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Identification and Characterization of Novel FSHR Copy Number Variations Causing Premature Ovarian Insufficiency

Anna Lokchine ^{1 2}, Anne Bergougnoux ^{3 4}, Nadège Servant ³, Linda Akloul ⁵, Erika Launay ², Laura Mary ², Laurence Cluzeau ², Mathieu Philippe ², Mathilde Domin-Bernhard ⁶, Solène Duros ⁶, Sylvie Odent ^{5 7}, Elena Tucker ^{8 9}, Françoise Paris ^{3 10 11}, Marc-Antoine Belaud-Rotureau ^{1 2}, Sylvie Jaillard ^{1 2}

¹ Univ Rennes, CHU Rennes, Inserm, EHESP, Irset (Institut de Recherche en Santé, Environnement et Travail)-UMR_S1085, Rennes, France.

² CHU Rennes, Service de Cytogénétique et Biologie Cellulaire, Rennes, France.

³ Service de Génétique Moléculaire et de Cytogénomique, CHU de Montpellier, Montpellier, France.

⁴ PhyMedExp, Université de Montpellier, INSERM U1046, CNRS UMR 9214, Montpellier, France.

⁵ CHU Rennes, Service de Génétique Clinique, CLAD Ouest, Rennes, France.

⁶ CHU Rennes, Département de Gynécologie Obstétrique et Reproduction Humaine, Rennes, France.

⁷ Institut de Génétique et Développement de Rennes, IGDR UMR 6290 CNRS, University of Rennes, Rennes, France.

⁸ Reproductive Development, Murdoch Children's Research Institute, Melbourne, Victoria, Australia.

⁹ Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia.

¹⁰ Unité d'Endocrinologie et Gynécologie Pédiatriques, Service de Pédiatrie I, Hôpital A. de Villeneuve, Montpellier, France.

¹¹ Développement Embryonnaire, Fertilité et Environnement, Université de Montpellier, INSERM, Montpellier, France.

Keywords:

FSHR; copy number variation; infertility; premature ovarian insufficiency.

Abstract

Follicle stimulating hormone (FSH) is a key pituitary gonadotropic hormone implicated in human fertility and is crucial for folliculogenesis and recruitment of new antral follicles. Variations in its receptor, FSHR, can lead to diverse reproductive phenotypes including ovarian hyperstimulation syndrome (OHSS) and premature ovarian insufficiency (POI). This study reports a novel case of FSHR-related ovarian insufficiency in a patient with primary amenorrhea, subnormal AMH levels, and delayed puberty. Genetic exploration revealed two compound heterozygous intragenic deletions of FSHR. Specifically, the patient inherited a maternally derived deletion spanning exons 5-10 and a paternally derived deletion involving exons 3-6. Through chromosomal microarray analysis (CMA), exome sequencing, long-range PCR, and Sanger sequencing, we characterized the breakpoints and confirmed the compound heterozygous deletions. The findings reveal a complete loss of function of both FSHR alleles, contributing to the patient's POI phenotype. This case emphasizes the complexity of genotype-phenotype correlations in FSHR-related disorders and the role of CNVs in POI phenotypes. Although these events are rare, our results advocate for the inclusion of CNV detection in the diagnostic workup of POI to ensure accurate diagnosis and better patient management.

1 | Introduction

Follicle stimulating hormone (FSH) is a key pituitary gonadotropic hormone implicated in human fertility. Upon binding to its receptor (FSHR), localized on the granulosa cells of the ovaries, FSH induces a conformational change in FSHR, which stimulates estradiol production, induction of luteinizing hormone (LH) receptor expression and recruitment of new antral follicles in the early follicular phase (Casarini and Crépieux 2019). FSHR is a glycoprotein hormone receptor (GPH) with a typical GPH structure, consisting of a large N-terminal extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular C-terminal portion (C- tail) (Jiang, Dias, and He 2014). The last and largest exon (exon 10) encodes the C-terminal part of the ECD hinge region, the TMD and the C-tail (Jiang et al. 2012). The rest of the exons (exons 1–9) encode the ECD, including a segment of the hinge region, pivotal in ligand recognition, binding of both FSH α - and β -subunits, receptor activation, and signal transduction. Activating FSHR heterozygous pathogenic variants, conferring higher responsiveness to FSH, cause ovarian hyper stimulation syndrome (OHSS) (Vasseur et al. 2003). Inactivating pathogenic variants, on the other hand, reduce FSHR function and are associated with autosomal recessive premature ovarian insufficiency (POI). Since the first report of a homozygous inactivating variant in 1995 (Aittomäki et al. 1995), over twenty inactivating homozygous or compound heterozygous single-nucleotide variants (SNVs) have been described (Desai, Roy, and Mahale 2013; Sassi et al. 2020; Yoo et al. 2023).

Copy number variations (CNVs) involving FSHR are however scarcely reported in literature. Moreover, phenotype to genotype correlation of CNVs is less straightforward as in vitro evaluation of FSHR activity is unavailable for larger deletion/insertion events. Based on a cohort of patients with ovarian deficiency, we describe a compound heterozygous POI patient with two partially overlapping FSHR intragenic deletions and discuss the implication of CNVs in female infertility.

2 | Patients, Material and Method

2.1 | POI Cohort

The patient was recruited as part of an ongoing cohort of patients with non-syndromic ovarian deficiency, POI, or diminished ovarian reserve (DOR), which are likely manifestations along a continuum of ovarian deficiency. Samples from 243 patients were analyzed after clinical examination from 2017 to 2024. The diagnosis of ovarian deficiency was established when a woman aged under 40 years had, for POI, elevated FSH (≥ 25 IU/L) and irregular menses or amenorrhea, and, for DOR, low AMH and/or low antral follicular count (AFC) after ultrasonographic assessment, according to age-based thresholds adapted from the literature data (Cohen, Chabbert-Buffet, and Darai 2015). All patients had a 46, XX karyotype, were negative for FMR1 premutation and 21-OH-antibodies, and benefited from additional analysis including microarray and exome sequencing. The patient's parents, both healthy and without relevant clinical history, were also analyzed after a genetic consultation related to their daughter's diagnosis. Written informed consent was obtained from the parents and the patient, in accordance with ethical standards of the Ethics Committee of Rennes University Hospital and the French law.

2.1.1 | Chromosomal Microarray Analysis (CMA)

Chromosomal microarray analysis was performed using the Agilent Human Genome CGH microarray custom 180 K (Agilent Technologies, Santa Clara, CA, USA) according to the protocol provided by the manufacturer. This custom array is enriched in 130 genes involved in ovarian function, including known POI genes (list of genes available on demand). This enrichment notably targets FSHR, with 39 probes covering the gene (instead of 15 probes for a classic 4×180 Agilent microarray). Identification of the probes with a significant gain or loss was based on the log₂ ratio plot deviation from 0 with cut-off values of 0.58 and -1 , for duplications and deletions, respectively. The Database of Genomic Variants (DGV) (<http://dgv.tcag.ca/dgv/app/home>) was used as a control population to identify polymorphic CNVs. The CNVs are described using the version GRCh37 of the human genome (hg19).

2.2 | Exome Sequencing (ES)

Genomic DNA underwent exome capture using an Agilent SureSelect XT Human All Exon V7 kit (Agilent Technologies Inc., Santa Clara, CA, USA), and sequenced on a NextSeq550 instrument (Illumina, San Diego, CA, USA). WES data were processed using C-GeVarA pipeline (Constitutional Genetic Variant Analysis, Bioinformatics Department, Rennes University Hospital) as previously described (Cospain et al. 2022). We performed two phases of analysis—the first focused on gene priority and the second focused on variant priority, as previously described (Tucker et al. 2016).

2.3 | Long-Range PCR and Sanger Sequencing

Long-range PCR primers were designed to frame both maternal and paternal deletion. We designed primers specific for the last regions known to be present before and after the deletion (telomeric and centromeric sides). Primers for maternal deletion were located in intron 5 (FSHR-int5-LR-F) and in the downstream region of FSHR (FSHR-downstream-LR-R). Primers for paternal deletion were located in intron 2 (FSHR-int2-F-LR2) and exon 8 (FSHR-Ex8-R-LR). PCR mix and reaction were performed using the Expand Long Range PCR kit (Roche Diagnostics GmbH, Mannheim, Germany). Migration of PCR products was performed on 2% agarose gel at 100 V for four hours. The detection of a PCR product affirmed the presence of the targeted regions. Sanger sequencing of the PCR product was then performed using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA). Primer sequences are available upon request.

2.4 | Quantitative Multiplex PCR of Short Fragments (QMPSF)

QMPSF was performed for exons 2 and 9, two regions of exon 10, and downstream region of FSHR, using dye-labeled primers with universal 5' FAM extensions. After PCR, amplicons were size differentiated by capillary electrophoresis using a 3500xL or Dx genetic analyzer (Applied Biosystems, Foster City, CA, USA). Data were analyzed using GeneMapper software (Applied Biosystems, Foster City, CA, USA). The areas of the peaks corresponding to the regions to be explored were compared between patient's sample and parental DNA.

Quantitative changes were detected by an increase or decrease in the areas of the corresponding fluorescent peaks. Primer sequences are available upon request.

3 | Results

Among the 243 patients, 17 exhibited one or more CNVs of interest (Table S1). These CNVs were selected due to their very low frequency in the general population, their size, gene content, or chromosomal mechanism. Two pathogenic CNVs were identified, in SYCE1 and FSHR. SYCE1 homozygous deletions are well described events (Zhe et al. 2020), and focus was directed towards the description of the FSHR CNVs.

3.1 | Patient Clinical and Biological Data

The patient is a 32-year-old woman with previously unexplored primary amenorrhea. Gynecological exploration revealed a T-shaped uterus (Grimbizis et al. 2013), small ovaries (right ovary: 25 mm on the major axis, left ovary: 20 mm on the major axis), with an AFC equal to 5, breast hypoplasia (Tanner S3), and hirsutism. Additional clinical findings include elevated body mass index (BMI = 37) and osteoporosis. Recent biological explorations found repeatedly elevated FSH (56.8 and 33.6 IU/L with > 4 months between measurement) and LH (26.6 IU/L). AMH (0.9 ng/mL) and estradiol (18 pg/mL) were decreased. Testosterone levels were normal (0.99 nmol/L) and thyroid function. FMR1 screening was in the intermediate range (51 CGG). The patient was born from a non-consanguineous union. Both the patient's sister and brother were able to conceive without difficulties. Her mother reports a normal age of menopause, and there was no other familial occurrence of POI.

3.2 | Genetic Analysis

CMA identified a large heterozygous deletion at 2p16.3 encompassing FSHR (MANE selected transcript NM_000145.4), with a central homozygous region, suggesting two overlapping deletions in trans. Familial testing confirmed this hypothesis, unveiling two separate deletions, inherited from each parent. On the one hand, the patient inherited a maternal interstitial heterozygous deletion spanning at least 29.9 kb of the 2p16.3 locus

(Figure 1A). This deletion involved exons 5–9 of FSHR. The last deleted probe targeted exon 9 and the first non-deleted probe was located outside of FSHR. On the other hand, there was a paternal interstitial deletion of at least 36.8 kb spanning exons 3–6. Hence, the patient carries a compound heterozygous deletion with an overlap in exons 5 and 6, leading to the homozygous loss of both exons on the array profile. ES did not find any SNV compatible with the phenotype. Reads were manually visualized using Integrative Genomic Viewer (IGV) and demonstrated the absence of reads mapping to exon 5 and 6.

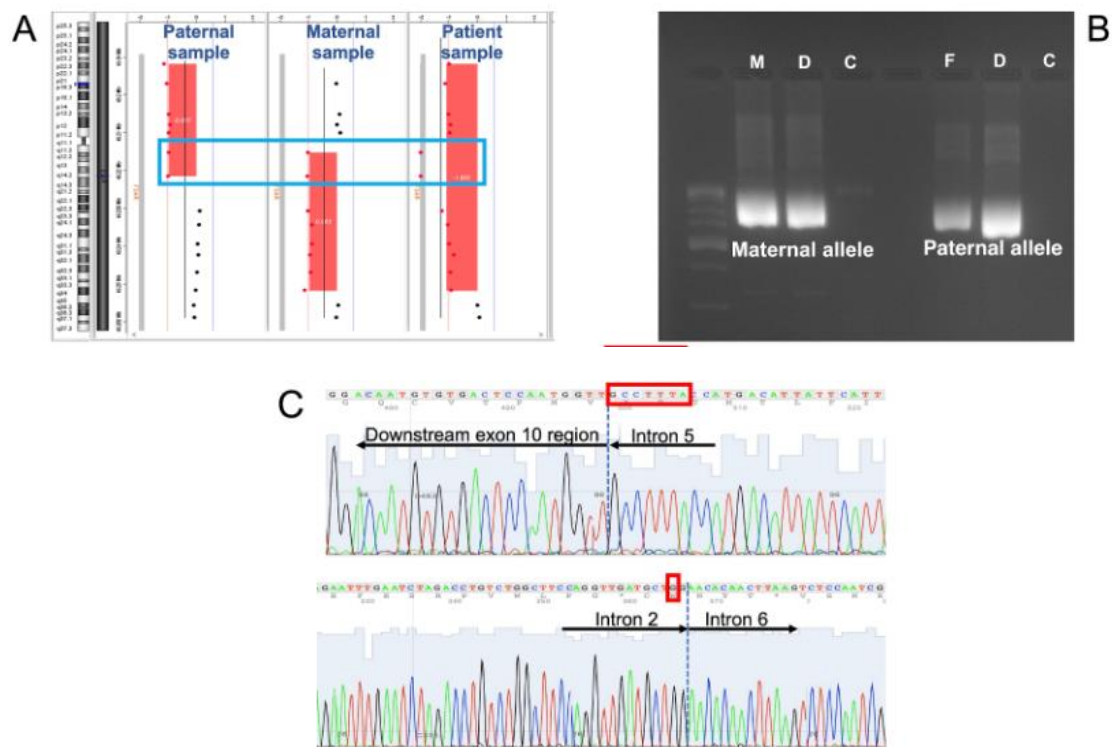


FIGURE 1 | A: CMA analysis (hg19) of the trio, focusing on the deletion. Profiles are shown for the father (left), mother (middle), and patient (right). The blue box indicates the region of homozygosity in the patient, due to overlapping heterozygous deletions from both parents. B: Long-range PCR sequencing for the mother, father, and patient. Primers were positioned in intron 2—exon 8 for the paternal allele and in intron 5—exon 10 for the maternal allele. PCR amplification products were detected in the father (F) and patient (daughter, D) for the paternal allele, and in the mother (M) and patient (daughter, D) for the maternal allele, confirming the presence of the deletion. No amplification occurred in the control sample (C). C: Sanger sequencing of the breakpoint regions in the parental alleles. The top panel shows the maternal allele and the bottom panel shows the paternal allele. Red lines frame the sequences indicating additional complexity at the breakpoint. The GCCTTTA motif, normally present in intron 5 and exon 10, has one copy deleted at the breakpoint. An additional guanine was found at the paternal breakpoint.

3.3 | Characterization of the Deletion Breakpoints

Delineation of the deletions was limited by CMA approach due to probe coordinates. To address this, we first performed long-range PCR (Figure 1B). The primers were located respectively, in intron 5 and in the downstream region of exon 10 to frame the maternal deletion, and in intron 2 and exon 8 for the paternal deletion. In both cases, PCR products were detected by migration on agarose, confirming the presence of both deletions and the absence of exon 10 on the maternal allele. Subsequent Sanger sequencing of both maternal and paternal PCR products (Figure 1C) allowed precise breakpoint mapping of both deletions

and thus the determination of their accurate size: 41388 bp for the maternal and 41,881 bp for the paternal deletions, with no modified breakpoints (BPs) between parents and patient. Additional base pairs were uncovered at BPs: A seven-base pair deletion for the maternal allele, corresponding to a GCCTTTA motif normally located in both exon 10 and intron 5, and a guanine addition for the paternal allele. QMPSF confirmed the heterozygous loss of exons 9 and 10, and downstream region of FSHR exon 10 of the maternal allele, while these regions were present twice as much in the paternal allele (Figure S1). Several repetitive elements are located at BPs, summarized in Table S2.

Transcript analysis was not possible because of non-availability of tissue expressing FSHR. Even if the recognition of a new coding sequence near the BPs could not be excluded, predictions using Alamut Visual did not show any favorable splicing environment on the sides of the BPs, both for the maternal or paternal variant allele. Therefore, maternal deletion is predicted to involve most of the protein as it spans the last 6 exons, from 5 to 10, resulting in a predicted amino-acid chain of only 124 residues (out of 695). The predicted loss of more than 10% of the protein (Abou Tayoun et al. 2018), conducive with high protein instability, combined with the biological importance of exon 10 (encoding the hinge domain, TMD and ICD) are strong arguments to suggest that the maternal deletion leads to complete inactivation of FSHR. The paternal deletion spans exons 3, 4, 5, and 6 and is predicted to encode a shorter in-frame protein lacking 100 amino acids, p.(Ile75_Leu175del).

4 | Discussion

In this manuscript, we present the first report of a patient with POI carrying two trans overlapping heterozygous intragenic deletions of FSHR, leading of biallelic absence of exon 5 and 6. The patient is additionally a carrier for an intermediate range FMR1 allele (51/30 CGG) inherited from her healthy mother. Although its impact cannot completely be excluded, recent works indicate the low significance of 45–54 repeats in reproductive phenotypes (Allen et al. 2021).

4.1 | FSHR Loss of Function (LoF)

FSHR LoF is associated with adverse reproductive phenotypes. Most of LoF FSHR variants are SNVs, while CNVs are very rare. To date, over 20 SNVs inactivating FSHR variants have

been identified, in all exons but exon 3 (Sassi et al. 2020; Ulloa- Aguirre et al. 2013). Inactivating biallelic variants affect quality or quantity of FSHR expressed at the plasmatic membrane and stop follicle maturation beyond the pre-antral stage (Oduwole, Huhtaniemi, and Misrahi 2021). Adequate FSHR protein expression indeed requires proper intracellular trafficking from the endoplasmic reticulum to the Golgi apparatus, N-linked glycosylation as well as the cleavage of the first 17 amino acids forming the signal sequence. The absence of proper FSHR signaling is associated with POI and is sometimes referred to as resistant ovary syndrome (ROS). The term ROS highlights the presence of low to normal AMH levels during clinical evaluation, in contrast to total follicular depletion observed in other cases of POI. Theoretically, FSHR *in vivo* activity correlates with phenotypic expression: Complete loss of FSHR function 4 of 7 American Journal of Medical Genetics Part A, 2024 is associated with primary amenorrhea and pubertal delay; whereas residual activity is associated with a less severe phenotype, although infertility remains constant (He et al. 2019; Katari et al. 2015; Liu et al. 2017). Overall, most patients exhibit primary amenorrhea or early secondary amenorrhea before the age of 20. Some variations have been observed regarding the development of secondary sexual characteristics, possibly due to differential timing of estrogenic impregnation, as well as AMH level and AFC, regardless of the degree of FSHR inactivation.

To evaluate FSHR *in vivo* bioactivity, *in vitro* functional studies of FSHR expression, cell surface localization and cAMP production have been developed. Nevertheless, the correlation between the phenotype and the level of *in vitro* FSHR bioactivity has not been substantially observed (Tucker et al. 2016), due to the fact that *in vitro* assays cannot exactly replicate *in vivo* conditions. Furthermore, *in vitro* assays are unable to adequately model CNVs and structural variants (SV) (Fowler and Rehm 2024). Hence, in the present case, variant pathogenicity was only assessed *in silico*. The maternal deletion is considered severe, because it probably leads to a transcript lacking the five last exons, including exon 10, which encodes the hinge domain, TMD and ICD. This putative shorten transcript likely leads to the complete absence of a maternal contribution of FSHR protein in the patient ovarian cells. The paternal deletion is predicted to be in-frame; therefore, the complete LoF of this allele cannot be directly assumed. Nonetheless, several of the deleted residues are highly conserved and required for interaction with FSHR through binding to the α -subunit, β -subunit or both subunits (Ulloa-Aguirre et al. 2018). Residue 160, included in the deletion, is crucial for the receptor membrane trafficking; a p.(Ile160Thr) missense variant was reported in a case of early secondary amenorrhea and was associated with the absence of cell surface expression, with dramatic decrease in FSH binding and cAMP production in COS-7 transfected cells (Beau et al. 1998). Taken together, genetic analysis as well as clinical and biological elements are conducive with a complete LoF of both FSHR alleles carrying these novel intragenic CNVs.

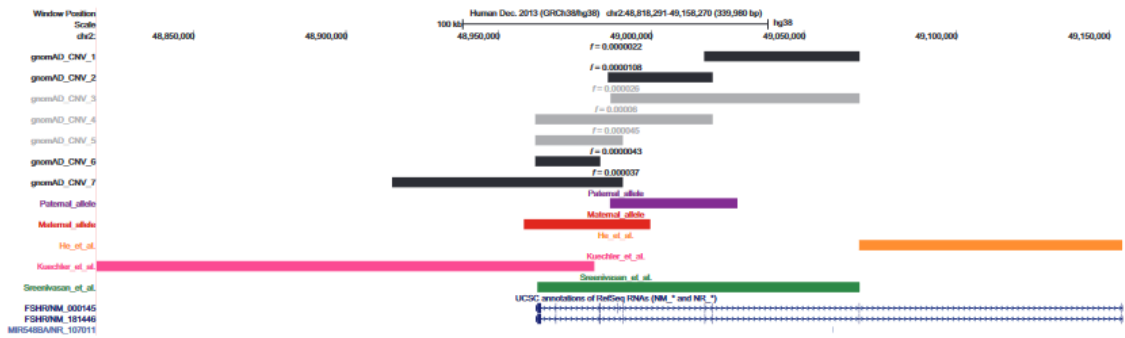


FIGURE 2 | Schematic representation of the FSHR gene structure and associated copy number variations (CNVs), adapted from the UCSC Genome Browser. This figure displays the exon/intron structure of the FSHR gene (in blue) alongside previously reported CNVs associated with primary ovarian insufficiency (POI). In gray: CNV polymorphisms from gnomAD CNV v4 are represented, with deletions shown in light gray and duplications in dark gray. The frequency (f) of these variations in the general population is indicated. In color: CNVs from the patient, as well as those previously described in the literature, are shown. Patient CNV coordinates were determined via Sanger sequencing. Exome analysis results are sourced from Sreenivasan et al. while breakpoints are based on FISH analysis from Kuechler et al. (with intervals shown). CNV coordinates for He et al. were unavailable. Coordinates are in the hg38 reference genome.

4.2 | CNVs in FSHR Gene

To our knowledge, only three CNVs involving FSHR and associated with POI have been reported in literature. These occurred either in a homozygous (Sreenivasan et al. 2022) or as compound heterozygous state (Figure 2) (He et al. 2019; Kuechler et al. 2010). Sreenivasan et al. reported a case of ROS with conserved AMH level (FSH 100 IU/L, AMH 1.4 ng/mL), primary amenorrhea and delayed puberty, associated with a homozygous FSHR CNV spanning 105 kb (hg19, chr2:49189845–49,295,452), and including exons 2–10 (Sreenivasan et al. 2022). He et al. reported FSHR compound heterozygous variants, c.44G > A p.(Gly15Asp) and heterozygous deletion of exons 1–2 (He et al. 2019) in a patient with a ROS phenotype, with only one cycle of menarche aged 13 and normal breast development. Basal endocrine testing revealed elevated FSH levels (57.3 IU/L), age-appropriate AMH level, and an AFC at 8. Finally, Kuechler et al. reported a patient with a paternally inherited c.1760C > A, p.(Pro587His) and a maternally inherited t (2;8) translocation (Kuechler et al. 2010). Initially appearing as balanced on karyotype analysis, microarray analysis later revealed a 162.7 kb deletion, spanning exons 9 and 10 of FSHR. Structural variants arise from different mechanisms including DNA recombination, replication, and repair-associated processes. These mechanisms are influenced by genomic architecture, such as regions of homology, repetitive elements, replication rate, or GC content (Carballo and Lupski 2016). FSHR CNVs show no breakpoint recurrency nor segmental duplications implication in reported CNVs (Figure 2), excluding non-allelic homologous recombination (NAHR) (Miyazaki et al. 2009; Verdin et al. 2013; Yatsenko et al. 2009). Other repetitive elements can also predispose to CNV formation by proving homology sequences forming non B-DNA conformation and overall genomic instability (Konkel and Batzer 2010). In this case, the proximal maternal BP interrupts a hAT Charlie DNA transposon, and distal BP is located 39 pb from (TTCCTA)_n/(TCTTTTC)_n simple repeats. Sequence alignment demonstrates a 7-

base pair TAAAGGC microhomology with both regions (Figure S2, Table S2). Microhomology mediated end joining (MMEJ) seems to be the most likely mechanism although a replicative mechanism such as fork stalling and template switching and microhomology-mediated break-induced replication (MMBIR) cannot be excluded (Schluth-Bolard et al. 2019). Insertion of guanine can be observed at the proximal paternal BP. The latter is characterized by a nearby T-stretch, while the distal breakpoint features a MIRb SINE. Although no clear mechanism can be identified, non-homologous end joining (NHEJ) or a replicative mechanism are the most plausible for the paternal allele (Schluth-Bolard et al. 2013).

4.3 | CNVs in POI

In contrast to the genetics of intellectual disability, CNVs appear to be infrequently documented as pathogenic in POI (Bestetti et al. 2019; Jaillard et al. 2016; Tšuiiko et al. 2016). In previous studies on POI and CNVs, candidate CNVs have generally not been functionally validated, and no follow-up studies were conducted after their initial publication (Ledig, Röpke, and Wieacker 2010). The improvement of CNV classification (Brandt et al. 2020) allows us today to reconsider some of the previously published candidates as polymorphisms (Ledig, Röpke, and Wieacker 2010; Norling et al. 2014). Most of the reported pathogenic CNVs were small intragenic events located in well-established candidate genes such as TP63 (Bestetti et al. 2019). Only a few recurring CNVs have been described in POI. SYCE1 (OMIM*611486), a crucial component of the synaptonemal complex, have been implicated in both female and male infertility (Adams and Davies 2023). SYCE1 heterozygous deletions are reported with a relatively high frequency in general population (> 0.1%) and are classified as polymorphism. The presence of a second hit (deletion or pathogenic SNV) can lead to pathological outcomes, POI or non-obstructive azoospermia (Wyrwoll et al. 2022; Zhe et al. 2020). Thus, polymorphism filtration should be performed carefully to avoid misleading classification of SYCE1 deletions. Another recurrent event mediated by NAHR is the 15q25.2 heterozygous deletion (Hyon et al. 2016), with CPEB1 (OMIM*607342) considered as the main candidate gene. The minimal critical region was recently reduced (Chen et al. 2020), suggesting that BCN1 (OMIM*601930) could be responsible for the phenotype, as heterozygous LoF variants in BNC1 have been reported in POI (Zhang et al. 2018). 6 of 7 American Journal of Medical Genetics Part A, 2024

5 | Conclusion

Although CNVs are rare in POI, they have been described in several genes associated with the monogenic inheritance of the condition. In this study, we report a novel case of FSHR-related ovarian insufficiency, caused by compound heterozygous intragenic deletions of FSHR. Our study underlines the importance of FSHR CNVs in the genetic diagnosis of POI patients and suggests that CNVs detection should not be overlooked, regardless of the method used (microarray or high-throughput sequencing).

Author Contributions

Conceptualization: A.L., A.B., and S.J. Methodology: A.L., A.B., S.J., N.S., and L.C. Formal analysis: A.L., A.B., E.L., L.M., and S.J. Resources: L.A., M.D.-B., S.D., S.O., and F.P. Investigation: N.S., L.C., and M.P. Writing – original draft: A.L., A.B., and S.J. Writing – review and editing: E.T., and M.-A.B.-R.; Supervision: A.B., M.-A.B.-R., and S.J.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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