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Inferring biallelism of two FSH receptor mutations associated with spontaneous ovarian hyperstimulation syndrome by evaluating FSH, LH and hCG cross-activity

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Abstract

Research question. Which is the cumulative effect of two FSHR mutations in sOHSS pathogenesis? Are these mutations in the mono- or biallelic state?

Design. Two follicle stimulating hormone-receptor (FSHR) mutations were found in a pregnant patient affected by spontaneous ovarian hyperstimulation syndrome (sOHSS) with no predisposing conditions. While the p.Asn106His mutation is novel, the p.Ser128Tyr was previously associated with the disease. Patient’s FSHR gene was analysed by Sanger sequencing and FSHR cDNAs carrying the single or both the point mutations were created by mutagenesis in vitro. cAMP activation by recombinant follicle-stimulating (FSH), luteinizing (LH), choriongonadotropin (hCG) and thyroid-stimulating hormone (TSH) was evaluated in transfected HEK293 cells, by bioluminescence resonance energy transfer (BRET).

Results. All mutations decreased FSH 50% effective concentrations (EC\textsubscript{50}) calculated for cAMP (Mann-Whitney’s U-test; p<0.05; n=6), resulting in 2- to 10-fold lower ligand potency. TSH failed in inducing FSHR-mediated intracellular cAMP increase, while LH was about 4-fold more potent than hCG in p.Ser128Tyr FSHR-expressing HEK293 cells in spite of lower cAMP plateau levels (Mann-Whitney’s U-test; p<0.05; n=5). p.Ser128Tyr FSHR mutation is responsible of LH/hCG-induced cAMP when it is in the biallelic heterozygous state with p.Asn106His, while no induced cAMP increase occurs in the monoallelic state (Mann-Whitney’s U-test; p≥0.05; n=5).

Conclusions. In vitro data support that, in the sOHSS pregnant patient, the two FSHR mutations have opposite effect on sOHSS pathogenesis and were in the biallelic heterozygous form, allowing p.Ser128Tyr FSHR-mediated cAMP increase by hCG.

Key messages:

- Novel p.Asn106His and known p.Ser128Tyr FSHR mutations were found in a pregnant sOHSS patients with unknown allelic setting

- In biallelic p.Asn106His/p.Ser128Tyr FSHR-expressing cells, LH/hCG induced cAMP in vitro
These mutations are informative of differences between LH and hCG, as well as of sOHSS and infertility.

**Key words:** FSH, FSHR, spontaneous Ovarian Hyperstimulation Syndrome, OHSS, gonadotropin

**Introduction**

Ovarian Hyperstimulation Syndrome (OHSS) is a medical condition characterized by bilateral, multiple ovarian cysts, ascites, abdominal distension and discomfort, low circulating protein levels, oliguria and electrolyte imbalance (Kaiser, 2003). OHSS may be iatrogenic, occurring as a complication of *in vitro* fertilization procedures (Mourad et al., 2017), or spontaneous (sOHSS), due to high circulating endogenous human chorionic gonadotropin (hCG) or thyroid-stimulating hormone (TSH) levels mimicking the follicle-stimulating hormone (FSH) action on granulosa cells (Lussiana et al., 2009).

Human FSH, LH and TSH are dimeric, pituitary glycoprotein hormones sharing a common α and having a specific β-subunit (Alevizaki and Huhtaniemi, 2002). hCG is the placental gonadotropin which binds the LH receptor (LHCG) and may cross-interact with the TSH receptor (TSHR) (De Leener et al., 2008). Similarly, TSH binding to LHCG was also demonstrated (Hidaka et al., 1993), suggesting the presence, at the receptor level, of a relatively low number of key-residues responsible for the ligand specificity (G. Smits et al., 2003). Within the physiological hormone range, β-subunit provides receptor-specific binding to the extracellular leucine-rich domain of their structurally similar receptors (Moyle et al., 2004), avoiding promiscuous activation of cAMP/PKA and other intracellular signalling cascades (Costagliola et al., 2005).

Several cases of sOHSS have been reported, and reviewed (Lussiana et al., 2008; Meduri et al., 2008), in women carrying a mutated form of FSH receptor (FSHR). These mutations may change the sensitivity to FSH or induce loss of binding specificity, leading to cross-interaction between the receptor and other glycoprotein hormones, mainly TSH and hCG (Montanelli et al., 2004).

In this study, we evaluated single and additive effects of two missense FSHR mutations carried by a sOHSS patient, measuring the cAMP production induced by treatment of a receptor-overexpressing cell line by recombinant FSH, LH, hCG and TSH, *in vitro*. However, DNA
sequencing did not reveal whether these heterozygous mutations are individually located on two different (biallelic) or on the same allele (monoallelic) (figure 1). At the protein level, mutations fall within the extracellular domain of FSHR and result in the asparagine to histidine amino acid change at position 106 of the protein chain (p.Asn106His), and in the serine to tyrosine change at position 128 (p.Ser128Tyr). While the p.Asn106His FSHR mutation is novel, the p.Ser128Tyr was previously associated with sOHSS and displayed hCG-, but not TSH-dependent cAMP increase in vitro and lack of constitutive activity (De Leenher et al., 2008). However, the effect of LH was not tested. Herein we administrated glycoproteins in molar terms, instead of IU/ml, providing a precise, quantitative comparison between the molecules and their effects mediated by mutant FSHRs.

[FIGURE 1]

Materials and Methods

sOHSS patient

A patient with a clinical picture of sOHSS came to our observation. She was a 30 years-old woman who asked for a legal, voluntary abortion at 11 weeks of pregnancy. She was admitted to the hospital with severe abdominal pain and distension, dyspnea, oliguria. The blood tests revealed increased haematocrit, increased serum creatinine, hyponatremia with increased potassium levels. TSH circulating levels were normal. The abdominal ultrasound showed severely enlarged ovaries (13 and 14 cm mean diameter) with multiple transonic cysts, and abundant ascites. Some pleural free fluid was also observed in the thorax radiography. The cytological examination of the abdominal fluid was negative for neoplastic cells. A brain magnetic resonance (MRI) was performed to exclude a pituitary adenoma. Blood hCG was monitored and it decreased normally after the abortion. The final diagnosis was severe sOHSS (Grade B according to previously published criteria) (Rizk and Aboulghar, 1991). Further ultrasound and hematological evaluations were scheduled every 15 days and documented the progressive reduction of ovarian volume and the complete disappearance of ascites with normalization of the blood exams.
Patient gave written consent for genetic screening.

**Patient's DNA extraction, amplification and sequencing**

DNA was isolated from EDTA-treated blood samples using the semi-automatic extractor ABI PRISM™ 6100 Nucleic Acid Prep Station (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. *FSHR* exons (NCBI reference sequence: NM_000145.3) were amplified using previously validated primer probes (Guillaume Smits et al., 2003). Each amplification reaction contained 50 ng of DNA, 10X GeneAmp PCR Gold Buffer (Thermo Fisher Scientific), 800 μM deoxynucleoside triphosphates (dNTPs), 1,5 mM MgCl2 (Thermo Fisher Scientific), 0.8 μM of primers mix and 2 U of AmpliTaq Gold DNA Polymerase® (Thermo Fisher Scientific), in a total volume of 50 μl. Reactions were performed on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). Each PCR contained 800 μM of deoxynucleoside triphosphates (dNTPs), 10X PCR buffer, 1,5 mM MgCl2, 0.4 μM primer and 2 U AmpliTaq Gold® in 50 μl distilled water. Denaturation at 95°C for 6 min was followed by 35 cycles at 94°C for 45 s, 49°C for 45 s, and 72°C for 90 s followed by a final elongation step at 72°C for 10 min.

PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Venlo, Netherlands) according to manufacturer’s instructions. Sanger sequencing reactions were performed for 4 ng of each amplicon using the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequence reactions included 28 cycles consisting in 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Products obtained from the sequence reactions were purified with the DyeEx® 2.0 Spin Kit (Qiagen) and then analyzed on the ABI PRISM ® 3100-Avant Genetic Analyzer (Applied Biosystems).

**Recombinant hormones**

*In vitro* experiments were performed by treating FSHR-overexpressing HEK293 cells by the following recombinant glycoproteins: FSH (Gonal-F; Merck KGaA, Darmstadt, Germany), LH (Luveris; Merck KGaA), hCG (Ovitrelle; Merck KGaA) and TSH (Thyrogen; Genzyme Corporation, Sanofi Aventis, Paris, France).
**Mutagenesis of FSHR cDNA**

Starting from the previously described wild-type FSHR cDNA inserted in a pTracer vector (Thermo Fisher Scientific, Waltham, MA, USA) (Casarini et al., 2016), mutant Asn106His and Ser128Tyr were created by PCR using the following primer sequences (NCBI Reference Sequence: NM_000145.3): 

**FSHR Asn106His** forward primer: 5'-GAAATTAGAATTGAAAAGGCCACAACCTGCTCTACATCAACC-3';  
**FSHR Asn106His** reverse primer: 5'-GGTTGATGTAGAGCGAGGGCCCTTTTCAATTCTAATTTC-3';  
**FSHR Ser128Tyr** forward primer: 5'-CCTCCCAACCTTCAATCTGTATAATAACACAGGTTATTAAG-3';  
**FSHR Ser128Tyr** reverse primer: 5'-CTTAATACCTGTGTATATATACGATATTGAAGGTTAGGGAAG-3'.

PCRs were performed in 1X Phusion Buffer GC (Thermo Fisher Scientific), 0.5 µM forward and reverse primers, 50 ng of template cDNA, 3% DMSO (Thermo Fisher Scientific), 200 µM dNTPs (Promega, Madison, WI, USA), 1 U of Phusion Polymerase (Thermo Fisher Scientific), in a total volume of 50 µl. Reactions were performed using the following settings: 30 s at 98°C; 30 s at 98°C, 30 s at 56°C and 9 min at 72°C, for a total of 35 cycles. PCR products were checked by 1% agarose-gel electrophoresis after 2 h at 37°C treatment with 5 U of the Dpn I restriction enzyme (Agilent Technologies, Santa Clara, CA, USA). Dpn I catalyzes the digestion of methylated DNA, as a remainder of template, non-mutated cDNA.

Mutant FSHR cDNAs inserted in the pTracer vector were amplified after thermal shock in super-competent XL1-Blue *Escherichia coli* transformation (Agilent Technologies) following the manufacturer's instruction. Transformed bacteria grown over-night in pH=7.4 Luria broth-agar plates (Sigma Aldrich), in the presence of 100 µg/ml ampicillin, at 37°C 8 h. Resistant clones were selected for growing over-night at 37°C and plasmids extracted by GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich) following the procedure indicated by the provider.

**Plasmid DNA sequencing**
Mutant FSHRs were screened by Sanger’s DNA sequencing using specific primer probes for sequence reactions (NCBI Reference Sequence: NM_000145.3). Forward *FSHR* primer: 5'-GCCCTGCTCCTGGTCTCTTT-3'; Reverse *FSHR* primer: 5'-TTGTGTATTTCTTGAATCCC-3'. Sequence reactions were performed in a total volume of 20 µl, including 3.5 pmol of forward and reverse primer, 200 ng of template, plasmid DNA, 2 µl BigDye® Terminator v1.1 (Thermo Fisher Scientific). PCRs were performed by 40 cycles with 10 s at 96°C, 15 s at 50°C and 4 min at 60°C. Products were sequenced by the ABI 3130 Genetic Analyzer (Applied Biosystems).

**HEK293 cell line and transfection protocol**

The HEK293 cell line was cultured in Petri dishes containing DMEM enriched by 10% fetal bovine serum (FBS), 100 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich Corporation), and maintained at 37°C and 5% CO₂ in an incubator. Confluent cells were separated by 0.5% trypsin and 0.2% EDTA diluted 1:10 in phosphate buffered saline (PBS) (Sigma-Aldrich Corporation).

The transfection protocol was previously described (Riccetti et al., 2017b). Briefly, 3.5x10⁴ cells were transiently transfected in 96-well plate using 100 ng of total plasmids and 0.5 µl of Metafectene PRO reagent per well (Biontex, München, Germany), following the manufacturer’s instructions. Plasmids consisted in 50 ng of mutant or wild-type FSHR-expressing vector and 50 ng of the cAMP biosensor (CAMYEL)-expressing pcDNA3.1 vector (OriGene Technologies, Rockville, MD, USA) carrying the yFP-RAPGEF3-rLuc8 fusion gene (Jiang et al., 2007).

Expression of mutant and wild-type FSHR proteins in HEK293 cells were checked by Western blotting, using a previously validated method and reagents (Casarini et al., 2014). Briefly, lysates from 3x10⁵, 48-h transiently transfected cells were subjected to SDS-page electrophoresis and transferred on nitrocellulose membrane. Membranes were incubated over-night at 4°C with a rabbit anti-FSHR (Thermo Fisher Scientific) diluted 1:500 in tris-buffered saline (TBS) (Sigma-Aldrich). The secondary antibody was a donkey anti-rabbit, horseradish peroxidase (HRP)-conjugated (1:10000 dilution in TBS; GE healthcare, Little Chalfont, UK). A rabbit anti-β-actin antibody HRP-conjugated (1:40000 dilution in TBS; Sigma-Aldrich Corporation) served as a loading control.
Signals were developed after treatment by ECL Advance Western Blotting Detection (Perkin Elmer Inc., Waltham, MA, USA), acquired using the VersaDoc system (BioRad Laboratories) and semi-quantified by the Quantity-One software (BioRad Laboratories).

Cell stimulations and Bioluminescence Resonance Energy Transfer (BRET) measurements

48-h transiently transfected cells were washed and maintained in 40 µl/well of 37°C PBS and 1 mM HEPES, in the presence or in the absence of 1 pM-1 µM FSH, LH, hCG or TSH. Cells treated by 50 µM of the adenylyl cyclase activator forskolin (Sigma-Aldrich Corporation) served as positive controls. BRET measurements were performed upon addition of 10 µl/well of 5 µM coelenterazine H (Interchim, Montluçon, France), using the CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). Donor and acceptor signals were detected at the wavelength of 480±20 and 540±20 nm, respectively, and represented as a ratio (BRET changes).

In order to extrapolate real cAMP concentrations from BRET change values, in FSH-stimulated cells, a cAMP standard curve was generated. To this purpose, HEK293 cells were transfected using the cAMP biosensor-expressing plasmid and exposed 20 min to increasing concentrations (0-10 µM range) of the analog 8-bromoadenosine 3,5-cAMP (8-br-cAMP; Sigma-Aldrich Corporation). Under these conditions, endogenous cAMP was undetectable. BRET change values were plotted on a X-Y graph, where 8-br-cAMP concentrations were on the logarithmic X-axis, while BRET change on the Y-axis. Data were interpolated using a non-linear regression, which was used for extrapolation of real cAMP concentrations.

Statistics

Analysis of cAMP dose-response data were represented as BRET changes over basal. Data were represented as mean±standard deviation (SD) in a graph with logarithmic X-axis and interpolated by non-linear regression. The 50% effective concentration (EC_50) was calculated for each dose-response curve. Departure from normality was evaluated by D’Agostino and Pearson test and comparisons between couples of EC_50 values were performed by Mann-Whitney’s U-test. Multiple comparisons between dose-response curves were performed using the two-way ANOVA and
Bonferroni post-test. Differences were considered significant for p<0.05. Statistical analysis was performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

Patient’s DNA and plasmid sequencing

DNA sequencing showed that the patient carries a heterozygous mutation in exon 4 of the FSHR gene, leading to the p.Asn106His amino acid substitution at the protein level (figure 2, panel A). Moreover, cytosine to adenine substitution, linked to the known p.Ser128Tyr mutation on exon 5 in heterozygosis, was also found (figure 2, panel B).

[FIGURE 2 A, B]

Mutant FSHRs were produced by mutagenesis and checked by DNA sequencing (figure 3). In this case, two mutant FSHRs, carrying the single mutation each, were produced starting from the wild-type receptor (FSHR p.Asn106His; FSHR p.Ser128Tyr; figure 3, panel A and B, respectively). To evaluate the additive effect of both the mutations, an additional receptor was produced (FSHR p.Asn106His+p.Ser128Tyr; figure 3, panel C).

[FIGURE 3 A, B, C]

Evaluation of mutant FSHR expression in transfected HEK293 cells

Expression levels of the wild-type and mutant FSHR proteins were evaluated by Western blot analysis in transiently transfected HEK293 cells. Untransfected HEK293 and COS-7 cells served as a negative control, while positive control was human primary granulosa cell lysates and β-actin was the normalizer (figure 4).

[FIGURE 4]

Wild-type and mutant FSHR cDNAs were similarly expressed, as a result of their insertion in front of the plasmid cytomegalovirus promoter. FSHR protein signal was detected at the level of granulosa cells lysate, while receptor expression was missing in untransfected HEK293 cells.

cAMP standard curve
A standard curve for extrapolating real cAMP values, expressed in molarity, from BRET change values was performed. To this purpose, HEK293 cells were transfected using a cAMP biosensor-expressing plasmid and exposed to nM-µM concentrations of 8-br-cAMP. Data were plotted in a X-Y graph as mean±SD (n=8) and interpolated by non-linear regression (figure 5).

[FIGURE 5]

Analysis of FSH-, LH-, hCG- and TSH-induced cAMP production

Wild-type and mutant FSHR-overexpressing HEK293 were stimulated by pM-µM concentrations of recombinant glycoprotein hormones. cAMP was measured by BRET technique, expressed as mean±SD and indicated in molarity (figure 6) after extrapolation of the concentration from the standard curve (figure 6). 50 µM forskolin was used to obtain the positive control.

FSH showed higher potency when its effect was mediated by the wild-type than by the mutant FSHRs (EC$_{50}$ wild-type FSHR=1.04±0.51 nM; p.Asn106His FSHR=4.31±0.16 nM; p.Ser128Tyr FSHR=2.02±0.13 nM; p.Asn106His+p.Ser128Tyr FSHR=10.2±0.43 nM; Mann-Whitney's U-test; p<0.05; n=6), in spite of comparable cAMP plateau levels (max cAMP level: wild-type FSHR=934.1x10$^{-9}$ ± 149.1x10$^{-9}$ M; p.Asn106His FSHR=764.3x10$^{-9}$ ± 264.6x10$^{-9}$ M; p.Ser128Tyr FSHR=633.3x10$^{-9}$ ± 264.6x10$^{-9}$ M; p.Asn106His+p.Ser128Tyr FSHR=529.3x10$^{-9}$ ± 374.4x10$^{-9}$ M; Mann-Whitney’s U-test; p≥0.05; n=6) (figure 6, panel A). In particular, the additive effect of the two mutations carried by the same receptor (biallelic homozygosity) consisted in decreasing the effectiveness of FSH in inducing cAMP rise. However, FSH effectively induced cAMP production within the 1-10 nM range, as demonstrated by EC$_{50}$s, acting through all FSHRs (figure 6, panel A).

Forskolin treatment induced activation of cAMP at levels significantly different than those of untreated cells (50 µM forskolin-induced cAMP levels in wild-type FSHR expressing HEK293 cells=88.2x10$^{-9}$ ± 23.0x10$^{-9}$ M; p.Asn106His FSHR=103.0x10$^{-9}$ ± 30.4x10$^{-9}$ M; p.Ser128Tyr FSHR=92.9x10$^{-9}$ ± 33.1x10$^{-9}$ M; p.Asn106His+p.Ser128Tyr FSHR=96.2x10$^{-9}$ ± 1.3x10$^{-9}$ M; Mann-Whitney’s U-test; p<0.05; n=6; data not shown).

[FIGURE 6]
Treatment of transfected FSHR-overexpressing HEK293 with 0-100 nM concentrations of recombinant TSH failed in inducing cAMP response (figure 6, panel B). It is worthy of note that treatment by the highest TSH concentration, 1 µM, results in weak increase of cAMP levels mediated by both FSHRs carrying the p.Asn106Hys mutation, although significantly different than the wild-type receptor-mediated response (max cAMP level: wild-type FSHR=27.4x10^{-9} ± 9.5x10^{-9} M; p.Asn106His FSHR=9.5x10^{-9} ± 9.5x10^{-9} M; p.Ser128Tyr FSHR=27.4x10^{-9} ± 9.5x10^{-9} M; p.Asn106His+p.Ser128Tyr FSHR=9.5x10^{-9} ± 9.5x10^{-9} M; Mann-Whitney’s U-test; p<0.05; n=4).

Cells were treated by equimolar concentrations of LH and hCG (figure 6, panel C and D, respectively). The two hormones act on the same receptor, LHCGR, however, hCG displays higher potency than LH on cAMP production (Casarini et al., 2012). Interestingly, although both gonadotropin treatments induced intracellular cAMP increase in p.Ser128Tyr FSHR-expressing HEK293 cells, LH displays lower EC_{50} than hCG (LH EC_{50}=26.4x10^{-9} ± 17.9x10^{-9} M; hCG EC_{50}=100.6x10^{-9} ± 32.3x10^{-9} M; Mann-Whitney’s U-test; p<0.05; n=5). However, in the same cells, higher plateau cAMP levels were achieved by hCG treatment than LH (LH-induced max cAMP levels=105.3x10^{-9} ± 19.7x10^{-9} M; hCG= 286.4x10^{-9} ± 27.4x10^{-9} M; Mann-Whitney’s U-test; p<0.05; n=5). Other FSHR mutants and the wild type mediate the achievement of lower cAMP levels than the p.Ser128Tyr FSHR (two-way ANOVA and Bonferroni post-test; p≥0.05; n=5).

Since it was unknown whether the sOHSS patient carried these FSHR mutations in the mono- or biallelic form, the cAMP response to hCG in biallelic heterozygosity was evaluated by transfecting HEK293 cells with both p.Ser128Tyr and p.Asn106Hys encoding plasmids, thus resulting in co-presence of the individual mutations. Results revealed that cAMP plateau levels achieved by treatment using the hCG µmolar concentration was similar between homozygous p.Ser128Tyr FSHR (figure 6, panel D) and biallelic heterozygous p.Ser128Tyr+p.Asn106Hys FSHR cells (figure 7; homozygous=286.4x10^{-9} ± 27.4x10^{-9} M; heterozygous=382.1x10^{-9} ± 19.7x10^{-9} M; Mann-Whitney’s U-test; p≥0.05; n=5). However, hCG resulted in about 2.8-fold lower EC_{50} in heterozygous than in homozygous cells (homozygous=100.6x10^{-9} ± 32.3x10^{-9} M; heterozygous=283.2x10^{-9} ± 28.3x10^{-9} M; Mann-Whitney’s U-test; p<0.05; n=5).

[FIGURE 7]
Since both the wild-type and the monoallelic p.Asn106His+p.Ser128Tyr FSHRs failed in mediating hCG-induced cAMP (figure 6), the effect in monoallelic heterozygous form, as a combination of these two conditions consisting in one wild-type FSHR and one FSHR allele carrying both the mutations, is reasonably expected to be the same (supplementary figure 1).

**Discussion**

In this study, we characterized single and additive effects of the missense FSHR mutations p.Asn106His and p.Ser128Tyr, that were found in a patient complaining of sOHSS. The functional characterization was carried out by treatment of transfected HEK293 cells, using recombinant gonadotropins and thyrotropin, and evaluating intracellular cAMP increase. While both the mutations appeared to be linked to decreased sensitivity of the receptor to the natural ligand FSH, the p.Ser128Tyr induced loss of specificity, resulting in LH- and hCG-induced cAMP increase. TSH did not induce significant effects, except at a relatively high concentration, 1 µM, which induced weak cAMP activation mediated by wild-type and p.Ser128Tyr mutant FSHR. LH and hCG cross-reactivity linked to the p.Ser128Tyr mutation was dampened by the presence of the amino acid histidine at position 106, reflected in lower EC\textsubscript{50} and plateau cAMP levels.

The p.Ser128Tyr amino acid change was previously associated to sOHSS (De Leener et al., 2008). This mutation falls within the exon 5 of the FSHR gene and is linked to increased affinity and sensitivity to hCG, without conferring constitutive activity to the receptor or promiscuous activation by TSH. We found this mutation in a pregnant women affected by sOHSS during the first trimester of pregnancy, when hCG achieves physiologically maximal levels (Mishell and Davajan, 1966). Taken together, these data suggest that the p.Ser128Tyr change may not be causative of FSH-dependent sOHSS development during the fertile windows of women, outside pregnancy. Interestingly, cAMP increase induced by 10-100 nM LH indicates cross-interaction with the p.Ser128Tyr mutant receptor, unlikely resulting in sOHSS risk during the midcycle LH peak, when the hormone serum concentration is about 10-100-fold lower and no significant FSHR expression occurs (Jeppesen et al., 2012). Most importantly, the LH results in about 4-fold lower EC\textsubscript{50} value than hCG, likely due to different binding affinity to the mutant receptor. Indeed, our results indicate
that tyrosine at position 128 is more selective for LH than for the structurally similar hCG. Previous data demonstrated the non-equivalence of the two hormones, revealing that hCG is about 5-fold more potent than LH in inducing cAMP production in human primary granulosa cells (Casarini et al., 2012), mouse Leydig cells (Riccetti et al., 2017a) and transfected cell lines (Riccetti et al., 2017b). These features are likely due to ligand-specific tridimensional conformations of the hormone-receptor complex (Grzesik et al., 2015), rather than different binding affinity for the natural receptor, LHCG.

The introduction of the amino acid histidine at position 106 of FSHR decreases binding affinity for the natural ligand, FSH, as well as LH, hCG and TSH cross-reactivity. These data indicate that asparagine at position 106 is important for the occurrence and specificity of FSH binding to its receptor. Moreover, these data suggest a lack of association of this novel FSHR mutation with sOHSS, since no results support hyper-responsiveness of the p.Asn106His mutant FSHR to endogenous glycoprotein hormones. On the contrary, this amino acid change may dampen the increased sensitivity to hCG, provided by the known p.Ser128Tyr FSHR mutation, when they are both carried by the same protein receptor but not in the biallelic heterozygous form.

In summary, our results suggest that, in our sOHSS patient, the two mutations occurred in biallelic heterozygous form, resulting in one allele with decreased sensitivity to glycoprotein hormones and one allele able to cross-interacts with hCG during pregnancy. These results provide an independent confirmation that p.Ser128Tyr FSHR induces hCG-induced sOHSS, while LH mid-cycle peak should not be a risk for developing the disease in women expressing the mutant receptor. Moreover, although asparagine and histidine share similar chemical properties, amino acid change at position 106 is crucial for proper FSHR functioning, suggesting that homozygous mutations falling within this region might be linked to infertility. Taken together, these findings reflect the individual-specific nature of the cell response to hormone stimulation and point out the relevance of proper pharmacogenetic approaches to be applied for clinical treatment. In this case, the evaluation of signaling activation using transfected cell line may provide data indicative of the hormonal treatment in vivo and a tool to be used for preliminary testing of the cell response associated to peculiar gonadotropin receptor genotypes.
Conclusions

We found a novel FSHR mutation, p.Asn106His, linked to decreased sensitivity to FSH, as well as a receptor carrying the known p.Ser128Tyr amino acid change, previously associated to increased affinity and sensitivity to hCG and resulting in sOHSS (De Leener et al., 2008). Interestingly, LH was about 4-fold more potent than hCG in inducing cAMP increase mediated by the p.Ser128Tyr mutant FSHR, oppositely to what observed in LHCGR expressing cells (Casarini et al., 2012). In HEK293 cells, hCG induced cAMP increase in biallelic heterozygous, but not in monoallelic or homozygous mutant FSHRs. Since p.Asn106His mutation did not result in damping of sOHSS symptoms, consistently with in vitro data, the two FSHR amino acid changes are likely in biallelic heterozygous form in our sOHSS pregnant patient. Finally, this study demonstrates that gonadotropin-induced cAMP production in vitro may be indicative of the allelic state of patients undergoing assisted reproduction, reflecting the relevance of performing genetic screening before clinical treatments in the era of personalized medicine.

Conflict of interests

The authors have no conflict of interests.

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Figure 1. Possible allelic combinations of sOHSS patient’s FSHR mutations. While no mutation occurs in the wild-type FSHR, heterozygosity may be sustained by mono- or biallelic mutations, depending on which allele they fall within. FSHR alleles are indicated by bars; red-coloured “X” = p.Asn106His mutation; green-coloured “X” = p.Ser128Tyr mutation.
Figure 2. OHSS patient's FSHR DNA sequencing. Genomic DNA was extracted from blood samples of the OHSS pregnant patient and analysed by Sanger's sequencing. Missense nucleotide changes corresponding to amino p.Asn106His and p.Ser128Tyr FSHR mutations were found. A) p.Asn106His heterozygous mutation. B) p.Ser128Tyr heterozygous mutation. Red arrows indicate nucleotide changes, as well as the “M” type, compared to the wild-type FSHR (NCBI Reference Sequence: NM_000145.3).
Figure 3. cDNA sequencing of mutant FSHRs plasmids. Vectors expressing the individual p.Asn106His or the p.Ser128Tyr FSHR mutation were produced by mutagenesis and bacterial transformation. A plasmid expressing a receptor carrying both the mutations was also produced.

FSHR cDNA mutations were confirmed in vectors by Sanger’s sequencing and reverse complementary sequences are shown for p.Asn106His FSHR samples. A) DNA sequencing of p.Asn106His FSHR-expressing plasmid. The indicated “G” nucleotide belongs to the GCG triplet, which is reverse complementary to the CAC triplet encoding the amino acid histidine. B) p.Ser128Tyr FSHR-expressing plasmid. It carries the nucleotide “A”, which forms the TAC triplet encoding the amino acid tyrosine. C) Plasmid expressing the FSHR carrying both the mutations.

Red arrows indicate nucleotide changes compared to the wild-type FSHR (NCBI Reference Sequence: NM_000145.3).
Figure 4. Analysis of FSHR expression in transfected cells. HEK293 cells were transfected using wild-type or mutant FSHR-expressing plasmids and receptor protein was detected by Western blotting. β-actin served as a loading control. Lane 1=untransfected COS-7 cells (negative control); 2=human primary granulosa cell lysates (positive control); 3=Untransfected HEK293 cells (negative control); 4=wild-type FSHR expressing HEK293; 5=p.Asn106His FSHR; 6=p.Ser128Tyr FSHR; 7=monoallelic p.Asn106His+p.Ser128Tyr FSHR. Image representative of three independent experiments.
Figure 5. cAMP standard curve. HEK293 cells were transfected using the CAMYEL cAMP biosensor-expressing plasmid and exposed 30 min to 0-1x10^{-6} M 8-br-cAMP. Induced BRET changes were measured and plotted against 8-br-cAMP concentration. Data were represented as means±SD and interpolated by non-linear regression (n=8).
Figure 6. FSH-, TSH-, LH- and hCG-induced cAMP production in wild-type and mutant FSHR expressing HEK293 cells. HEK293 cells were transfected using wild-type or mutant FSHR- and CAMYEL-expressing plasmids, then treated by pM-µM hormone concentrations. Induced BRET changes were measured and intracellular cAMP concentrations were extrapolated from the standard curve (figure 4). Data are means±SD and were interpolated by non-linear regressions. A) FSH dose-response curves (n=6). B) TSH (n=4). C) LH (n=5). D) hCG (n=5). Decreased potency of FSH in inducing cAMP are due to the presence of mutations, especially in the biallelic heterozygous form, and were evaluated by comparing EC$_{50}$s (Mann-Whitney's $U$-test; $p<0.05$).
Higher potency of LH than hCG in inducing cAMP activation, mediated by the p.Ser128Tyr FSHR, was evaluated as well (Mann-Whitney’s U-test; p<0.05). Statistical analysis, panel A: EC₅₀ wild-type FSHR=1.04±0.51 nM is significantly lower than other EC₅₀s (p.As106His FSHR=4.31±0.16 nM; p.Ser128Tyr FSHR=2.02±0.13 nM; p.As106His+p.Ser128Tyr FSHR=10.2±0.43 nM; means±SD; Mann-Whitney’s U-test; p<0.05; n=6). cAMP plateau levels mediated by the wild-type FSHR are not significantly different than others (wild-type FSHR=934.1x10⁻⁹ ± 149.1x10⁻⁹ M; p.As106His FSHR=764.3x10⁻⁹ ± 264.6x10⁻⁹ M; p.Ser128Tyr FSHR=633.3x10⁻⁹ ± 264.6x10⁻⁹ M; p.As106His+p.Ser128Tyr FSHR=529.3x10⁻⁹ ± 374.4x10⁻⁹ M; means±SD; Mann-Whitney’s U-test; p≥0.05; n=6). Statistical analysis, panel B: recombinant TSH failed in inducing cAMP response except at the concentration of 1 µM (wild-type and p.Ser128Tyr FSHRs versus p.As106Hys carriers; max cAMP level: wild-type FSHR=27.4x10⁻⁹ ± 9.5x10⁻⁹ M; p.As106His FSHR=9.5x10⁻⁹ ± 9.5x10⁻⁹ M; p.Ser128Tyr FSHR=27.4x10⁻⁹ ± 9.5x10⁻⁹ M; p.As106His+p.Ser128Tyr FSHR=9.5x10⁻⁹ ± 9.5x10⁻⁹ M; means±SD; Mann-Whitney’s U-test; p<0.05; n=4). Statistical analysis, panels C and D: LH displays lower EC₅₀ than hCG (LH EC₅₀=26.4x10⁻⁹ ± 17.9x10⁻⁹ M; hCG EC₅₀=100.6x10⁻⁹ ± 32.3x10⁻⁹ M; means±SD; Mann-Whitney’s U-test; p<0.05; n=5), as well as lower plateau cAMP levels (LH-induced max cAMP levels=105.3x10⁻⁹ ± 19.7x10⁻⁹ M; hCG= 286.4x10⁻⁹ ± 27.4x10⁻⁹ M; Mann-Whitney’s U-test; p<0.05; n=5).
Figure 7: hCG-induced cAMP production in biallelic homozygous and heterozygous mutant FSHR settings. Mutant FSHRs- and CAMYEL-expressing HEK293 were treated by pM-µM hCG concentrations and cAMP levels calculated stating from induced BRET change values. Data were represented as means±SD and hCG EC50s, as well as cAMP plateau levels were compared. hCG µmolar concentration induced similar cAMP plateau levels between homozygous p.Ser128Tyr FSHR (figure 6, panel D) and biallelic heterozygous p.Ser128Tyr+p.Asn106Hys FSHR cells (homozygous=286.4x10^{-9} ± 27.4x10^{-9} M; heterozygous=382.1x10^{-9} ± 19.7x10^{-9} M; means±SD; Mann-Whitney’s U-test; p≥0.05; n=5). hCG displayed lower EC50 in heterozygous than in homozygous cells (homozygous=100.6x10^{-9} ± 32.3x10^{-9} M; heterozygous=283.2x10^{-9} ± 28.3x10^{-9} M; means±SD; Mann-Whitney’s U-test; p<0.05; n=5).