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Molecular autopsy by proxy in preconception counseling

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Abstract

Monogenic diseases that result in early pregnancy loss or neonatal death are genetically and phenotypically highly variable. This often poses significant challenges in arriving at a molecular diagnosis for reproductive planning. Molecular autopsy by proxy (MABP) refers to the genetic testing of relatives of deceased individuals to deduce the cause of death. Here, we specifically tested couples who lost one or more children/pregnancies with no available DNA. We developed our testing strategy using whole exome sequencing data from 83 consanguineous Saudi couples. We detected the shared carrier state of 50 pathogenic variants/likely pathogenic variants in 43 families and of 28 variants of uncertain significance in 24 families. Negative results were seen in 16 couples after variant reclassification. In 10 families, the risk of more than one genetic disease was documented. Secondary findings were seen in 10 families: either genetic variants with potential clinical consequences for the tested individual or a female carrier for X-linked conditions. This couple-based approach has enabled molecularly informed genetic counseling for 52% (43/83 families). Given the predominance of autosomal recessive causes of pregnancy and child death in consanguineous populations, MABP can be a helpful approach to consanguineous couples who seek counseling but lack molecular data on their deceased offspring.

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KEYWORDS
consanguinity, molecular autopsy by proxy, neonatal deaths, recurrent pregnancy loss, whole exome sequencing

1 | INTRODUCTION

Advances in genomic data utilization have encouraged their adoption in increasingly diverse clinical settings. Reproductive medicine is one of the areas where genomic tools have proven to be useful. Genetic tests in reproductive medicine are typically pursued for three main purposes: to identify infertility causes, determine genetic diseases transmissible to offspring, and optimize assisted reproductive technology (ART).[1]

Many monogenic disorders have a lethal phenotype either early in utero or later in life. Genetic and chromosomal disorders contribute significantly to neonatal and infant mortality and morbidity with congenital malformations and metabolic crisis are leading causes of death.[2–4] Indeed, 28% of deaths in neonatal intensive care unit (NICU) are caused by confirmed genetic diagnoses, a third of which are only diagnosed post-mortem, which tend to lead to more time spent in the NICU in Boston Children's Hospital.[4] Furthermore, the incidence of inborn errors of metabolism (IEM) in the population of patients admitted to the PICU was 2.2%–3% in different populations, a figure quite similar to the reported incidence for patients with septic shock.[5] Wojcik et al. demonstrates the mortality burden of genetic diseases in infancy using NGS technology in prenatal, postnatal and post-mortem samples. This study reveals a higher prevalence of genetic disorders up to 22% in 573 deceased infants; 54% had chromosomal disorders and 47% had monogenic disorders with one infant had both chromosomal disorder and monogenic disorder. The proportion of genetic diagnoses made by NGS technologies increased over the years. For counseling purpose, a confirmed molecular diagnosis is required to provide a family with the reproductive options.[6]

Additionally, early pregnancy loss can represent the severe end of phenotypic spectrum of several monogenic disorders although this remains poorly addressed in the literature.[7–10] The incidence of pregnancy loss from implantation to clinically recognized spontaneous abortion (SAB) has been reported to be approximately 30%. Pregnancy loss includes SAB or fetal death prior to 20 weeks (miscarriage) and fetal death at 20 weeks of gestation or greater (stillbirth or intrauterine fetal demise [IUFD]). Approximately 50% of the spontaneous pregnancy loss results from chromosomal abnormalities such as aneuploidy.[11] It has been suggested that 86% of these abnormalities are numerical chromosomal abnormalities, 6% are structural abnormalities, and 8% are due to other genetic mechanisms, such as chromosomal mosaicism and molar pregnancies.[9] Due to the significant psychological consequences of recurrent pregnancy loss on families, determining the underlying genetic etiologies helps in providing the family with informed reproductive options for normal future pregnancies as well as minimizing the guilt felt among those losing pregnancies.[12,13]

Consanguinity is known to be a possible risk factor for birth defects, as it results in the expression of rare and deleterious genes causing autosomal recessive disorders. Saudi Arabia is considered to be among the countries with the highest rates of consanguineous marriages, leading to high rates of birth defects and even neonatal deaths.[14] Many families in Saudi Arabia prefer consanguineous marriages in hopes of having already been acquainted with the spouse and as a way to keep the property within the family and the tradition alive.[15] Warsy et al. studied the consanguinity prevalence in well-educated Saudi females in two generations. The study concludes that even though there is an awareness that certain genetic disorders occur at a higher frequency in cousin marriages, there is no decrease in the prevalence of consanguinity over a generation.[16] Although the contribution of genetics to neonatal deaths and IUFD is not fully understood, NGS has made it possible to increase awareness of monogenic diseases in embryonic stages, in addition to uncovering novel genes in embryonic lethality in humans.[8,10,12]

Several strategies have been established to provide families, particularly high-risk families, with accurate risk estimates of having a child with a genetic condition. Preconception exome-based parental screening is the process of testing couples for their risk of having a child with a genetic disease, particularly autosomal recessive and X-linked recessive conditions.[17] Sallevelt et al. proposed exome-based preconception carrier screening (PCS) and a filtering strategy to rapidly identify the majority of relevant pathogenic mutations.[17] Additionally, several studies have investigated the utilization of WES in the prenatal setting in cases of structurally abnormal pregnancies revealed by prenatal scans as well as in cases of pregnancy loss and developmental disorders. These studies demonstrated the clinical applications of WES in pregnancy loss or IUFD and structurally abnormal pregnancy and have revealed the lethal Mendelian genes that might contribute to RPL. The few studies using WES in both parents looked for carrier status of genetic mutation and established a risk in each family.[8,18–20]

MABP is a term we coined to describe genetic testing of couples or relatives with a deceased offspring (other relatives) which indicates a priori increased risk of having a child with a recessive genetic disease before they attempt to conceive.[8] In most families, there is no clear genetic etiology for the phenotype of early loss without genetic testing or incomplete genetic testing and therefore it is difficult to characterize the phenotype early in life or in utero. Furthermore, early neonatal death without a genetic diagnosis requires parental testing and family counseling. Therefore, MABP through PCS aims to reveal the genetic cause for an identified phenotype to provide families with informed reproductive options.[21]

In this study, we describe a couple-based approach using WES in 83 consanguineous Saudi couples with early pregnancy loss, IUFD, neonatal death or family history of an unidentified genetic condition without established genetic diagnosis to determine familial monogenic diseases. Our goal is to provide high-risk couples with variable reproductive options, including prenatal diagnosis, preimplantation genetic diagnosis, acceptance of the genetic risk and preparation for the possibility of having a child with a certain disease, and avoidance of further conception.

2 | METHODS

2.1 | Human subjects

We counseled 83 Saudi consanguineous couples who sought medical genetics services with a previous history of neonatal and infantile death, IUFD, and/or pregnancy loss without an established genetic diagnosis to determine the familial monogenic diseases that could have been due to a recessive disorder. Detailed clinical information was gathered, including family history of monogenic disease, consanguinity, past medical history, and death reports. As applicable, couples and their children were recruited by written informed consent forms approved by the Internal Review Board of King Saud University Medical Center and King Faisal Specialist Hospital and Research Centre.

2.2 | Whole exome sequencing

Double-stranded DNA capture baits against approximately 36.5 Mb of the human coding exome (targeting >98% of the coding RefSeq and Gencode v28 regions, which were obtained from the human genome build GRCh37/hg19 on May 2018) were used to enrich target regions from fragmented genomic DNA with the Twist Human Core Exome Plus kit (Twist Bioscience). The generated library was sequenced on an Illumina platform to obtain at least 20× coverage depth for >98% of the targeted bases. An in-house bioinformatics pipeline, including read alignment to GRCh37/hg19 genome assembly, variant calling and annotation, and comprehensive variant filtering, was applied. All disease-causing variants reported in HGMD® in ClinVar as well as all variants with minor allele frequencies (MAFs) below 1% in the genomAD database were considered.

The investigation for relevant variants was focused on coding exons and flanking ±20 intronic bases. All potential modes of inheritance patterns were considered. In addition, family histories and clinical information were used to evaluate identified variants with respect to their pathogenicity and causality; the variants were categorized as diagnostic, inconclusive and unremarkable. All variants related to the phenotype of the patient, except benign or likely benign variants, were reported.

Our lab has established stringent quality criteria and validation processes for variants detected by NGS. Low-quality single nucleotide variants and all relevant deletion/insertion variants were confirmed by Sanger sequencing. Consequently, we warrant a specificity of >99.9% for all reported variants.

2.3 | Interpretation strategy

Causative pathogenic variants that were detected in both partners in the same autosomal recessive gene are classified as diagnostic. This indicates an increased risk for their progeny to be affected by autosomal recessive disease if both are heterozygous.

Variants of uncertain clinical significance (VUS) detected in both partners in the same autosomal recessive gene are classified as inconclusive. According to the American College of Medical Genetics (ACMG) recommendation, VUS were studied further by family segregation and/or clinical consistency (Figure 3). In selected cases, we have performed deletion and duplication analysis in the other partner in the case of a pathogenic variant detected in one partner consistent with the phenotype for further clarification.

The variants in the latter category were carefully chosen to qualify as much as possible as pathogenic, likely pathogenic if the genes were established in the online Mendelian inheritance in man database. Regarding the genetic variation ~~to~~ in candidate research genes, we have considered only genes with compelling biological candidacy (special emphasis was made on animal models, but other lines of evidence were also pursued).

Variants with no clinical relevance to the described phenotype and/or VUS excluded by family segregation or by lack of clinical consistency were classified as unremarkable.

2.4 | Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) is a technique used to identify variations in the copy number of genes and if there are deletions or duplications in specific genes. We have used specific MLPA probes to recognize adjacent target-specific sequences, and only in the presence of a perfect match without a single gap, after hybridization, the probes ligated and amplified after which PCR amplification is performed using only one PCR primers pair, which is fluorescently labeled followed by separation by size by capillary electrophoresis.[22] In one of the family, we have found a pathogenic variant in *PEX12* gene in the father and negative maternal exome. So, we performed quantitative PCR assay (qPCR) by using six gene-specific amplicons encompassing the coding exons 1, 2, 3 (or part of it) of the PEX12: NM_000286.2 genes.

3 | RESULTS

3.1 | Human subjects

All 83 counseled families were consanguineous couples. Clinical information was completed for 77% of the families, and 61% of the families had recurrent neonatal deaths with a reported phenotype.

As demonstrated in Figure 1, couples with nephews or nieces who died with of undetermined genetic diseases represented 7% ($N = 6$ couples) of the cases; RPL including IUFD and miscarriages represented 24% ($N = 20$ couples) of the cases; neonatal deaths in the offspring represented 60% ($N = 50$ couples) of cases; both neonatal death and RPL represented about 9% of our cohort ($N = 7$ couples).

3.2 | Whole exome sequencing and interpretation of the results

Duo WES was performed in 83 Saudi consanguineous parents and showed high-diagnostic yield (65% total). Eighty-one variants were found in 67 families, including pathogenic, likely pathogenic variants and VUS.

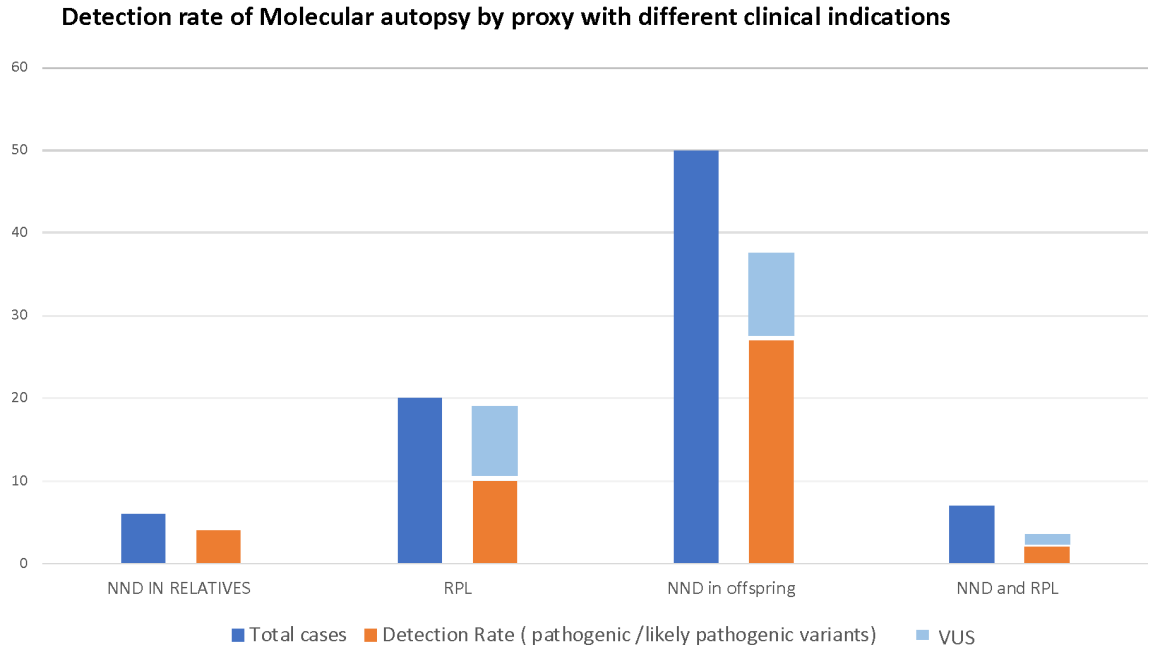

FIGURE 1 Clinical summary for indication of parental exome testing and the detection rate for pathogenic variants and VUS [Colour figure can be viewed at wileyonlinelibrary.com]

3.2.1 | Diagnostic

Pathogenic or likely pathogenic variants that segregated in the family and showed clinical consistency were found in 52% of the families ($N = 43$ couples) (Figure 2). Diagnostic result with the variants in both parents were identified in the following genes: *POLR3A*, *ECHS1*, *CTU2*, *ACAD9*, *GUSB*, *MMUT*, *IBA57*, *BCS1L*, *STXBP2*, *HSPG2*, *CANT1*, *MMAB*, *MALT1*, *EVC2*, *FRAS1*, *CEP290*, *TCTN2*, *NPHS1*, *ISPD*, *CRIPT*, *MKS1*, *CC2D2A*, *EML1*, *PEX26*, *LGI4*, *KLHL7*, *TMEM231*, *GAA*, *DNAH5*, *CDT1*, *LAMB2*, *MRAP*, *ACADVL TRIP11*, *PAX1*, *AK2*, *and SLC26A3 KIAA0586*, *LZTR1*, *MPDZ*, *NPHP3* (Table 1).

3.2.2 | Inconclusive

VUS were found in 32% of the families ($N = 27$ couples) (Figure 2). Segregation, clinical consistency and/or duplication, and deletion analyses were performed to confirm the pathogenicity of these variants and resulted in the reclassification of these variants as follows: in 41% of the families with inconclusive variants ($N = 11$ couples), the variants (*DOCK7*, *CHAT*, *KIAA0586*, *NDUFAF3*, *GBA*, *PEX1*, *NDUFAF5*, *HTRA2*, *CCDC88C*, *CPS1*, *POLR3A*) were reclassified as diagnostic; in 11% of the families ($N = 3$ couple), the variants were reclassified as benign; c.1768G > A:p.(Val590Met) at *KIAA0556* gene, c.4552C > G p.(Arg1518Gly) at *DCHS1* gene, and c.685C > G:p.(Pro229Ala) at *TMEM231* gene; and in 48% of the families ($N = 13$ couples), the variants remained uncertain (*KIAA0556*, *WDR34*, *CC2D2A*, *AGRN*, *GORAB*, *QARS*, *KLHL40*, *FH*, *ACACA*, *PAX1*, *FKRP*, *DOCK6*, *FKTN*, *UNC80*) (Figure 2 and Table 2). Therefore, the diagnostic yield increased to 65% ($N = 54$) (Figure 3).

Further analysis revealed two variants in candidate genes, ZMIZ2 and DHX34 (novel genes), that might cause RPL.

3.2.3 | Negative

Fifteen percentage of the families had negative results ($N = 13$ couples) (Figure 2, Table 1), which accounted for the lack of variants relevant to the phenotype and/or that only one of the partners was a carrier (no family segregation). Table 1 summarizes the WES findings for the 83 families, the phenotypes, whether the exome findings matched the phenotype or not, and the novel variants discovered. Including the three families with benign variants makes the undiagnosed percentage 19% ($N = 16$ couples).

3.3 | Secondary findings

Few families showed secondary findings, even though these cases were solved, and, therefore, need counseling in the future. We detected 10 couples who shared the carrier status of several autosomal recessive disease-associated genes, which indicates a risk of offspring having more than one genetic disease of 12%. In family 4, a well-known pathogenic variant for congenital adrenal hyperplasia, *CYP21A2*: c.92C > T, was identified, and even though it segregated in the family, it was not consistent with the phenotype, as the child's death was due to persistent lactic acidosis, hypertrophic cardiomyopathy, and high-liver enzyme, which is associated with the *ACAD9* gene. Female carriers for an X-linked condition were seen in three families (1, 43, and 65), and there was a common pathogenic variant found in

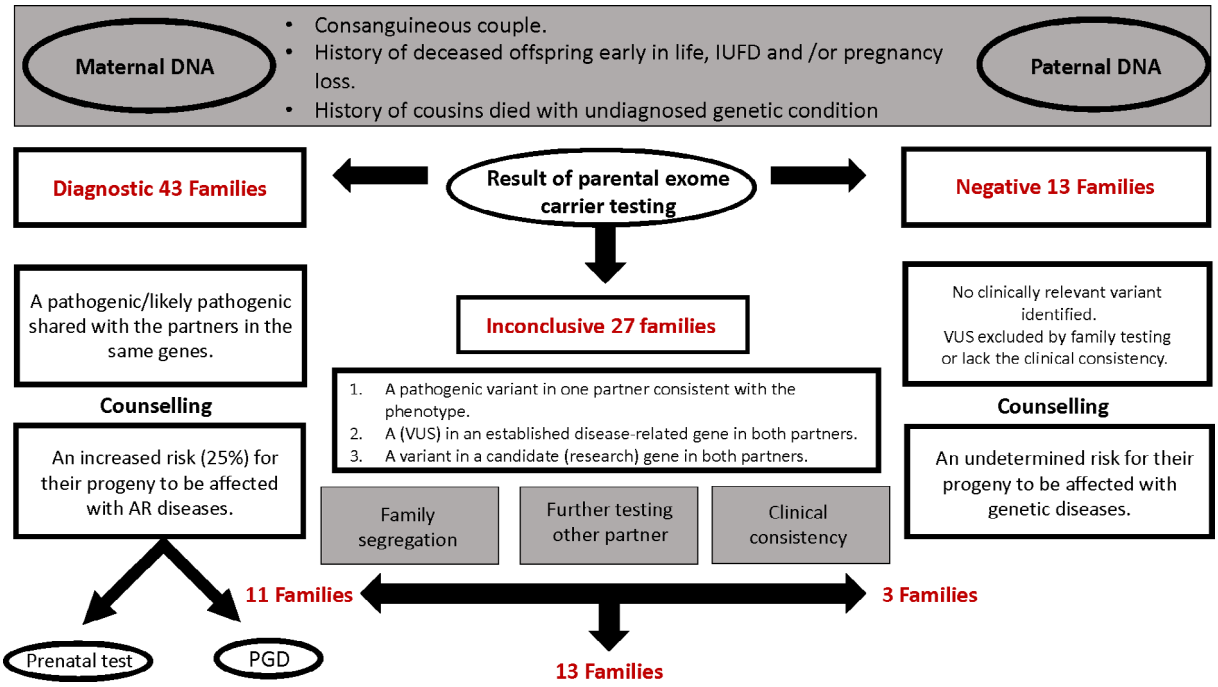

FIGURE 2 Workflow for all cases with different indications, including the result and the analysis of the result [Colour figure can be viewed at wileyonlinelibrary.com]

both families, *G6PD*: c.233T > C p. (Ile78Thr); where only the female was a carrier, we performed further counseling regarding the 50% male offspring risk of G6PD deficiency. In family 71, ES of both parents revealed that the female partner carried an X-linked condition AR gene variant p.(Gln58Leu), but no detected variants could explain recurrent acrania and IUFD. Furthermore, autosomal-dominant mutations with potential clinical consequences for the tested individuals were found in five couples (family 36, 52, 65, 66) which changed our approach to presymptomatic testing for the affected individuals (Table 3).

Furthermore, MABP identify a carrier status of a pathogenic genetic variant in one partner that might explain a phenotype in several families, but the other partner was not a carrier; for example, *MCPH1* c.2595-1G > C/likely pathogenic p.(Arg497*) was found in a female partner and *DDX11* NM_001257144.1:c.1489C > T likely pathogenic in a male partner, but the variants were not found in the other partner. Also, WES revealed a heterozygous variant in the PEX12 gene, c.616C > T p.(Gln206*) in the male partner only. Consequently, we performed MLPA to exclude deletions or duplications in the other partner that were negative in the couple (Table 3).

3.4 | Diseases-causing genes observed

The most observed genetic etiology of RPL was complex dysmorphology disorders; eight genes—including *CTU2*, *KLHL7*, *DCHS1*, *CTD1*, and *DOCK6*—were identified in five families. Additionally, variants in the *TMEM231*, *DNAH5*, and *CC2D2A* genes associated with ciliopathy, which is a specific group of multisystem disorders, were identified in two cases each. Diagnostic results with the detection of variants in the *LGI4* gene causing neurogenic arthrogryposis were identified in two couples, and *AGRN* and *CHAD*, which are associated with congenital myasthenic syndrome, were identified in two couples each. Additionally, two couples each was carrier of *DOCK7* or *CCDC88C*. Congenital muscular dystrophy genes *FKRP* and *FKTN* in other two families, each is carrier for one variant. Variants in three genes—*GAA*, *FH*, and *AK2*—associated with enzyme and metabolic diseases were noted in three cases. Variants were also identified in other disease categories, including myopathy (*KLHL40*), skeletal disorders (*TRIP11*), and gastrointestinal disorders (*SLC26A3*), in four cases.

Additionally, candidate genes that are not consistent with a given phenotype were identified that might be of clinical significance, including *ZMIZ2* and *DHX34*.

On the other hand, our analyses showed that inborn errors in metabolism are the most common causes of neonatal deaths, including mitochondrial disorders associated with *CPS1*, *HTRA2*, *NDUFAF5*, *PEX1*, *GBA*, *NDUFAF3*, *PEX26*, *MMAB*, *BCSIL*, *IBA57*, *MMUT*, *GUSB*, *ACAD9*, *ECHSI*, *ACADVL*, and *AK2*. Ciliopathy, associated with *EVC2*, *CEP290*, *TCTN2*, *MKS1*, *CC2D2A*, *TMEM231*, *DNAH5*, *KIAA0586*, and *TCTN2*, was the second most common category. The third category involved neurologic phenotypes (*POLR3A*, *DOCK7*, *CCDC88C*, *EML1*, *MACF1*, *MPDZ*). Several variants were identified in different disease categories, including neuromuscular diseases (*NEB*, *ISPD*, *CHRNG*, *KLHL40*), complex dysmorphology syndrome (*CRIPT*, *LZTR1*, *FRAS1*), and renal (*NPHS1*, *NPHP3*, *LAMB2*), skeletal (*CANT1*, *HSPG2*, *TRIP11*), hematological (*STXBP2*), and immunological (*MALT1*) diseases (Table 1).

TABLE 1 Pathogenic/likely pathogenic variants with the reported phenotype

	Phenotype	Indications	Gene	Variants	Classification	Serial no.
1	Hx of second- and third-degree relative died with GDD, hypotonia, and cardiac condition.	ND	*POLR3A*	c.1909 + 22G > A	Pathogenic	1
2	Parent of two children deceased with lactic acidosis and severe hypotonia.	ND	*ECHS1*	c.88þ5G	Pathogenic	2
			NEB	c.17358T > A:p.N5786K	Likely pathogenic	3
3	VSD, kidney malformation abnormal limb posture, cerebellar hypoplasia, hypertelorism and severe IUGR.	IUFD	*CTU2*	c.1086 + 5G > A	Pathogenic	4
4	Persistent lactic acidosis, hypertrophic cardiomyopathy, high-liver enzyme level.	ND	*ACAD9*	c.1240C > T (p.Arg414Cys)	Pathogenic	5
			CYP21A2	c.92C > T	Pathogenic	6
5	Fetal hydrops fetalis, pleural effusion, pericardial effusion, ascites ended by fetal demise.	ND	*GUSB*	c.1429C > T (p.Arg477Trp)	Pathogenic	7
6	Second- or third-degree nephews/nieces who died early in life (2nd or 3rd day of life).	ND	*MMUT*	c.329A > G (p.Tyr110Cys)	Pathogenic	8
7	Recurrent neonatal deaths.	ND	*IBA57*	c.316A > G (p.Thr106Ala)	Pathogenic	9
8	Recurrent neonatal deaths.	ND	*BCS1L*	c.385G > A (p.Gly129Arg)	Pathogenic	10
9	Recurrent neonatal deaths	ND	*STXBP2*	c.1485 + 1G > A	Pathogenic	11
10	Recurrent neonatal deaths.	ND	*HSPG2*	c.790C > T (p.Arg264*)	Likely pathogenic	12
11	Recurrent neonatal deaths.	ND	*CANT1*	c.902_906dup (p.Ser303Alafs*21	Pathogenic	13
12	Recurrent neonatal deaths.	ND	*MMAB*	c.197-1G > T	Pathogenic	14
13	Recurrent neonatal deaths.	ND	*MALT1*	c.1240G > A (p.Gly414Arg)	Likely pathogenic	15
14	Dysmorphic, short neck, narrow restricted chest, faint heart sound, short four limbs with polydactyly of the upper limb.	ND	*EVC2*	c.2017_2021del (p.Thr673Glufs*14)	Likely pathogenic	16
15	Early neonatal death with bilateral renal agenesis.	ND	*FRAS1*	c.1226dup (p.Gln411Thrfs*26)	Likely pathogenic	17
16	Early death, microcephaly, Potter-like facies, sloping head, microphthalmia, low-set ears, short webbed neck, occipital encephalocele, polycystic kidneys, ambiguous genitalia, polydactyly (suspected diagnosis - Meckel-Gruber syndrome).	ND	*CEP290*	c.613C > T (p.Arg205*)	Pathogenic	18
17	Microcephaly, Potter-like facies, sloping head, microphthalmia, low-set ears, hypertelorism, occipital encephalocele, polycystic kidneys, oligohydramnios, talipes, polydactyly.	ND	*TCTN2*	c.1506-2A > G	Pathogenic	19
18	Recurrent neonatal death due to polycystic kidney disease.	ND	*NPHS1*	c.2540_2543delCTAA (p.Thr847ArgfsTer57)	Likely pathogenic	20
19	Severe hydrocephalus, encephalocele and lissencephaly in two pregnancies; mother is healthy apart from hypothyroidism with no other medical issues.	ND	*ISPD*	c.1186G > T (p.Glu396*)	Pathogenic	21
20	Characteristic facies, hypoplastic terminal phalanges, osteopenia, albinoid fundus, markedly impaired retinal function, recurrent infections, persistent anemia with anisopoikilocytosis.	ND	*CRIPT*	c.141delT (p.Phe47Leufs*84)	Pathogenic	22

(Continues)

TABLE 1 (Continued)

	Phenotype	Indications	Gene	Variants	Classification	Serial no.
21	Anencephaly, polydactyly, cystic kidney (suspected diagnosis Meckel syndrome).	ND	*PEX26*	c.228C > T (p.Gly76Alafs*5)	Pathogenic	23
22	Microcephaly, agenesis of corpus callosum, occipital encephalocele, multicystic kidney, hypotonia, IUFR (suspected Meckel syndrome).	ND	*MKS1*	c.261 + 2T > A	Pathogenic	24
23	Meckel syndrome-affected fetuses.	IUFD	*CC2D2A*	c.3084delG (p.Lys1029Argfs*3)	Pathogenic	25
24	Four neonatal deaths with congenital hydrocephalus.	ND	*CC2D2A*	c.3084delG (p.Lys1029Argfs*3)	Pathogenic	26
25	Bilateral genu recurvatum, narrow chest with mild to moderate inspiratory stridor, no organomegaly, generalized hypotonia, hyporeflexia, mild microcephaly, respiratory distress.	ND	*PEX26*	c.228C > T (p.Gly76Alafs*5)	Pathogenic	27
26	Hypotonia, dysmorphic feature, low-set ears, micrognathia, high-arched palate, bilateral talipes, equinovarus, poor feeding.	ND	*EML1*	c.2233G > A (p.Val745Ile)	Pathogenic	28
27	Recurrent miscarriage.	RPL	*LGI4*	c.834del (p.Ser279Alafs*191)	Likely pathogenic	29
28	Recurrent spontaneous abortion. Abnormal facial shape; CHD, atrial septal defect; CNS abnormality; cerebellar hypoplasia; congenital onset; depressed nasal bridge; dilation of lateral ventricles; enlarged cisterna magna; hernia of the abdominal wall; high, narrow palate; hypertonia; low-set ears; microcephaly; optic atrophy; optic disc hypoplasia; overlapping fingers; ventriculomegaly.	RPL+ IUFD	*KLHL7*	c.807C > A	Likely pathogenic	30
29	Abnormal vertebral morphology, renal abnormality, aplasia/hypoplasia of the cerebellar vermis, clinodactyly, hydrocephalus, low-set ears, micrognathia, multiple renal cysts, oligohydramnios, polydactyly, rocker bottom foot and ventriculomegaly. Asymptomatic parents are consanguineous, and they lost two children at the ages 33 and 32 G.W.	IUFD	*TMEM231*	c. 930-5_930-2delinsTGTC	Likely pathogenic	31
30	Recurrent pregnancy loss.	RPL	*CHRNG*	c.1019C > T	Likely pathogenic	32
			GAA	c.1430delT	Likely pathogenic	33
31	One child died with unilateral renal agenesis, recurrent spontaneous abortion. Hx of previous hydatidiform mole.	RPL	*DNAH5*	c.5503C > T(p.Gln1835Ter)	Likely pathogenic	34
			AMHR2	c.994C > T	Pathogenic	35
			PTPRQ	c.4155 + 1G > A	Pathogenic	36
			PCDH15	c.4604_4608dup	Pathogenic	37
32	Recurrent miscarriages and IUFD 3X skeletal phenotype in the aborted fetus.	RPL	*TRIP11*	c.3082C > T (p.Arg1028*)	Pathogenic	38

TABLE 1 (Continued)

	Phenotype	Indications	Gene	Variants	Classification	Serial no.
33	Multiple malformations.	IUFD	*CDT1*	c.1393dupT (p.V464fs)	Likely pathogenic	39
34	Recurrent neonatal deaths with renal phenotype.	ND	*LAMB2*	c.4276dupG (p.Ala1426Glyfs*6)	Likely pathogenic	40
35	Second- or third-degree cousin died with phenotype consistent with liver disease, and hypoglycemia	ND	*MRAP*	c.105_106 + DEL P.(His36Phefs*84)	Pathogenic	41
36	Recurrent failed implantation after IVF and 2 miscarriages, one died with recurrent infection	RPL	*SLC26A3*	c.79G > C p.(Gly27Arg)	Pathogenic	42
			AK2	c.559G > T p.(Gly187*)	Likely pathogenic	43
37	Abnormal stomach, congenital arthrogryposis multiplex, talipes equinovarus.	ND	*LGI4*	c.639G > A P.(Trp213*)	Likely pathogenic	44
38	Son 1: Intracranial cystic lesion, Neonatal death, Premature birth; Son 2: Abnormal heart morphology, Abnormality of the kidney, Neonatal death, Premature birth.	ND	*NPHP3*	c.2694-2_2694-1del	Pathogenic	45
39	Two previous IUFD at 24 weeks and 27 weeks with scan showing skeletal changes	ND	*TRIP11*	c.763C > T p.R255X	Pathogenic	46
40	Two offspring passed away with 1- Coarctation of aorta; Hydrocephalus and brain anomalies 2- Atrial septal defect, Hydrocephalus; Severe, other anomalies + recurrent pregnancy loss.	ND IUFD	*MPDZ*	c.628C > T. p.(Gln210*)	Pathogenic	47
41	Hx of cousins with early death with cardiomyopathy.	ND	*LZTR1*	c.639del P.(Cys214Alafs*38)	Likely pathogenic	48
42	Recurrent neonatal deaths.	ND	*KIAA0586*	c.78dup (p.Lys27fs*)	Likely pathogenic	49
43	Neonatal death at 3 DOL with positive NBS VLCAD	ND	*ACADVL*	c.65C > A;p.Ser22X	Pathogenic	50

Abbreviations: RPL: recurrent pregnancy loss including miscarriage and IUFD; ND: neonatal death; PCS: preconception screening in the presence of cousins with genetic condition.

TABLE 2 Variants of unknown clinical significance likely related to the phenotype and shared by both partners

Case ID	Phenotype	Indication	Gene	Variants	OMIM disease	Serial no.
1	Recurrent neonatal deaths	ND	*WDR34*	c.544C > T p.R182W	Short-rib thoracic dysplasia 11 with or without polydactyly	1
2	Oligohydramnios, dysmorphic facial features, midline cleft lip, agenesis of corpus callosum, occipital encephalocele.	ND	*CC2D2A*	c.4531T > C (p.Trp1511Arg)	Joubert syndrome-9	2
3	IUGR, subcutaneous edema, abnormal position of upper and lower limbs with no movement.	RPL	*AGRN*	c.5948C > T (p.Thr1983Met)	Congenital myasthenic syndrome-8	3
4	Intrauterine fetal death, IUGR.	RPL	*ZMIZ2/KIAA1886*	c.1270C > T (p.Gln424*)		4
5	Recurrent neonatal deaths.	ND	*GORAB*	c.306dup (p.Pro103Thrfs*20)	Gerodermia osteodysplastica	5
6	Recurrent neonatal deaths.	ND	*QARS1*	c.316G > A (p.Asp106Asn)	Progressive microcephaly with seizures and cerebral and cerebellar atrophy (MSCCA)	6
			ACACA	c.81A > G (p.Ile27Met)	Acetyl-CoA carboxylase deficiency	7
7	Central hypotonia, scoliosis, cerebellar hypoplasia, 2–3 toe syndactyly, atrial septal defect, PDA, abnormal ear morphology, high palate.	ND	*PAX1*	c.95C > T p.(A32V)	Otofaciocervical syndrome 2	8
			MSRB3	c.2T > G/likely pathogenic	Autosomal recessive deafness-74	9
			MOCOS	c.894CA (p.Y298*) likely pathogenic	Molybdenum cofactor sulfurase	10
8	2X Hydrops fetalis, polyhydramnios, multiple congenital anomalies.	RPL	*KLHL40*	c.35G > T p.(Arg12Leu)	AR nemaline myopathy type 8	11
9	1: Dandy-Walker malformation, polyhydramnios, stillbirth; 2: IUFD with CNS anomalies. 3: Absent septum pellucidum; agenesis of corpus callosum; aplasia/hypoplasia of the corpus callosum; cerebellar vermis hypoplasia; Dandy-Walker malformation; dilated third ventricle; echogenic fetal bowel; enlarged cisterna magna; ventriculomegaly.	RPL	*FH*	c.1043G > A p.(Gly348Asp)	Fumarate hydratase	12
			FKRP	c.1061G > A p.(Gly354Glu)	Congenital muscular dystrophy-dystroglycanopathy with or without impaired intellectual development	13
10	Transposition of great arteries, abnormal heart valves, agenesis of corpus callosum, holoprosencephaly, hydrocephalus, IUGR.	IUFD	*FKTN*	c.44T > G P.(Leu15Arg)	Muscular dystrophy-dystroglycanopathy (congenital with brain and eye anomalies), type A, 4	14
			DOCK6	c.356A > G P.(Asp119Gly)	Adams-Oliver syndrome-2	15
11	IUFD with increased NT, ventriculomegaly, multicyclic dysplastic kidney and short long bone.	IUFD	*DHX34*	c. 1399G > A P. D467N		16
12	Brain anomalies, FTT, dilated lateral ventricles, partial agenesis of the corpus callsum, EEG abnormalities, IUGR, bilateral hearing loss, optic atrophy.	IUFD	*DOCK7*	c.1591G > A	Developmental and Epileptic Encephalopathy-23 (DEE23)	17

TABLE 2 (Continued)

Case ID	Phenotype	Indication	Gene	Variants	OMIM disease	Serial no.
13	5X; IUFD at 7 months, boy with short limbs and hydrocephalus; IUFD at 6 months with short limbs; IUFD at 24 weeks with massive fetal edema, abnormal skeletal system and bilateral dilated renal pelvis; IUFD at 27 weeks, fetal edema, ascites and short limbs.	IUFD	CHAT	c.1300G > A (p.Gly434Ser)	Myasthenic syndrome, congenital, 6, presynaptic	18
14	Recurrent spontaneous abortions. Couple had 2 children affected with seizures, hypotonia, global developmental delay, recurrent aspiration pneumonia, constipation, both children deceased.	ND RPL	*KIAA0556*	c.1777T > C p.(Tyr593His)	Joubert syndrome-26	19
15	Recurrent neonatal deaths.	ND	*NDUFAF3*	c.481C > G (p.Arg161Gly)	Mitochondrial respiratory chain complex I	20
16	Recurrent neonatal deaths.	ND	*GBA*	c.520T > A (p.Tyr174Asn)	Gaucher disease	21
17	Abnormal VLCFA (? diagnosis-Zellweger syndrome).	ND	*PEX1*	c.1240_1359del (p.Ile414_Leu453del)	Peroxisomal biogenesis disorders 1A	22
18	Agenesis of corpus callosum associated with white matter and brainstem abnormal signal.	ND	*NDUFAF5*	c.737T > A (p.Leu246Gln)	Mitochondrial respiratory chain complex I deficiency nuclear type 16	23
19	Microcephaly, hypotonia, encephalopathy, increased CSF lactate level and 3-methylglutaconic aciduria.	ND	*HTRA2*	c.818_820del (p.Leu273del)	3-Methylglutaconic aciduria type VIII (MGCA8	24
20	Neonatal death with hyperammonemia.	ND	*CPS1*	c.211T > C p.(Ser71Pro)	Carbamoyl phosphate synthetase I	25
21	Recurrent miscarriage.	RPL	*CCDC88C*	c.5059-2A > G	congenital hydrocephalus-1	26
22 23	2X early neonatal death with hypotonia and recurrent respiratory infections and respiratory distress.	ND	POLR3A	c.1895G > T (p.Cys632Phe)	Leukodystrophy, hypomyelinating, 7 (HLD7)	27
24	Abnormal facial features; Failure to thrive; feeding difficulty,Laryngomalacia; Motor delay; passed away with Respiratory compromise. 1: Stillbirth; Brother 2: Stillbirth; Brother 3: Stillbirth; Mother: Spontaneous abortion; Sister 1: Stillbirth; Sister 2: Stillbirth; Sister 3: Stillbirth Siblings affected.	ND	*UNC80*	c.5254C > T p.(Leu1752Phe)	Infantile hypotonia with psychomotor retardation and characteristic facies-2	28
15	Recurrent neonatal deaths.	ND	*TMEM231*	c.685C > G:p.(Pro229Ala)	Ciliopathic diseases Meckel syndrome 11	29
25	Previous 3X IUFD Multiple congenital anomalies and neonatal death with brain atrophy, esophageal atresia, heterotaxy; hypoplastic right heart IUFD	ND	*DCHS1*	c.4552C > G p.(Arg1518Gly)	Multiple congenital anomalies	30
26	Multiple congenital anomalies and early death.	ND	*KIAA0556*	NM_015202.3:c.1768G > A:p.(Val590Met)	AR Joubert syndrome type 26	31

Abbreviations: RPL: recurrent pregnancy loss including miscarriage and IUFD; ND: neonatal death; OMIM, online Mendelian inheritance in man; PCS: preconception screening in the presence of cousins with a genetic condition.

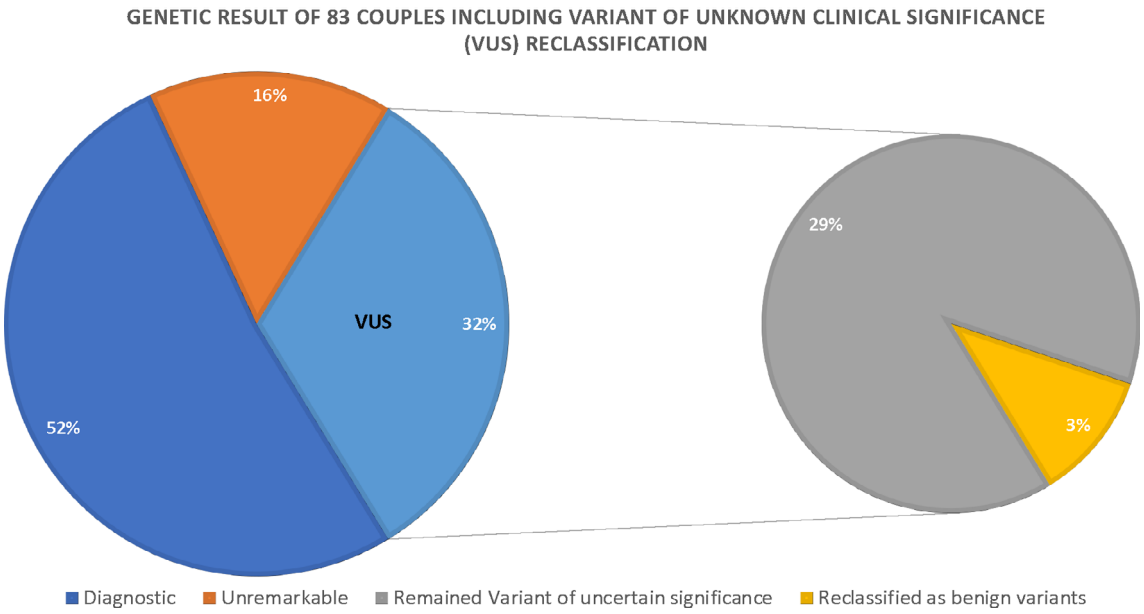

FIGURE 3 Parental exome sequencing results and VUS reclassification (pie chart) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Autosomal-dominant mutations with potential clinical consequences for the tested individual

Gene	Partner	Variants	Classification	OMIM phenotype
SLC5A2	Male partner	c.1035_1062del.p.(Val346Alafs*1)	Pathogenic	Renal glucosuria
FLT4	Female partner	c.2740G > C p.(Gly 914Arg)	Pathogenic	Pedal edema
TTN	Male partner	c.60451delp.(Ile20151Serfs*12)	Likely pathogenic	Cardiomyopathy, dilated
ASCC1	Female partner	c.495del p.(Ala166Profs*14)	Likely pathogenic	Barrett esophagus/esophageal adenocarcinoma[23]
HTRA2	Both partners	c.818_820del (p.Leu273del)	Likely pathogenic	Susceptibility to the development of autosomal dominant Parkinson disease-13
GBA	Both partners	c.520T > A (p.Tyr174Asn)	Likely pathogenic	Susceptibility of Parkinson disease

Abbreviation: OMIM, online Mendelian inheritance in man.

4 | DISCUSSION

WES was first introduced for clinical diagnostic purposes in 2009 and has since been applied in different clinical settings as a highly valuable diagnostic approach mainly in postnatal and prenatal genetic diagnosis of Mendelian disorders. WES has provided an opportunity to affordably screen a patient's exome to establish the genetic basis of diseases.[24–26] The reported diagnostic yield of WES generally ranges between 25% and 35%, with a maximum yield of 40% in trio analysis[27–29] and a high-diagnostic yield of 43%–49% in large consanguineous cohorts from Saudi Arabia.[30,31] Another study also reported a diagnostic yield of 60% in Middle Eastern patients from Qatar.[32]

Our results provide 51% of the families with a genetic diagnosis, with an additional 13% of the families if we consider VUS with potential clinical usefulness. VUS in both parents were found in 31% of our cohort, and after further analysis of the reports describing the phenotype and segregation of the family, we could exclude three (11%) and consider 12 variants (46%) as potentially disease causing. A negative result was seen in 16 families (19%). We have counseled our families who consented to pretesting for the potential for identifying and reporting incidental (or secondary) findings, which are results that are not related to the indication for ordering the sequencing but that may nonetheless be of medical value or utility to the physician and the patient. In nine families, the results identified a risk of more than one genetic condition in the family; in three families, the female partner was determined to carry an X-linked genetic disease; and in six families, we discovered that one of the partners carried a heterozygous mutation with potential clinical consequences for the tested individual.

The first report of the yield of WES of a couple (Duo WES) of 44 families with at least one death or lethal fetal malformation at any stage of in utero development and this strategy identified pathogenic/likely pathogenic variants that was shared by both of the couple and resulted in cause embryonic or perinatal lethality.[8] Further utilization of WES for trio analysis using cultured amniocytes or product of conception from the affected fetuses determined a genetic cause in four

of seven cases of IUFD,[13] and compound heterozygous variants in *DYNC2H1* and *ALOX15* were identified in miscarriages from two of four families with RPL.[33] More recent trio-WES studies in fetuses with ultrasound anomalies that resulted in IUFD or pregnancy termination identified positive variants in 20%, possible variants in 45%, and candidate variants in 9% of 84 fetal deaths with ultrasound anomalies.[34] In a similar work, WES in 15 of 19 POC cases with missed abortion revealed novel variants potentially associated with early embryonic lethality.[35] A study using different filtering strategies proved the applicability of parental WES in eight consanguineous and 25 nonconsanguineous couples for identifying the genetic variants shared by the couples.[17] These results supported the clinical utility of ES in reproductive medicine to assess in couples planning a pregnancy the risk of those couples having children affected with a genetic disease as well as to detect the monogenic etiology of pregnancy loss. The identification of disease-associated variants provided information for follow-up genetic counseling regarding recurrence risk and management of subsequent pregnancies. The discovery of novel variants could provide insight into the underlying molecular mechanisms of fetal death.

Several disease categories were noted for the 21 genes carrying variants of diagnostic value. The most prevalent disease category with recurrent pregnancy loss was multisystem disorders; eight genes—including *CTU2*, *KLHL7*, *DCHS1*, *CTD1*, and *DOCK6*—were identified in five families. Additionally, variants in the *TMEM231*, *DNAH5* and *CC2D2A* genes of ciliopathy, which is a specific group of multisystem disorders, were identified in two cases. The second most common category was neurological disorders, including neurogenic arthrogryposis in two couples with the *LGI4* gene, congenital myasthenic syndrome in two families with *AGRN* and *CHAD*, other neurological disorders associated with genes including *DOCK7* and *CCDC88C* in two couples, and muscular dystrophy associated with *FKRP* and *FKTN* in two couples. In contrast, in another study, the second most common cause of RPL after multisystem disorders was cardiac anomalies.[36] In another study, a molecular panel of 70 genes associated with cardiac channelopathies and cardiomyopathies in stillbirth cases was applied to identify pathogenic variants in 12% of 290 cases of IUFD, which indicates that cardiac anomalies are one of the known causes of IUFD.[37] Variants in three genes—*GAA*, *FH*, and *AK2*—associated with enzyme and metabolic diseases were noted in three cases. Variants were also identified in other disease categories, including myopathy (*KLHL40*), skeletal disorders (*TRIP11*), and gastrointestinal disorders (*SLC26A3*), in three cases.

Two novel genes that might have clinical implications and embryonic lethality are *ZMIZ2*: c.1270C > T; p. (Gln424*), which causes embryonic lethality in mouse (https://www.mousephenotype.org/data/genes/MGI:106374#phenotypesTab), and *DHX34*: c.1399G > A P.(D467N), which is predicted to be deleterious in silico. Dhx34 protein deficiency in zebrafish has been shown to result in severe neurodevelopmental defects and embryonic lethality.[38]

Among the solved cases, the most observed causes of neonatal death were inborn errors in metabolism, mainly mitochondrial disorders due to enzyme deficiencies, followed by ciliopathy and congenital anomaly disorders.

Other similar efforts with different strategies to reduce the neonatal morbidity and mortality as well as the pregnancy loss were conducted in different populations. Variable premarital carrier screening programs were established in different populations based on carrier frequency and disease frequency in that population either as obligatory or voluntary screening.[39] In Saudi Arabia, the premarital screening program that was instituted in 2002, includes sickle cell anemia and thalassemia as a mandatory screening, as well as in Iran and Tunisia the premarital test is a mandatory program.[39] Preconception carrier screening program was established in the Jews population since 2013. This program includes several fatal diseases, with a carrier frequency of at least 1:60 and/or a disease frequency of 1:15 000 live births.[40] The program resulted in a significant reduction of these diseases as shown in the Singer study 2020.[41] Furthermore, Preconception Carrier Screening Programs were also started in the Netherlands since 2016 for the couples wishing to start a family, to know their genetic carrier status for 50 severe genetic diseases.[40]

Some ethical issues that were considered during the counseling of these couples included stigmatization and discrimination, knowing that the individual is a carrier for the autosomal recessive genetic condition and at risk of having affected offspring might cause a negative view to and about people with those traits. As well as, potential findings of a dominant trait in the tested individual that harbored a reduced penetrance or variable expressivity, however, it is not yet expressed clinically. Additionally, as expected this strategy influences how the couples perceive the planning of a pregnancy and making a family in a positive light but some undesirable consequences might impact the family like divorce or remarriage of another wife. Another concern is that if this test is routine or are they obligated to do it? Are they guilty if they opt not to?. Some of these ethical dilemmas were also considered in preconception carrier screening in different populations.[42]

We acknowledge several limitations of MABP. First, this approach only works to reveal the carrier status of familial variants. Although our work focused on single gene cases, we note that MABP can also reveal the carrier status of balanced chromosomal rearrangements, which were found in a recent very large study to account for a substantial fraction of recurrent pregnancy loss.[43] Second, MABP may only identify the carrier status for autosomal recessive diseases in one of the couples, although this is less of a problem in consanguineous couples who tend to share the same variants. Third, as with other diagnostic applications of ES, VUS remain a formidable challenge. However, it is hoped that data sharing efforts will contribute to the successful reclassification of these variants and it is hoped that this study will contribute toward this goal.

In conclusion, we show that MABP is a highly effective testing strategy in consanguineous populations where autosomal recessive variants tend to be a more common cause of premature death among offspring of couples seeking preconception counseling. Our work highlights the additional benefit of uncovering additional pathogenic variants that can empower couples to make reproductive choices for diseases beyond the ones they are seeking preconception counseling for.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the article material and references of this article.

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