were screened for the given clinical indications. The non-immune hydrops panel genes were fully covered and analyzed; coverage of critical genes including *NEB* (90.60%) and *SLCO2A1* (100.00%) is provided in the supplementary appendix of the clinical exome report. Low-quality and low-depth variants were first removed. Common variants were filtered based on allele frequencies in public population databases, including 1000 Genomes Phase 3, gnomAD (v2.1), EVS, dbSNP (v151), 1000 Japanese Genome, and an internal Indian database. Functional filtering retained only protein-altering variants, including non-synonymous and splice-site changes, while synonymous variants were excluded. Variants were annotated using Ensembl VEP against Ensembl release 99 and cross-referenced with ClinVar, OMIM, HGMD, GWAS, and SwissVar databases.

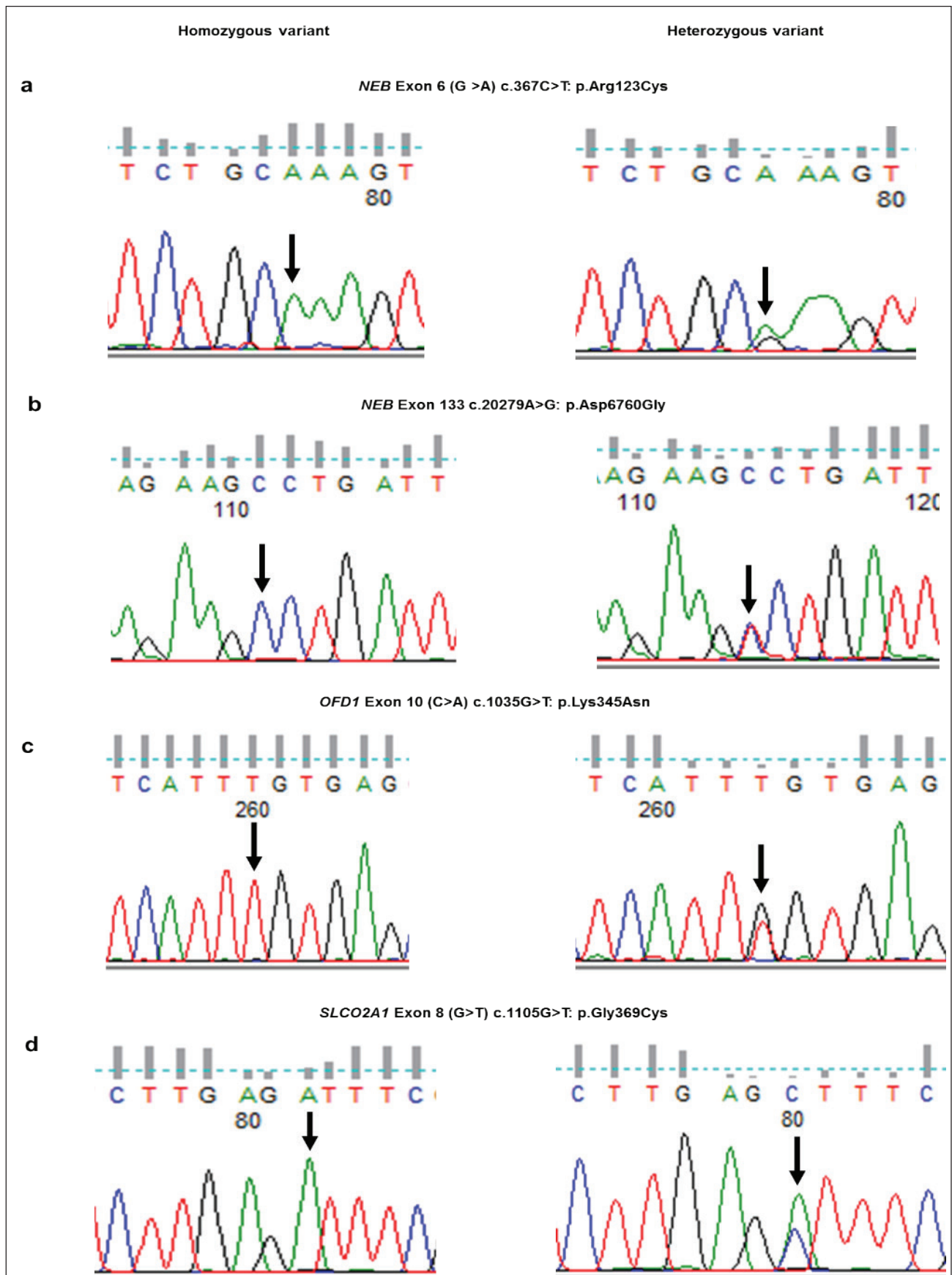
Functional impact and clinical interpretation

The functional impact of nonsynonymous variants was assessed using multiple in silico prediction tools, including *SIFT*, *PolyPhen-2*, *MutationTaster2*, *LRT*, *CADD*, and *REVEL*. Clinically relevant variants were prioritized based on the patient phenotype and interpreted according to ACMG/AMP guidelines. Only actionable variants in coding regions and splice sites were reported, while silent variants without predicted functional impact were excluded.

Confirmation of trio-clinical exome sequencing-identified variants by Sanger sequencing

The candidate gene variants identified by trio-clinical exome sequencing were validated using Sanger sequencing. Selected exons of *NEB* (exons 6 and 133), *OFDI* (exon 10), and *SLCO2A1* (exon 8) were amplified using gene-specific primers. PCR primers were designed using the reference sequences from the NCBI database, and Primer3 was used to generate variant-specific primers. Sanger sequencing was performed using the ABI BigDye Terminator Cycle Sequencing Kit v3.1

277	(Applied Biosystems, Foster City, CA) according to the	carriers. All identified variants were confirmed by Sanger	333
278	manufacturer's protocol. Sequencing was carried out on	sequencing (Figure 1).	334
279	an ABI PRISM 3500 Genetic Analyzer (Tokyo, Japan),		
280	and the results were analyzed using BioEdit and FinchTV		
281	software.		
282	Functional analysis of the impact of identified		
283	variants		
284	The functional impact of the identified nonsynonymous	Sequencing of extended family members, including	337
285	variants was evaluated using multiple in silico prediction	siblings and grandparents, revealed that the maternal	338
286	tools. Sorting Intolerant From Tolerant (<i>SIFT</i>) predicts	grandmother, paternal grandfather, and siblings B2	339
287	whether amino acid substitutions are likely to affect	(paternal sibling of the father) and B3 (maternal sibling	340
288	protein function based on sequence homology and the	of the mother) were heterozygous carriers of <i>NEB</i> and	341
289	physical properties of amino acids (Kumar et al., 2009),	<i>SLCO2A1</i> variants. The unrelated maternal grandfather,	342
290	while Polymorphism Phenotyping v2 (PolyPhen-2)	paternal grandmother, and paternal sibling (B1) did not	343
291	assesses the potential impact of these substitutions	carry any variants. The maternal grandmother was also	344
292	on protein structure and stability using sequence,	found to be heterozygous for the X-linked <i>OFDI</i> variant	345
293	phylogenetic, and structural information (http://genetics.	(Figure 2).	346
294	bwh.harvard.edu/pph2/).		
295	Combined Annotation Dependent Depletion (CADD)	The amino acid residues affected in <i>NEB</i> (p.Arg123 and	347
296	scores were obtained to estimate the deleteriousness	p.Asp6760), <i>OFDI</i> (p.Lys345), and <i>SLCO2A1</i> (p.Gly369)	348
297	of SNVs by integrating multiple genomic annotations	were highly conserved across species, indicating that	349
298	(https://cadd.bihealth.org/snv/), and Rare Exome Variant	these variants may disrupt critical functional regions of	350
299	Ensemble Learner (REVEL) was used as an ensemble	the proteins (Figure 3).	351
300	method combining several computational tools to predict		
301	the pathogenicity of missense variants (https://sites.	Variant interpretation	352
302	google.com/site/revelgenomics/contact).		
303	Population frequency data were retrieved from publicly	The two homozygous variants identified in the <i>NEB</i>	353
304	available databases, including the 1000 Genomes Project	gene (p.Arg123Cys and p.Asp6760Gly) showed high	354
305	(1KGP) and gnomAD, and gene-level constraint metrics	<i>PolyPhen-2</i> scores (0.975 and 0.917, respectively),	355
306	such as missense Z-scores from gnomAD were used	indicating probable damaging effects, along with	356
307	to assess intolerance to variation. Additional details on	elevated CADD scores (26.7 and 32), suggestive of	357
308	prediction tool parameters and score interpretation have	functional impact. Although <i>SIFT</i> scores (0.269 and	358
309	been described previously (Richards et al., 2015; Genet	0.424) were within the tolerated range, the combined	359
310	Med. 17:405–424).	interpretation of multiple tools, including REVEL scores	360
311	Results	(0.269 and 0.397), supports a possible deleterious effect.	361
312	Trio-clinical exome sequencing identifies novel	Additionally, the <i>NEB</i> gene demonstrated moderate	362
313	variants in the <i>NEB</i>, <i>OFDI</i>, and <i>SLCO2A1</i> genes	intolerance to missense variation (Z-score = 2.25), further	363
314	Trio-clinical exome sequencing of the proband and	indicating that amino acid substitutions in this gene	364
315	couple revealed two nonsynonymous variants in <i>NEB</i>	are less likely to be tolerated and may have functional	365
316	(NM_001164507.2) and one variant each in <i>OFDI</i>	consequences.	366
317	(NM_003611.3) and <i>SLCO2A1</i> (NM_005630.3). In <i>NEB</i> ,	The hemizygous variant in the <i>OFDI</i> gene (p.Lys345Asn)	367
318	the proband carried two homozygous nonsynonymous	showed a high <i>PolyPhen-2</i> score (0.966) and moderate	368
319	variants: One in codon 123, c.367C>T (p.Arg123Cys)	REVEL score (0.501), suggesting a potentially damaging	369
320	and the second in codon 6760, c.20279A>G	effect, while the <i>SIFT</i> score (0.682) indicated tolerance.	370
321	(p.Asp6760Gly), corresponding to genomic positions	The missense Z-score for <i>OFDI</i> was 0, suggesting that	371
322	chr2:g.151725488G>A and chr2:g.151547517T>C.	the gene is relatively tolerant to missense variation,	372
323	Both variants were present in a heterozygous state in	thereby reducing the strength of computational evidence	373
324	the couple. Interestingly, a hemizygous nonsynonymous	supporting pathogenicity.	374
325	variant, c.1035G>T (p.Lys345Asn), mapping to chrX:	Similarly, the homozygous variant in the <i>SLCO2A1</i> gene	375
326	g.13751348G>T was in codon 345 of the X-linked	(p.Gly369Cys) demonstrated a high <i>PolyPhen-2</i> score	376
327	<i>OFDI</i> gene in the proband; this variant was absent in	(1.000), elevated CADD score (35), and high REVEL	377
328	the father but present in the mother in a heterozygous	score (0.726), indicating a potentially damaging effect,	378
329	state. Additionally, the proband carried a homozygous	although the <i>SIFT</i> score (0.726) suggested tolerance. The	379
330	nonsynonymous variant, c.1105G>T (p.Gly369Cys),	missense Z-score for <i>SLCO2A1</i> was 1.11, indicating only	380
331	corresponding to chr3:133948536C>A in <i>SLCO2A1</i>	mild constraint, which limits the strength of evidence for	381
332	(NM_005630.3), with the couple being heterozygous	functional intolerance.	382
		Overall, while all variants exhibited computational	383
		evidence suggestive of potential deleterious effects,	384
		the combination of multiple prediction tools along	385
		with gene-level constraint metrics indicates that the	386
		<i>NEB</i> variants have comparatively stronger support for	387
		functional impact. In contrast, the <i>OFDI</i> and <i>SLCO2A1</i>	388
		variants show less consistent evidence, particularly due to	389



390 **Figure 1.** Sanger sequencing chromatograms confirming variants identified by trio-clinical exome sequencing in the proband and
 391 couple. a. A homozygous nonsynonymous variant in exon 6 of the *NEB* gene, c.367C>T, was identified in the proband, and the
 392 couple were heterozygous. b. Exon 133 of the *NEB* gene c.20279A>G variant was homozygous in the proband, and the couple
 393 were heterozygous. c. The nonsynonymous variant identified in exon 10 of the *OFD1* gene c.1035G>T was in a hemizygous state
 394 in the proband. The mother was heterozygous for this variant. No variant was detected in the proband's father. d. A homozygous
 395 nonsynonymous variant in exon 8 of the *SLCO2A1* gene c.1105G>T, was detected in the proband, and the couple were carriers.

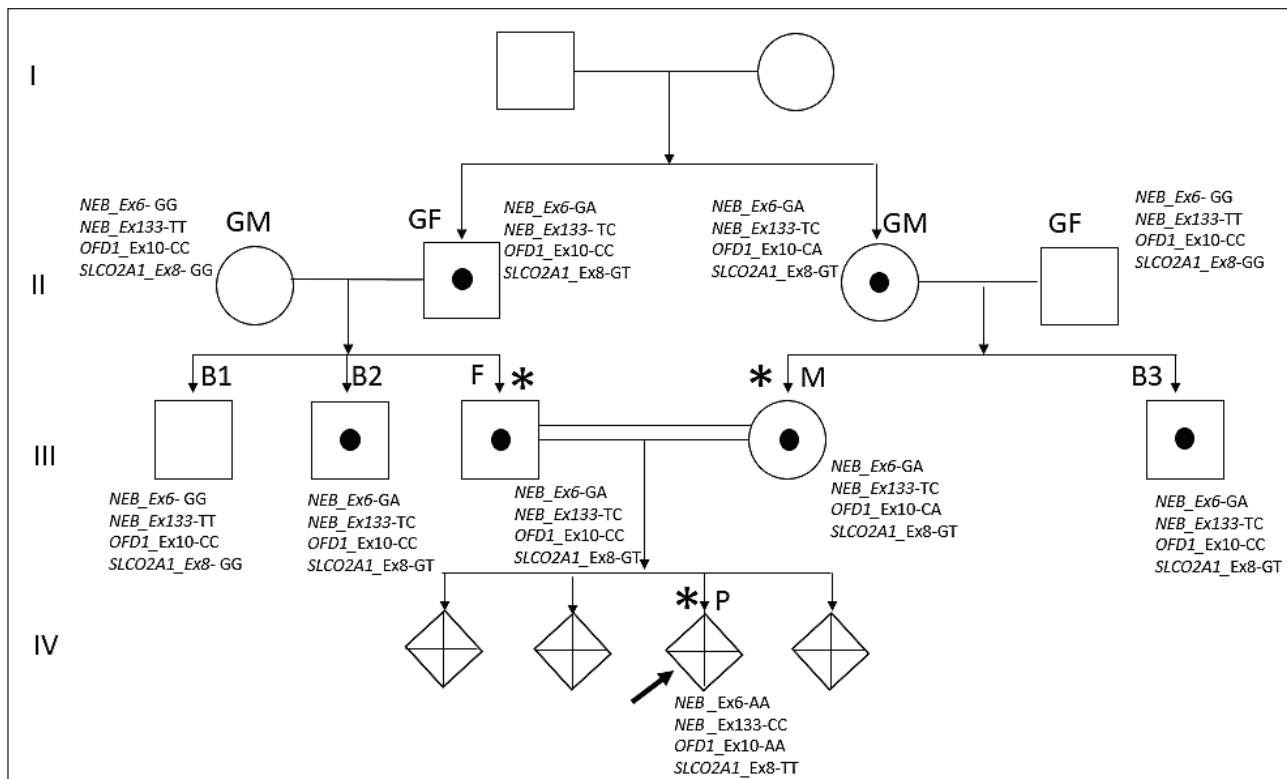


Figure 2. Pedigree chart of the proband's family showing segregation of identified variants. The black dotted circles (females) and squares (males) indicate family members carrying the gene variants, whereas the blank circles or squares indicate unaffected members. Asterisks indicate the DNA samples subjected to exome sequencing. The arrow indicates the proband. Abbreviations: P: Proband; F: Father; M: Mother; B1, B2: Paternal siblings (father's siblings); B3: Maternal sibling (mother's sibling); GF: Grandfather; GM: Grandmother.

gene-level tolerance and limited phenotypic correlation (Tables 1 and 2).

Phenotype–genotype correlation

The fetus was evaluated at 13 weeks of gestation and presented with non-immune hydrops fetalis, including generalized edema, pleural effusion, ascites, and absent cardiac activity. Early gestational age and autolytic changes precluded detailed autopsy and histopathological assessment, including confirmation of skeletal muscle abnormalities. Despite these limitations, the combination of recurrent IUFD, early-onset hydrops, consanguinity, and candidate *NEB* variants (VUS) are possibly consistent with a severe prenatal nemaline myopathy phenotype. It is acknowledged that hydrops fetalis and IUFD alone are not pathognomonic for *NEB*-related disease, and the PP4 criterion is applied with caution in the absence of histopathological confirmation of nemaline rods. Overall, even with limited histological data, the observed clinical pattern is compatible with *NEB*-related disease, providing a supportive phenotype–genotype correlation that is consistent with, but not diagnostic of, *NEB*-related prenatal myopathy. Histopathological confirmation of nemaline rods was not possible due to complete autolysis of fetal tissue at 13–14 weeks gestational age; this represents a key limitation of the phenotypic interpretation.

Discussion and Conclusion

We investigated the genetic cause of fetal loss in a proband from a 28-year-old woman with a history of multiple miscarriages. As the molecular basis of this unique miscarriage case was previously unknown, we performed trio-based clinical exome sequencing, which identified two novel homozygous nonsynonymous variants, p.Arg123Cys and p.Asp6760Gly, in the *NEB* gene. Both parents were confirmed to be carriers of these variants. Nebulin plays an essential role in actin filament-based movement, cardiac muscle cell development, muscle contraction, and muscle tissue formation. The histopathological assessment was not done.

Previous studies have linked *NEB* variants with Arthrogryposis Multiplex Congenita-6 (AMC6), a severe congenital myopathy characterized by fetal akinesia and multiple joint contractures (15). Yonath et al. (2012) reported abnormal prenatal ultrasound findings in fetuses affected by AMC6, while Abdalla et al. (2017) demonstrated that *NEB* variants contribute to fetal akinesia deformation sequence and lethal multiple pterygium syndrome (15,16). Ahmed et al. (2018) and Rocha et al. (2021) further confirmed the association of homozygous or compound heterozygous *NEB* variants with severe prenatal phenotypes (17,18). Formal haplotype phasing of the two *NEB* variants was not performed in this study; given the consanguineous background, these variants may reside on the same ancestral haplotype (identity

a. NEB c.367C>T: p.Arg123Cys			b. NEB c.20279A>G: p.Asp6760Gly			c. OFD1 c.1035G>T: p.Lys345Asn			d. SLCO2A1 c.1105G>T: p.Gly369Cys		
Consensus	Conservation	DTPELRRIKKV	Consensus	Conservation	LIYKSDFFKMQ	Consensus	Conservation	ETYDRKLLKNEL	Consensus	Conservation	ANFLIGA VNL P
Homo sapiens	113	DTPELRRIKKV 123	Homo sapiens	6755	LIYKSDFFKMQ 6765	Homo sapiens	340	ETYDRKLLKNEL 350	Lemur catta	355	ANFLIGA VNL P 365
Pan paniscus	117	DTPELRRIKKV 128	Pan troglodytes	7241	LIYKSDFFKMQ 7251	Pan troglodytes	292	ETYDRKLLKNEL 302	Cavia porcellus	537	ANFLIGA VNL P 547
Pan troglodytes	117	DTPELRRIKKV 128	Gorilla gorilla gorilla	6755	LIYKSDFFKMQ 6765	Pan paniscus	340	ETYDRKLLKNEL 350	Rhinopithecus bieti	288	ANFLIGA VNL P 298
Gorilla gorilla gorilla	117	DTPELRRIKKV 128	Pan paniscus	6755	LIYKSDFFKMQ 6765	Cercopithecus atys	341	ETYDRKLLKNEL 351	Heterocephalus glaber	364	ANFLIGA VNL P 374
Pongo abelii	117	DTPELRRIKKV 128	Nomascus leucogenys	6264	LIYKSDFFKMQ 6274	Nomascus leucogenys	341	ETYDRKLLKNEL 351	Callithrix jacchus	364	ANFLIGA VNL P 374
Trachypithecus francoisi	117	DTPELRRIKKV 128	Pongo abelii	6750	LIYKSDFFKMQ 6760	Hyllobates moloch	340	ETYDRKLLKNEL 350	Acomys russatus	363	ANFLIGA VNL P 373
Rhinopithecus roxellana	117	DTPELRRIKKV 128	Rhinolophus ferrumequinum	5781	LIYKSDFFKMQ 5791	Gorilla gorilla gorilla	292	ETYDRKLLKNEL 302	Tupaia chinensis	362	ANFLIGA VNL P 401
Nomascus leucogenys	113	DTPELRRIKKV 123	Aotus nancymae	5778	LIYKSDFFKMQ 5788	Pongo abelii	292	ETYDRKLLKNEL 302	Cebus imitator	364	ANFLIGA VNL P 374
Ptilocolobus tephroscels	117	DTPELRRIKKV 128	Trachypithecus francoisi	5783	LIYKSDFFKMQ 5793	Macaca thibetana thibetana	340	ETYDRKLLKNEL 350	Octodon degus	353	ANFLIGA VNL P 392
Colobus angolensis palliatus	117	DTPELRRIKKV 128	Rhinopithecus roxellana	5783	LIYKSDFFKMQ 5793	Rhinopithecus roxellana	292	ETYDRKLLKNEL 302	Propithecus coquereli	364	ANFLIGA VNL P 374
			Callithrix jacchus	5778	LIYKSDFFKMQ 5788	Macaca fascicularis	233	ETYDRKLLKNEL 243	Sciurus carolinensis	364	ANFLIGA VNL P 374
			Puma yagouaroundi	5787	LIYKSDFFKMQ 5797	Macaca mulatta	200	ETYDRKLLKNEL 210	Sapajus apella	364	ANFLIGA VNL P 374
			Panthera pardus	6273	LIYKSDFFKMQ 6283	Theropithecus gelada	371	ETYDRKLLKNEL 381	Aotus nancymae	364	ANFLIGA VNL P 374
			Macaca mulatta	5783	LIYKSDFFKMQ 5793	Macaca nemestrina	200	ETYDRKLLKNEL 210	Pongo abelii	364	ANFLIGA VNL P 374
			Macaca thibetana thibetana	5783	LIYKSDFFKMQ 5793	Mandrilus leucophaeus	341	ETYDRKLLKNEL 351	Colobus angolensis palliatus	362	ANFLIGA VNL P 372
			Chlorocebus sabaeus	5783	LIYKSDFFKMQ 5793	Trachypithecus francoisi	340	ETYDRKLLKNEL 350	Microcebus murinus	364	ANFLIGA VNL P 374
			Macaca fascicularis	5783	LIYKSDFFKMQ 5793	Rhinopithecus bieti	340	ETYDRKLLKNEL 350	Chlorocebus sabaeus	364	ANFLIGA VNL P 374
						Callithrix jacchus	340	ETYDRKLLKNEL 350	Macaca mulatta	364	ANFLIGA VNL P 374
						Saimiri boliviensis boliviensis	340	ETYDRKLLKNEL 350	Papio anubis	364	ANFLIGA VNL P 374
						Aotus nancymae	233	ETYDRKLLKNEL 243	Ptilocolobus tephroscels	364	ANFLIGA VNL P 374
						Chlorocebus sabaeus	339	ETYDRKLLKNEL 349	Mandrilus leucophaeus	364	ANFLIGA VNL P 374
						Cebus imitator	340	ETYDRKLLKNEL 350	Macaca nemestrina	364	ANFLIGA VNL P 374
						Ptilocolobus tephroscels	233	ETYDRKLLKNEL243	Oryctolagus cuniculus	363	ANFLIGA VNL P 373
						Callithrix jacchus	346	ETYDRKLLKNEL 356	Rhinopithecus roxellana	364	ANFLIGA VNL P 374
									Pan paniscus	364	ANFLIGA VNL P 374
									Hipposideros armiger	364	ANFLIGA VNL P 374
									Pan troglodytes	364	ANFLIGA VNL P 374
									Trachypithecus francoisi	334	ANFLIGA VNL P 344
									Gorilla gorilla gorilla	364	ANFLIGA VNL P 374
									Cercopithecus atys	364	ANFLIGA VNL P 374
									Nomascus leucogenys	364	ANFLIGA VNL P 374

Figure 3. Evolutionary conservation of mutated amino acid residues in NEB, OFD1, and SLCO2A1. Clustal alignment showing 100% conservation of the amino acid Arginine (R) in codon 123 and Asparagine (D) in codon 6760 of the NEB gene (a & b), in the OFD1 gene amino acid residue Lysine (K) in codon 345 (c), in the SLCO2A1 gene amino acid Glycine (G) in codon 369 (d) across the vertebrate species. Conserved amino acid residues are marked in red.

Table 1. Genetic variants identified by trio-clinical exome sequencing.

Gene	Ref Seq transcript / Protein ID	cDNA change	Protein change	Chromosome position/ dbSNP	Zygosity	Inheritance	Variant type
<i>NEB</i> (Exon 6)	NM_001164507.2/ NP_001157980.2	c.367C>T	p.Arg123Cys	Chr2:151725488/ rs200555425	Homozygous	Autosomal recessive	Nonsynonymous
<i>NEB</i> (Exon 133)	NM_001164507.2/ NP_001157980.2	c.20279A>G	p.Asp6760Gly	Chr2:151547517	Homozygous	Autosomal recessive	Nonsynonymous
<i>OFD1</i> (Exon 10)	NM_003611.3/ NP_003602.1	c.1035G>T	p.Lys345Asn	ChrX:13751348	Hemizygous	X-linked	Nonsynonymous
<i>SLCO2A1</i> (Exon 8)	NM_005630.3/ NP_005621.2	c.1105G>T	p.Gly369Cys	Chr3:133948536	Homozygous	Autosomal recessive	Nonsynonymous

Trio clinical exome sequencing was performed in the proband and both parents. This table summarizes the identified variants, including gene name, RefSeq transcript and protein identifiers (NM/NP), cDNA and protein changes, chromosomal position, zygosity, inheritance pattern, and variant type. All chromosomal positions are reported according to the human reference genome GRCh38.p13. NM, RefSeq mRNA accession number; NP, RefSeq protein accession number; cDNA, complementary DNA

by descent) or separate haplotypes, which cannot be determined from the current exome data.

In addition, we detected a hemizygous nonsynonymous variant (p.Lys345Asn) in exon 10 of the *OFD1* gene in the proband. The mother was heterozygous for this variant, while the father carried no variant, suggesting an X-linked inheritance pattern. The *OFD1* gene is known to play a crucial role in cilium assembly, membrane docking, and organelle localization via membrane tethering, and variants in this gene are often associated with X-linked male lethality. *OFD1*-related male lethality typically occurs during the first or second trimester, as previously reported in women with recurrent male miscarriages (9). However, given the absence of *OFD1*-specific phenotypic features in the proband, the *OFD1* variant identified in this study is interpreted as a possible contributory or modifying factor of uncertain relevance, noted for completeness and for genetic counselling purposes, rather than as a contributor to male fetal lethality. In our study, the hemizygous variant was maternally inherited; fetal sex was not determined by the sequencing laboratory as per regulatory requirements (Pre-Natal Diagnostic Techniques Rules, 1994), but is inferred to be male based on the hemizygous state of this X-linked variant.

Clinical features such as macrocephaly, nasal bridge abnormalities (depressed or broad), and lip malformations (cleft lip, pseudo-cleft lip, full lips, and prominent philtrum) have been observed in families carrying *OFD1* variants (9). Moreover, orofacioidigital syndrome type I (*OFD1*) is inherited as an X-linked dominant condition with male lethality, usually manifesting in the first or second trimester. The variant identified in our case was most likely maternally inherited. The *OFD1* gene lies within regions that escape X-chromosome inactivation (XCI), resulting in higher gene expression levels in females, approximately twice that of males (19). Therefore, heterozygosity for this gene in females is typically non-lethal.

In addition, we have also identified a novel homozygous nonsynonymous variant (p.Gly369Cys) in the *SLCO2A1* gene in the proband, which was heterozygous in the couple. Homozygous or compound heterozygous variants in *SLCO2A1* have been frequently associated with autosomal recessive primary hypertrophic osteoarthropathy-2 (*PHOAR2*) and autosomal dominant primary hypertrophic osteoarthropathy (*PHOAD*), characterized by digital clubbing, periostosis, and pachydermia (11,20). However, none of these phenotypic features were observed in the proband or the couple in the present case. This suggests that the identified *SLCO2A1* variant may have heterogeneous phenotypic effects and could be involved in later developmental stages rather than early embryogenesis.

Several studies have validated the functional importance of *NEB*. Bang et al. (2006) demonstrated that *NEB* knockout mice (*NEB*^{-/-}) exhibited 25% shorter skeletal muscle thin filaments, and isometric contractility assays revealed significantly reduced force generation in nebulin-deficient skeletal muscles, leading to

Table 2. *In-silico prediction, population frequency, and ACMG classification of variants identified by trio clinical exome sequencing.*

Gene	PolyPhen-2	SIFT	CADD	REVEL	SAS	gnomAD MAF	ACMG Evidence	ACMG Classification	Inheritance/current phenotype
NEB (Exon 6)	0.975	0.269	26.7	0.269	0.00006601	0.00005476	PM2 + PP3 + PP1+PP4	VUS	Not reported in ClinVar; dbSNP rs200555425. Autosomal recessive, homozygous in proband, heterozygous in parents. VUS – candidate contributor to phenotype.
NEB (Exon 133)	0.917	0.424	32	0.397	0.0000112	0.000001376	PM2 + PP3 + PP1 + PP4	VUS	Not reported in ClinVar or dbSNP (novel). Autosomal recessive, homozygous in proband, heterozygous in parents. VUS – candidate contributor to phenotype.
OFD1 (Exon 10)	0.966	0.682	21.8	0.501	Novel	Novel	PM2 + PP3	VUS	Not reported in ClinVar or dbSNP (novel). X-linked, hemizygous in proband, heterozygous in mother. VUS—uncertain relevance to current phenotype; noted for completeness and genetic counselling.
SLCO2A1 (Exon 8)	1.000	0.726	35	0.726	Novel	Novel	PM2 + PP3	VUS	Not reported in 1000 Genomes, gnomAD, or internal MedVarDb. Autosomal recessive, homozygous in proband, heterozygous in parents. VUS—incidental finding; no PHOAR2 features observed.

This table presents in silico prediction scores (*PolyPhen-2*, *SIFT*, *CADD*, and *REVEL*), population frequencies from publicly available databases including the 1000 Genomes Project (1KGP) and gnomAD, and variant classification according to guidelines of the American College of Medical Genetics and Genomics. ACMG evidence codes applied include PM2 (low or absent population frequency), PP3 (computational evidence), PP1 (co-segregation with disease), and PP4 (phenotype specificity). All variants were classified as variants of uncertain significance (VUS).

PolyPhen-2, Polymorphism Phenotyping v2; *SIFT*, Sorting Intolerant From Tolerant; *CADD*, Combined Annotation Dependent Depletion; *REVEL*, Rare Exome Variant Ensemble Learner; SAS, South Asian population frequency (gnomAD/1000 Genomes); 1KGP-1000 Genomes Project; gnomAD-Genome Aggregation Database; ACMG, American College of Medical Genetics and Genomics; PM, moderate evidence of pathogenicity; PP, supporting evidence of pathogenicity; MAF, minor allele frequency; VUS, variant of uncertain significance.

518 compromised cardiac contractile function and death of
519 the knockout mice (21).

520 Based on the genetic findings in the context of inheritance
521 pattern, gene function, and phenotypic correlation, we
522 consider the two homozygous variants identified in the
523 *NEB* gene (c.367C>T; p.Arg123Cys and c.20279A>G;
524 p.Asp6760Gly) to be the most likely candidate
525 contributors to the observed phenotype, rather than an
526 established primary cause, given that both remain variants
527 of uncertain significance (VUS) and the functional
528 testing or confirmatory skeletal muscle histopathology
529 was not available. These variants are consistent with
530 an autosomal recessive mode of inheritance, segregate
531 appropriately within the family (homozygous in the
532 proband and heterozygous in both parents), and are
533 supported by established literature linking *NEB* variants
534 to severe congenital myopathies, including fetal akinesia
535 and IUFD.

536 The hemizygous variant in the *OFDI* gene (c.1035G>T;
537 p.Lys345Asn), inherited from the mother, is noted given
538 its association with X-linked disorders and male lethality.
539 However, due to the absence of definitive phenotypic
540 features specific to *OFDI*-related conditions in the
541 proband, its role in the present case remains uncertain
542 and is therefore interpreted as a possible contributory or
543 modifying factor rather than a primary cause.

544 In addition, a homozygous variant (c.1105G>T;
545 p.Gly369Cys) in the *SLCO2A1* gene was identified
546 in the proband, with both parents being heterozygous
547 carriers. Although variants in *SLCO2A1* are known to be
548 associated with primary hypertrophic osteoarthropathy,
549 the classical phenotype typically manifests during
550 childhood or adolescence rather than in the prenatal
551 period. In the present case, no clinical features consistent
552 with this condition were observed in the proband or the
553 parents, suggesting a lack of phenotypic correlation.
554 Therefore, the direct role of this variant in the observed
555 IUFD remains uncertain. It is possible that this variant
556 exhibits variable expressivity or incomplete penetrance,
557 or that it may not be causally related to the fetal outcome.
558 Accordingly, this variant is reported as a variant of
559 uncertain clinical significance in the context of the current
560 phenotype and is presented as an incidental finding, as
561 no phenotypic features consistent with *PHOAR2* were
562 identified in the proband or the couple.

563 Overall, *in silico* analysis of the identified variants suggests
564 a potentially deleterious effect for the two homozygous
565 variants identified in the *NEB* gene and demonstrates the
566 strongest evidence for pathogenic relevance, supported
567 by their segregation pattern, extremely low population
568 frequency, and their well-established association
569 with severe congenital myopathies and fetal akinesia
570 leading to IUFD. In contrast, the variants identified
571 in *OFDI* and *SLCO2A1*, although predicted to be
572 damaging, lack consistent phenotypic correlation and
573 are typically associated with conditions manifesting
574 later in development or with distinct clinical features not
575 observed in the present case.

576 Therefore, based on the combined *in silico* predictions,
577 inheritance pattern, and genotype–phenotype correlation,
578 the *NEB* gene variants are the most likely contributors

to the observed fetal phenotype, while the *OFDI* and
SLCO2A1 variants are interpreted as variants of uncertain
or possible modifying significance. The findings enabled
informed genetic counseling and supported reproductive
risk assessment and decision-making for the affected
couple.

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List of Abbreviations

1KGP	1000 Genomes Project	596
GnomAD	Genome Aggregation Database	597
ACMG	American College of Medical Genetics and Genomics	598
AMC6	Arthrogryposis Multiplex Congenita-6	599
CADD	Combined Annotation Dependent Depletion	600
cDNA	complementary DNA	601
CMA	chromosomal microarray analysis	602
CNV	copy number variation	603
DNA	deoxyribonucleic acid	604
HGMD	Human gene mutation database	605
IUFD	Intrauterine fetal demise	606
LMP	Last menstrual period	607
MAF	Minor allele frequency	608
MCC	Maternal cell contamination	609
NEB	Nebulin	610
NGS	Next-generation sequencing	611
NM	Reference sequence mRNA accession number	612
NP	Reference sequence protein accession number	613
OFD1	Oral-Facial-Digital Syndrome Type 1	614
PCR	Polymerase chain reaction	615
PGD	Preimplantation Genetic Diagnosis	616
PHOAD	Primary Hypertrophic Osteoarthropathy, Autosomal Dominant	617
PHOAR2	Primary Hypertrophic Osteoarthropathy-2	618
PM	moderate evidence of pathogenicity	619
PolyPhen-2	polymorphism phenotyping v2	620
PP	supporting evidence of pathogenicity	621
REVEL	Rare Exome Variant Ensemble Learner	622
SAS	South Asian population frequency (gnomAD/1000 Genomes)	623
SIFT	Sorting Intolerant from Tolerant	624
SLCO2A1	Solute Carrier Organic Anion Transporter Family Member 2A1	625
SNV	single nucleotide variants	626
VEP	variant effect predictor	627
VUS	variant of uncertain significance	628
WES	whole-exome sequencing	629

Conflict of interest

The authors of this article have no affiliations with or
involvement in any organization or entity with any financial

interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Consent to participate

The Institutional Ethics Committee of Madras Medical College (MMC) approved the study (Approval No: 34042021; Dated: 07.04.2021). Written informed consent was obtained from the couple for genetic testing of fetal tissue, and separately from all extended family members (siblings and grandparents) who provided blood samples for segregation analysis.

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Consent for publication

Written informed consent for publication of the fetal clinical, autopsy, pedigree, and genetic findings, including identifying pedigree information, was obtained from the proband's parent.

Author contributions

Gopinath Padmavathi and Arasambattu Kannan Munirajan were involved in the study conception and design. Gopinath Padmavathi, Ravi Sudesh, and Arasambattu Kannan Munirajan assisted in the analysis and interpretation of the data. Gopinath Padmavathi, Arasambattu Kannan Munirajan, and Avaniyapuram Kannan Murugan drafted and edited the manuscript.

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