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ORIGINAL ARTICLE

MONOGENIC FINDINGS IN EARLY PREGNANCY LOSS: WHOLE-EXOME SEQUENCING STUDY OF EUPLOID PRODUCTS OF CONCEPTION

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ABSTRACT

Early pregnancy loss (EPL), particularly when recurrent, represents a profoundly distressing experience for affected couples. Although chromosomal abnormalities are the most common cause of EPL, a substantial proportion of cases, especially those involving euploid embryos, remain unexplained. In this study, we investigated the potential contribution of rare monogenic variants to euploid EPL using whole-exome sequencing (WES).

WES was performed on 66 euploid products of conceptions (POCs) from EPLs occurring before 12 gestational weeks. A molecular diagnosis with a high level of confidence, defined as the presence of pathogenic or likely pathogenic (P/LP) variant(s) consistent with the expected mode of inheritance, was established in 13/66 POCs (19.7%). These included one large 21q22.12-q22.3 duplication encompassing *DYRK1A* and *RUNX1*. P/LP small variants were detected in *CPLANE1*, *DHCR7*, *DSG2*, *DVL1*, *F5*, *NF1*, *RBM8A*, *SLC6A1*, and *VWF*, representing genes with variable degrees of prior association with developmental phenotypes and, in some cases, limited or no evidence for embryonic lethality.

In an additional 9/66 POCs (13.6%), findings were suggestive but not conclusive for a monogenic contribution. These included four cases with compound heterozygosity involving a pathogenic variant and a variant of uncertain significance (VUS) in autosomal recessive genes (*GBA1*, *PAH*, *PKHD1*, and *RPGRIP1L*), as well as five cases harboring single heterozygous VUS in autosomal dominant genes (*MYH3*, *PRDM6*, *SCN5A*, *TBX18*, and *TSC1*). The pathogenic relevance of these variants remains uncertain, particularly in the absence of functional validation.

The implicated genes were clustered in biological categories: 1) genes plausibly associated with prenatal or early embryonic lethality, 2) genes causing severe congenital disorders not typically considered embryonically lethal, and 3) genes linked to later-onset or susceptibility phenotypes. These observations are consistent with a spectrum model in which highly deleterious variants may act as primary drivers of embryonic demise, whereas variants with reduced penetrance, later-onset associations or uncertain significance may contribute in a multifactorial context, potentially interacting with additional genetic, maternal or environmental factors.

In conclusion, our findings suggest that monogenic variants may contribute to a subset of euploid EPL cases, although the strength of evidence varies considerably across detected variants. The integration of WES into the evaluation of recurrent euploid pregnancy loss holds promise but should be interpreted with caution. Further studies incorporating functional analyses, larger cohorts, and parental data are needed to clarify causality and to define the clinical utility of such approaches in genetic counseling, recurrence-risk assessment, and reproductive planning.

Keywords: early pregnancy loss (EPL); product of conception (POC); whole-exome sequencing (WES); monogenic disease; euploid early pregnancy loss; genetic diagnosis

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INTRODUCTION

Early pregnancy loss (EPL) is defined as spontaneous pregnancy loss before 12 completed gestational weeks. Its etiology is multifactorial, involving genetic, anatomic, endocrine, immunological, and environmental factors. Chromosomal abnormalities account for approximately 50–60% of EPLs, with trisomy being the most common, followed by monosomy X and triploidy [1-3]. These aberrations are typically de novo, and recurrence risk is generally not elevated in the couples.

Recurrent pregnancy loss (RPL), defined as two or more consecutive early losses, affects up to 5% of couples. Compared with sporadic losses, chromosomal abnormalities are less frequent in RPL, suggesting that additional genetic mechanisms may contribute [4].

While the contribution of chromosomal anomalies is well established, the role of monogenic disorders in euploid EPL remains incompletely defined. The advent of next-generation sequencing technologies, particularly whole-exome sequencing (WES), has expanded the capacity to detect pathogenic variants underlying Mendelian disease. WES enables identification of the majority of coding disease-causing variants and has become an essential tool in rare disease diagnostics [5, 6].

However, only a limited number of studies have applied WES to POCs from early losses, and many included heterogeneous gestational ages or selected fetuses with ultrasound-detected anomalies. Consequently, the prevalence and spectrum of monogenic causes in typical first-trimester euploid EPL remain insufficiently characterized.

Recent studies that applied WES to POCs have reported pathogenic or likely pathogenic variants in genes implicated in a broad spectrum of disorders, including multisystem developmental syndromes, cardiac malformations, skeletal dysplasia, kidney disorders, and central nervous system abnormalities [7–11]. These findings support the hypothesis that a subset of early pregnancy losses may be attributable to rare, deleterious variants in genes critical for early embryonic development.

In this study, we analyzed 66 euploid POCs from EPL occurring exclusively before 12 gestational weeks using WES. By focusing on this carefully defined cohort, we aimed to assess the prevalence and biological spectrum of potentially pathogenic variants contributing to early embryonic demise.

MATERIALS AND METHODS

EPL tissue samples

WES was performed on 66 euploid POCs derived from 60 unrelated couples. In six families, two losses were analyzed. Gestational age ranged from 6 to 12 weeks (mean 8.23 ± 1.25), and maternal age ranged from 20 to 42 years (mean 30.9 ± 5.18). Couples reported 2–13 prior losses (mean 3.56 ± 2.14) and 0–2 live births (0.28 ± 0.57). Of the POCs, 34 were male and 32 female (M:F = 1.09:1); 32 originated from Macedonian and 34 from Albanian families. Full demographic and clinical details are provided in Supplementary Table S1.

DNA extraction, maternal-cell contamination testing, and chromosomal screening

Genomic DNA was extracted from two to three chorionic villus fragments or fetal tissue of approximately 2 mm^2 using the MagCore Super automated nucleic-acid extractor (RBC Bioscience). An in-house QF-PCR assay, targeting short tandem repeats on chromosomes 13, 18, 21, X, and Y, was applied to exclude maternal-cell contamination and detect triploidy [12-14]. MLPA (MRC-Holland) subtelomeric probe mixes were used to screen for numerical or large structural chromosomal anomalies [2, 3]. Samples negative by both assays proceeded to WES.

Whole-exome sequencing, variant prioritization, interpretation strategy, and confirmation

Libraries were prepared with the Twist Human Core + RefSeq + Mitochondrial panel (Twist Bioscience) and sequenced on an Illumina NovaSeq 6000 with 2 x 100 bp paired-end reads. Reads were aligned to the GRCh38 reference genome using BWA-MEM v0.7.15 [15]. The mean on-target depth exceeded 100x, with at least 98% of bases covered at >=10x. Single-nucleotide variants (SNVs) and small insertions/deletions (indels) were called using GATK v4.3 HaplotypeCaller with 25 bp exon padding and annotated with Ensembl VEP v106 [16,17]. CNV analysis was performed on exome depth-of-coverage data using the CeGaT pipeline; only high-confidence, clinically relevant CNVs were considered reportable, and clinically relevant CNVs were validated by array comparative genomic hybridization (4x180k, Oxford Gene Technologies/Agilent Technologies) and/or MLPA.

Variants were classified according to American College of Medical Genetics and Genomics ACMG/AMP guidelines as pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, or benign [18]. Analysis was performed exome-wide rather than being restricted to a predefined gene list, with prioritization of rare coding and splice-site variants in genes. Variant review incorporated population frequency, predicted functional consequence, known gene-disease validity, expected inheritance model, segregation data when available. Population-frequency assessment relied on gnomAD v2.1.1 and v4.0 and an internal dataset of 1513 WES samples. Variants above the allele-frequency thresholds used in our diagnostic pipeline were generally deprioritized unless previously established as pathogenic.

Clinical significance was assessed using Franklin (Genoox) and VarSome, followed by manual curation. All reported P/LP variants and selected VUS discussed as possible contributors were confirmed by Sanger sequencing. Primers designed with Primer3 and PCR conditions are listed in Supplementary Tables S2 and S3. Parental studies were performed in all cases, using Sanger sequencing, MLPA, or arrayCGH as appropriate. QF-PCR was the principal method used to exclude maternal-cell contamination and triploidy.

RESULTS

Diagnostic yield

Whole-exome sequencing (WES) was performed on 66 euploid products of conception (POCs) from early pregnancy losses (EPLs) occurring before 12 gestational weeks. A molecular diagnosis, defined as pathogenic or likely pathogenic (P/LP) variant(s) consistent with the expected mode of inheritance, was established in 13/66 POCs (19.7%), including one large de novo 21q22.12-q22.3 duplication encompassing *DYRK1A* and *RUNX1*.

An additional 9/66 POCs (13.6%) harbored findings considered possible monogenic contributors rather than definitive diagnoses. The remaining 44/66 cases (66.7%) had no reportable variant. Figure 1 summarizes the overall classification of cases represented among reportable findings.

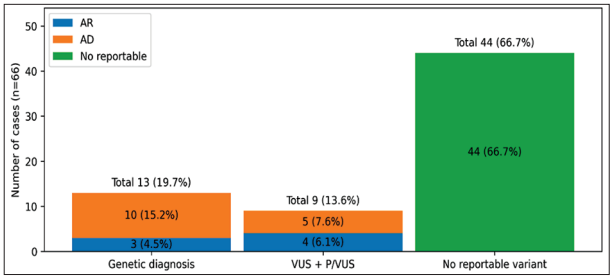

Figure 1. Overview of molecular findings in 66 euploid early pregnancy losses. Diagnostic summary showing cases with a genetic diagnosis, possible monogenic contribution (VUS + P/VUS), and no reportable variant, with autosomal recessive (AR) and autosomal dominant (AD) findings indicated where applicable

Interpretive categorization

To reduce overinterpretation, reportable findings were further stratified into three categories: (1) genes plausibly associated with prenatal or early embryonic lethality, (2) genes causing severe congenital disorders not typically considered embryonically lethal, and (3) genes linked to later-onset or susceptibility phenotypes. Detailed molecular data and this interpretive framework are summarized in Tables 1 and 2. Figure 2 provides an overview of the reportable findings according to interpretive category and the major developmental or organ systems represented.

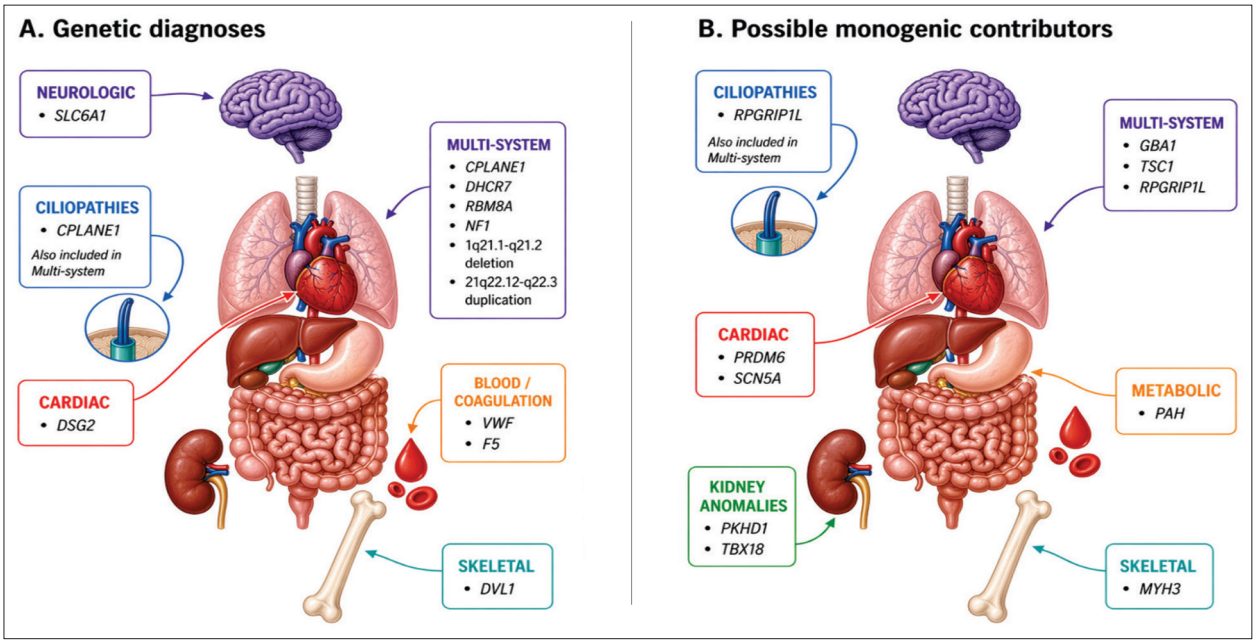

Figure 2. Overview of the identified genetic findings grouped by major developmental or organ-system association. (A) Genetic diagnoses, including definitive molecular diagnoses, and the pathogenic copy-number finding 21q22.12-q22.3 duplication. (B) Possible monogenic contributors. Genes are mapped to their principal affected systems, including neurologic, cardiac, blood/coagulation, kidney anomalies, skeletal, metabolic, ciliopathies, and multi-system involvement. *CPLANE1* and *RPGRIP1L* are shown in both ciliopathy and multi-system categories because of their broader phenotypic effects.

Table 1. Detailed overview of variants detected by WES in euploid EPL and their molecular characteristics

Case	Gene	Reference sequence	Variant	Protein change	Zygosity	Inheritance	Type of variant	Known / Novel	ACMG classification	ACMG criteria
1. Genes plausibly associated with prenatal or early embryonic lethality										
Definitive molecular diagnoses										
Abp-411	*CPLANE1*	NM_001384732.1	c.1819delT;7817T>A	p.Tyr607ThrfsTer6; p.Leu2624Ter	hom	M/F	Frameshift	Known	Pathogenic	PVS1; PM2; PP5/PVS1; PM2; PP5
Abp-445[1]	*CPLANE1*	NM_001384732.1	c.1819delT;7817T>A	p.Tyr607ThrfsTer6; p.Leu2624Ter	het	F	Frameshift; Nonsense	Known	Pathogenic	PVS1; PM2; PP5/PVS1; PM2; PP5
			c.5820+3_5820+6del	exon 29 skipping	het	M	Splice site	Novel	Pathogenic	PS3, PM2; PM3; PM4; PP3
Abp-494[2]	*DHCR7*	NM_001360.3	c.452G>A	p.Trp151Ter	het	F	Nonsense	Known	Pathogenic	PVS1; PM2; PP5
			c.964-1G>C	altered splicing	het	M	Splice site	Known	Pathogenic	PVS1; PM2; PP5
Abp-545[2]	*DHCR7*	NM_001360.3	c.452G>A	p.Trp151Ter	het	F	Nonsense	Known	Pathogenic	PVS1; PM2; PP5
			c.964-1G>C	altered splicing	het	M	Splice site	Known	Pathogenic	PVS1; PM2; PP5
Abp-551[1]	*CPLANE1*	NM_001384732.1	c.1819delT;7817T>A	p.Tyr607ThrfsTer6; p.Leu2624Ter	het	F	Frameshift; Nonsense	Known	Pathogenic	PVS1; PM2; PP5/PVS1; PM2; PP5
			c.5820+3_5820+6del	exon 29 skipping	het	M	Splice site	Novel	Pathogenic	PVS1; PM2; PP5/PVS1; PM2; PP5
Possible monogenic contributors										
Abp-501	*GBA1*	NM_000157.4	c.1444G>T	p.Asp482Tyr	het	M	Missense	Known	VUS	PM2; PM3; PP3
			c.1226A>G	p.Asn409Ser	het	F	Missense	Known	Likely pathogenic	PM1; PM2; PM5; PP2; PP3; PP5
Abp-694	*PKHD1*	NM_138694.4	c.107C>T	p.Thr36Met	het	M	Missense	Known	Likely pathogenic	PM2; PM5; PP3; PP5
			c.10883C>T	p.Thr3621le	het	F	Missense	Known	VUS	PM2; PM3
Abp-825	*RPGRIP1L*	NM_015272.5	c.2771G>A	p.Ser924Asn	het	M	Missense	Known	VUS	PM2, PM3
			c.3295-2A>G	/	het	F	Splice site	Known	Likely pathogenic	PVS1; PM2; PP5
2. Genes causing severe congenital disorders not typically considered embryonically lethal										
Definitive molecular diagnoses										
Abp-251	*SLC6A1*	NM_003042.4	c.740C>A	p.Pro247His	het	de novo	Missense	Novel	Likely pathogenic	PM2; PM5; PP2; PP3
Abp-716	*RBM8A*	NM_005105.5	c.-21G>A	/	het	M	Missense/ noncoding	Known	Pathogenic, low penetrance	PS3, PM3
	1q21.1-q21.2	hg19	chr1:143,767,833-149,400,542	/	het	F	Deletion	Known	Pathogenic	2A; 3B; 4L >1 point
Abp-799	*NF1*	NM_001042492.3	c.4600C>T	p.Arg1513Ter	het	F	Nonsense	Known	Pathogenic	PVS1; PM2; PP5
Abp-801	*DSG2*	NM_001943.5	c.2315del	p.Leu772Ter	het	M	Nonsense	Novel	Likely pathogenic	PVS1; PM2
Abp-825	*DVL1*	NM_001330311.2	c.1961dup	p.Pro657AlafsTer50	het	F	Frameshift	Novel	Likely pathogenic	PVS1; PM2
Possible monogenic contributors										
Abp-80	*PAH*	NM_000277.3	c.842C>T	p.Pro281Leu	het	M	Missense	Known	Pathogenic	PS3, PM2; PM5; PP2; PP3; PP5
			c.*19G>T	/	het	F	Missense/ noncoding	Known	VUS	PM3; BS1; BS2; BP7
Abp-87	*PRDM6*	NM_001136239.4	c.1057G>A	p.Asp353Asn	het	M	Missense	Known	VUS	PM2
Abp-166	*TBX18*	NM_001080508.3	c.1570C>T	p.His524Tyr	het	M	Missense	Known	VUS	PM2; PP3; PP5
Abp-233	*SCN5A*	NM_000335.5	c.3911C>T	p.Thr1304Met	het	F	Missense	Known	VUS	PM2, PP3, PP5
Abp-577	*TSC1*	NM_000368.5	c.3113_3119del	p.Ser1038ThrfsTer51	het	F	Frameshift	Novel	VUS	PVS1(moderate); PM2
Abp-781	*MYH3*	NM_002470.4	c.3137G>A	p.Arg1046Gln	het	M	Missense	Known	VUS	PM2; PP3
3. Genes linked to later-onset or susceptibility phenotypes										
Abp-668	*VWF*	NM_000552.5	c.3797C>T	p.Pro1266Leu	het	M	Missense	Known	Likely pathogenic	PM1; PM2; PM5; PP5
Abp-809	*F5*	NM_000130.5	c.1601G>A	p.Arg534Gln	hom	M (hom)/F (het)	Missense	Known	Pathogenic, low penetrance	PS3, PS4
Additional distinct pathogenic copy-number finding										
Abp-972	21q22.12-q22.3dup	hg19	chr21:33,398,108-43,587,648	/	het	de novo	Duplication	Known	Pathogenic	3C; 4L >1 point

[1,2] fetuses from same family. Abp-825 harbored two distinct reportable findings assigned to different interpretive categories: a heterozygous likely pathogenic *DVL1* variant and a biallelic *RPGRIP1L* finding composed of one likely pathogenic splice-site variant and one missense VUS.

Table 2. Zygosity, inheritance, OMIM-associated diseases, and interpretive grouping of the detected genes

Case	Gene	Variant	Accession number	AF (gnomAD)	Internal frequency	OMIM disease/s; Inheritance	Major developmental / organ system
1. Genes plausibly associated with prenatal or early embryonic lethality							
Definitive molecular diagnoses							
Abp-41[1]	*CPLANE1*	c.1819del[T];7817T>A	rs777686211; rs749523755	0.0001554; 0.00002390	0.0077	614615, Joubert Syndrome 17, AR; 277170, Orofaciodigital syndrome VI, AR	Multi-system
Abp-445[1]	*CPLANE1*	c.1819del[T];7817T>A	rs777686211; rs749523755	0.0001554; 0.00002390	0.0077	614615, Joubert Syndrome 17, AR; 277170, Orofaciodigital syndrome VI, AR	Multi-system
		c.5820+3_5820+6del	/	/	0.0017		
Abp-494[2]	*DHCR7*	c.452G>A	rs11555217	0.0007759	0.0084	270400, Smith-Lemli-Opitz syndrome, AR	Multi-system
		c.964-1G>C	rs138659167	0.003854	0.0042		
Abp-545[2]	*DHCR7*	c.452G>A	rs11555217	0.0007759	0.0084	270400, Smith-Lemli-Opitz syndrome, AR	Multi-system
		c.964-1G>C	rs138659167	0.003854	0.0042		
Abp-551[1]	*CPLANE1*	c.1819del[T];7817T>A	rs777686211; rs749523755	0.0001554; 0.00002390	0.0077	614615, Joubert Syndrome 17, AR; 277170, Orofaciodigital syndrome VI, AR	Multi-system
		c.5820+3_5820+6del	/	/	0.0017		
Possible monogenic contributors							
Abp-501	*GBA1*	c.1444G>T	/	/	0	608013, 230800, 230900, 231000, 231005, Gaucher disease types perinatal death, I, II, III, IIIC, AR	Multi-system
		c.1226A>G	rs76763715	0.002235	0.0067		
Abp-694	*PKHD1*	c.107C>T	rs137852944	0.0005094	0.0014	263200, Polycystic kidney disease 4, with or without hepatic disease, AR	Kidney anomalies
		c.10883C>T	rs147700643	0.00005312	0		
Abp-825	*RPGRIP1L*	c.2771G>A	rs142234650	/	0	611561, Meckel syndrome 5, AR; 611560, Joubert syndrome 7, AR	Ciliopathies/multi-system
		c.3295-2A>G	rs1258182460	/	0		
2. Genes causing severe congenital disorders not typically considered embryonically lethal							
Definitive molecular diagnoses							
Abp-251	*SLC6A1*	c.740C>A	/	/	0	616421, Myoclonic-atonic epilepsy, AD	Neurologic
Abp-716	*RBM8A*	c.-21G>A	rs139428292	0.01794	>2%	274000, Thrombocytopenia-absent radius syndrome, AR	Multi-system
	1q21.1-q21.2	chr1:143,767,833-149,400,542	/	/	0		
Abp-799	*NF1*	c.4600C>T	rs760703505	0.000007957	0	162200, Neurofibromatosis, type 1, AD	Multi-system
Abp-801	*DSG2*	c.2315del	/	/	0	610193, Arrhythmogenic right ventricular dysplasia 10, AD	Cardiac
Abp-825	*DVL1*	c.1961dup	/	/	0	616331, Robinow syndrome, autosomal dominant 2, AD	Skeletal
Possible monogenic contributors							
Abp-80	*PAH*	c.842C>T	rs5030851	0.0001026	0.0010	261600, Phenylketonuria, AR	Metabolic
		c.*19G>T	rs372637021	0.002029	0		
Abp-87	*PRDM6*	c.1057G>A	rs202224762	0.0002604	0.0010	617039, Patent ductus arteriosus 3, AD	Cardiac
Abp-166	*TBX18*	c.1570C>T	rs760905589	0.000008061	0.0010	143400, Congenital anomalies of kidney and urinary tract 2, AD	Kidney anomalies
Abp-233	*SCN5A*	c.3911C>T	rs199473603	0.0001649	0	601144, Brugada syndrome 1, AD; 601154, Cardiomyopathy, dilated, 1E, AD; 603830, Long QT syndrome 3, AD	Cardiac
Abp-577	*TSC1*	c.3113_3119del	/	/	0.00035	191100, Tuberous sclerosis-1 , AD	Multi-system
Abp-781	*MYH3*	c.3137G>A	rs142002449	0.0004031	0.0010	193700, Arthrogryposis, distal, type 2A (Freeman-Sheldon), AD	Skeletal
3. Genes linked to later-onset or susceptibility phenotypes							
Abp-668	*VWF*	c.3797C>T	rs61749370	0.0008322	0.0010	193400, von Willebrand disease, AD/AR	Blood
Abp-809	*F5*	c.1601G>A	rs6025	0.01752	>3%	188055, Thrombophilia 2 due to activated protein C resistance, AD; 614389, {Pregnancy loss, recurrent, susceptibility to, 1}, AD	Blood
Additional distinct pathogenic copy-number finding							
Abp-972 [U]	21q22.12-q22.3	chr21:33,398,108–43,587,648	/	/	0	/, 21q22 Duplication Syndrome	Multi-system

[1,2] fetuses from same family. Abp-825 harbored two distinct reportable findings assigned to different interpretive categories: a heterozygous likely pathogenic *DVL1* variant and a biallelic RPGRIP1L finding composed of one likely pathogenic splice-site variant and one missense VUS.

Segregation findings according to interpretive categorization

Among genes plausibly associated with prenatal or early embryonic lethality, molecular diagnoses were identified in cases with biallelic pathogenic variants in *CPLANE1* and *DHCR7*, while additional potential monogenic contributors included biallelic combinations in *GBA1*, *PKHD1*, and *RPGRIP1L*. Parental testing was available in all cases and confirmed biparental inheritance in recessive findings. Pedigree analysis of families with definitive molecular diagnoses and, in selected cases, recurrent affected pregnancy losses (Figure 3).

Among genes causing severe congenital disorders not typically considered embryonically lethal, molecular diagnoses consisted of heterozygous variants in *SLC6A1*, *NF1*, *DSG2*, *DVL1*, and one *RBM8A*-associated case with the characteristic combination of the low-penetrance regulatory variant and the recurrent 1q21.1-q21.2 deletion. The potential contributory subset included heterozygous VUS in *MYH3*, *PAH, PRDM6*, *SCN5A*, *TBX18*, and *TSC1*. All heterozygous variants were inherited from one parent, with exception of *SLC6A1:* c.740C>A which occurred de novo; parental phenotypic information was not available.

Among genes linked to later-onset or susceptibility phenotypes, reportable findings included homozygous *F5:* c.1601G>A and heterozygous *VWF:* c.3797C>T variants.

One de novo 21q22.12-q22.3 duplication represented a distinct pathogenic copy-number diagnosis.

Variant spectrum and predicted molecular consequences according to interpretive categorization

Genes plausibly associated with prenatal or early embryonic lethality.

This category was dominated by biallelic truncating and splice-disrupting variants in *CPLANE1* and *DHCR7,* which led to molecular diagnosis. A notable finding was the novel *CPLANE1:* c.5820+3_5820+6del variant, previously shown to disrupt splicing and cause exon skipping. Potential contributory cases included compound-heterozygous P/LP and VUS combinations in *GBA1*, *PKHD1*, and *RPGRIP1L*, highlighting missense variants such as *GBA1:* p.Asp482Tyr and p.Asn409Ser, *PKHD1:* p.Thr36Met and p.Thr362Ile, and *RPGRIP1L:* p.Ser924Asn.

Genes causing severe congenital disorders not typically considered embryonically lethal

This category was dominated by simple heterozygous findings. Several novel variants were identified, including *SLC6A1:* p.Pro247His, *DSG2:* p.Leu772Ter, *DVL1:* p.Pro657AlafsTer50, *NF1*: p.Arg1513Ter, while the RBM8A-associated case reflected the expected combination of the low-penetrance regulatory variant and the recurrent 1q21.1-q21.2 deletion. Additional potential contributory findings were mainly heterozygous missense VUS variants, including *MYH3:* p.Arg1046Gln, *PRDM6:* p.Asp353Asn, *TBX18:* p.His524Tyr, and *SCN5A:* p.Thr1304Met, *TSC1*: p.Ser1038ThrfsTer51 and *PAH*: p.Pro281Leu.

Genes linked to later-onset or susceptibility phenotypes

This category included missense variants in *F5* and *VWF*, namely *F5:* c.1601G>A (p.Arg534Gln) and *VWF:* c.3797C>T (p.Pro1266Leu).

Copy number variants (CNV)

Structural alterations across the reportable cohort also included the 1q21.1-q21.2 deletion in the *RBM8A*-associated case and the de novo 21q22.12-q22.3 duplication involving *DYRK1A* and *RUNX1*.

Predicted molecular consequences

Predicted effects were consistent with variant class: truncating variants are expected to cause loss of function, and splice-site variants such as *DHCR7:* c.964-1G>C, *RPGRIP1L:* c.3295-2A>G, and the *CPLANE1:* c.5820+3_5820+6del are predicted to disrupt normal splicing. Missense variants were more difficult to interpret, but those retained were prioritized based on rarity, in silico prediction, ACMG-based classification, and gene-disease relevanceThe functional effects of the identified variants at the RNA or protein level were not directly evaluated and therefore remain inferential, except for the *CPLANE1:* c.5820+3_5820+6del variant, which has previously been demonstrated to cause exon 29 skipping [7]. Protein change, variant class, classification, and allele frequencies for all reportable variants are provided in Tables 1 and 2.

DISCUSSION

Over the past decade, whole-exome sequencing (WES) has become an important tool in clinical genetics laboratories. With steadily decreasing costs, WES has been increasingly adopted as a first-tier approach in postnatal rare-disease diagnostics [19]. In addition, improvements in analytical pipelines have extended its potential utility to include CNV inference from exome data [20]. In prenatal genetics, WES has shown relatively high diagnostic yields in fetuses with ultrasound-detected anomalies, supporting its expanding role in prenatal evaluation [21].

In EPL, chromosomal abnormalities remain a major genetic contributor, but the potential role of monogenic causes in euploid POCs is still incompletely defined. Recent reports have started to describe single-gene findings

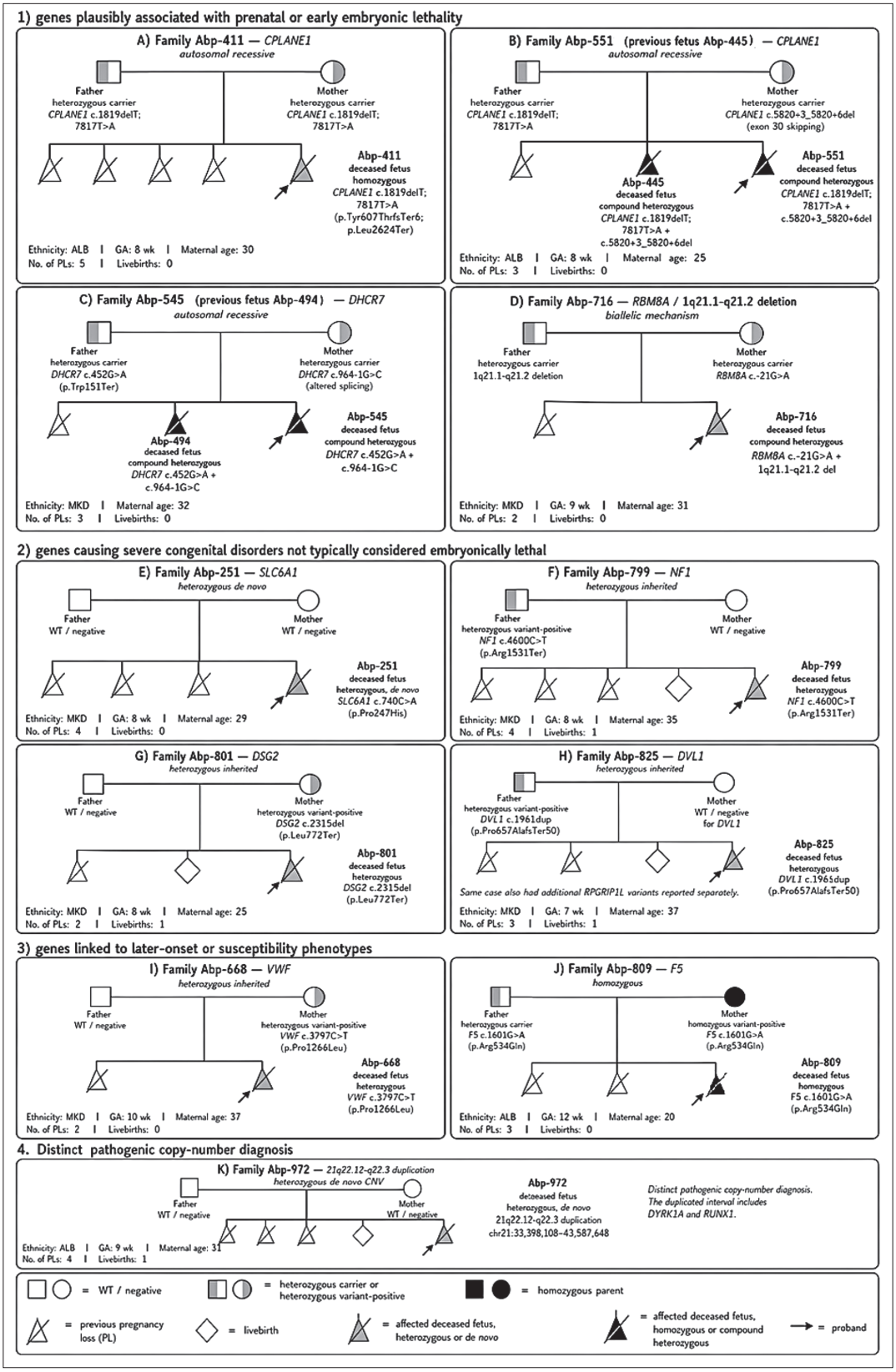

Figure 3. Pedigrees of families with definitive molecular diagnoses in the euploid early pregnancy loss cohort, grouped according to interpretive category. Previous losses, livebirths, and affected fetuses are shown for each family, and recurrent affected fetuses are integrated into the same pedigree when present.

in euploid fetal material; however, many studies include wide gestational ranges extending beyond the first trimester and apply heterogeneous sequencing and interpretation strategies, which may limit direct comparisons across cohorts and may complicate conclusions for losses occurring specifically before 12 gestational weeks [22,23]. Published data increasingly suggest that genes essential for early development and multiple organ systems may be represented among genetic findings in EPL cohorts [8,24].

In this study, we analyzed 66 euploid POCs from EPL occurring exclusively before 12 gestational weeks using WES and identified clinically relevant monogenic findings in a substantial subset of cases. A molecular diagnosis in the conceptus was established in 13/66 POCs (19.7%), and in an additional 9/66 POCs (13.6%) we identified findings that may represent a possible monogenic contribution, including compound heterozygosity with a pathogenic variant plus a VUS in autosomal recessive genes and VUS findings in autosomal dominant genes.

A key interpretive distinction in this study is between identifying a molecular diagnosis in the conceptus and establishing that the diagnosis plausibly explains the pregnancy loss. Not all pathogenic or likely pathogenic findings carry the same degree of etiologic relevance to EPL. This distinction is particularly important for genes associated with later-onset, variably expressive, incompletely penetrant, or susceptibility phenotypes. Accordingly, our interpretation framework emphasizes levels of causal confidence rather than treating all P/LP findings as equally explanatory for first-trimester loss.

Our molecular diagnostic yield of 19.7% is close to the 22% pathogenic/likely pathogenic abnormality-detection rate reported by Zhao et al. in the largest directly comparable exome study of chromosomally preselected products of conception [8]. However, comparisons across studies should be interpreted cautiously, because published cohorts differ substantially in specimen type, gestational window, prior exclusion of aneuploidy/CNVs, sequencing design, and the definition of a "positive" result. Several other reports used exploratory variant-prioritization or burden-based frameworks rather than ACMG-style diagnostic classification, and some included recurrent miscarriage or broader fetal/perinatal death cohorts rather than first-trimester euploid POCs. These methodological differences likely explain much of the variability in reported yields and reinforce the need to compare studies within the context of cohort design rather than by percentage alone [10-11,24,36-39]. A likely strength of the present study is the deliberate restriction to euploid losses before 12 gestational weeks, which reduces heterogeneity and allows a more focused assessment of monogenic findings in early miscarriage tissue. At the same time, our yield should be interpreted in light of our distinction between a molecular diagnosis in the conceptus and evidence that the finding explains EPL, as not all diagnosed conditions are equally likely to be directly causal for first-trimester loss.

To contextualize this heterogeneous gene set, we interpreted implicated genes within three biological categories: (1) genes plausibly associated with prenatal or early embryonic lethality, (2) genes causing severe congenital disorders not typically considered embryonically lethal, and (3) genes linked to later-onset or susceptibility phenotypes. This framework allows a more explicit separation of molecular diagnosis from etiologic inference.

Category 1: Genes plausibly associated with prenatal or early embryonic lethality

Molecular diagnosis in the conceptus (CPLANE1, DHCR7)

Among the autosomal recessive findings, *CPLANE1* and *DHCR7* represent some of the most plausible candidates for involvement in euploid EPL because biallelic pathogenic variants in these genes are associated with severe developmental disorders and have been discussed in the setting of fetal or perinatal lethality in other reports [25]. In our cohort, recurrent biallelic findings in unrelated families strengthen the biological plausibility of these genes as contributors to EPL.

For *CPLANE1*, associated with Joubert syndrome and related ciliopathy phenotypes, we observed biallelic variants in unrelated families, including a complex allele (c.1819delT;7817T>A) that appears enriched in our population and may warrant further investigation, particularly in families with recurrent EPL and Albanian ancestry [7]. While definitive causality in EPL cannot be inferred from sequencing alone, repeated observations of biallelic disruption in genes linked to severe developmental phenotypes support a role in early developmental non-viability.

Similarly, for *DHCR7*, we identified compound heterozygosity involving well-established pathogenic alleles associated with severe Smith-Lemli-Opitz syndrome (SLOS). Severe SLOS phenotypes have been reported with major developmental abnormalities, and both variants have been previously associated with pregnancy loss and/or severe pre-/neonatal phenotype [25].

Potential monogenic contribution (RPGRIP1L, PKHD1, GBA1)

Additional genes in this category include *RPGRIP1L, PKHD1, GBA1* in which we identified compound heterozygosity involving a pathogenic variant and a VUS (AR genes). While these genes have been implicated in severe prenatal disorders, the specific variant combinations identified in our cohort lack sufficient evidence for definitive pathogenic classification [26, 27]. Therefore, these findings should be interpreted as possible contributors, pending functional validation and replication in independent cohorts.

Category 2: Genes causing severe congenital disorders not typically considered embryonically lethal

Molecular diagnosis in the conceptus (DSG2, DVL1, NF1, RBM8A, SLC6A1)

A second group comprised genes associated with severe developmental or multisystem disorders that are more often recognized postnatally than in the setting of very early pregnancy loss. This group included *DSG2*, *DVL1*, *NF1*, *RBM8A*, and *SLC6A1*. Detection of such findings establishes a molecular diagnosis in the conceptus but does not, by itself, prove etiologic attribution for EPL. Their contribution may range from biologically relevant to incidental or context-dependent, depending on the gene, variant type, penetrance, and the presence of additional fetal, placental, maternal, or environmental factors.

For *DSG2* and other genes related to cardiac development or electrophysiology, a mechanistic connection to first-trimester loss remains indirect. Similarly, *NF1* and *SLC6A1* are well-established disease genes, but neither is classically regarded as a standard explanation for very early embryonic demise. We therefore interpret these findings as clinically meaningful molecular diagnoses with variable and often uncertain explanatory power for EPL. [28–30]

The *RBM8A*-associated TAR genotype identified in our cohort merits particularly careful interpretation. TAR syndrome results from compound inheritance of a null allele, typically the 1q21.1 deletion, in trans with a hypomorphic *RBM8A* regulatory allele such as c.-21G>A. Thus, c.-21G>A is not independently causative and becomes clinically relevant only in the presence of the deletion. In our cohort, this combined genotype supports a bona fide molecular diagnosis in the conceptus, although its precise contribution to early gestational loss remains to be clarified in larger datasets [31].

Possible monogenic contribution (MYH3, PAH, PRDM6, SCN5A, TBX18, TSC1)

The potential monogenic contribution subset in this category comprised genes with roles in cardiac development (*PRDM6*), fetal arrhythmia (*SCN5A*) or kidney anomalies (*TBX18*), skeletal development (*MYH3*), metabolic disease (*PAH*) and multisystem growth regulation (*TSC1*) [32,33]. Although there is biological plausibility for some of these findings, particularly those involving cardiac developmental pathways, the available evidence is insufficient for definitive pathogenic attribution in the present cohort. These variants are therefore interpreted conservatively as possible contributors rather than established causes of EPL.

Category 3: Genes linked to later-onset or susceptibility phenotypes

This group included findings in *F5* and *VWF*, both of which were classified as P/LP at the molecular level but are more difficult to interpret as direct causes of first-trimester loss. These variants may reflect susceptibility, maternal-fetal interaction, placental factors, or other context-dependent mechanisms rather than classic monogenic embryonic lethality.

The identification of fetal homozygosity for Factor V Leiden in a case with maternal homozygosity is noteworthy, but it should be regarded as hypothesis-generating rather than directly explanatory for EPL. Likewise, the mechanistic link between a *VWF* finding and early embryonic demise remains uncertain. Accordingly, these results are best interpreted as clinically relevant findings of uncertain etiologic weight in relation to EPL [34].

More broadly, the diagnostic variants in our cohort spanned multiple disease categories, mirroring patterns reported in previous studies [35-42]. The repeated recovery of similar functional categories across independent cohorts supports the view that disruption of core developmental pathways contributes to a subset of euploid EPL. At the same time, the presence of susceptibility or later-presenting diagnoses in early losses highlights that some findings may reflect allele-specific severity, variable expressivity, reduced penetrance, or coincident maternal and/or environmental factors rather than direct monogenic causation.

Recurrent biallelic findings in *CPLANE1* and *DHCR7* in unrelated families represent some of the strongest candidates for involvement in euploid EPL because they affect genes linked to severe developmental disorders and were observed more than once in the cohort as well as in other previously published studies [7, 25]. In contrast, the *RBM8A* case highlights the importance of integrated SNV/CNV interpretation, because the diagnosis depends on the combination of the regulatory c.-21G>A allele with the 1q21.1 deletion in trans. Finally, de novo findings such as *SLC6A1* c.740C>A and the 21q22.12-q22.3 duplication clearly establish molecular diagnoses in the conceptus, but their direct explanatory value for first-trimester loss remains more uncertain and remains to be clarified.

Limitations and future directions

This study has several limitations. The cohort size is moderate and may not capture the full spectrum of rare variation contributing to EPL. Moreover, fetal-only WES, while practical in a diagnostic setting, limits the ability to identify all de novo variants, detect parental mosaicism, and confidently phase biallelic findings in every case; trio-based sequencing, when feasible, may improve interpretation [43]. In addition, detailed fetal phenotyping is

inherently limited in first-trimester pregnancy-loss tissue, which restricts robust genotype–phenotype correlation.

Functional validation remains particularly important for novel variants and VUS, and future studies incorporating detailed family segregation data, transcriptomic, proteomic, model-system, and bioinformatic approaches will be needed to strengthen causal inference and further clarify the molecular effects of these variants [40–43].

CONCLUSION

Whole-exome sequencing identified a molecular diagnosis in the conceptus in 19.7% (13/66) of euploid early pregnancy losses occurring before 12 gestational weeks. Our findings suggest that monogenic variants may contribute to a subset of euploid EPL cases, although the strength of evidence varies considerably across detected variants. The integration of WES into the evaluation of recurrent euploid pregnancy loss holds promise but should be interpreted with caution. Further studies incorporating functional analyses, larger cohorts, and parental data are needed to clarify causality and to define the clinical utility of such approaches in genetic counseling, recurrence-risk assessment, and reproductive planning.

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Ethics Committee Approval

The study was approved by the Ethics Subcommittee for Medicine, Pharmacy, Veterinary Medicine, and Dentistry of the Macedonian Academy of Sciences and Arts (03-203/2 from January 25, 2024). Written informed consent was obtained from all participants.

Author Contributions

Concept- Gj.B.; D.P.K; Design-D.P.K; Supervision-D.P.K; Materials-K.K.S.; Data Collection- Gj.B, P.N, M.T.; K.K.S.; Analysis or Interpretation- Gj.B, P.N., M.T.; D.P.K.; Literature Review- Gj.B, D.P.K.; Writing- Gj.B, D.P.K.; Critical Review- Gj.B, P.N., K.K.S., D.P.K.

Conflict of Interest

The authors declared no conflict of interest.

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Supplemental Table S1. Detailed overview of the demographic and clinical characteristics of the EPL studied group

	Sample ID	Fetal sex	Ethnic origin	Gestational age (weeks)	Maternal Age	No. of PLs (n)	Livebirths (n)
1	Abp-2	F	MKD	8	42	4	1
2	Abp-26	F	ALB	7	30	5	0
3	Abp-76	M	MKD	8	31	3	0
4	Abp-80	F	ALB	6	26	6	0
5	Abp-87	M	ALB	9	29	4	1
6	Abp-166	F	ALB	7	38	12	0
7	Abp-233	F	MKD	8	40	4	0
8	Abp-251	M	MKD	8	29	4	0
9	Abp-258[1]	F	MKD	8	31	11	0
10	Abp-266	F	MKD	7	32	4	0
11	Abp-272[2]	M	ALB	11	28	4	0
12	Abp-278	F	ALB	9	24	4	2
13	Abp-303	F	ALB	8	29	2	0
14	Abp-312	M	ALB	12	27	3	1
15	Abp-357	M	MKD	9	41	3	0
16	Abp-367	M	ALB	8	30	3	0
17	Abp-372[2]	M	ALB	9	29	6	0
18	Abp-395	M	MKD	9	27	3	1
19	Abp-404	M	ALB	7	28	3	0
20	Abp-407	M	ALB	8	35	3	0
21	Abp-411	M	ALB	8	30	5	0
22	Abp-444[3]	F	MKD	9	27	2	0
23	Abp-445[4]	F	ALB	10	24	2	0
24	Abp-457[5]	M	ALB	9	27	3	0
25	Abp-488	M	ALB	8	34	3	1
26	Abp-494	M	MKD	7	32	3	0
27	Abp-501	F	ALB	8	31	7	0
28	Abp-517[6]	F	ALB	8	25	1	0
29	Abp-551[4]	F	ALB	8	25	3	0
30	Abp-562	M	ALB	6	32	3	0
31	Abp-577	F	MKD	8	41	3	1
32	Abp-589[7]	F	MKD	8	36	2	0
33	Abp-590[1]	M	MKD	8	35	13	0
34	Abp-591	F	MKD	8	35	4	0
35	Abp-601[3]	F	MKD	8	28	3	0
36	Abp-604	M	ALB	9	29	4	0
37	Abp-642	F	ALB	9	25	3	0
38	Abp-656[5]	M	ALB	7	29	4	0
39	Abp-665[7]	M	MKD	7	37	3	0
40	Abp-666	M	MKD	7	33	4	0
41	Abp-668	M	MKD	10	37	2	0
42	Abp-677	F	MKD	8	31	4	0
43	Abp-682	F	ALB	9	26	2	0
44	Abp-694	M	ALB	9	23	2	0
45	Abp-699	M	MKD	12	29	2	0
46	Abp-715	F	ALB	8	26	4	0
47	Abp-716	F	MKD	9	31	2	0
48	Abp-722	M	MKD	6	34	3	0
49	Abp-729	F	MKD	8	36	2	0
50	Abp-734	M	ALB	7	24	3	1
51	Abp-741	M	ALB	9	22	3	0
52	Abp-746	M	MKD	8	32	2	1
53	Abp-781	F	MKD	9	33	2	0
54	Abp-786	F	ALB	8	27	2	0
55	Abp-799	M	MKD	8	35	4	1
56	Abp-801	F	MKD	8	25	2	1
57	Abp-809	M	ALB	12	20	3	0
58	Abp-812	F	MKD	6	36	3	1
59	Abp-813[6]	M	ALB	8	30	3	0
60	Abp-825	F	MKD	7	37	3	1
61	Abp-833	F	MKD	7	39	2	0
62	Abp-859	M	ALB	8	31	2	3
63	Abp-864	M	ALB	9	24	3	0
64	Abp-866	F	MKD	8	42	3	1
65	Abp-900	M	MKD	8	37	2	0
66	Abp-972	F	ALB	9	31	4	1

[1-7] fetuses from same family

SUPPLEMENTARY TABLES

Supplemental Table S2. Designed PCR primer sequences used in the confirmation and segregation analyses.

Gene	Variant	Primer name	Primer nucleotide sequence (5`>3`)	PCR length (nt)
CPLANE1	c.1819delT	CPLANE1_c.1819_F	CCACCAATGAGTCTTGAGCTG	732
		CPLANE1_c.1819_R	AAGAACGCCAAAGTGATGCTAT	
	c.7817T>A	CPLANE1_c.7817_F	TGGGTTTGTAGGAGGAGAGGT	471
		CPLANE1_c.7817_R	CATACTTCCTGCTCCTTTTCCT	
	c.5820+3_5820+6del	CPLANE1_c.5820_F	GCCACACAGCATGGCTATATT	431
		CPLANE1_c.5820_R	TCTCAAGGCTCATCTGGGAT	
DHCR7	c.452G>A	DHCR7_c.452_F	GTGAAGCAAGTTCCATCCCC	586
		DHCR7_c.452_R	GCAGAACCAAAGGATGGACTC	
	c.964-1G>C	DHCR7_c.964-1_F	GCAGAACACGCTCTTGACAG	721
		DHCR7_c.964-1_R	CAGGTAGAAGGCAGGTAGAGTT	
RBM8A	c.-21G>A	RBM8A_ex1_F	TGAAGGGGGCGGAATCTCTA	353
		RBM8A_ex1_R	TGCGTGTTTTTACCGTGCAG	
NF1	c.4537C>T	NF1_ex35_F	TGGTCCTGAGGTCTTTTTGG	560
		NF1_ex35_R	TGTTGTCTTCACTCCCTGGT	
F5	c.1601G>A	FV-Leiden_F	TGATGCCCAGTGCTTAACAA	265
		FV-Leiden_R	TCACACTGGTGCTAAAAAGGA	
TSC1	c.3113_3119del	TSC1_ex23_F	GGCTCTCAGAAAGGCTACTGG	436
		TSC1_ex23_R	CATCCTCCGAATGTGGACAG	
VWF	c.3797C>T	VWF_ex28_F	ACCGGGATCACAATGACCTT	632
		VWF_ex28_R	GTAGGGCTCAGAAGTGTCCA	
SLC6A1	c.740C>A	SLC6A1_ex8_F	AAATGTGAGCTGGTTGGCTC	432
		SLC6A1_ex8_R	AAACCTGGTCTACAGTGAGGG	
DSG2	c.2315del	DSG2_ex14_F	GCCCACTTGACTCAGATCCT	590
		DSG2_ex14_R	TGGGTCCCATTTCTCTTTCCTTA	
DVL1	c.1961dup	DLV1_ex15_F	GGTTGTTCTGGACGTGGC	722
		DLV1_ex15_R	GGTCTTCCTCATCCCAGGAG	
RPGRIP1L	c.2771G>A	RPGRIP1L_2771G_A_F	GGGGTGGCAGCTTAGTTCTT	389
		RPGRIP1L_2771G_A_R	CCTGGCTAGTTCACATGGTAG	
	c.3295-2A>G	RPGRIP1L_3295-2_F	AGGCCAATGGGCTTCTTTTCT	3211
		RPGRIP1L_3295-2_R	GATGGTGATGTCATCGGCTG	
		RPGRIP1L_3295-2_seq	GCAGAGGTGGGCGGATCATGAG	
PAH	c.842C>T	PAH_ex7_F	GCCAGCAATGAACCCAAACC	239
		PAH_ex7_R	TCTTTTCATCCCAGCTTGCAC	
	c.*19G>T	PAH_ex13_F	ACAAGTGGCCCATTTTGATGGT	402
		PAH_ex13_R	GGCCCATTTTGATGGTGTTTT	
GBA	c.1444G>T	GBA_ex10_F	CTGCCTCTCCCACATGTGA	391
		GBA_ex10_R	CAAAAGGGGATGGGTGTGC	
	c.1226A>G	GBA_EX9_F	CTTTTCTGCATCGCAGTCCA	459
		GBA_EX9_R	TCCCACATGTGACCCTTACC	
PKHD1	c.107C>T	PKHD1_ex3_F	CAGGCCCACTTTTACACCTG	423
		PKHD1_ex3_R	GGGGCTTCTGATGATGTGTTT	
	c.10883C>T	PKHD1_ex61_F	ATCAGCCCTCATTTGGATGTGA	537
		PKHD1_ex61_R	TTCCATTCACTTGGCCCTCA	
PRDM6	c.1057G>A	PRDM6_ex5_F	ATTGGTTGCTGGGGACAATC	380
		PRDM6_ex5_R	AGTGAACCACGTTTCATGAGT	
TBX18	c.1570C>T	TBX18_ex8_F	GCAACTGGATGAAACAGGGG	1074
		TBX18_ex8_R	CTCCAACCCTTGCCTTGTAAC	
SCN5A	c.3911C>T	SCN5A_ex22_F	CACGGCCATAGGACATCAGA	268
		SCN5A_ex22_R	TGTTCCCATCCTCCCCATTT	
MYH3	c.3137G>A	MYH3_ex25_F	TCTTCTGAAACTGGAGGCCC	345
		MYH3_ex25_R	CTTGCAAAGCATTTGTTCCCA	

Supplemental Table S3. PCR mixture and cycling conditions used for Sanger sequencing for confirmation and phasing of the detected variants on WES analysis.

PCR master mix content:	Volume (ul)
H_2O	16.1
10xB2 buffer	2.5
25mM $MgCl_2$	1.3
2.5 mM nucleotide mix	2
10mM Forward primer	1
10 mM Reverse primer	1
HotFire Polymerase 1U/ul	0.1
DNA (100ng/ul)	1
Total volume:	25
PCR cycling conditions:	
95°C/15 min	x1 cycle
95°C/30 sec	x33 cycles
59°C/30 sec	
72°C/45 sec	
72°C/10 min	x1 cycle