European Journal of Obstetrics and Gynecology 297 (2024) 221–226

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.journals.elsevier.com/european-journal-of-obstetrics-and-gynecology-and-reproductive-biology

Full length article

Exome sequencing in genuine empty follicle syndrome: Novel candidate genes

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ARTICLE INFO

Keywords:
Genuine empty follicle syndrome (gEFS)
Whole Exome sequencing (WES)
Trigger
Ovarian stimulation

ABSTRACT

Objective(s): Empty follicle syndrome (EFS) is a condition in which no oocytes are retrieved in an IVF cycle despite apparently normal follicular development and meticulous follicular aspiration following ovulation induction. The EFS is called genuine (gEFS) when the trigger administration is correct. The existence of gEFS is a subject of controversy, and it is quite rare with an undetermined etiology. Genetic defects in specific genes have been demonstrated to be responsible for this condition in some patients. Our objective was to identify novel genetic variants associated with gEFS.

Study Design: We conducted a prospective observational study including 1,689 egg donors from July 2017 to February 2023. WES were performed in patients suffering gEFS.

Results: Only 7 patients (0.41 %) exhibited gEFS after two ovarian stimulation cycles and we subsequently performed whole exome sequencing (WES) on these patients. Following stringent filtering, we identified 6 variants in 5 affected patients as pathogenic in new candidate genes which have not been previously associated with gEFS before, but which are involved in important biological processes related to folliculogenesis. These genetic variants included c.603_618del in *HMMR*, c.1025_1028del in *LMNB1*, c.1091-1G > A in *TDG*, c.607C > T in *HABP2*, c.100 + 2 T > C in *HAPLN1* and c.3592_3593del in *JAG2*.

Conclusion: As a conclusion, we identified new candidate genes related to gEFS that expand the mutational spectrum of genes related to gEFS.This study show that WES might be an efficient tool to identify the genetic etiology of gEFS and provide further understanding of the pathogenic mechanism of gEFS.

Introduction

Empty follicle syndrome (EFS) is a female fertility disorder characterized by the complete failure to retrieve oocytes despite apparently normal ovarian follicular development and successful ovarian puncture [1]. Its prevalence ranges from 0.045 % to 7 % [2]. False EFS (fEFS) occurs due to inadequate levels of circulating HCG or LH, often indicating errors in pharmaceutical preparation, administration, physician procedures during oocyte retrieval, or patient-related factors [3]. In contrast, genuine EFS (gEFS) occurs despite a correctly administered ovulation trigger. The physiopathology of gEFS remains poorly understood, with potential causes including dysfunctional folliculogenesis, ovarian aging, or genetic defects. Notably, some patients experience recurrent gEFS despite modifications to ovarian stimulation protocols and trigger, oocyte retrieval timing and rescue strategies, suggesting a genetic basis [4].

Currently, only a limited number of disease-causing genes have been associated with gEFS, including the luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) [5] and the zona pellucida glycoproteins (*ZP1*, *ZP2* and *ZP3*) [6–8]. In cases with *LHCGR* mutations, neither

Abbreviations: EFS, Empty follicle syndrome; gEFS, genuine empty follicle syndrome; WES, whole exome sequencing; fEFS, false genuine empty follicle syndrome; NGS, Next-generation sequencing; ACMG, American College of Medical Genetics and Genomics; HA, Hyaluronic acid; ECM, extracellular matrix components; POI, Premature ovarian insufficiency; SNVs, single nucleotide variations; VCF, Variant Call Format files; MAF, minor allele frequency.

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https://doi.org/10.1016/j.ejogrb.2024.04.029
Received 1 November 2023; Received in revised form 18 February 2024; Accepted 21 April 2024
Available online 23 April 2024

oocytes nor cumulus-oocyte complex (COC) are retrieved. Conversely, ZP mutations result in the retrieval of a few COCs, but the oocytes within them are often degenerated or collapsed. It is reasonable to speculate that other genetic defects within the folliculogenesis or ovulation pathways may contribute to gEFS.

Next-generation sequencing (NGS) is contributing to the rapid evolution of the infertility genetics field [9]. When evaluating individuals with complex phenotypes suggesting a genetic origin, the American College of Medical Genetics and Genomics (ACMG) acknowledges the potential utility of whole exome sequencing (WES) [10]. WES can provide valuable insights into understanding the etiology of the disorder, recurrence risks, and prognosis. Additionally, it can aid in establishing novel disease-gene relationship. Moreover, through an adequate genetic counselling family screening may detect relatives affected by the disorder. This approach has rapidly evolved in the diagnosis of male factor genetic conditions [11] and premature ovarian failure [12,13].

The primary objective of our study was to employ WES to identify genetic variants within novel candidate genes that could elucidate the genetic underpinnings of gEFS, thereby expanding our knowledge beyond the currently known causative genes.

Materials and methods

Study design and population

We conducted a prospective observational cohort study that included a total of 1,689 egg donors between July 2017 and February 2023. This study focused on egg donors as they represent an ideal model for evaluating ovarian stimulation due to their young age, normal ovarian function, and rigorous selection criteria. All donors were Hispanic Caucasian and met legal requirements in Spain. Both ASRM and ESHRE guidelines for oocyte donors are followed. In addition, a complete gynecological examination is carried out, including follicular basal count, Pap test, and screening for infectious diseases such as HIV, hepatitis B and C, gonoccocia, and syphilis. In addition, karyotyping and expanded carrier screening tests were performed. We also conducted clinical WES on seven patients suffering from gEFS.

All work was conducted with formal approval of the Institutional Review Board (IBB42-2023). Informed consent was obtained from all participants prior to the study.

Ovarian stimulation in oocyte donors

Following the Spanish Fertility Act requirements, all donors received controlled ovarian stimulation with tailored doses of urinary FSH or recombinant FSH. Stimulation began on day 2 of menstrual cycles, with doses adjusted based on the donor's age, body mass index (BMI), and antral follicle count (AFC). To prevent a premature LH peak, the GnRH antagonist cetrorelix (Cetrotide, Merck-Serono, Paris) or natural micronized progesterone (Utrogestan; SEID, Spain) was introduced as needed. Triggering was performed with subcutaneous triptorelin (Decapeptyl; Ipsen, Spain) when at least three follicles reached a diameter of 18 mm or more. Ovarian response was monitored by transvaginal ultrasound and plasma estradiol concentrations. Oocytes were aspirated 36 h after analogue administration by transvaginal, ultrasound-guided needle aspiration under sedation, and LH levels were monitored to confirm the action of the trigger, excluding fEFS when the oocyte retrieval was unsuccessful.

Whole-exome sequencing

Genomic DNA was extracted from peripheral blood from all donors suffering gEFS using the MagMAX DNA Multi-Sample Ultra 2.0 kit (Thermo Fisher Scientific, Colchester, UK) on a KingFisher™ Duo Prime system (Thermo Fisher Scientific, Colchester, UK). WES of the genomic DNA was performed using Trusight One Expanded Sequencing Panel (Illumina®) which covers about 16.5 Mb genomic content (~6700 genes) on the NextSeq 550 (Illumina®). The sequenced data were aligned to the human genome 19 (hg19) using BWA tool and GATK algorithm was used for single nucleotide variations (SNVs)/InDel identification. Variant Call Format files (VCF) were recorded using Variant Interpreter software.

The following criteria were used for filtering and annotating of genetic candidate variants: [1] minor allele frequency (MAF) < 0.05 in the gnomAD and 1000 genomes project for the European population, [2] variants in genes previously associated with gEFS, genes involved in oocyte maturation, folliculogenesis, meiosis and cell division processes, [3] exonic/splicing bound-aries ($\pm$50 bp) variants in genes, [4] variants having potentially strong/moderate functional effects on the protein (nonsense, frameshift, inframe deletion, splice region and missense variants). Within the variants with a potentially moderate effect, the missense variants were evaluated using three in silico prediction algorithms (SIFT, PolyPhen-2 and MutationTaster). Other moderate variants, as inframe deletions or variants in splice regions were considered separately. Moreover, the variants were assessed according to the guidelines from the American College of Medical Genetics and Genomics (ACMG) [14]. The candidate genetic variants identified by WES, were confirmed by Sanger sequencing.

Results

Clinical characteristics and ovarian stimulation of the affected individuals

Within the cohort of 1,689 egg donors, there was a failure to recover oocytes from 7 subjects in two consecutive cycles, meeting the criteria for gEFS. Consequently, the incidence of gEFS in our study was 0.41 %. Table 1 presents the clinical characteristic of these patients. Overall, the mean age of the study participants was 22.14 years, their mean AFC was 15, and their mean BMI was 22.98 kg/m2. These data align with the profile of a healthy, young woman with normal ovarian reserve, which is typical for egg donors.

Table 2 provides an overview of the ovarian stimulation parameters for the egg donors affected by gEFS. The ovarian stimulation cycles in the affected gEFS individuals started with a mean starting dose of 262 IU and the total doses of gonadotropin used was 2,726 IU. The number of follicles in the last ultrasound corresponds to a normal ovarian response (12.43 ± 4.65) with a number of leading follicles that apparently suggests normal development of ovarian response. Approximately an equal percentage of patients used an antagonist (43 %) vs progestin-primed (57 %) protocol for ovarian stimulation. However, a lower proportion of affected gEFS patients used urinary rather than recombinant FSH (35.71 % vs 64.29 %) for ovarian stimulation.

Variant detection

The WES results from seven donors revealed the presence of 24 pathogenic or likely pathogenic variants in genes potentially associated with the phenotype mechanism. Variants were manually registered, and 18 genetic variants were discarded based on likely benign annotations from computational prediction tools. Among the remaining 6 candidate variants, 3 were frameshift, 2 were splicing and 1 was a non-sense mutation. Within the variants, all of them were predicted to have deleterious effects by at least two of the three in silico algorithms used and according to the ACMG classification, were classified as pathogenic. Detailed characteristics of the genetic variants identified are shown in Table 3. All variants were heterozygous. The first variant identified was a frameshift mutation p.Arg201SerfsTer5 detected in the *HMMR* gene, which encodes the Hyaluronic acid (HA)-mediated motility receptor. Additionally, 3-pb deletion creating a frameshift mutation p. Lys342ArgfsTer7 was detected in *LMNB1* gene. The last frameshift mutation p. Ser1198ThrfsTer33 was identified in the *JAG2* gene. Moreover, two splicing alteration mutations were identified in *TDG*

Table 1
Egg donors suffering gEFS clinical characteristics.

	Patients (n = 7) Mean ± SD	Oocyte donor 1	Oocyte donor 2	Oocyte donor 3	Oocyte donor 4	Oocyte donor 5	Oocyte donor 6	Oocyte donor 7
Age (years)	22.71 ± 4.42	32	23	23	19	22	21	19
Height (m)	1.65 ± 0.08	1.66	1.55	1.72	1.77	1.59	1.63	1.61
Weight (Kg)	62.39 ± 10.36	73	54.3	80	57	60	60.9	51.5
BMI (Kg/m^2)	22.98 ± 3.22	26.5	22.6	27.0	18.2	23.7	22.9	19.9
Total AFC	15 ± 2.90	12	12	14	17	20	16	14
Right Ovary AFC	8 ± 2	6	7	7	7	12	9	8
Left Ovary AFC	6.86 ± 1.67	6	5	6	10	8	7	6
Smokers (%)	28.57	Yes	No	No	No	Yes	No	No

Table 2
Ovarian stimulation parameters of gEFS oocyte donors.

	Cycles mean ± SD	Oocyte donor 1 cycles	Oocyte donor 2 cycles	Oocyte donor 3 cycles	Oocyte donor 4 cycles	Oocyte donor 5 cycles	Oocyte donor 6 cycles	Oocyte donor 7 cycles
	(n = 14)	mean						
Duration of stimulation (days)	10.54 ± 1.41	9	12	10.5	10.5	11	11	11
Starting dose of gonadotrophins (IU)	262.5 ± 40.13	300	225	262.5	262.5	225	225	275
Total dose of gonadotrophins (IU)	2,746.43 ± 442.55	2400	2475	2900	2900	2475	2350	2900
No. of follicles in the last ultrasound	12.50 ± 4.60	10.5	19	10.5	10	19	22	11
No. of leading follicles								
Left ovary (≥17 mm)	3.21 ± 1.76	2.5	5	2.5	2.5	5	7	3
Right ovary (≥17 mm)	2.71 ± 2.16	3	5	1	1	4	6	4
Type of protocol	(%)							
Antagonist	43	100	100	50	50	0	0	0
Progestin-primed	57	0	0	50	50	100	100	100
Type of gonadotropin used	(%)							
Urinary FSH (%)	35.71	100	100	50	0	0	0	0
Recombinant FSH (%)	64.29	0	0	50	100	100	100	100

(c.1091-1G > A) and *HAPLN1* (c.100 + 2 T > C) genes. Finally, a nonsense mutation that creates a stop codon; the p.Arg203Ter in *HABP2* gene. *HABP2* gene encodes the Hyaluronan-binding protein 2. All the genes in which variants were identified are expressed in ovary and testis among other tissue. All the variants were located in conserved regions according to the conservative score based on multiple alignments of 99 vertebrate genome sequences. The six genetic variants were found in five donor. Therefore, the average detection rate of genetic variants potentially explaining gEFS in these patients was 57.2 % (4/7).

Table 4 provides general information on the genes harboring variants identified in our study, including gene descriptions and biological processes.

Discussion

gEFS, causing distress in IVF due to unretrieved oocytes despite proper procedures, is a complex reproductive challenge. Probable causes include folliculogenesis errors and oocyte apoptosis. We examined egg donors' genomic DNA using WES to explore genetic factors underlying gEFS. This method allowed comprehensive analysis of genes tied to oocyte maturation, folliculogenesis, meiosis, and cell division. Our aim was to broaden this gene spectrum. In our study, we unveiled six potentially harmful genetic variants in five egg donors' exomes, connected to diverse oocyte development processes, shedding light on gEFS origins.

In the present study the gEFS incidence was 0.41 %, suggesting that gEFS is extremely rare. Previous studies reported a relatively low incidence of EFS, ranging from 0.045 % to 7 %. The largest study, based on 15,729 IVF cycles, reported a gEFS prevalence of 0.045 %, considering only cases with confirmed adequate follicular development [15]. In contrast, a previous study in an egg donor population showed a higher prevalence of 3.5 % in 2034 cycles [16]; however, it did not exclude cases of fEFS from the analysis. This contrasts with the 0.16 % incidence reported by Blazquez et al. (2014) in egg donors [17]. These discrepancies may attributed to differences in exclusion criteria for categorizing fEFS vs. gEFS, the threshold of HCG concentration used, and the characteristics of the patient population. In our study, we rigorously confirmed the action of the ovulation trigger in oocyte donors with gEFS, including only those with repeated occurrences, thereby enhancing the reliability of the possible genetic basis.

Ovulation is a complex process that is not fully understood and the pathogenesis of gEFS remain largely elusive. To date, few genes have been reported to be responsible for gEFS: luteinizing hormone/chorionic gonadotropin receptor (LHCGR) [18], zona pellucida glycoprotein 1 (ZP1), 2 (ZP2) and 3 (ZP3) [8]. With advancements in NGS, we anticipate the discovery of more genetic variants associated with gEFS. Using WES we moved forward, to expand the spectrum of genes related to gEFS.

During folliculogenesis, immature oocytes arrested in prophase I progressively acquire meiotic and developmental competence while granulosa cells proliferate and differentiate [19]. Folliculogenesis involves intricate oocyte-granulosa cell interaction stabilised by extracellular matrix components (ECM). The primary component of the ECM in the follicles is hyaluronan (or hyaluronic acid, HA). Both oocytes and

Table 3
Pathogenic selected variants prioritised from the exome sequencing data in the gEFS oocyte donor.

Oocyte donor	Gene	Localization	Transcript	dsSNP	Nucleotide change	Aminoacid Change	Effect	GnomAD MAF	Conservation Score	Zygosity	ACMG score
1	*HMMR*	chr5:162898422	NM_001142556.1	rs765297224	c.603_618del	p.(Arg201SerfsTer5)	*Frameshift Indel*	0.000106	4.67	Het	PM2/ PVS1
2	*LMNB1*	chr5:126154698	NM_005573.3	rs763314150	c.1025_1028del	p.(Lys342ArgfsTer7)	*Frameshift Indel*	0.0000176	10.00	Het	PM2/ PVS1
3	*TDG*	chr12:104380725	NM_003211.4	rs1452810794	c.1091-1G > A	–	*Splice acceptor*	0.000423	7.45	Het	PM2/ PVS1
5	*HABP2*	chr10:115338424	NM_004132.4	rs41292628	c.607C > T	p.(Arg203Ter)	*Stop gained*	0.0000462	1.22	Het	PM2/ PVS1
7	*HAPLN1*	chr5:82969241	NM_001884.3	rs773738875	c.100 + 2 T > C	–	*Splice acceptor*	0.00003	6.24	Het	PM2/ PVS1
	JAG2	chr14:105609156	NM_002226.4	rs1566757489	c.3592_3593del	p.(Ser1198ThrfsTer33)	*Frameshift Indel*	–-	4.21	Het	PM2/ PVS1

GnomAD MAF, minor allelic frequency in the genomAD database for non-Finish European population; *ACMG*, American College of Medical Genetics.
Conservation score was calculated with PhyloP100way.

cumulus cells produce HA during folliculogenesis, which is crucial for successful ovulation and fertilization [20]. In the present study, genetic variants classified as pathogenic have been identified in three genes related to HA. These variants included c.603_618del in *HMMR* gene coding the Hyaluronic acid (HA)-mediated motility receptor, c.607C > T in *HABP2* gene coding the Hyaluronan-binding protein 2 and c.100 + 2 T > C in *HAPLN1* gene coding the Hyaluronan and proteoglycan link protein 1. The HA action is mediated by hyaluronan binding receptors. One of the major surface receptor for HA is the HMMR [21]. In cell models, HMMR linked to centrosomal and acentrosomal spindle assembly, as well as spindle orientation functions [22]. Studies in animal models showed mice exhibit impaired fertility [23]. Deletion of the HMMR *in vivo* resulting in impaired spindle orientation in the dividing granulosa cells, folliculogenesis defects and subsequent female hypofertility [24]. In humans, *HMMR* gene has been identified as a novel candidate gene related to premature ovarian insufficiency [25]. The second gene related to HA in which a pathogenic mutation was identified was *HABP2* gene. The protein HABP2 is an extracellular serine protease, which binds hyaluronic acid. HABP2 degrades the extracellular matrix and functions in coagulation [26]. Numerous studies have demonstrated the involvement of coagulation proteins in ECM turnover and modifications to the pre- and postovulatory ECM [27]. In the female reproductive system, HABP2 acts as an angiogenesis promoter in the ECM, which varies cyclically during menstrual cycle. Moreover, studies in animal models showed that HABP2 plays a role in the integrity of mouse cumulus ECM [28]. Finally, the last gene related to HA where pathogenic variant was identified in our study population was *HAPLN1* gene. Hyaluronan and proteoglycan link protein 1 (HAPLN1), a component of follicular matrix that link HA, was shown to enhance cumulus-oocyte complex expansion *in vitro*. Studies in rat ovary showed that the LH surge induces HAPLN1 expression in periovulatory granulosa and cumulus cells and HAPLN1 promotes the survival of periovulatory granulosa cells [29]. In summary, proper ECM formation in a periovulatory follicle is requisite for successful ovulation, corpus luteum formation, and fertilization [30]. Altogether, the pathogenic variants identified in three genes related to HA could explain the gEFS in three oocyte donors in our study.

In one of our donors suffering gEFS a frameshift mutation p.(Lys342ArgfsTer7) was identified in *LMNB1* gene. This null mutation leads to protein truncation and loss of function. LMNB1 encodes the Lamin B1, a component of the interphase nuclear lamina, which is required to maintain nuclear shape and mechanical integrity [31]. Lamin B1 also plays a role in spindle assembly; thus depletion of lamin B leads to defects in spindle assembly [32]. LMNB1 contributes to granulosa cell survival by resisting apoptosis during folliculogenesis [33]. Altogether, this mutation's effects, its low population frequency, and the gene's involvement in oocyte maturation may explain gEFS in this oocyte donor.

Genome-wide DNA methylation reprogramming occurs during mammalian gametogenesis and early embryogenesis. The *TDG* gene encodes Thymine DNA Glycosylase responsible for removing thymine moieties from G/T mismatches [34]. TDG is the only DNA glycosylase whose germline deletion results in embryonic lethality [35]. Aberrant TDG expression can reduce the 5-methylcytosine levels in oocyte genomic DNA, causing abnormal epigenetic modifications and meiotic cell cycle arrest in mouse oocytes [36]. Therefore, abnormal TDG expression can block oocyte maturation, impede meiotic cell cycle progression, polar body emission, and affect spindle shape and chromosomal organization [36]. Patient 3 carries the c.1091-1G > A variant in the *TGD* gene. This variant is classified as a pathogenic genetic variant based on in silico predictions. This mutation induces a splicing effect that could alter gene expression, possibly contributing to the failure to obtain oocytes in this patient.

Lastly, a frameshift mutation (p.Ser1198ThrfsTer33) classified as pathogenic, was found in the *JAG2* gene. The *JAG2* gene encodes a Jagged transmembrane protein that is a ligand for the Notch family and

Table 4
Gene description of variants identified from the exome sequencing data in the gEFS oocyte donors.

Individual	Gene	Cytogenetic location	Protein encoded	Gene Function	Infertility association
1	*HMMR*	*5q34*	Hyaluronic acid (HA)-mediated motility receptor	Hyaluronan mediated motility receptor (HMMR) is a hyaluronan receptors. When hyaluronan binds to HMMR, the phosphorylation of a number of proteins, including PTK2/FAK1 occurs. HMMR is an intracellular, microtubule-associated, spindle assembly factor that localizes protein complexes to augment the activities of mitotic kinases, like polo-like kinase 1 and Aurora kinase A, and control dynein and kinesin motor activities.	RHAMM deficiency disrupts folliculogenesis resulting in female hypofertility (23, 243)
2	*LMNB1*	*5q23.2*	Lamin B1	Lamins are the major components of the nuclear lamina which underlies the nuclear envelope of eukaryotic cells, is required to maintain nuclear shape and mechanical integrity. Lamin B1 promotes repair of the DNA breaks, as well as cell survival, by maintaining the level of the RAD51 protein that is employed in homologous recombinational repair. Lamin B1 has a role in spindle assembly.	Chromatin configuration during meiosis resumption in the oocyte (40)
3	*TDG*	*12q23.3*	Thymine-dna glycosylase	TDG initiates repair of G/T and G/U mismatches, commonly associated with CpG islands, by removing thymine and uracil moieties. TDG associates with transcriptional coactivators CBP and p300 and that the resulting complexes are competent for both the excision step of repair and histone acetylation.	Aberrant TDG expression causes epigenetic modifications and meiotic cell cycle arrest of oocytes (35)
5	*HABP2*	*10q25.3*	Hyaluronan-binding protein 2	HABP2 is an extracellular serin-protease that binds to hyaluronic acid and participates in cell adhesion. Activates extrinsic coagulation pathways and the fibrinolysis-inducing enzyme urokinase. It does not initiate the formation of the fibrin clot and does not cause the fibrinolysis directly but converts the inactive single chain urinary plasminogen activator (pro-urokinase) to the active two chain form.	HABP2 is associated with unexplained female infertility [41]
7	*HAPLN1*	*5q14.3*	Hyaluronan and proteoglycan link protein 1	Stabilizes the aggregates of proteoglycan monomers with hyaluronic acid in the extracellular matrix. Predicted to be an extracellular matrix structural constituent conferring resistance.	HAPLN1 may promote periovulatory granulosa cell survival, which would facilitate their differentiation into luteal cells (28)
	JAG2	*14q32.33*	Jagged 2	The *JAG2* gene encodes a transmembrane protein that is a ligand for the Notch family of transmembrane receptors that are critical for various cell fate decisions.	Jagged2 and Notch1, are involved in primordial folliculogenesis, in the ovary (39)

are critical for various cell fate decisions. The highly conserved Notch signalling pathway is emerging as an important means of intrafollicular communication [37]. Notch pathway activation occurs in both the embryonic and postnatal ovary, playing significant roles in events such as follicle assembly and growth, meiotic maturation, ovarian vasculogenesis, and steroid hormone production [38]. As a cell contact-dependent pathway, Notch signalling requires binding of membrane bound Notch receptors as Jagged on adjacent cells [39]. Jagged2 and Notch are essential molecules for completion of folliculogenesis and a good ovarian response [40]. In our patient, we identified the previous described variant in the *HALPN1* gene (c.100 + 2 T > C) and the variant in the *JAG2* gene which is a null variant (frameshift) where the protein is truncated causing loss of function. Both of these findings could be the cause of the gEFS in this patient.

In conclusion, while gEFS is complex, this study identified potential genetic factors beyond the oocyte-cumulus complex. Understanding these genes could lead to new strategies for prevention and management of gEFS, but further research is needed to confirm these findings and discover additional involved genes.

Funding

There is no funding for the project.

Author contributions

Experiment conception and design: BLL, RM, JG; Experiment performed: FML, MH, JG; Data analysis: JJP, JAO; Manuscript writing: BLL, JJP; Paper final edition: BLL, RM, JAO, AB, RB.

CRediT authorship contribution statement

Belen Lledó: Conceptualization, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing. **Juan J. Piqueras:** Conceptualization, Investigation, Methodology. **Francisca M. Lozano:** Formal analysis, Investigation. **Mónica Hortal:** Formal analysis, Investigation. **Ruth Morales:** Formal analysis, Investigation, Supervision, Validation. **José A. Ortiz:** Formal analysis, Investigation. **Jaime Guerrero:** Conceptualization, Investigation. **Andrea Benabeu:** Resources, Supervision. **Rafael Bernabeu:** Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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