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GENETICS

Successful in vitro maturation of oocytes in a woman with gonadotropin-resistant ovary syndrome associated with a novel combination of FSH receptor gene variants: a case report

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Abstract

Infertility due to Gonadotropin-Resistant Ovary Syndrome (GROS) is a rare type of hypergonadotropic hypogonadism. Here, we report an original case of GROS, associated with compound heterozygous follicle-stimulating hormone receptor (FSHR) variants, in a woman who achieved a live birth by in vitro maturation (IVM) of her oocytes. This 31-year-old woman consulted our assisted reproduction center for a second opinion after having been advised, because of pervasive high serum follicle-stimulating hormone (FSH) levels, to pursue in vitro fertilization (IVF) with donor oocytes. She presented with primary infertility and progressively prolonged menstrual cycles. Her serum FSH levels were indeed found to be high, but in discordance with a normal anti-Müllerian hormone (AMH) level and antral follicle count. Genetic investigation found the patient to be compound heterozygous for two FSHR variants: I160T, a known pathologic variant, and N558H, which has never been previously reported. As there was no ovarian response to high daily doses of exogenous gonadotropins, IVM was proposed to the patient with success and she finally delivered at term a healthy boy. Effects of the receptor variants were analyzed in heterologous cells. Whereas the I160T mutation blocked FSHR membrane trafficking and FSH-stimulated cAMP-dependent signaling in transfected CHO cells, the novel variant, N558H, functioned equivalently to wild-type FSHR in the assays employed. In conclusion, IVM should always be offered as a first-line therapy to infertile women presenting with GROS. The N558H variant discovered in FSHR is novel, but its functional significance, if any, is unresolved and merits further investigation as it may be associated with a recessive FSHR-related disorder.

Keywords In vitro maturation (IVM) · Ovarian resistance · Gonadotropins · FSH receptor · Follicle-stimulating hormone (FSH)

Introduction

First described almost 50 years ago, Gonadotropin-Resistant Ovary Syndrome (GROS) is a rare type of hypergonadotropic hypogonadism that remains over time a diagnostic and therapeutic challenge in the context of infertility [1–3]. It is often caused by genetic mutations in the follicle-stimulating hormone receptor (FSHR) [4, 5]. Clinically, affected patients present with amenorrhea or progressively prolonged menstrual cycles, along with elevated serum levels of FSH [6]. In contrast to a true primary ovarian insufficiency (POI), antral follicle count (AFC) and serum levels of anti-Müllerian hormone (AMH) are most often within the normal range [7, 8].

The treatment of the infertility in those patients is often a challenge. Here, we describe a case of a woman with infertility due to GROS, presenting clinically with an unusual desynchronized and erratic folliculogenesis, likely caused by

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compound heterozygous missense variants in the *FSHR* gene. One of these variants, mapping to the third intracellular loop of the receptor, has not previously been reported in the literature. Eventually, this woman conceived after treatment with in vitro maturation of her own oocytes (IVM) and delivered at term a healthy baby, after an uneventful pregnancy.

Materials and methods

Informed consent

As this manuscript contains personal medical information about living individuals, our patient and her husband read the final version of the manuscript and gave their explicit written consent before it was submitted to the journal for consideration.

Oocytes collection and IVM

Aspiration of immature follicles, under 10 mm of diameter, was completed with a 19-gauge IVM needle (K-OPS-7035-RWHET-US, Cook Medical) using a negative pressure of 80–90 mmHg. Oocytes were collected and evaluated for maturation by sliding method. Immature oocytes were placed in IVM medium (SAGE In vitro Maturation Media®, Origio, USA) supplemented with hMG (Menopur®) at a concentration of 0.075 IU/ml for 24 h.

In vitro studies

Site-directed mutagenesis

Site-directed mutagenesis of the FSHR was performed using the QuikChange protocol using pSVL-hFSHR WT as a template [9]. For the I160T mutation, the primers used were: forward: 5'-TCCACACAACTGAAAGAAATTCTTTC-3′, and reverse: 5'-GAAAGAATTTCTTTCAGTTGTGTGGA-3′. For the N558H mutation, the primers used were: forward 5'-AGTGCGGCACCCCAACATCGT-3′, and reverse 5'-ACGATGTTGGGGTGCCGCACT-3′. Sanger sequencing was performed at GenomeQuebec to confirm the mutation.

Whole cell ELISA

CHO cells were plated at a density of 70,000 cell per well on poly-D-lysine (1 mg/ml)-coated 24-well plates in DMEM/F12 medium containing 10% FBS. The next day, cells were either left untransfected (mock), or transiently transfected with 500 ng of empty vector, pSVL-hFSHR wild-type (WT), pSVL-hFSHR bearing the I160T mutation (I160T), pSVL-hFSHR bearing the N558H mutation (N558H), or with 250 ng of each variant in combination (I160T + N558H). Forty-eight hours post-transfection, cells were fixed with PFA and whole cell ELISA was performed as previously described [10]. Briefly, cells were rinsed three times with PBS and incubated for 2 h with blocking solution at room temperature. Cells were then incubated with human FSHR antibody mAb 106.105 for 2 h at room temperature [11]. Cells were then washed and incubated with an HRP-conjugated goat anti-mouse antibody. Next, cells were washed five times for 5 min each with PBS, and then incubated in 500 μl of 3,3′,5,5′-tetramethylbenzidine substrate for 15 min. The reaction was stopped by adding 2 N sulfuric acid. Finally, absorbance was measured at 450 nm using Biochrom Asys UVM 340 microplate reader. Data represent the mean ± SEM of three independent experiments performed in triplicate.

Luciferase assays

CHO cells were seeded at a density of 40,000 cell per well in 48-well plates in DMEM/F12 medium containing 10% FBS. The next day, cells were co-transfected with 50 ng of pSVL-hFSHR WT or variants along with 225 ng pCRE-Luc reporter vector. In conditions were both variants were expressed in combination, 25 ng of each plasmid was transfected. Twenty-four hours post-transfection, cells were starved overnight with serum-free medium. The following day, cells were either left untreated or treated with 10, 40, 100, or 400 IU/L of human FSH (R&D Systems) for 6 h. Finally, luciferase activity was measure as described in Tran et al. 2013 and Lamba et al. 2006 [12, 13].

Statistical analyses

One-way ANOVA test with Tukey post hoc test was performed for whole cell ELISA and two-way ANOVA test followed by multiple comparison test for luciferase assays. p value < 0.05 was considered as statistically significant. The GraphPad Prism software was used. The SNPs3D and the PMut online analysis tools were used to predict FSHR variants pathogenicity http://mmb.irbbarcelona.org/PMut/ and http://www.snps3d.org.

Results

Case description

The Caucasian couple, a 31-year old woman and her husband, a 36-year old man, initially consulted a fertility clinic for a history of primary infertility for 1 year. The woman noted progressively prolonged menstrual cycles, and repeated measurements of serum FSH were consistently elevated, ranging from 43 to 62 IU/L (normal range 3.9–8.8 IU/L). She was then told she was developing POI and was therefore advised to

pursue in vitro fertilization (IVF) with donor oocytes. The couple purchased oocytes abroad with this treatment in mind; but, they were still unsure and hesitant, and therefore consulted our assisted reproductive centre for a second opinion. At this time, a deeper investigation into the couple's infertility was initiated.

Fertility investigations

The woman's ovarian reserve testing was repeated, including serum hormone levels in the early follicular phase. Again, her FSH was found to be elevated at 55 IU/L (normal range 3.9–8.8 IU/L) as was her luteinizing hormone (33 IU/L; normal range 2.1–10.9 IU/L). Her thyroid-stimulating hormone (TSH) level was normal (1.84 mIU/L; normal range 0.34–5.50 mIU/L) under a daily dose of 50 μg of levothyroxine, as was her prolactin (9 μg/L; normal range 5.2–26.5 μg/L). Despite the confirmed elevation of FSH, the patient's AMH level was normal (3.24 ng/mL; normal range 0.9–95 ng/ml). Transvaginal ultrasound was performed on day five of the patient's menstrual cycle, and surprisingly revealed an antral follicle count of 19; however, several of these were abnormally large for day five of the cycle. The follicle diameters (mm) were as follows: 18, 18, 14, 13, 13, 11, and 13 follicles < 10 mm. The uterus and ovaries were otherwise normal, and the endometrial stripe was 6 mm in thickness. In the presence of chronic anovulation, this clinical presentation was considered to be very unusual with dominant follicles too early in the patient's follicular phase, suggesting a very disturbed folliculogenesis.

Karyotype analysis and molecular testing for Fragile X were normal. Blood testing for autoimmune disorders was normal, including absence of anti-adrenal, anti-nuclear, anti-DNA, anti-thyroglobulin, anti-21-hydroxylase, and anti-thyroid peroxidase antibodies. The patient's calcium, albumin, and cortisol levels were normal as well. Magnetic resonance imaging of the hypothalamus and pituitary gland was normal.

Genetic analysis of the FSH receptor

At this point, GROS was considered the most likely diagnosis for this woman. A consultation with a medical geneticist was arranged, at which time a search was undertaken for genetic mutations that could be responsible for this patient's phenotype. To this end, a panel of 11 genes was sequenced and analyzed: *BMP15*, *CYP17A1*, *CYP10A1*, *FIGLA*, *FSHR*, *GDP9*, *LHCGR*, *NOBOX*, *NR5A1*, *POR*, and *PSMC3IP*. The results were normal for all of these genes, except for two heterozygous variants in the *FSHR* gene. The first variant, I160T (NM_000145.3: exon 6: c.479 T>C: p.Ile160Thr, rs121909659), is a mutation located in the extracellular ligand binding domain (Fig. 1). It has already been identified as pathologic in a previous patient with GROS [17]. The second variant, N558H (NM_000145.3: exon 10: c.1672 A>C: p.Asn558His), had not previously been reported (Fig. 1). Both variants are very rare but with comparable frequency as reported in the Genome Aggregation Database (gnomAD) is 4.065e-5 for I160T and 2.443e-5 for N558H [18]. The substitution also occurs at a position that is conserved across mammalian species, and in silico analysis predicted this variant would most probably be damaging to the protein structure/function. Based on currently available information, the report stated it was unclear whether this variant was a pathogenic variant or a rare benign variant. Sanger sequencing confirmed the patient's *FSHR* variants were in a *trans* relationship, with each being present on a separate parental allele of the *FSHR* gene. These *FSHR* variants were thus consistent with our clinical diagnosis of GROS and allowed us to better counsel the couple for their reproductive options.

Attempted ovarian stimulation and conversion to IVM

While waiting for the results of their genetic analysis, the couple decided to attempt an autologous cycle of IVF, with a probability to be converted into a cycle of IVM. As described in the fertility investigation, the patient still presented a dysfunctional folliculogenesis with spontaneous development of large anovulatory follicles much too early in her menstrual cycle. An oocyte retrieval was attempt on day 4 of the cycle, but even if cumulus cells were identified in the aspiration follicular fluid, no oocytes were found.

Due to the encouraging presence of cumulus cells in the follicular fluid, it was decided that a second autologous IVF cycle would be attempted without further delay. The patient was immediately put on monophasic oral contraceptive pill (Marvelon®) for 21 days. Fifteen days after starting oral contraceptive, serum estradiol was at 41 pmol/L; FSH and LH were downregulated at 7 and 6 IU/L, respectively; and progesterone lower than 1 nmol/L. A single shot of intramuscular injection of triptorelin 1.25 mg was then administered, as previously described [19]. After cessation of the oral contraceptive pill, a transvaginal ultrasound was performed on the fifth day of the subsequent withdrawal bleeding. This revealed a total of 11 follicles, all under 10 mm in diameter, as well as an endometrial stripe thickness of 4 mm, attesting adequate ovarian downregulation. Daily subcutaneous injections of hMG (Menopur®) were initiated, beginning at 300 IU. The patient has had a regular ultrasound follicle tracking and the medication was increased to 450 IU on the sixth day of stimulation due to a lack of follicular growth. On the thirteenth day of stimulation, the dose of gonadotropins was increased further to 600 IU (Menopur® 300 IU plus recombinant FSH, Gonal F® 300 IU). On day 16 of stimulation, lack of ovarian response (all follicles were < 10 mm) prompted the decision to convert the cycle to IVM. As the endometrial thickness was 4 mm, gonadotropins were continued at the same dose for another 4 days. The addition of oral estradiol (Estrace®)

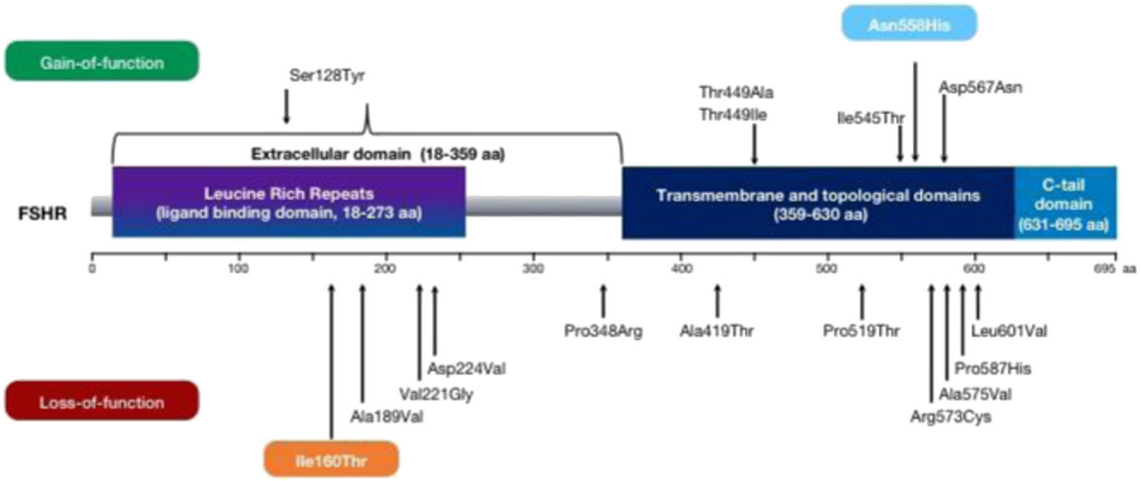

Fig. 1 FSH receptor, showing the locations of the patient's variants. Representation of allelic variants of *FSHR* as described in OMIM (Online Mendelian Inheritance in Man). The information regarding loss-of-function and gain-of-function was taken from the NCBI database (www.pubmed.com – the ClinVar section). Note that only variants with demonstrated clinical significance were included. On top of the figure are shown variants leading to ovarian hyperstimulation (gain-of-function). Under the figure are variants leading to loss-of-function (ovarian dysgenesis). The two variants sequenced in our patient: I160T variant (in orange) localized in exon 6 and N558H variant (in blue) localized in exon 10. The different domains of the receptor were based on Fan 2005, Jiang 2012 and Ulloa-Aguirre 2016. [14–16]

at a dose of 2 mg twice daily for the next 8 days was successful in achieving an endometrial thickness of 8 mm. A subcutaneous injection of hCG 10,000 IU (Pregnyl®) was administered and oocyte retrieval was scheduled 36 h later. Vaginal progesterone (Endometrin® 100 mg TID) was started the day of fertilization.

IVM

Six oocytes were collected. All of them showing germinal vesicle, they were placed in the IVM medium for 24 h. After denuding the oocytes, three had already reached the metaphase II stage (MII). To allow for complete cytoplasmic maturation, the intracytoplasmic sperm injection (ICSI) was performed 3 h after denudation of these oocytes. Of the remaining three immature oocytes, one was in the metaphase I (MI) stage, and the other two were at the germinal vesicle (GV) stage. After incubating these immature oocytes in the IVM medium a further 24 h, one reached MII stage and ICSI was performed (Fig. 2a). The four micro-injected oocytes were incubated in maintenance medium for a total of 3 days. At the 18-h point after ICSI, successful fertilization was confirmed for all four oocytes by visualization of the expulsion of the second polar body and the two pronuclei. The embryos were examined at days 2 and 3 post-ICSI for assessment of embryonic cell division.

Embryo transfer and follow-up

On day 3, two embryos from the first maturation time displayed optimal cell division with eight cells and less than 5% fragmentation (Fig. 2b). One of these was transferred to the patient's uterus. The other stopped developing after reaching the compacted morula stage, and therefore was not cryopreserved. Serum beta-human chorionic gonadotropin (B-hCG) was positive (247 U/L) 14 days after embryo transfer. A transvaginal ultrasound confirmed viability of a normal single intrauterine pregnancy at 6 weeks and 6 days gestational age. Both oral estradiol and vaginal progesterone were continued up to 10 weeks of pregnancy. Subsequently, a combined first trimester prenatal screening was performed at 12 weeks and 2 days, which was normal and indicated a low risk for aneuploidy and maternal adverse events. After an uneventful 39-week pregnancy, the patient spontaneously delivered a healthy boy of 2885 g.

Functional characterization of the FSHR variants

To understand the functional significance of the newly identified variant N558H, we generated separate expression vectors for FSHR containing N558H and the previously characterized I106T mutation. When transfected into heterologous Chinese hamster ovary (CHO) cells, wild-type and N558H forms of the FSHR were expressed at equivalent levels at the plasma membrane, as assessed by ELISA (Fig. 3). As previously described, I160T mutant was not detected at the cell surface [17]. When the two variants were expressed together, membrane expression was equivalent to that when N558H was expressed alone.

FSH signaling via its receptors induces cAMP accumulation and CREB protein phosphorylation. We therefore examined whether FSH activation of cAMP/CREB signaling was altered downstream of the N558H receptor. CHO cells were

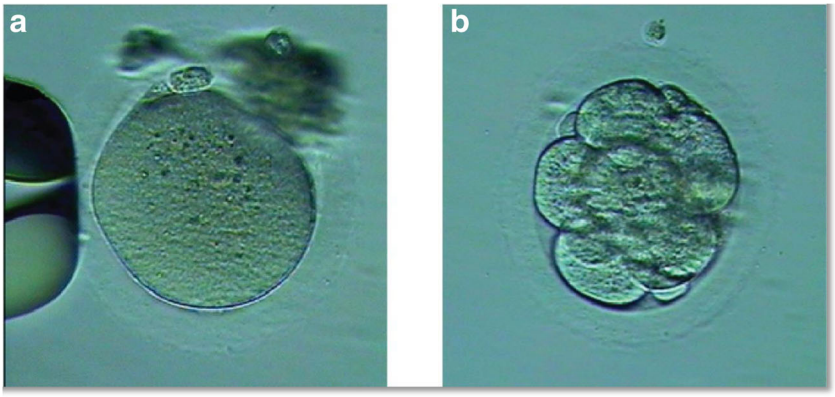

Fig. 2 Oocyte maturation and embryo development. **a** Expulsion of first polar body confirming MII maturation. **b** Embryo used for transfer on day 3 after ICSI

co-transfected with the FSHR expression vectors described above as well as the pCRE-Luc reporter plasmid. Cells were treated for 6 h with 10 to 400 IU/L of human FSH. FSH stimulated reporter activity in a concentration-dependent manner via the wild-type and N558H receptors (Fig. 4). In contrast, FSH failed to stimulate the reporter via the I160T receptor at any of the FSH concentrations tested. When I160T and N558H were co-expressed, FSH stimulated luciferase activity at a comparable level to that seen when the N558H receptor was expressed alone.

Discussion

We describe here a case of a patient suffering from infertility caused by gonadotropin-resistant ovary syndrome (GROS), associated with compound heterozygous variants in the FSH

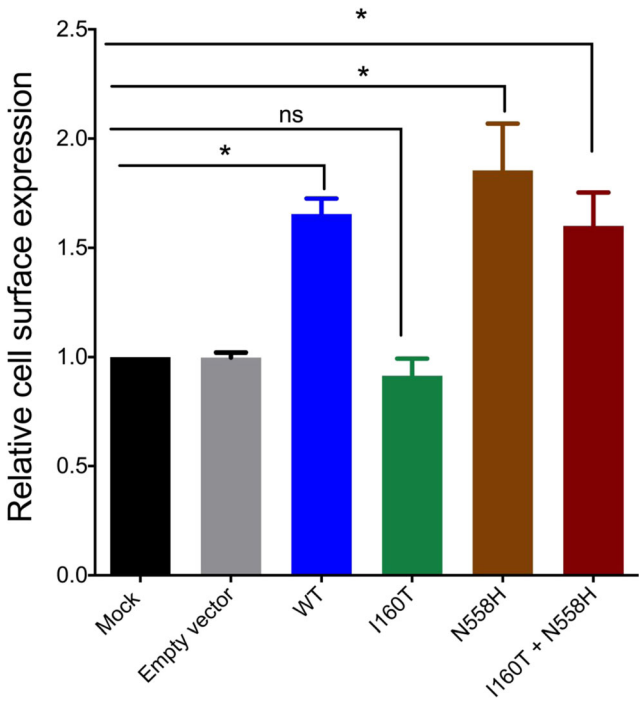

Fig. 3 Cell surface expression of wild-type and variant human FSH receptors. CHO cells were either left untransfected (mock), or transiently transfected with empty vector, or expression vectors encoding wild-type (WT), I160T, N558H, or I160T and N558H human FSHR receptor(s). Forty-eight hours post-transfection, cells were fixed and whole cell ELISAs were performed using mAb 106.105 against human FSH receptor to measure relative surface receptor expression. Results are shown as ratio of OD 450 nm for expression vectors over the value for non-transfected cells. Data represent the mean ± SEM of three independent experiments, each performed with three technical replicates. *$p < 0.05$, *ns* non-significant

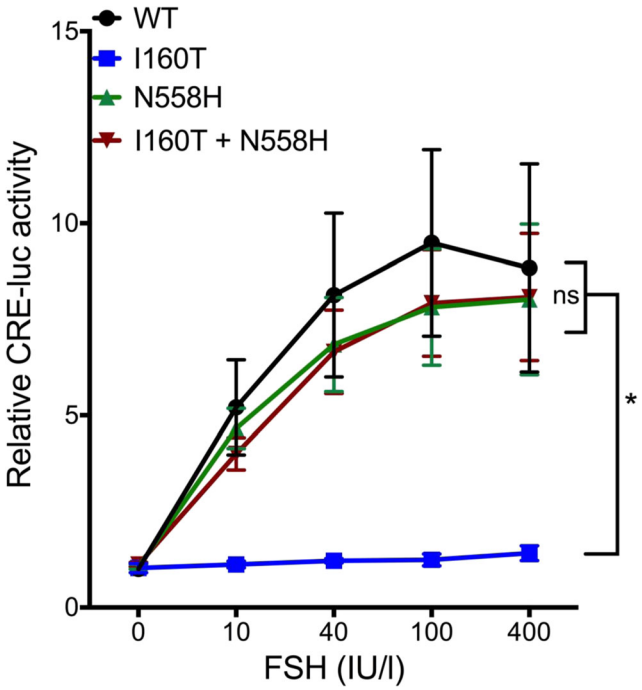

Fig. 4 FSH activation of a cAMP responsive reporter via wild-type and variant human FSH receptors. CHO cells were transfected with pCRE-Luc plasmid along with expression vectors encoding wild type (WT), I160T, N558H, or with both I160T and N558H FSHR receptor(s). Twenty-four hours post-transfection, cells were either left untreated or treated with human FSH at the indicated concentrations. Six hours post-treatment, cells were lysed and luciferase activity was assessed. Results are shown as the fold induction of treated conditions over the untreated condition expressing the WT receptor. Data represent the mean ± SEM of three independent experiments, each performed with two technical replicates. *$p < 0.05$, *ns* non-significant

receptor, one of which was not previously reported in the literature. Fortunately, this patient, who was initially advised to proceed with donor oocytes, was able to conceive with her own eggs due to an autologous in vitro maturation cycle. She delivered a healthy baby.

This case highlights the importance of completing a thorough investigation of infertile patients presenting with oligomenorrhea or amenorrhea who have persistently high levels of serum FSH and to not categorize all such patients as having primary ovarian insufficiency. Measures of ovarian reserve, including the measure of serum levels of FSH, AMH, and transvaginal ultrasound measurement of antral follicle count (AFC) should always be included in the complete evaluation of a patient's ovarian reserve [20]. In cases of POI, there is generally concordance among results of FSH, AMH, and AFC [21]. However, in the rarer cases with discordance between ovarian reserve markers, where FSH is high but AMH and AFC are normal, a diagnosis of GROS should always be considered and, in such patients, IVM, already a fertility option, should be offered as a first-line therapy before resorting to the use of donor oocytes [22].

Beside this success with IVM, this case is also interesting for its clinical features: resistance to gonadotropins, chronic anovulation, and abnormal presence of multiple dominant follicles early in the follicular phase. The fact that one allele has a loss-of-function and the other might affect the functionality of FSHR made this case particularly interesting. Indeed, the I160T mutation of the FSHR that was found in our patient has previously been associated with GROS [17]. In addition, as we observed here, functional assays confirmed that this mutation reduces membrane expression and FSH-dependent signaling via the FSHR compared to wild-type [17]. This (heterozygous) variant is however not always reported to significantly affect the ovarian response to gonadotropins [23, 24].

The second genetic FSHR variant found in our patient, N558H, is novel. In this variant, asparagine is substituted with histidine, which is normally highly conserved among mammals, and bioinformatics analyses predicted that this alteration would be deleterious. However, when expressed in heterologous cells, the FSHR harboring the N558H variant was expressed at the cell surface equivalently to the wild-type FSHR, and was able to equivalently broker FSH stimulation of a cAMP-dependent reporter. Co-expression of the I160T variant did not affect membrane expression or signaling by the FSHR-N558H variant, at least not in the assays used here.

The N558H mutation occurs in the third intracellular loop (ICL3) of the receptor. This loop is important for G protein coupling and signaling [25–27]. N558H is now the third mutation mapped to ICL3 in the FSHR. One mutation, R573C, was reported in a patient with a similar clinical presentation to the patient described here, with progressive oligomenorrhea, infertility, and lack of response to exogenous gonadotropins [17]. Remarkably, this patient was also heterozygous for the I160T mutation. Like N558H, the R573C mutation did not block membrane trafficking of the receptor (nor did it affect ligand binding); however, it did impair FSH-stimulated cAMP accumulation. R573 occurs in a motif involved in G protein coupling and receptor activation, whereas N558 has not previously been implicated in either process. Indeed, our observation that FSH can stimulate a cAMP-dependent reporter via the N558H mutant receptor suggests that this residue is not required for Gs coupling. A second mutation in ICL3 of the FSHR, D567G, was described in a hypophysectomized man, who maintained testis size and spermatogenesis without exogenous gonadotropin treatment [28]. The mutation caused constitutive (ligand-independent) receptor activation. It is tempting to speculate that N558H, just 9 residues N-terminal to D567, might change the configuration of the loop, impact the conformation of the sixth transmembrane helix and, as D567G, provoke constitutive activity of the FSHR, which could explain the enlarged follicles observed early in the menstrual cycle in our patient. However, follicle development was blocked when the patient was downregulated with a GnRH agonist and we did not detect constitutive receptor activity in the reporter assay. It is possible, however, that this assay lacks the sensitivity needed to detect such activity. It is also possible that the mutation led to a gain of function (e.g., coupling to additional G proteins) that we failed to detect in our analyses. Indeed, one might predict altered signaling by FSHR-N558H given the patient's anomalous follicle development and impaired sensitivity to exogenous gonadotropins.

Our experience, as well as two other reported cases of GROS but without FSHR pathologic variants, confirms that it is possible to obtain multiple embryos with autologous eggs from a woman with GROS [19, 22]. In retrospect, although our patient underwent several days of attempted gonadotropin stimulation, it is likely that a much shorter ovarian stimulation could have been undertaken; for example 3–5 days, if at all, before proceeding with oocyte retrieval. Failure to respond to prolonged gonadotropin exposure, however, did confirm without a doubt that our patient was clinically affected by GROS. We still do not know the fertility potential of the patient under these conditions; however, this patient was never able to conceive spontaneously before or after this reported IVM. So, she underwent recently, in September 2018, a second IVM attempt during which 7 immature oocytes were retrieved and 5 embryos obtained, all from follicles < 10 mm, with no follicular development after 10 days of hMG.

Clinical and biological aspects of IVM have been well documented and it has already led to the birth of several thousand babies, with no apparent risk of increased anomalies [29, 30]. Nowadays, there still is a restricted list of candidates which includes women at risk for ovarian hyperstimulation syndrome, women with polycystic ovary syndrome (PCO) or PCO-like ovaries, women with estrogen-sensitive cancers,

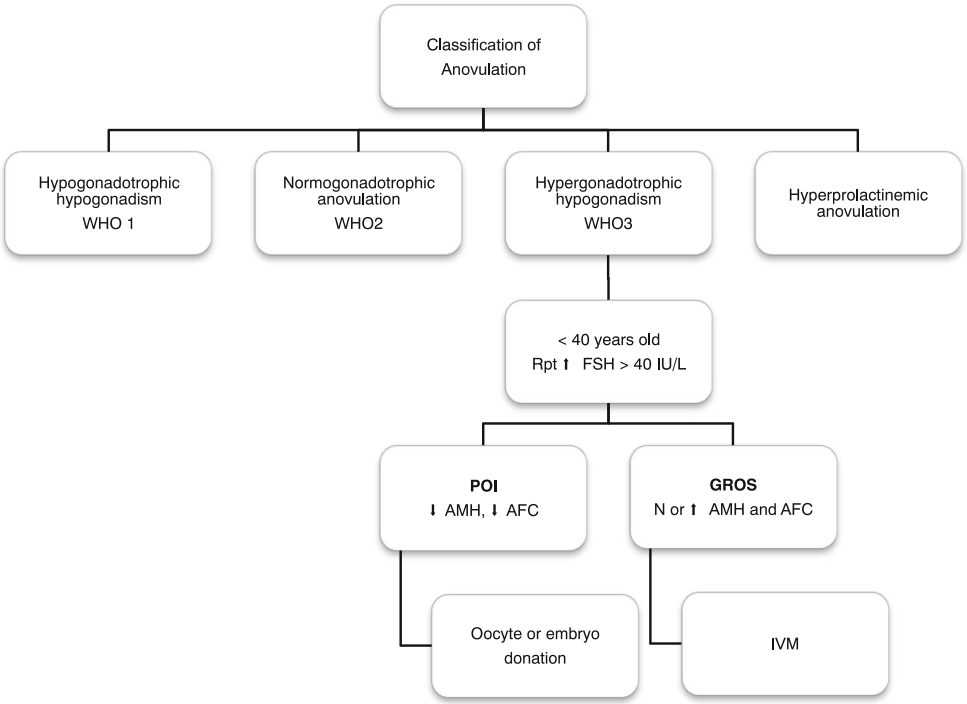

Fig. 5 Modifications proposed to WHO classification for chronic anovulation

and those with limited time prior to initiating gonadotoxic treatments for fertility preservation [31]. Based on our own experience, it is our recommendation that IVM indications should also be extended to GROS, especially in patients with FSHR mutations.

In conclusion, in the presence of a presumed POI in patients seeking fertility treatment, one should always be careful in considering a complete differential diagnosis, including the diagnosis of GROS. In order to confirm and clarify this diagnosis, AFC, AMH, genetic counseling, and finally next-generation gene sequencing analysis prove helpful. Before resorting to the use of donor oocytes, IVM should also always be offered first to infertile women suffering from GROS, especially in the presence of FSHR mutations. The N558H variant discovered in ICL3 of FSHR is novel, but its functional significance, if any, is unresolved and merits further investigation. Finally, as medical consensuses are usually established not only to help physicians for diagnostic purposes but also to guide them for therapeutic options, we also suggest WHO classification for chronic anovulation to be revisited by the ESHRE Capri Workshop Group in order to take into consideration, among others, AMH and AFC in its diagnostic algorithm, particularly for WHO class 3, as well as IVM as a therapeutic option for GROS [32–35] (Fig. 5).

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Author's roles CF was responsible for the laboratory procedures, literature review, writing, and coordinating the submission of the manuscript. VB developed the IVM procedure and participated in the laboratory procedures. SC provided genetic consultation, interpretation of genetic testing, and contributed to manuscript writing. SA participated in the reflection and writing of the manuscript. MB supervised the IVM procedures. CT and DJB studied the in vitro effects of the identified mutations on FSHR function and wrote parts of the manuscript. PM was responsible for the diagnostic evaluation and clinical management of the couple, wrote parts of the manuscript, and coordinated its preparation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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