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ORIGINAL ARTICLE

A new enrichment approach for candidate gene detection in unexplained recurrent pregnancy loss and implantation failure

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Abstract

Recurrent pregnancy loss (RPL) and implantation failure (RIF) are obstacles to livebirth and multifactorial conditions in which nearly half of the cases remain unexplained, and we aimed to identify maternal candidate gene variants and pathways for RPL and RIF by analyzing whole-exome sequencing (WES) data via a new detailed bioinformatics approach. A retrospective cohort study was applied to 35 women with normal chromosomal configuration diagnosed with unexplained RPL and/or RIF. WES and comprehensive bioinformatics analyses were performed. Published gene expression datasets ($n=46$) were investigated for candidate genes. Variant effects on protein structure were analyzed for 12 proteins, and BUB1B was visualized *in silico*. WES and bioinformatics analyses are effective and applicable for studying URPL and RIF to detect mutations, as we suggest new candidates to explain the etiology. Forty-three variants in 39 genes were detected in 29 women, 7 of them contributing to oligogenic inheritance. These genes were related to implantation, placentation, coagulation, metabolism, immune system, embryological development, cell cycle-associated processes, and ovarian functions. WES, genomic variant analyses, expression data, and protein configuration studies offer new and promising ways to investigate the etiology of URPL and RIF. Discovering etiology-identifying genetic factors can help manage couples' needs and develop personalized therapies and new pharmaceutical products in the future. The classical approach with chromosomal analysis and targeted gene panel testing is insufficient in these cases; the exome data provide a promising way to detect and understand the possible clinical effects of the variant and its alteration on protein structure.

Keywords Pregnancy loss · Implantation failure · Variant analyses · Protein structure · Exome sequencing

Introduction

Recurrent pregnancy loss (RPL) and recurrent implantation failure (RIF) are crucial issues, and many researchers are trying to find an answer to explain the etiology in unexplained cases, 0.5–2.3% of all populations. Karyotyping and Y microdeletions are investigated in routine laboratory studies to rule out chromosomal abnormalities in couples with RPL and RIF. Although it is still accepted as a golden standard in molecular genetics studies, Sanger sequencing is considered an old-fashioned technique since long-read sequencing is used in clinical studies as the third generation. However, screening only chromosomes and a few genes causing monogenic disorders is insufficient due to the limitation of classical techniques, and nearly half of the cases remain unexplained. Next-generation sequencing (NGS) made it possible to sequence the whole genome (WGS), whole exome (WES), or targeted hundreds of genes (gene

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panel testing) in one run and given opportunities to study and clarify the genomic changes which are associated with the disease phenotypes.

RPL is defined as two or more miscarriages. It is a common multifactorial health condition with chronic maternal diseases, infections, and endocrinological, chromosomal, anatomical, immunological, and environmental factors (Bender Atik et al. 2018). Primary infertility (PI) is "the failure of pregnancy for at least one year," and secondary infertility (SI) is used when a new pregnancy cannot develop for at least 12 months after one or more live-born, which is not uncommon in patients with RPL (Mascarenhas et al. 2012). To overcome this problem, assisted reproductive techniques (ART), such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or transferring blastocyst attempts, are suggested as empirical therapies which allow to transfer embryos with higher quality and increase the success rate of implantation (Kirshenbaum and Orvieto 2019). RIF has been observed more frequently in cases with PI or SI, and the commonly accepted definition of RIF is ≥ 2 failures at the implantation after ART trials (Coughlan et al. 2014). Biochemical pregnancies are also investigated under implantation failure; ~ 30% of spontaneous and 10% of clinically identified pregnancies are predicted to be aborted (Coulam and Roussev 2003; Larsen et al. 2013). Some of the underlying etiological mechanisms in RIF are similar to those of RPL, such as intrauterine abnormalities, endometriosis, and immunological factors. Endometrial and associated functional defects are the most apparent causative factors in unexplained early RPL and RIF. Four main approaches stand out in the studies of RPL cases via WES: i. analyzing samples of miscarriage and parents in a trio; ii. investigating abortus material and segregation analysis via Sanger sequencing of parents and family members (Filges et al. 2014; Wilbe et al. 2015; Dohrn et al. 2015; Qiao et al. 2016; Bondeson et al. 2017; Cristofoli et al. 2017); iii. studying couples to determine heterozygous gene variants that could be associated with intrauterine fetal loss (Ellard et al. 2015; Stals et al. 2018); and iv. WES analysis of females only.

To our knowledge, only two serial studies have been published using the WES technique in women with RPL and RIF who have no chromosomal abnormalities. Quintero-Ronderos et al. reported the results of 49 women with early RPL, and 27 variants were detected in 22 genes in 20 women (Quintero-Ronderos et al. 2017). These genes functioned in cellular adhesion, coagulation, matrix remodeling, and immunological, hormonal, cellular, and metabolic activities. In another study, including 75 women with RPL ($n = 61$) and primary infertility ($n = 14$), variants were identified in 14 cases in genes associated with female infertility (i.e., *TLE6*, *NLRP5*, *NLRP7*, *FSHR*, *ZP1)*. Primary ciliary dyskinesia (*DNAH11*, *CCNO*) and homozygous variants were found in several genes (*CCDC68*, *CBX3*, *CENPH*, *PABPC1L*, *PIF1*, *PLK1*, and *REXO4*), which were interpreted as candidates for female PI (Maddirevula et al. 2020).

Here, we present the results of the WES analysis in 35 Turkish women diagnosed with unexplained RPL (URPL) and/or RIF, revealing 43 variants, including 7 novel in 39 genes in 29 women. Some family members demanded to participate in segregation studies, which might be associated with the phenotype. Next, a comparison of wild type (WT) versus mutant revealed energetically destabilizing or stabilizing variants that could affect protein function and result in a disease phenotype. Additionally, phenotypically associated Gene Expression Omnibus (GEO) datasets were collected, analyzed, and compared to our results. Furthermore, we investigated the BUB1B variant by analyzing its protein structure in the mitotic checkpoint complex to determine its effect on protein–protein interactions. In our study, WES and bioinformatic analyses were beneficial for researching and detecting new candidate pathways and monogenic/oligogenic effects on etiology.

Materials and methods

Cohort Our study group was formed with 35 women with URPL ($n = 24$), URPL and RIF ($n = 5$), PI and RIF ($n = 6$), who were referred to the Department of Medical Genetics of Istanbul Faculty of Medicine, Istanbul University, between 2011 and 2019 for genetic diagnosis and counseling (Fig. 1). All couples were investigated for medical and family histories (Table 1). Couples without cytogenetic, anatomical, endocrinological, and autoimmune abnormalities were included. This study was approved by the Istanbul Medical Faculty Clinical Research Ethics Committee of Istanbul University (2018–44 and 2018–249). All participants signed the informed consent form.

Sequencing The genomic DNA of the cases was extracted from 400 µl peripheral venous blood samples (MagPurix® Blood DNA Extraction Kit 200; OP02001). Library preparation was performed with the xGen Exome Research Panel IDT Kit, and samples were sequenced on the Illumina NextSeq® platform. An average reading depth of 65 × was obtained for the target exome regions. The data were aligned with the reference genome data using the Burrows–Wheeler Aligner, and the shooting of the variants was done with the GATK Unified Genotyper. The alignment corrections in the in/del regions with realignment and filtering processes were carried out according to the percentage of variant detection (≥ 30%) and reading depth (≥ 10x). Sanger sequencing was performed for confirmation and segregation studies. Sequence data were analyzed byusing Seq Scape v3 and Chromas v2.6.6 analysis programs. DNA extraction,

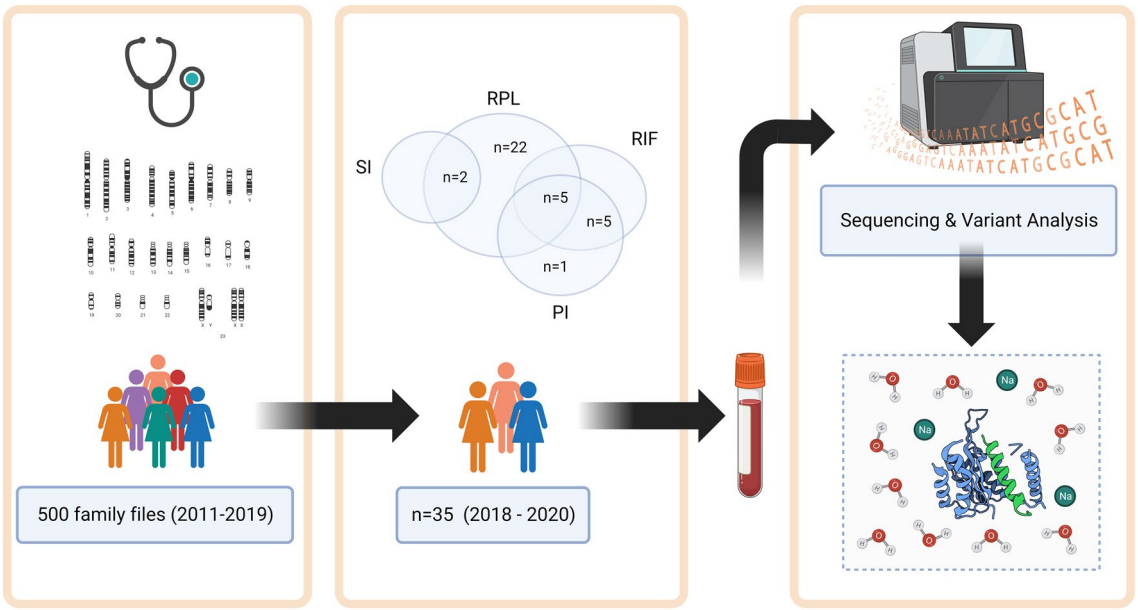

Fig. 1 Steps of study: 1. examination of the family/case files, 2. choosing the cases according to selection criteria, 3. exome sequencing and analyses and segregation studies, 4. bioinformatical analyses and conformational studies (created with BioRender.com)

sequencing, primer designation, and variant analysis steps were carried out at Istanbul University Medical Faculty, Department of Medical Genetics, Turkey.

Bioinformatics analyses The initial step of the bioinformatic analysis was to create a list of active pathway genes in the first trimester of pregnancy through data mining. Genes related to RPL and female infertility were examined in our cohort as the first filtering step. Secondly, variants classified as pathogenic or possible pathogenic were evaluated ($\geq 20\times$ of reading depth, minor allele frequency (MAF) of < 0.01 The variant analysis criteria from the American College of Medical Genetics and Genomics (ACMG) were taken into consideration (Green et al. 2013). The databases and tools used to determine the functional effects are given in Supplementary Tables 1a, b, and the bioinformatics workflows are shown in Fig. 2.

Forty-six published Gene Expression Omnibus (GEO) datasets of expression studies for four mammalian organisms [*Homo sapiens* ($n=26$), *Macaca mulatta* ($n=2$), *Bos taurus* (n = 7), and *Mus musculus* ($n=11$)] associated with pregnancy loss, implantation failure, infertility, and female reproductive system were analyzed using GEO2R and compared with our cohort (Table 1) (Barrett et al. 2013).

In silico protein structural analyses Confirmation analyses of variants with some of the cases could not be performed due to the COVID pandemic. Data from open-access databases and WES data were interpreted together for the patients that could not be invited and their families. We also tried to explain the impact of changes in protein structure and molecular interactions with bioinformatics tools to examine the effect of candidate variants on function. Protein structure affecting variants were filtered, and configuration stabilization analyses via web-based bioinformatics tools applied 15 variants in 12 proteins in 9 cases (HFM1, WNT4, BUB1B, CBS, ACE, EGFR, CYP17A1, ANXA6, GNRHR, MMP11, MCM2, MCM4). The available PDB files were collected, mutation-specific files were created via CHARMM-GUI, and stabilization patterns were calculated. Furthermore, we investigated wild-type and mutant BUB1B protein structures and compared residue–residue distances via the CoCoMaps tool (Crystal Structure of the N-terminal Domain of BubR1; PDB ID: 2WVI). Structural and possible functional differences were identified for TYR162 residue, visualized with the VMD program. They predicted potentially affected contacts with other proteins in the cell cycle complex (Cryo-EM structure of the Anaphase-promoting complex/Cyclosome, complex with the Mitotic checkpoint complex (APC/C-MCC) at 3.8-angstrom resolution; PDB ID: 6TLJ).

Results

During the study design, we examined 500 family files, and after the cases were evaluated according to the selection criteria, 100 candidate families suitable for the study were identified. Sample collection between 2018 and 2020, and the study involved 35 participants. The cases originally

Table 1 Data of our cohort

Case	Age; first pregnancy age/first ART	PL < 24th GW[a]	PL > 24th GW[a]	ART[a]	Live births[a]	Therapeutic termination[a]	Ectopic pregnancies[a]	Consanguinity	Diagnosis
C-1	29; 20	5	None	None	None	None	None	None	RPL
C-2	35; 18	13	None	None	1	None	None	1. ° cousins	RPL
C-3	34; 24	5	None	None	1	None	None	1. ° cousins	RPL
C-4	37; 29	4	None	None	None	None	None	None	RPL
C-5	32; 25	4	None	None	None	None	None	None	RPL
C-6	38; 23	4	None	2	2	None	None	1.5. ° cousins	RPL, SI
C-7	39; 30	6	None	None	1	None	None	None	RPL
C-8	42; 24	4	None	None	1	None	1	None	RPL
C-9	33; 21	6	1	None	1	None	1	None	RPL
C-10	43; 26	8	None	1	2	None	None	None	RPL, SI
C-11	37; 23	5	None	None	1	1[b]	None	1. ° cousins	RPL
C-12	29; 19	10	None	None	None	None	None	1.5. ° cousins	RPL
C-13	21; 17	4	None	None	1	None	None	none	RPL
C-14	23; 19	3	None	None	None	None	1	1.5. ° cousins	RPL
C-15	35; 30	4	None	None	1	None	None	1. ° cousins	RPL
C-16	38; 24	17	none	None	None	None	None	2. ° cousins	RPL
C-17	30; 22	5	None	None	1	None	None	None	RPL
C-18	25; 23	4	None	None	None	None	None	None	RPL
C-19	30; 23	None	None	4	None	None	None	1. ° cousins (P)	PI
C-20	43; 19	10	None	2	None	None	None	1.5. ° cousins	RPL, PI, RIF
C-21	35; 23	8	None	None	None	1[c]	None	None	RPL
C-22	36; 23	5	1	None	1	None	None	None	RPL
C-23	42; 22	3	None	3	None	None	None	1.5. ° cousins	RPL, PI, RIF
C-24	30; 18	3	None	None	None	None	2	None	RPL
C-25	37; 33	4	None	4	None	None	1	None	RPL, PI, RIF
C-26	34; 31	11	None	None	None	None	None	2. ° cousins (P)	RPL
C-27	40; 33	2	None	2	None	None	1	None	RPL, PI, RIF
C-28	31; 27	2	None	7	None	None	None	None	PI, RIF
C-29	42; 25	None	None	4	None	None	None	None	PI, RIF
C-30	41; 28	1	None	5	None	None	None	None	PI, RIF
C-31	42; 34	None	None	4	None	None	None	None	PI, RIF
C-32	60; 30	1	None	5	None	None	None	None	PI, RIF
C-33	45; 25	7	None	None	2	None	None	1. ° cousins	RPL
C-34	39; 30	9	None	7	None	None	None	None	RPL, PI, RIF
C-35	45 ; 24	6	None	1	None	None	None	None	RPL

Colored values mentions the values as in numbers. The biggest numbers are colored as dark

RPL: recurrent pregnancy loss; RIF: recurrent implantation failure; PI: primary infertility; SI: secondary infertility, P: parents of the case, ART: assisted reproductive techniques

[a]Numbers of the content

[b]Absence of distal bones in the extremities, acrania, single umbilical artery, omphalocele, and polydactyly

[c]Lumbar spina bifida, kyphoscoliosis

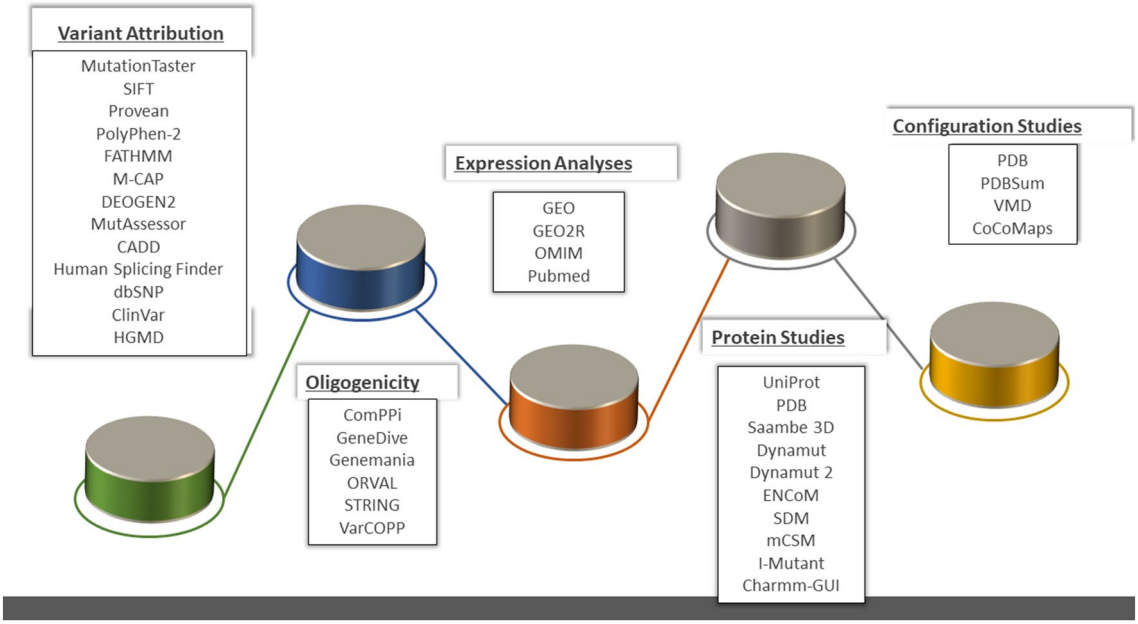

Fig. 2 Bioinformatics workflow and compared databases

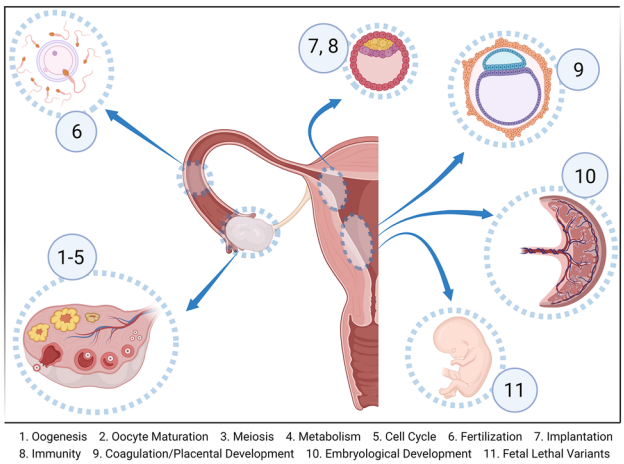

Fig. 3 The step in which the detected gene variants may be related (created with BioRender.com)

came from all regions of Turkey and resided in the Marmara region. All participants in the study had their first pregnancies < 35 years old. According to the case follow-up gestation records, 30 couples achieved spontaneous pregnancy (85.7%), and we diagnosed 11 cases with PI and 2 patients with SI (Table 1). One case (C-19) was diagnosed with primary infertility and empty follicle syndrome after four ART trials.

Mutations might affect the female reproductive system

In this study, 43 variants in 33 genes were detected in 29 women (Fig. 3). These genes were associated with various pathways for placental development, implantation, coagulation, metabolism, immunity, embryological development, cell cycle, DNA repair systems, oogenesis, and oocyte maturation (Table 2), and according to the ACMG classification via VarSome were classified as likely benign ($n = 4$), VUS ($n = 27$), likely pathogenic ($n = 5$), and pathogenic ($n = 7$) (Table 3).

Analyzing expression study datasets showed that 12 of our genes correlated with the studies' findings (*ACE*, *ANXA6*, *CBS*, *EGFR*, *GNRHR*, *H6PD*, *HTRA4*, *IGF2*, *VEGFA*, *WNT4*, *WNT6*, *ZAR1*) (Supplementary File 1). As a result of protein stabilization analyses, the destabilizing frequency of ΔΔG scores was found as > 0.8 in five variants encoding four different peptides (MCM4, EGFR, CYP17A1, BUB1B) (Supplementary Table 2).

Candidate oligogenic mutations

Complex disorders can be attributed to oligogenic inheritance. To understand this phenomenon, we need some key points: protein interactions with DNA or other proteins, tissue-specific expression studies, family segregation analyses, and model organism experiments (Schäffer 2013). In seven cases (Supplementary File 1), the oligogenic effects

Table 2 Candidate genes and pathways

Gene	Biological function	Associated pathway/s	Possible effects on reproductivity^	Detected phenotype (case)
ACE	Blood pressure regulation, electrolyte balance	Placental development	RPL	RPL, PI, RIF (C-20) PI, RIF (C-29)
ALPPL2	Dephosphorylation	Placental development	ND	PI, RIF (C-29)
EGFR	Receptor for cell signaling	Placental development Implantation	abortion	RPL, PI, RIF (C-20) RPL, PI, RIF (C-34)
FLT1	Vascular cell proliferation and differentiation	Placental development	RPL	RPL (C-18)
HSF1	Thermotolerance	Placental development Oogenesis	mice embryos from *Hsf1* $^{-/-}$ females died after the zigotic stage	RPL (C-16)
HTRA4	Ligomeric chaperone protease	Placental development	pre-eclampsia	RPL (C-7)
PROKR1	Regulation of reproduction	Placental development Implantation	RPL	PI, RIF (C-28)
PROKR2	Regulation of reproduction	Placental development Implantation	Hypogonadotropic hypogonadism 3 with/without anosmia (MIM #244,200), RPL	RPL (C-26)
VEGFA	Angiogenesis	Placental development	RPL	PI, RIF (C-31)
ANXA6	Ca2 + -regulated phospholipid membrane binding	Implantation	upregulated in RIF	RPL (C-33)
GNRHR	Receptor for pituitary hormones	Implantation	Hypogonadotropic hypogonadism 7 without anosmia (MIM #146,110)	RPL, PI, RIF (C-34)
MMP10	Degradation of the extracellular matrix	Implantation	RPL	RPL (C-16) RPL, PI, RIF (C-20)
MMP11	Degradation of the extracellular matrix	Implantation	ND	RPL, PI, RIF (C-34)
MMP7	Degradation of the extracellular matrix	Implantation	RPL, RIF	RPL (C-8)
F5	Coagulation	Coagulation	Susceptibility to RPL (MIM #614,389)	RPL (C-12)
CBS	Transsulfuration	Coagulation metabolism	Hyperhomocysteinemic thrombosis (MIM #236,200)	RPL (C-13) RPL, PI, RIF (C-27)
CPT1B	Transporting long-chain fatty acyl-CoAs	Metabolism	Heterozygous mice were more susceptible to spontaneous abortion	RPL (C-17, C-24)
CYP17A1	Synthesize 17-alpha-hydroxylated glucocorticoids and sex steroids	Metabolism	17-Alpha-hydroxylase/17,20-lyase deficiency (MIM #202,110)	PI, RIF (C-29)
H6PD	Oxidize glucose-6-P, glucose, galactose-6-P 2-deoxyglucose-6-P	Metabolism	Cortisone reductase deficiency 1 (MIM #604,931)	RPL (C-22)
CXCL8	Activation and migration of neutrophils	Inflammation immunity	RPL	RPL (C-22)
PIBF1	Antiabortive	Immunity	Low expression in women with premature pregnancy termination	RPL (C-4)
IGSF10	GnRH neuronal migration	Embryological development	Delayed puberty, premature ovarian insufficiency	RPL (C-17)
NOBOX	Folliculogenesis	Embryological development	Premature ovarian failure 5 (MIM #611,548)	PI, RIF (C-31)
WNT4	Female sexual development	Embryological development	Mullerian aplasia and hyperandrogenism (MIM #158,330)	RPL (C-1)
WNT6	Expressed in the reproductive system	Embryological development	RPL	RPL (C-21)

Table 2 (continued)

Gene	Biological function	Associated pathway/s	Possible effects on reproductivity^	Detected phenotype (case)
ZAR1	Oocyte-to-gamete genomic transition	Embryological development	Mice embryos from *Zar1* $^{-/-}$ females arrested at the 1-cell stage	RPL, SI (C-6)
ZSCAN4	Telomere maintenance, genomic stability	Embryological development	Increased karyotype abnormalities, sister chromatid exchange	RPL, PI, RIF (C-25)
BUB1B	Checkpoint protein for prophase I arrest and prometaphase progression in oocytes	Cell cycle	Premature chromatid separation trait (MIM #176,430)	RPL (C-5)
MCM2	DNA replication and cell division	Cell cycle	Embryonic lethality in female mice embryos	RPL (C-35)
MCM4	DNA replication and cell division	Cell cycle	Embryonic lethality in female mice embryos	RPL (C-35)
MTOR	Regulating cell growth and proliferation	Cell cycle	RPL	PI, RIF (C-31)
SPICE1	Centriole duplication, bipolar spindle formation, chromosome congression	Cell cycle	ND	RPL (C-14)
EXO1	DNA mismatch repair	Meiosis DNA repair system	In mice, *Exo1*$^{-/-}$ oocytes failed proceeding metaphase II	RPL, PI, RIF (C-23)
HFM1	Crossover and synapsis	Meiosis DNA repair system	Premature ovarian failure 9 (MIM #615,724)	RPL (C-1)
MCM8	DNA replication and cell division	Meiosis DNA repair system	Premature ovarian failure 10 (MIM #612,885)	RPL (C-1, C-11)
EIF4ENIF1	Developing germ cells in the ovary, transporting translation factors to the nucleus	Oogenesis	Premature ovarian failure	RPL (C-9)
IGF2	Regulation of cell proliferation, growth, migration, differentiation, and survival	Oogenesis	Polycystic ovary syndrome	RPL, PI, RIF (C-23)
PATL2	Expressed in the human germinal vesicle, metaphase I, and polar body I oocytes	Oogenesis Oocyte maturation	Oocyte maturation defect 4 (MIM #617,743)	RPL (C-15)
ZP1	Human zona pellucida	Oocyte maturation, fertilization	Oocyte maturation defect 1 (MIM #615,774)	PI (C-19)

of gametogenesis, implantation, and placentation-associated genes/proteins were predicted as a result of pathway analyses, shared external molecular interactions, biological processes, and phenotypic effects, which are shown in Fig. 4, and gene–gene interactions are mentioned in Table 4.

WES studies as carrier genetic screening for lethal fetal disorders

Carrier genetic screening (CGS) has been proposed to determine potential autosomal or X-linked recessive disorders and, depending on their risk, makes it available to prefer to go through ART with preimplantation genetic testing (PGT), exploit gamete donation, get pregnant spontaneously or disregard bearing a child (Delatycki et al. 2020). It was first applied for Tay–Sachs disease in the 1970s (Kaback et al. 1993); likewise, thalassemia, sickle cell disease, and cystic fibrosis are primarily examined for carrier screening. Generally, gene panel testing customized for ethnicity is used to identify the disease-associated variants (Cannon et al. 2019).

Thirteen heterozygous variants in 11 genes associated with autosomal recessive inherited rare diseases/syndromes (AR) were determined in ten women (VUS: $n=4$, likely pathogenic: $n=3$, pathogenic: $n=5$). These genes were related to ciliopathy ($n=7$) and metabolic disorders ($n=3$) and were active during embryogenesis ($n=1$) (Table 5). Principally, ciliopathy-related genes, *KIF7*, *TTC21B*, *CEP290*, *DYNC2H1*, *PKHD1*, *CELSR1,* and *PKD1L1*, were notable within the study. Pathogenic variants of *KIF7* have been linked to hydrolethalus syndrome-2 (MIM #614,120) and acrocallosal syndrome (MIM #200,990). In addition, the monoallelic form has also been detected in many ciliopathy phenotypes (Putoux et al. 2011; Dafinger et al. 2011). *KIF7* NM_198525.2:c.3409 T > C was in the heterozygous form

Table 3 Mutations that affect the female reproductive system

CaseGene	Nucleotide	Transcript ID	Uniprot	Peptide	Type	MutationTaster (score)	Provean (score)	SIFT (score)	PolyPhen (score)	HumVar (score)	FATHMM	M-CAP (score)	DEOGEN2 (score)	Mutation Assessor	DANN (score)	CADD (score)	Human Splicing Finder	Consensus score of pathogenicity	GnomAD frequency (female)	ACMG classification	Zygosity; dbSNP; ClinVar
C-1 *HFM1*	c.2521C>T	NM_001017975.4	A2PYH4	p.Arg841Trp	Missense	Disease causing (0.9077)	Deleterious (– 4.55)	Damaging (0.002)	Probably damaging (1)	Probably damaging (1)	Tolerated	Possibly Pathogenic (0.049)	Benign (0.26)	2.645 Medium	0.9941	24	NA	9/11 (0.81)	0.000009045	VUS	Heterozygous; rs775049733
MCM8	c.2132G>T	NM_032485.6	Q9UJA3	p.Gly671Val	Missense	Disease causing (0.999)	Deleterious (– 2.88)	Damaging (0.009)	Possibly damaging (0.772)	Benign (0.326)	Tolerated	Likely Benign (0.20)	Benign (0.08)	1.43 Low	0.9951	25.6	NA	6/11 (0.54)	0.0002780	Likely benign	Heterozygous; rs150257637
WNT4	c.896C>T	NM_030761.4	P56705	p.Thr299Met	Missense	Disease causing (1)	Deleterious (– 4.76)	Damaging (0)	Possibly damaging (0.840)	Benign (0.360)	Tolerated	Possibly Pathogenic (0.299)	Deleterious (0.95)	3.845 High	0.999	24.2	NA	9/11 (0.81)	NA	VUS	Heterozygous; rs1264065222
C-4 *PIBF1*	c.1214G>A	NM_006346.4	Q8WXW3	p.Arg405Gln	Missense	Disease causing (0.999)	Deleterious (– 3.43)	Damaging (0)	Probably damaging (1)	Probably damaging (0.996)	Tolerated	Possibly Pathogenic (0.046)	Benign (0.31)	2.56 Medium	0.9996	29.7	NA	9/11 (0.81)	0.01663	VUS	Heterozygous; rs17089782; VCV000217689.3
C-5 *BUB1B*	c.485A>G	NM_001211.5	O60566	p.Tyr162Cys	Missense	Disease causing (0.9927)	Deleterious (– 3.97)	Damaging (0.016)	Probably damaging (1)	Probably damaging (0.992)	Tolerated	Possibly Pathogenic (0.052)	Benign (0.28)	2.035 Medium	0.998	26.6	NA	9/11 (0.81)	NA	VUS	Heterozygous; rs1555381180
C-6 *ZAR1*	c.1257C>G	NM_175619.3	Q86SH2	p.Ser419Arg	Missense	Disease causing (0.9997	Deleterious (– 4.73)	Damaging (0.001)	Probably damaging (1)	Probably damaging (0.998)	No weights	Possibly Pathogenic (0.027)	Deleterious (0.69)	1.935 Low	0.9964	22.8	NA	9/11 (0.81)	0.0003244	VUS	Heterozygous; rs150750718
C-7 *HTRA4*	c.1333G>C	NM_153692.4	P83105	p.Asp445His	Missense	Disease causing (1)	Deleterious (– 5.24)	Damaging (0.001)	Probably damaging (1)	Probably damaging (0.999)	Damaging	Possibly Pathogenic (0.258)	Benign (0.1)	3.08 Medium	0.994	25.4	NA	10/11 (0.9)	0.0009260	VUS	Heterozygous; rs139374339
C-8 *MMP7*	c.55C>T	NM_002423.5	P09237	p.Pro19Ser	Missense	Disease causing (0.9979)	Deleterious (– 4.84)	Damaging (0.011)	Probably damaging (0.999)	Possibly damaging (0.874)	Tolerated	Likely Benign (0.013)	Deleterious (0.56)	2.67 Medium	0.9981	25.3	NA	9/11 (0.81)	0.00002322	VUS	Heterozygous; rs148198672
C-9 *EIF4ENIF1*	c.569G>A	NM_001164501.2	Q9NRA8	p.Glu33Lys	Missense	Disease causing (0.9942)	Deleterious (– 3.55)	Damaging (0.003)	Probably damaging (0.994)	Probably damaging (0.991)	No weights	Likely Benign (0.016)	Deleterious (0.58)	2.64 Medium	0.9992	-	NA	8/10 (0.8)	0.003481	VUS	Heterozygous; rs11544091
C-12*F5*	c.1340C>T	NM_000130.5	P12259	p.Pro447Leu	Missense	Disease causing (0.9999)	Deleterious (– 7.03)	Damaging (0.028)	Probably damaging (0.997)	Possibly damaging (0.857)	Damaging	Possibly Pathogenic (0.399)	Benign (0.43)	2.585 Medium	0.9974	24.7	NA	10/11 (0.9)	0.001090	VUS	Heterozygous; rs140044814
C-13*CBS*	c.599C>T	NM_000071.2	P35520	p.Pro200Leu	Missense	Disease causing (1)	Deleterious (– 9.25)	Damaging (0.001)	Probably damaging (0.993)	Possibly damaging (0.761)	Damaging	Possibly Pathogenic (0.811)	Deleterious (0.98)	2.095 Medium	0.9984	24.4	NA	11/11 (1)	0.000008739	Likely pathogenic	Heterozygous; rs758712880; VCV000642590.1
C-14*SPICE1*	c.2508delA	NM_144718.4	Q8N0Z3	p.Ala837GlnfsTer21	Frameshift	Disease causing (0.99)	NA	NA	NA	NA	Tolerated	NA	NA	NA	NA	24	NA	2/3 (0.66)	NA	Pathogenic	Heterozygous; NA

Table 3 (continued)

CaseGene	Nucleotide	Transcript ID	Uniprot	Peptide	Type	MutationTaster (score)	Provean (score)	SIFT (score)	PolyPhen (score)	HumVar (score)	FATHMM	M-CAP (score)	DEOGEN2 (score)	Mutation Assessor	DANN (score)	CADD (score)	Human Splicing Finder	Consensus score of pathogenicity	GnomAD frequency (female)	ACMG classification	Zygosity; dbSNP; ClinVar
C-15*PATL2*	c.839G > A	NM_001145112.1	C9JE40	p.Arg280Gln	Missense	Disease causing (0.9964)	Deleterious (– 2.38)	Tolerated (0.11)	Probably damaging (1)	Probably damaging (0.979)	Tolerated	Possibly Pathogenic (0.039)	Benign (0.03)	2.605 Medium	0.999529	NA		8/11 (0.72)	0.00003480	VUS	Heterozygous; rs569729547
C-16*HSF1*	c.430G > A	NM_005526.4	Q00613	p.Val144Met	Missense	Disease causing (0.9999)	Deleterious (– 2.49)	Damaging (0.003)	Probably damaging (1)	Probably damaging (0.923)	No weights	Possibly Pathogenic (0.042)	Benign (0.42)	2.425 Medum	0.998926.1	NA		9/11 (0.81)	0.000008663	VUS	Heterozygous; rs1564620538
MMP10	c.497-2G > A	NM_002425.3		p.(?)	Splice site	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.9624	NA	Splicing affected (acceptor site)	2/3 (0.66)	0.01271	VUS	Heterozygous; rs17860955
C-17*CPT1B*	C.1237G > A	NM_004377.4	Q92523	p.Glu413Lys	Missense	Disease causing (1)	Deleterious (– 3.65)	Damaging (0.001)	Probably damaging (1)	Probably damaging (0.997)	Damaging	Possibly Pathogenic (0.185)	Deleterious (0.90)	3.99 High	0.999329.3	NA		11/11 (1)	0.000007810	VUS	Heterozygous; rs745702877
IGSF10	c.352C > T	NM_178822.5	Q6WRI0	p.Arg118Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.997939	NA		3/3 (1)	0.00006751	VUS	Heterozygous; rs142596318
C-18*FLT1*	c.3720 + 1G > A	NM_002019.4		p.(?)	Splice site	Disease causing (1)	NA	NA	NA	NA	0.98163	NA	NA	NA	0.994735	Splicing affected (donor site)		5/5 (1)	NA	Pathogenic	Heterozygous; rs1158779072
C-19*ZP1*	c.628C > T	NM_207341.3	P60852	p.Gln210Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	Likely Benign (0.007)	NA	NA	0.993532	NA		3/4 (0.75)	NA	Pathogenic	Homozygous; rs776515172
C-20*ACE*	c.596C > T	NM_000789.3	P12821	p.Pro199Leu	Missense	Disease causing (1)	Deleterious (– 3.65)	Damaging (0.027)	Probably damaging (0.999)	Possibly damaging (0.834)	Tolerated	Possibly Pathogenic (0.102)	Benign (0.24)	3.54 High	0.998522.7	NA		9/11 (0.81)	0.00004662	VUS	Heterozygous; rs553520266
EGFR	C.500 T > C	NM_005228.5	P00533	p.Ile167Thr	Missense	Disease causing (0.9999)	Deleterious (– 3.75)	Damaging (0.03)	Probably damaging (0.991)	Probably damaging (0.993)	Damaging	Possibly Pathogenic (0.413)	Deleterious (0.92)	3.46 Medium	0.998325.5	NA		11/11 (1)	0.00001731	VUS	Heterozygous; rs765416003
MMP10	c.497-2G > A	NM_002425.3		p.(?)	Splice site	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.9624	NA	Splicing affected (acceptor site)	2/3 (0.66)	0.01271	VUS	Heterozygous; rs17860955
C-21*WNT6*	c.208C > G	NM_006522.4	Q9Y6F9	p.Arg70Gly	Missense	Disease causing (0.6575)	Deleterious (– 3.32)	Tolerated (0.061)	Possibly damaging (0.895)	Possibly damaging (0.650)	Tolerated	Possibly Pathogenic (0.253)	Deleterious (0.87)	2.01 Medium	0.995925	NA		9/11 (0.81)	NA	VUS	Heterozygous; rs142171369
C-22*H6PD*	c.1171_1181del	NM_004285.4	O95479	p.Ser391Alafs*Ter102	Frameshift	Disease causing (1)	NA	NA	NA	NA	Damaging	NA	NA	NA	NA	10–15	NA	2/3 (0.66)	NA	Pathogenic	Heterozygous; NA
CXCL8	c.231 T > A	NM_000584.4	P10145	p.Cys77Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.991936	NA		3/3 (1)	0.000008725	VUS	Heterozygous; rs536774132

Table 3 (continued)

CaseGene	Nucleotide	Transcript Uniprot ID	Peptide	Type	MutationTaster (score)	Provean (score)	SIFT (score)	PolyPhen (score)	HumVar (score)	FATHMM	M-CAP (score)	DEOGEN2 (score)	Mutation Assessor	DANN (score)	CADD (score)	Human Splicing Finder	Consensus score of pathogenicity	GnomAD frequency (female)	ACMG classification	Zygosity; dbSNP; ClinVar
C-23*EXO1*	c.2222_2223 delTA	NM_006027 Q9UQ84	p.Tyr742Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	NA	28	NA	2/2 (1)	NA	Pathogenic	Heterozygous; NA
IGF2	c.97C > T	NM_001127598.3 P01344	p.Gln33Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.9969	36	NA	3/3 (1)	0.0006455	Benign	Heterozygous; rs200441006; VCV000710647.1
C-24*CPT1B*	c.167C > T	NM_004377.4 Q92523	p.Pro56Leu	Missense	Disease causing (0.9999)	Deleterious (– 8.77)	Damaging (0)	Probably damaging (1)	Probably damaging (1)	Damaging	Possibly Pathogenic (0.540)	Deleterious (0.93)	2.96 Medium	0.9988	28.4	NA	11/11 (1)	0.00002637	VUS	Heterozygous; rs374480938
C-25*ZSCAN4*	c.412C > T	NM_152677.2 Q8NAM6	p.Gln138Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.9969	37	NA	3/3 (1)	NA	VUS	Heterozygous; NA
C-26*PROKR2*	C.518 T > G	NM_144773.4 Q8NFJ6	p.Leu173Arg	Missense	Disease causing (0.9999)	Deleterious (– 3.03)	Damaging (0.006)	Probably damaging (0.989)	Probably damaging (0.951)	Tolerated	Possibly Pathogenic (0.067)	Benign (0.45)	2.645 Medium	0.9976	25.8	NA	9/11 (0.81)	0.002085	Likely pathogenic	Heterozygous; rs74315416; VCV000003449.7
C-27*CBS*	c.1105C > T	NM_001 178008.2 P35520	p.Arg369Cys	Missense	Disease causing (1)	Deleterious (– 6.19)	Damaging (0)	Probably damaging (1)	Probably damaging (0.998)	Damaging	Possibly Pathogenic (0.798)	Deleterious (0.99)	2.34 Medium	0.9994	31	NA	11/11 (1)	0.003105	Likely pathogenic	Heterozygous; rs117687681; VCV000212860.11
C-28*PROKR1*	c.886 T > C	NM_138964.4 Q8TCW9	p.Cys296Arg	Missense	Disease causing (1)	Deleterious (– 10.53)	Damaging (0.001)	Probably damaging (1)	Probably damaging (0.999)	Tolerated	Possibly Pathogenic (0.100)	Deleterious (0.74)	4.04 High	0.9972	28.2	NA	10/11 (0.9)	0.00004327	VUS	Heterozygous; rs200892456
C-29*ACE*	c.1739C > A	NM_000789.3 P12821	p.Pro580His	Missense	Disease causing (0.9997)	Deleterious (– 3.89)	Damaging (0.001)	Benign (0.093)	Benign (0.344)	Tolerated	Possibly Pathogenic (0.035)	Benign (0.14)	2.865 Medium	0.9959	22.6	NA	7/11 (0.63)	NA	Likely benign	Heterozygous; NA
ALPPL2 (*ALPG*)	c.68-1G > C	NM_031313.3	p.(?)	Splice site	Disease causing (1)	NA	NA	NA	NA	0.63141	NA	NA	NA	0.5748	23.7	Splicing affected (acceptor site)	3/4 (0.75)	0.00008661	Likely benign	Heterozygous; rs201675300
CYP17A1	c.1487G > A	NM_000102.4 P05093	p.Arg496His	Missense	Disease causing (0.9999)	Deleterious (– 4.09)	Damaging (0)	Probably damaging (1)	Probably damaging (1)	Damaging	Possibly Pathogenic (0.332)	Deleterious (0.89)	3.34 Medium	0.9994	29.3	NA	11/11 (1)	0.000009021	Likely pathogenic	Heterozygous; rs763398879
C-31*MTOR*	c.307C > T	NM_004958.4 P42345	p.Arg103Ter	Nonsense	Disease causing (1)	NA	NA	NA	NA	NA	NA	NA	NA	0.9974	36	NA	3/3 (1)	NA	Pathogenic	Heterozygous; rs768374086
NOBOX	c.1067G > A	NM_001080413.3 O60393	p.Arg356Gln	Missense	Disease causing (0.9397)	Deleterious (– 3.94)	Damaging (0)	Probably damaging (1)	Probably damaging (0.999)	Damaging	Possibly Pathogenic (0.657)	Deleterious (0.73)	2.625 Medium	0.9994	29	NA	11/11 (1)	0.000008810	VUS	Heterozygous; rs749172175
VEGFA	c.100C > T	NM_001025366.3 J3KPA4	p.Pro34Ser	Missense	Disease causing (1)	Neutral	Damaging (0)	NA	NA	Tolerated	Possibly Pathogenic (0.320)	NA	0.805 low	0.9987	NA	NA	4/7 (0.57)	NA	VUS	Heterozygous; rs749282093
C-33*ANXA6*	c.1117C > T	NM_001155.5 P08133	p.Arg373Trp	Missense	Disease causing (0.9999)	Deleterious (– 3.44)	Damaging (0.004)	Probably damaging (1)	Probably damaging (0.997)	Tolerated	Possibly Pathogenic (0.065)	Benign (0.40)	3.38 Medium	0.9991	27.8	NA	9/11 (0.81)	NA	VUS	Heterozygous; rs1249929192

Table 3 (continued)

CaseGene	Nucleotide	Transcript ID Uniprot	Peptide	Type	MutationTaster (score)	Provean (score)	SIFT (score)	PolyPhen (score)	HumVar (score)	FATHMM	M-CAP (score)	DEOGEN2 (score)	Mutation Assessor	DANNCADD (score)	DANNCADD (score)	Human Splicing Finder	Consensus score of pathogenicity	GnomAD frequency score of (female)	ACMG classification	Zygosity; dbSNP; ClinVar
C-34*EGFR*	c.2884C > T	NM_005228.5 P00533	p.Arg962Cys	Missense	Disease causing (1)	Deleterious (– 5.96)	Damaging (0)	Probably damaging (1)	Probably damaging (0.987)	Damaging	Possibly Pathogenic (0.180)	Deleterious (0.92)	Low	0.999332		NA	10/11 (0.9)	0.00002317	Likely pathogenic	Heterozygous; rs17337451
GNRHR	c.317A > G	NM_000406.3 P30968	p.Gln106Arg	Missense	Disease causing (0.9999)	Deleterious (– 3.66)	Damaging (0.001)	Probably damaging (0.997)	Probably damaging (0.992)	Tolerated	Possibly Pathogenic (0.050)	Deleterious (0.71)	Low	0.995823.6		NA	9/11 (0.81)	0.002674	Pathogenic	Heterozygous; rs10 4893836;VC V000016023.12
MMP11	c.232C > T	NM_005940.5 P24347	p.Pro78Ser	Missense	Disease causing (0.9999)	Deleterious (– 6.05)	Damaging (0.001)	Possibly damaging (0.914)	Benign (0.404)	Tolerated	Likely Benign (0.016)	Deleterious (0.57)	Medium	0.998922.2		NA	8/11 (0.72)	0.001008	Likely benign	Heterozygous; rs61752251
C-35*MCM2*	c.1670C > G	NM_004526.4 P49736	p.Thr557Arg	Missense	Disease causing (1)	Deleterious (– 5.61)	Damaging (0)	Probably damaging (1)	Probably damaging (0.998)	Tolerated	Possibly Pathogenic (0.046)	Benign (0.41)	4.705 High	0.995	26.9	NA	9/11 (0.81)	NA	VUS	Heterozygous; NA
MCM4	c.1970C > T	NM_005914.4 P33991	p.Ala657Val	Missense	Disease causing (1)	Neutral	Tolerated (0.665)	Benign (0.003)	Benign (0.014)	Tolerated	Possibly Pathogenic (0.028)	Benign (0.06)	– 0.455 neutral	0.988222.6		NA	4/11 (0.36)	NA	VUS	Heterozygous; NA

Mutation Assessor functional impact of a variant: predicted functional (high, medium), predicted non-functional (low, neutral), *RVIS* Residual Variation Intolerance Score, *CADD* 10 indicates substitutions predicted to be the 10% most deleterious, and a score of greater than or equal to 20 indicates the 1% most deleterious, *NA* not applicable

in C-6. Her partner, who also had phenotypically normal children, was concordant with the AR inheritance and could be a candidate to explain the etiology.

TTC21B, which encodes the IFT139 (THM1) protein that involves in the flagellar transport A complex, has been associated with nephronophthisis-12 (MIM #613,820) and asphyxiating thoracic dysplasia-4 (MIM #613,819) (Davis et al. 2011). *TTC21B* NM_024753.5:c.1546C > T was observed in C-11 and her partner and might be closely related to the etiology, since the case had a history of pregnancy loss with signs of ciliopathies (absence of distal bones in the extremities, acrania, single umbilical artery, omphalocele, polydactyly). We could not study this variant in the terminated fetus due to a lack of DNA.

CELSR1 is an active gene in the neurulation, proliferation, and differentiation of neuronal precursor cells, especially axonal development and ventricular and outer granular layers (Tissir et al. 2002; Copp et al. 2003). We thought that *CELSR1* NM_014246.4:c.2110A > G might be the factor in our couple (C-21), who were from the same village and had a high probability of consanguineous marriage, and a therapeutic terminated fetus with total spina bifida in their history. Unfortunately, this variant could not be studied in the male partner and fetus.

Effect of variation on BUB1B structure and interactions/*in silico* structural analysis of BUB1B

By analyzing the mutation in BUB1B, our strategy was to understand how the cell cycle complex and structural changes could affect the function and alter the pathway.

BUB1B encodes a protein that participates in mitotic checkpoints, controls prophase I arrest, and is associated with premature chromatid separation trait (AD, MIM #176,430) and shortened metaphase duration in cases with recurrent miscarriages (Bajnóczky and Gardó 1993). BUB1B has three TPR motifs (helix-loop-helix), and in *Homo sapiens*, the residues of 57–200 contact with Blinkin (Bolanos-Garcia et al. 2011). Furthermore, Y162 is located close to the binding interface between BUB1B and CDC20 in the cell cycle complex.

Analyzing the structure of the anaphase-promoting complex/cyclosome (6TLJ), BUB1B (chain S) has been observed to interact with CDC20 (chain Q and R), MD2L1 (chain Z), and ANC2 (chain N) proteins (Alfieri et al. 2020). The TYR162 residue (S), in which we have detected a heterozygous variation at that position, is in interaction with 6 (Q) and 14 (R) residues of CDC20 protein at distances between 6 and 10 Å (Fig. 5a, b).

With the TYR162CYS mutation (NM_001211.5:c.485A > G), the interresidual contacts of

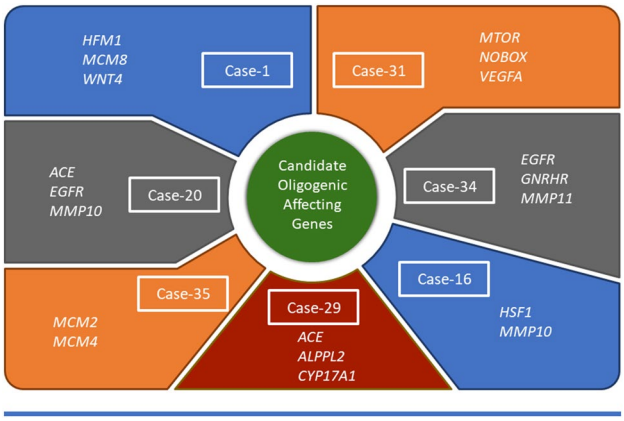

Fig. 4 Oligogenic effect attributed genes and detected cases

BUB1B were examined, and the distances with ASP137 and SER140 increased more than eight (2WVI) (D'Arcy et al. 2010). In WT, ASP137 interacts with ARG286 in CDC20 € with the hydrogen bond at a distance of 2.85 Å and the salt bridge at 2.70 Å. Withal, SER140 connects with SER268 in CDC20 (R) with the hydrogen bond at a distance of 3.27 Å in the complex. ARG165 (S), which is in a close neighborhood of TYR162 (S), interacts with the LYS155 residue (Q) with hydrogen bonds and nonbonded contacts; additionally, ALA164 (S) interacts with TYR152 (Q) and GLU161 (S) with LYS155 (Q). ARG165 (R) and GLU 166 (S) are located in the complex close to PRO225 (R), and ASN 167 (S) has contacts without bonds with GLN224 (R). These results suggested that this mutation leads to increased distances from residues of the interface site and destabilizes interactions between the proteins of the anaphase-promoting complex in the cell cycle.

Discussion

Unexplained pregnancy losses show multifactorial etiology and involve many embryological, developmental, and reproductive pathways. Therefore, the most practical tool for examining candidate genes is exome sequencing with an individualized mixture of bioinformatics and medical genetics approaches. Clinical exome sequencing is limited to already known and mostly possible gene variant analyses. However, there are a few known single gene variants for URPL/RIF. With our new enrichment approach, we consider more than 40 variants in 33 genes. Here, variants with consensus pathogenicity scores > 0.8 according to *in silico* tools were studied (*IGF2*, *H6PD*, *EXO1*, *SPICE1*, *CXCL8*, *ANXA6*, *MMP7*, *FLT1*, *HTRA4*, *PROKR1*, *PROKR2*, *F5*, *CBS*, *ZSCAN4*, *ZAR1*, *WNT6*, *CPT1B*).

A nonsense heterozygous variant of *IGF2* (c.97C > T) was detected in a noncanonical transcript (NM_001127598.2), and in the intra-familial segregation analysis, the father of the case was the carrier (C-23). The *IGF2* gene is linked to Beckwith–Wiedemann syndrome (MIM #130,650), and the maternal allele is imprinted; therefore, the paternal allele remains active. In the female reproductive system, IGF2 protects granulosa cells from apoptosis and ensures a smooth microenvironment for oocytes (Bøtkjær et al. 2019), stimulating the proliferation of luteinized granulosa cells and producing estradiol/progesterone (Perrier d'Hauterive et al. 2004; Dória et al. 2010). IGF1 and IGF2 can behave like hCG in regulating endometrial LIF expression during pregnancy; similarly, in the absence of IGF2, granulosa cells cannot produce sufficient estrogen in response to LH release and cannot maintain corpus luteum continuity (Wang et al. 2006; Baumgarten et al. 2015). We analyzed human cumulus cell expression datasets cultured in vitro; *IGF2* was highly expressed on the 15th day, lower in the 24th hour and 7th day, and the lowest on the 30th day (GSE149033). In our case, since the paternal allele would be the active one, we considered this variant as a strong candidate.

Biallelic pathogenic variants in *H6PD* are associated with cortisone reductase deficiency type 1 (MIM #604,931), which is caused by the inability to produce active cortisol from cortisone with 11β hydroxysteroid dehydrogenase (HSD11B1). ACTH-mediated adrenal hyperandrogenism occurs due to the failure of the cortisol cycle, pseudo-pubertas praecox in men, hirsutism, oligomenorrhea, and infertility in women (Lavery et al. 2008). A novel intergenic deletion (NM_004285.4:c.1171_1181del) resulting in a frameshift and early termination of *H6PD* (p.S391Afs*102) was observed in our case (C-22), and we foresaw that this might cause the loss-of-function effect. We investigated the endometrial tissue expression of women with RIF; in line with our results, *H6PD* expression was found to be lower in three RIF cases than in three controls (GSE103465).

Implantation is fundamental for early pregnancy, and by altering this biological stage, mutations in *ANXA6* (NM_001155.5:c.1117C > T) and *MMP7* (NM_002423.5:c.55C > T) were found. The MMP7 protein is released from syncytiotrophoblasts and cytotrophoblasts when the blastocyst contacts the endometrial cavity (Wilson and Matrisian 1996; Vettraino et al. 1996; Zhang and Nothnick 2005). Annexins (ANXA-), which are phospholipid-binding proteins regulated by Ca^{+2} ions, are involved in membrane movements, proliferation, and apoptosis (Grewal et al. 2017). Decidualized endometrial stromal cells are mobile at the implantation site; together with movement impairment might cause implantation failure, and this mobility was significantly increased in RPL (Garrido-Gómez et al. 2014; Dhaenens et al. 2019). The expression profiles

Table 4 Analyses of gene–gene interactions

Case	Genes	VarCoPP classification score (0–1)	VarCoPP Support Score (0–100)	ORVAL digenic effect predictor	External proteins in gene–gene interaction (Genemania)	External proteins in pro–pro interaction (comPPi)	External proteins in pro–pro interaction (GeneDive)	STRING	Common biological process	Identified phenotypes/processes
C-1	*WNT4-MCM8*	0.95 (disease causing)	100 (disease causing)	True digenic (0.872)	SFRP1, CDC7	HNRNPL	BRCA1 estradiol	NA	Female gamete generation	Ovarian reserve Leiomyoma
	HFM1-MCM8	0.69 (disease causing)	96.80 (disease causing)	NA	LSM6, MCM-ORC2, CDC7	NA	REC8 RAD51	Neighborhood in genome Co-expression Co-mentioned	DNA recombination	Premature ovarian insufficiency
	WNT4-HFM1	0.60 (disease causing)	84.60 (disease causing)	NA	KL, LSM6	NA	GAPDH	NA	NA	Ovarian reserve
C-16	*HSF1-MMP10*	0.74 (disease causing)	98.80 (disease causing)	True digenic (0.671)	NTS	NA	AKT1 P53	NA	NA	Placentation
C-20	*MMP10-ACE*	0.83 (disease causing)	100 (disease causing)	True digenic (0.646)	CAV2	NA	MMP9, MMP12 MET, CD44 VEGFA	NA	Metallopeptidase, serine-type peptidase, endopeptidase activities; zinc ion binding	Angiogenesis regulation Placental development Breast neoplasms
	EGFR-ACE	0.75 (disease causing)	98 (disease causing)	True digenic (0.857)	CAV2, CDH1 SHC1	CSNK2A1, ACTB LMNA, EWSR1 PSITPS8P1, MYH9	CD44 JNK1	NA	Regulation of vasoconstriction, peptidyl-tyrosine phosphorylation, kinase activity and hormone levels; peptide metabolic progress	Placentation Fibrosis
	EGFR-MMP10	0.65 (disease causing)	95.60 (disease causing)	Dual molecular diagnosis (0.435)	ABL2, TGFA EPS8, CAV2	MYH9	MMP2 MMP9	Co-mentioned	Signal transduction MAPK family signaling cascades	Implantation
C-29	*CYP17A1-ACE*	0.69 (disease causing)	98.20 (disease causing)	True digenic (0.834)	SP1, AGTR2	NA	ACTH, MTHFR CY11B2 CYP19A1	Experimental data Co-expression Co-mentioned	Hormone metabolic process	Ovarian and placental functions
	CYP17A1-ALPPL2	0.62 (disease causing)	94.20 (disease causing)	True digenic (0.694)	ALPL	NA	NA	NA	Metabolic pathways	Placental functions

Table 4 (continued)

Case	Genes	VarCoPP classification score (0–1)	VarCoPP Support Score (0–100)	ORVAL digenic effect predictor	External proteins in gene–gene interaction (Genemania)	External proteins in pro–pro interaction (comPPi)	External proteins in pro–pro interaction (GeneDive)	STRING	Common biological process	Identified phenotypes/processes
C-31	*MTOR-NOBOX*	0.87 (disease causing)	100 (disease causing)	NA	TRMT61A	NA	PI3K	NA	Cell signaling pathways	Folliculogenesis
	VEGFA-MTOR	0.60 (disease causing)	86.20 (disease causing)	True digenic (0.623)	AKT1	NA	AKT1	Co-mentioned	Regulation of cell proliferation and migration; cardiac muscle cell and post-embryonic development	Placentation Angiogenesis
C-34	*EGFR-MMP11*	0.84 (disease causing)	100 (disease causing)	True digenic (0.613)	Directly	NA	VEGFA	Co-expression Co-mentioned	NA	Implantation
	GNRHR-MMP11	0.79 (disease causing)	100 (disease causing)	Monogenic + modifier (0.523)	CEBPA	NA	EGFR, PRL	NA	Hormone metabolism	Implantation
	GNRHR-EGFR	0.73 (disease causing)	99.60 (disease causing)	True digenic (0.818)	CEBPA, MMP11 SH2B1	NA	ERBB1	NA	Signal transduction	Implantation Placentation
C-35	*MCM2-MCM4*	0.42 (neutral)	24.20 (neutral)	NA	Directly	NA	FANCD2, PCNA MCM 2–7 complex	Co-expression Co-mentioned Experimental data Association in databases	MCM complex DNA replication G1/S transition of mitotic cell cycle	Cell cycle

Table 5 Autosomal recessive diseases/syndromes or fetal-lethal disorder-associated variants

DANN (score)	CADD	Consensus score of pathogenicity	GnomAD frequency (total)	ACGM classification	dbSNP ClinVar
0.9959	24.9	9/11 (0.81)	NA	VUS	rs745809521 VCV000499405.1
946	15.69	2/3 (0.66)	0.00001545	Pathogenic	rs368387623
0.9977	41	3/3 (1)	0.000003978	Pathogenic	rs281875373 VCV000140512.1
0.9903	21.3	8/10 (0.8)	0.0001078	Likely pathogenic	rs144562769 VCV000291519.2
NA	NA	1/1 (1)	0.00001138	Pathogenic	rs781310385
0.9944	26	9/10 (0.9)	0.000004245	VUS	rs372499560 VCV000939916.1
0.9488	22.3	3/11 (0.27)	0.00005336	Likely pathogenic	rs373643848
0.9909	24.5	6/10 (0.6)	NA	VUS	NA
0.9991	24.9	10/10 (1)	0.00003189	Likely pathogenic	rs763457259
NA	NA	1/2 (0.5)	NA	Pathogenic	NA
0.9987	39	7/11 (0.63)	0.00003602	VUS	rs777356082
NA	NA	1/2 (0.5)	0.00002236	Pathogenic	rs386834158 VCV000056739.4

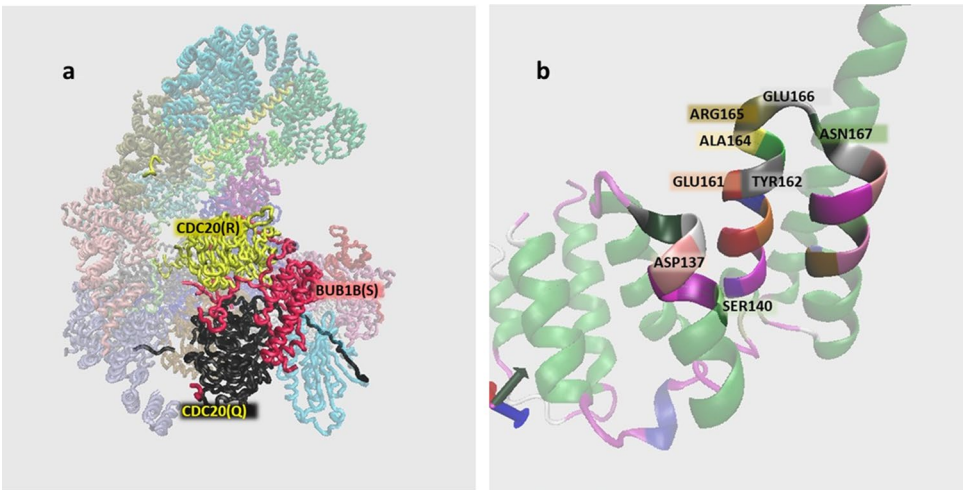

Fig. 5 **a** In the anaphase-promoting complex, TYR162, the residue of BUB1B is close to the interaction site between CDC20's two chains (6TLJ). BUB1B is represented in red (chain S). CDC20 is visualized as black (chain Q) and yellow (chain R). **b** TYR162 has contact with residues that are in interaction with CDC20. TYR162 and these residues are shown in different colors on the peptide structure of BUB1B (2WVI)

of *ANXA6* have shown that applying various amounts of progesterone in cases with luteal deficiency, 10 and 40 mg progesterone treatment were strongly connected with higher expression (GSE56980). Second, endometrial receptivity and *ANXA6* expression were evident in the early stages of the luteal phase, such as the 2nd or 3rd day rather than the 4th (GSE13027).

The formation of vessels, the development of the placenta, and the provision of fetal circulation are other necessary steps in pregnancy. We observed variants in *FLT1* (NM_002019.4:c.3720 + 1G > A), *HTRA4* (NM_153692.4:c.1333G > C), and *PROKR1* (NM_138964.4:c.886 T > C) genes which are related to this pathway. Increased *HTRA4* expression has been found in pregnancies with pre-eclampsia (Inagaki et al. 2012). In an analysis of the transcriptomic dataset of natural and IVF pregnancy placentas, the expressions of *HTRA4* were distinguishably higher in spontaneous ones. This finding strengthened the idea that *HTRA4* was essential for placental development in early pregnancies (GSE122214). The vascular endothelial growth factor protein family (VEGF-) has basic functions in placental angiogenesis (Demir et al. 2004). VEGFA and FLT1 are also involved in oocyte maturation, implantation, fetal development, and placentation; herewith, pregnancy loss, intrauterine fetal death, growth retardation, and pre-eclampsia can be diagnosed in function failure (Vuorela et al. 2000; Zygmunt et al. 2003; Semczuk et al. 2013). *VEGFA* was also identified as expressed higher

in IVF blastocyst cells than in degenerative IVF embryos, which indicated its necessity in early pregnancy and embryogenesis (GSE24936) (Huang et al. 2010). The VEGF protein family interacts with PROKR1 and PROKR2, too; blood circulation is provided by the effect of PROK1, and *PROK1*, *PROKR1,* and *PROKR2* mutations are reported to be connected with pregnancy loss (Hoffmann et al. 2009; Salker et al. 2010; Su et al. 2014; Traboulsi et al. 2015).

Monoallelic pathogenic variants in *PROKR2* lead to hypogonadotropic hypogonadism (MIM #244,200) and may show digenic inheritance with *KAL1* or incomplete penetrance (Dodé et al. 2006). In addition, in a study conducted with 98 women with RPL and 142 female controls, it was shown that *PROKR2* p.V331M (NM_144773.4:c.991G > A) polymorphism was associated with an increased risk of pregnancy loss (Su et al. 2014). Although *PROKR2* NM_144773.4:c.518 T > G is classified as likely pathogenic, our case did not have symptoms of hypogonadotropic hypogonadism, and we considered this variant a candidate because it has examples of various phenotypes and hypothalamic amenorrhea (Caronia et al. 2011; Reynaud et al. 2012). Despite possible effects on reproductive function and being active in the embryo's central nervous system, this variant has been transferred through ancient times with the founder effect (Avbelj Stefanija et al. 2012). The conservative transmission of the variant depends on variable expressivity and presents a classical hypogonadotropic hypogonadism phenotype in the digenic or oligogenic inheritance pattern.

We distinguished variants in *F5* (susceptibility to RPL, AD, MIM #614,389) and *CBS* (hyperhomocysteinemic thrombosis, MIM #236,200). The FVL substitution of the *F5* gene (p.R534Q), a well-known variation for hereditary thrombophilia, is frequently examined in RPL; on the other hand, the population frequency of this variant is ~ 4% in the monoallelic form (Lucotte and Mercier 2001). In this study, variants with < 0.01 MAF score were evaluated, and we identified variants in *F5* (NM_000130.5:c.1340C > T) and *CBS* (NM_000071.2:c.599C > T and NM_000071.2:c.1105C > T). Cystathionine beta-synthase combines homocysteine and serine amino acids to form cystathionine. High homocysteine levels may lead to pregnancy loss, pre-eclampsia, intrauterine growth retardation, neural tube defects, and infertility. Hyperhomocysteinemia impairs decidualization at the implantation site and leads to subfertility by increasing endoplasmic reticulum stress in uterine cells (Guzmán et al. 2006; Nuño-Ayala et al. 2012). In mice, heterozygous pathogenic variants of *Cbs* cause a reduction in mRNA and enzyme activity and increase plasma homocysteine levels by approximately 50% (Watanabe et al. 1995). Lastly, the expression dataset analysis showed that *CBS* expression was more detectable in cumulus cells at metaphase II than metaphase I in PCOS patients (GSE40400).

Moreover, we found variants in genes that are active in embryonic development: *WNT6* (NM_006522.4:c.208C > G), *ZAR1* (NM_175619.3:c.1257C > G), and *ZSCAN4* (NM_152677.2:c.412C > T). After fertilization, stored maternal RNA and proteins in the oocyte are used until the embryo's genome gets active, and this process is called "zygotic genome activation (ZGA)" (DePamphilis et al. 2002). One of the best-known genes in ZGA is *Zscan4*; along with its absence, blastocyst growth was intervented, and implantation was not successful in mice (Falco et al. 2007; Assou et al. 2012). In the oocyte, ~ 10% of maternal gene products remain active until the blastocyst stage, and the maternal genome is more effective than the paternal genome (Sutovsky and Schatten 2000; Bell et al. 2008; Li et al. 2010; Jiao and Woodruff 2013). The importance of maternal influence genes stands out in the success of implantation in ART trials (Patrizio et al. 2007). *ZAR1* is one of the oocyte-specific maternal genes and effectively develops oocytes and embryogenesis. When *Zar1* was knocked out, there was no difference in histological findings between heterozygous and homozygous embryos, but homozygous females were infertile (Wu et al. 2003). Furthermore, we analyzed transcriptomic datasets of the bovine ovary, *ZAR1* was found highly expressed in immature oocytes rather than theca, granulosa, and cumulus cells (GSE149151), and *Zar1* expression was higher both in unfertilized and fertilized oocytes than in embryos in mice (GSE39897). *WNT6* is expressed in the decidualization of the endometrium and the eight-cell stage; therefore, in a study conducted on 100 women with RPL, its variants were related to etiology (Wang et al. 2013; Zhang et al. 2015; Vlismas et al. 2016). We found signatures of relatedness between *Egfr*, *Wnt4,* and *Wnt6* in the expression profiles of the trophoblast cells (GSE95666). *CPT1B*, in which two different variants were revealed in two cases (NM_004377.4:c.1237G > A and NM_004377.4:c.167C > T), is active in the mitochondrial fatty acid β oxidation pathway, and pathogenic variants may have variable effects in a range of asymptomatic to sudden death. When *Cpt1b* was knocked out, the embryos could not tolerate the absence of this energy pathway and died in the preimplantation stage (Ji et al. 2008).

Also, we detected the *MMP10* c.497-2G > A (rs17860955) variant in two different cases and determined it as possible polygenic, affecting predisposing risk increasing due to this variant's gnomAD population frequency of 0.01271 in females. To understand this splice site variant's possible impact on the structure, further studies are needed, and to support this result of being predisposing, cohort studies with higher participant numbers can help.

To the best of our knowledge, this study is the first series in Turkey and one of the pioneers in the world, investigating maternal genetic factors in URPL and URIF via WES.

Due to our findings, WES was found quite successful in determining new candidate genes except for the associated gene studies or gene panel testing. The limitations of the technique were the inability to examine long sequences and repeats and not detecting submicroscopic deletions and duplications. Besides, the data obtained from URPL and URIF cases were compared and found to share effective pathways.

In the literature, there are few studies to investigate the relationship between biochemical markers of reduced ovarian reserve and RPL. It has been declared that there is a link between RPL and decreased ovarian reserve regardless of maternal age; likewise, the effect of biological ovarian age on oocyte quality and ovarian reserve is more remarkable than chronological age (Atasever et al. 2016). In a retrospective study with a similar cohort, AMH and estradiol levels were examined. The decrease in ovarian reserve was strongly related to RPL, particularly in the unexplained group (Pils et al. 2016). As reported by Maddirevula et al., RPL and infertility can arise from the same genetic etiology (Maddirevula et al. 2020). Consequently, we concluded that genes related to POI or defects in oocyte maturation could cause RPL, and disparate reproductive problems can be rooted in similar pathways.

The effective use of bioinformatics tools is valuable in interpreting cases, predicting variants' single or multiple effects, determining new candidate genes, and guiding future functional studies. Investigation of physiopathology obtains a deeper look into the problem. In most complex cases, solutions remain in the angle change of a specific amino acid within a protein domain.

Applying the WES for CGS makes further detailed data approachable for genetic counseling. In a study it is reported that AR genetic disorder risk was detected in 37 of 776 IVF couples, 2.3% of the cohort was the carrier of ≥ 1 pathogenic variant, and provided to investigate secondary health conditions (Capalbo et al. 2019). In this study, we discovered pathogenic variants not only responsible for RPL, infertility, or RIF, but also capable of causing rare and fatal diseases/syndromes in utero. We detected ten women as heterozygous carriers of recessive disorders that could be lethal or disrupt intrauterine development, and three of them are discussed below.

Identifying the genetic etiology may help apply more effective treatment options to patients, such as ART, PGT, and counseling. WES is advantageous for communities with a high rate of consanguinity, such as in Turkey, rather than panel tests. In addition, it may be beneficial to examine the maternal genome initially if fetal materials are unavailable and family members do not want to participate in research.

This paper presents important information regarding unexplained recurrent pregnancy loss and implantation defects. We believe that further studies will significantly examine the candidate genes we identified. The study showed that this approach, which includes detailed and enriched bioinformatics analyses, is helpful and promising for understanding the effect of the variants and alterations on the protein structure, while WES gives us a lot of data to handle.

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Author contributions EGB: literature search, genomic study design, providing clinical data and blood samples, laboratory experiments, bioinformatics analyses, drafting the manuscript, preparing tables and figures, critique, and critical discussion of the manuscript. CVŞ: bioinformatics study design, bioinformatics analyses, protein structure studies, drafting the manuscript, preparing tables and figures, critique, and critical discussion of the manuscript. TK: providing clinical data and blood samples. ZOU: supervising laboratory experiments and variant analyses, critique, and critical manuscript discussion. G.BA: supervising protein structure studies and analyses, critique, and critical discussion of the manuscript, tables, and figures. SB: genomics study design, supervising the study, critique, and critical manuscript discussion.

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Data availability The data underlying this article will be shared on reasonable request to the corresponding author. The analyzed GEO datasets underlying this article and its online supplementary material are available in the paper.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval This study was approved by the Istanbul Medical Faculty Clinical Research Ethics Committee of Istanbul University (2018-44 and 2018-249). All participants signed the informed consent form.

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