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Diverse genetic causes of amenorrhea in an ethnically homogeneous cohort and an evolving approach to diagnosis

Shabnam Bakhshalizadeh [a,b], Fateme Afkhami [c], Katrina M. Bell [d], Gorjana Robevska [a], Jocelyn van den Bergen [a], Sara Cronin [e], Sylvie Jaillard [f,g], Katie L. Ayers [a,b], Pramod Kumar [h], Christian Siebold [h], Zhangping Xiao [i], Edward W. Tate [i], Shahla Danaei [j], Laya Farzadi [j], Shirin Shahbazi [c], Andrew H. Sinclair [a,b,1], Elena J. Tucker [a,b,*,1]

[a] *Murdoch Children's Research Institute, Melbourne, Australia*
[b] *Department of Paediatrics, University of Melbourne, Melbourne, Australia*
[c] *Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[d] *Department of Bioinformatics, Murdoch Children's Research Institute, Melbourne, Australia*
[e] *Cyto-Molecular Diagnostic Research Laboratory, Victorian Clinical Genetics Services and Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, 3052, Victoria, Australia*
[f] *Univ Rennes, CHU Rennes, INSERM, EHESP, IRSET (Institut de Recherche en Santé, Environnement et Travail), UMR_S 1085, F-35000, Rennes, France*
[g] *CHU Rennes, Service de Cytogénétique et Biologie Cellulaire, F-35033, Rennes, France*
[h] *Division of Structural Biology, Wellcome Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, UK*
[i] *Department of Chemistry, Imperial College London, 82 Wood Lane, London, W12 0BZ, UK*
[j] *Department of Obstetrics and Gynecology, Tabriz University of Medical Sciences, Tabriz, Iran*

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ABSTRACT

Research question: Premature ovarian insufficiency (POI) is characterised by amenorrhea associated with elevated follicle stimulating hormone (FSH) under the age of 40 years and affects 1–3.7% women. Genetic factors explain 20–30% of POI cases, but most causes remain unknown despite genomic advancements.
Design: We used whole exome sequencing (WES) in four Iranian families, validated variants via Sanger sequencing, and conducted the Acyl-cLIP assay to measure HHAT enzyme activity.
Results: Despite ethnic homogeneity, WES revealed diverse genetic causes, including a novel homozygous nonsense variant in *SYCP2L*, impacting synaptonemal complex (SC) assembly, in the first family. Interestingly, the second family had two independent causes for amenorrhea – the mother had POI due to a novel homozygous loss-of-function variant in *FANCM* (required for chromosomal stability) and her daughter had primary amenorrhea due to a novel homozygous *GNRHR* (required for gonadotropic signalling) frameshift variant. WES analysis also provided cytogenetic insights. WES revealed one individual was in fact 46, XY and had a novel homozygous missense variant of uncertain significance in *HHAT*, potentially responsible for complete sex reversal although functional assays did not support impaired HHAT activity. In the remaining individual, WES indicated likely mosaic Turners with the majority of X chromosome variants having an allelic balance of ~85% or ~15%. Microarray validated the individual had 90% 45,XO.
Conclusions: This study demonstrates the diverse causes of amenorrhea in a small, isolated ethnic cohort highlighting how a genetic cause in one individual may not clarify familial cases. We propose that, in time, genomic sequencing may become a single universal test required for the diagnosis of infertility conditions such as POI.

* Corresponding author. Murdoch Children's Research Institute The University of Melbourne The Royal Children's Hospital, 50 Flemington Road, Melbourne, Victoria, 3052, Australia.
E-mail address: elena.tucker@mcri.edu.au (E.J. Tucker).
[1] Co-senior authors of the study.

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1. Key message

Genetic cause of POI, characterised by amenorrhea and elevated gonadotropins in women under 40 years, largely eludes identification. Despite ethnic homogeneity, whole exome sequencing reveals diverse genetic causes of amenorrhea. Our study highlights the complexity of amenorrhea's genetic landscape, suggesting genomic sequencing as a universal diagnostic tool for infertility conditions.

2. Introduction

Premature ovarian insufficiency is a leading cause of infertility in women associated with elevated gonadotropins reflecting impairment of ovarian development or function (European Society for Human et al., 2016). Global prevalence of POI is 3.7% as reported by a recent meta-analysis (Golezar et al., 2019).

Based on current American and European established guidelines, the diagnosis of POI is confirmed in women <40 years through elevated serum gonadotropin measurement on two sequential occasions at least 4 weeks apart (follicle stimulating hormone (FSH) > 25 IU/ml), with concomitant low oestradiol (E2) levels (<50 pg/ml), and amenorrhea or oligomenorrhea for 4–6 months (European Society for Human et al., 2016, Hewitt and Gerancher 2018).

Recently the known genetic spectrum of POI has broadly expanded, however most cases remain without a clarified genetic cause (Table 1). Identifying POI aetiology has implications for the management of symptoms and/or accompanying conditions. Single gene variants may have pleiotropic effects and cause Mendelian disorders such as Fragile X syndrome, mitochondrial disorders (e.g. Perrault syndrome), and other multi-organ syndromes (e.g. cerebellar ataxia) (Tucker et al., 2016).

The most frequent genetic cause of POI is arguably premutation of the CAG triplet repeat in the 5' untranslated region of the *fragile-X messenger ribonucleoprotein (FMR1)* gene (Wittenberger et al., 2007; Sullivan et al., 2011). Given that the incidence of *FMR1* premutation is 2–5% in sporadic POI cases and 11.5–16% in familial POI (Murray et al., 2014, Turkyilmaz et al., 2022), *FMR1* premutation testing is indicated in POI women according to European Society of Human Reproduction and Embryology (ESHRE) Guidelines (European Society for Human et al., 2016).

Abnormalities in the X chromosome are another entrenched genetic cause of POI, and their frequency is approximately 12–14% (Janse et al., 2010; Qin et al., 2012), however, they are more frequent in patients with primary amenorrhea or familial POI (Rebar et al., 1982). Turner syndrome (TS), with incidence of 1 in 2500–3000 females (Sybert and McCauley 2004), is due to complete or partial loss of one X chromosome (Ford et al., 1959) and typically manifests as short stature, short neck, cardiovascular disorders, kidney malformations as well as ovarian dysgenesis and/or loss of ovarian reserve before puberty (Zinn et al., 1993). While 45, X is the most common cytogenetic cause of primary amenorrhea, individuals with mosaic 45, X/46, XX often have a milder phenotype of secondary amenorrhea (Fechner et al., 2006; Barros et al., 2020). Given chromosomal aberrations are a well-established cause of POI, cytogenetic analysis is recommended in patient diagnosis (European Society for Human et al., 2016). Traditionally this was done via high-resolution banding, but subsequently by fluorescent in situ hybridization (FISH). More recently, comparative genomic hybridization (CGH) array is the preferred approach for cytogenetic analysis due to its higher resolution and cost-effectiveness (Barros et al., 2020). CGH arrays currently identify a genetic aetiology in ~10% of POI patients (Qin et al., 2015; Jaillard et al., 2016; Franca et al., 2022).

The genetic technologies employed to identify the pathogenic variants causing POI have evolved significantly over time (Table 2). Up to the early 2000s, monogenic causes of POI were established predominantly using linkage analysis of familial cases or by single gene sequencing of candidate genes based on studies in animal models (Laissue 2018).

In the last decade, significant advances in next generation sequencing (NGS) technologies have enabled breakthrough in the discovery of the genetic cause of POI, due to the capability of sequencing numerous genomic regions simultaneously at competitive costs. NGS has revolutionised medical genomics research, identifying many new genes and variants related to hundreds of monogenic and heterogenous complex diseases including female infertility disorders such as POI (Qin et al., 2015; Tucker et al., 2016).

To date, there have been over 100 genes in which variants have been described in association with POI and amenorrhea (Supplementary File S1). These genes are implicated in diverse processes such as ovarian development, chromosome stability (for example, *FANCM*, required for DNA damage repair), meiosis (including *SYCP2L* required for synaptonemal complex formation for alignment of chromosomes during cell division), and hormone signalling (such as *GNHRH* required for regulating the reproductive axis) (Chapman et al., 2015; Qin et al., 2015; Tucker et al., 2016). Although NGS has accelerated the rate of identifying causative genes associated with disease, the analysis of NGS data can be challenging, and functional validation is imperative to accurate

Table 1
Genetic causes of POI.

Genetic cause	Frequency	References
Chromosomal aberrations	10–14%	(Janse et al., 2010; Qin et al., 2012; Qin et al., 2015; Jaillard et al., 2016)
FMR1 premutation	2–5% sporadic POI 11.5–16% familial POI	(Murray et al., 2014, Turkyilmaz et al., 2022)
Monogenic causes[a]	50% familial POI 16–29% POI	(Liu et al., 2020; Heddar and Misrahi 2022, Rouen et al., 2022; Vogt et al., 2022)

[a] The varied percentage attributed to monogenic causes reflects the stringency of criteria used for assigning pathogenicity. Often ACMG criteria are inappropriately applied in a research context, leading to overstatement of diagnostic yields.

Table 2
Timeline of changing genomic technologies for identifying genetic causing of POI.

Genomic technologies	Timeline	Gene discovery examples	Reference
Conventional karyotyping	1959	X chromosome defects	(Ford et al., 1959)
Fluorescence in situ hybridization (FISH)	1994, 1998		(Powell et al., 1994; Devi et al., 1998)
Direct sequencing of PCR products	1995, 1996, 1998, 1999, 2000	*FSHR, LHR*	(Aittomäki et al., 1995; Latronico et al., 1996; Beau et al., 1998; Touraine et al., 1999; Shelling et al., 2000)
Comparative genomic hybridization (CGH) array	2008, 2009		(Tachdjian et al., 2008; Aboura et al., 2009)
Genome-Wide Association Studies (GWAS)	2008, 2009, 2012		(Kang et al., 2008; Knauff et al., 2009; Pyun et al., 2012)
Whole-exome sequencing (WES) in syndromic POI	2010, 2013, 2014	*HSB17B4, LARS2, CLPP, TWNK*	(Pierce et al., 2010; Jenkinson et al., 2013; Pierce et al., 2013; Morino et al., 2014)
Whole-exome sequencing (WES) in non-syndromic POI	2014	*HFM1, STAG3, MCM9, SYCE1*	(Caburet et al., 2014; de Vries et al., 2014; Wang et al., 2014; Wood-Trageser et al., 2014)

variant curation.

In the present study, we aimed to evaluate the genetic causes of amenorrhea via WES in four independent Iranian families. We showed the diverse causes of amenorrhea in this small cohort of homogeneous ethnicity. Independent genetic causes of amenorrhea in a single pedigree highlighted that the identification of cause in one individual does not always generalise to familial cases. Although 46, XY sex reversal was identified as the cause of amenorrhea in one individual, the molecular cause of sex reversal remains unknown with a novel homozygous missense variant in *HHAT* remaining a variant of uncertain significance after functional experiments failed to demonstrate an overt impact of the variant on protein function. WES detected chromosomal aberrations, circumventing the need for microarray. This study emphasises the capacity of genome sequencing as a single assay for the diagnosis of female infertility, such as POI.

3. Materials and methods

3.1. Participants and ethical adherence

Six amenorrheic patients from four Iranian families were included in this study – five diagnosed with POI with elevated FSH (>20 mIU/ml) and one diagnosed with hypogonadotropic hypogonadism. Table 3 includes the available family and medical history. No patient reported a history of autoimmune disease, although ovarian autoantibodies were not assessed. All procedures were approved by the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne (HREC# 22,073). Written informed consent was obtained from all participants.

3.2. General molecular techniques

Genomic DNA from the patients was extracted from EDTA-blood samples using the NucleoSpin® Blood XL kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA quality was assessed by NanoDrop™ 1000 spectrophotometer and Qubit dsDNA BR Assay (Thermo Fisher Scientific, Waltham, MA, USA).

3.3. Sanger sequencing

Selected variants were validated by Sanger sequencing using BigDye v3.1 Terminators (Applied Biosystems) and ABI 3130*X*, according to manufacturer's protocol. Primer sequences and cycling conditions are available on request.

3.4. Clinical microarray

For molecular karyotyping of POI patients, a standard SNP microarray platform was performed on the DNA samples of individuals (500 ng of genomic DNA) by the Victorian Clinical Genetics Services (VCGS) using Illumina Infinium Omni2.5 high density microarrays. Data were processed and analysed using Illumina GenomeStudio 2.0 and Illumina KaryoStudio v1.4.

3.5. Whole-exome sequencing (WES)

Whole-exome sequencing of DNA from the patients was performed at VCGS on the NovaSeq 6000 (Illumina) with SureSelect Human All Exon V6 (Agilent) exome capture. Variant detection was performed using Cpipe (Sadedin et al., 2015). WES data were analysed using SeqR software (https://seqr.broadinstitute.org/) through two phases of analysis (Table 4) including gene-centric and variant-centric approaches as reported previously (Tucker et al., 2019). Gene priority using POI candidate genes (Tucker et al., 2023) (Supplementary File S1), differences of sex development candidate gene list from Eggers et al. (Eggers et al., 2016), Differences of Sex Development gene list and Kidneyome SuperPanel gene list from PanelApp-AUS (https://panelapp.agha.umccr.org/) have been used for gene-centric analysis. For gene-centric analysis we filtered for variants with MAF<0.005 and high quality score (Q > 20, allele balance>25). Variant-centric analysis focused on

Table 4
Genes identified with variants of interest after filtration.

		Patient 1, 2	Patient 3, 4	Patient 5
	Median coverage	76, 46	72, 59	62
	Percentage of bases > x10	98.4, 98.3	98.8, 98.3	98.5
	Mod-high (all)	399	155	293
Gene centric	Mod-high (POI)	25 variants (25 genes)	10 variants (10 genes)	–
	Mod-high (DSD)	–	–	2variants (2 genes)
	Mod-high (Kidneyome)	–	–	11 variants (11 genes)
Variant centric	Recessive-type	9 variants (7 genes)	23 variants (17 genes)	24 variants (20 genes)
	Loss of function (LOF)	44 variants (40 genes)	11 variants (11 genes)	30 variants (30 genes)
	Pathogenic/likely pathogenic	SYCP2L	FANCM and GNRHR	HHAT

Table 3
Summary of patient clinical details.

Patient	Gene	Karyotype	Diagnosis	Age of diagnosis	Menstrual status	Hormones	Imaging	Gestation status	Other
Patient 1	SYCP2L	46XX	POI	23	Secondary amenorrhea	FSH: 117.8 IU/I AMH: 0.01 ng/ml	Both ovaries smaller than normal	G0	–
Patient 2	SYCP2L	46XX	POI	19	Hypomenorrhea	FSH: 18 IU/I AMH: 0.33 ng/ml		G0	Low ovarian reserve and infertility
Patient 3	FANCM	46XX	POI	31	Secondary amenorrhea	FSH: 65 IU/L	Both ovaries smaller than normal	G1P1 Alive1	–
Patient 4	GNRHR		Amenorrhea	17	Hypothalamic amenorrhea	FSH: 0.1 IU/L	Nonvisible ovaries and absent uterus	G0	Hypogonadotropic hypogonadism
Patient 5	HHAT	46XY	Amenorrhea	19	Primary amenorrhea	FSH:	Atrophic ovaries/ myometer smaller than normal	G0	Nephrotic Syndrome/kidney transplantation at 16 years old
Patient 6	Mosaic Turner	45,X/46,X, r(X)	POI	32	Secondary amenorrhea	FSH: 71.8 IU/I AMH: 0.01 ng/ml	Normal ovaries	G0	–

high quality (Q > 20, allele balance >25) high-priority variants (likely leading to loss of function: nonsense, splice spite, frameshift) with minor allele frequency (MAF) < 0.0001 in any gene and with any inheritance or high quality (Q > 20, allele balance>25) biallelic variants with predicted moderate-high impact (missense, in-frame indels, frameshift, nonsense or splice spite) and MAF<0.005. We chose to exclude variants with >0.005 MAF because POI affects ~1% of the general population and is caused by variants in >100 genes. Each genetic cause of POI identified to date is rare. The rationale for MAF filtering thresholds for the different phases of analyses reflects the tolerance of recessive variants in the population (in heterozygous state) in contrast to the intolerance of dominant variants (that therefore must be rarer in the general population). We chose a higher MAF for gene-centric analysis to ensure we didn't overlook variants that were in clinically-relevant genes. The public database gnomAD v4 (https://gnomad.broadinstitute.org/) was used to assess MAF and the tolerance of genes to missense and/or loss-of-function variation. Heterozygous "high" priority variants were discounted if they affected 1) genes tolerant of LoF based on gnomAD data, 2) genes not expressed in gonadal tissue as per GTEx data, 3) transcripts with minimal expression that have not been validated or 4) genes that are not related to ovarian biology or development.

We predicted missense variant pathogenicity in silico using several online tools including Mutation Taster (http://www.mutationtaster.org/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), CADD (Combined Annotation-Dependent Depletion) (https://cadd.gs.washington.edu/snv), SIFT/Provean (http://provean.jcvi.org/) and Decipher database (https://www.deciphergenomics.org/). We assessed the conservation of affected residues by using Multiz Alignments of 100 vertebrates (UCSC Genome Browser https://genome.ucsc.edu/).

3.6. Data availability

Patient variants were submitted to ClinVar and given accession IDs SCV004217775 – SCV004217778. Additional sequencing data are available from the corresponding author upon request and in accordance with human research ethics approval.

3.7. Acyl-cLIP assay

The Acyl-cLIP assay was applied to measure the enzymatic activity of HHAT wild type (wt) or R424C mutant. HHAT wildtype and mutant proteins for the enzymatic assay were produced following an established procedure described in (Coupland et al., 2021). The Acyl-cLIP assay has been described before (Lanyon-Hogg et al., 2019; Andrei et al., 2022). Briefly, a 7.5 μM palmitoyl-CoA (Sigma Aldrich) was prepared in reaction buffer (100 mM MES, 20 mM NaCl, 1 mM DTT, 1 mM TCEP, 0.1% BSA, pH 6.5). Next, FAM-labelled SHH-peptide was diluted in reaction buffer to a concentration of 2 μM. Recombinant HHAT (about 0.1 mg/ml, obtained by following the published protocol (Coupland et al., 2021) was diluted 30 times in storage buffer (20 mM HEPES, 350 mM NaCl, 1% DDM, 5% glycerol, pH 7.3). Diluted HHAT (220 μL) and SHH peptide (2 ml) were mixed to prepare enzyme-substrate mixture. To start the reaction, SHH and HHAT mixture (12 μL) was mixed with palmitoyl-CoA (4 μL) and reaction buffer (4 μL) in to each well of a 384 well plate, and the fluorescence polarization (Excitation 480 nm, emission 535 nm) measured for 1 h at 2-min intervals on an EnVision Xcite 2104 (PerkinElmer). The slope of the resulting curves was calculated for each well using Prism 9.1.2 (GraphPad) and each well was background subtracted by subtracting the average of values from wells without palmitoyl-CoA. The background subtracted data were then normalized to plot Michaelis–Menten kinetics using Prism with 95% confidence intervals (n = 4 replicates).

4. Results

4.1. Novel variants associated with amenorrhea in four families

4.1.1. A homozygous nonsense SYCP2L variant: c.1528C > T p. (Gln510Ter)

Patient 1 was born to consanguineous parents (first cousins) of Iranian descent. At age 23, she was diagnosed with POI after presenting with secondary amenorrhea. FSH was elevated at 117.8 IU/I, and AMH was low at 0.01 ng/ml. Ultrasound identified ovaries that were smaller than normal. Patient 1 had a similarly affected sister (Patient 2) who was diagnosed with POI at age 19 (Fig. 1a) (Table 4).

WES of the two affected siblings and parents identified a homozygous stop-gain *SYCP2L* variant: Chr6:10,930,409 (hg38), NM_001040274.3(SYCP2L):c.1528C > T p. (Gln510Ter) shared by both sisters and heterozygous in both parents (Fig. 1b). This variant is detected only once in gnomAD v4 (allele frequency 0.0000012) and predicted to be likely deleterious by online algorithms (Table 5). Sanger sequencing confirmed segregation of the *SYCP2L* variant with disease in the family (Fig. 1c). Since the variant is located in a central exon (exon 19 out of 30) and causes a premature stop codon, it is likely that the transcript is degraded by nonsense-mediated decay (NMD) resulting in loss of SYCP2L protein (Fig. 1d). If residual mRNA persisted, any truncated protein is likely to be non-functional. The variant falls within a region encoding compositional bias (polar residues 507–527). Regions of polarity often have crucial roles in protein structure, stability, and function, often participating in interactions with other polar or charged molecules (Ayuso-Tejedor et al., 2011; Illergård et al., 2011). Protein truncation at this site would also remove three downstream regions of compositional bias that are highly conserved and likely critical for protein structure/function (570–614 polar, 615–631 basic/acidic, 639–656 basic/acidic) (Fig. 1d).

4.1.2. A homozygous frameshift FANCM variant: c.2215_2216delTG p. (Trp739AlafsTer11) and a homozygous frameshift GNRHR variant: c.248delT p. (Leu83ArgfsTer3)

A homozygous frameshift FANCM variant: c.2215_2216delTG p. (Trp739AlafsTer11) and a homozygous frameshift GNRHR variant: c.248delT p. (Leu83ArgfsTer3).

Patient 3, also from a consanguineous Iranian pedigree was diagnosed with POI at the age of 31 due to secondary amenorrhea and high FSH (65 IU/I), with pelvic ultrasound showing small ovaries (Fig. 2a). Her daughter (Patient 4) was diagnosed with primary amenorrhea at the age of 16, with low FSH (0.1 IU/I), indicating hypothalamic amenorrhea. Pelvic ultrasound could not detect a uterus or ovaries.

WES was performed for the mother and her daughter. Given both mother and daughter were affected by amenorrhea, dominant inheritance was considered, but no likely causative variants were identified. Data analysis instead identified two different genetic causes of amenorrhea; variants in *FANCM* in the mother and variants in *GNRHR* in the daughter. These genes are in keeping with the disparate hormonal status of each affected individual – the mother having elevated FSH and the daughter having low FSH. Patient 3 (mother) had a novel homozygous loss-of-function *FANCM* variant: Chr14:45,173,106 (hg38), NM_020937.4(FANCM):c.2215_2216delTG p. (Trp739AlafsTer11), whereas Patient 4 (daughter) had a novel homozygous frameshift *GNRHR* variant: Chr4:67,754,087 (hg38), NM_000406.3 (GNRHR): c.248delT p. (Leu83ArgfsTer3). (Figs. 2b and 3a). Sanger sequencing confirmed the WES findings, demonstrating that the mother was homozygous for the deletion in *FANCM*, and the daughter was heterozygous for the same variant (Fig. 2c). The *GNRHR* variant segregated with the disorder in the family, with the daughter being homozygous and her parents being heterozygous (Fig. 3b). Both variants were absent in public databases, such as gnomAD v.4 (Table 5) and were predicted to be likely damaging by online tools. The *FANCM* variant falls within exon 13 out of 23 and the region encoding the highly conserved FANCM-MHF

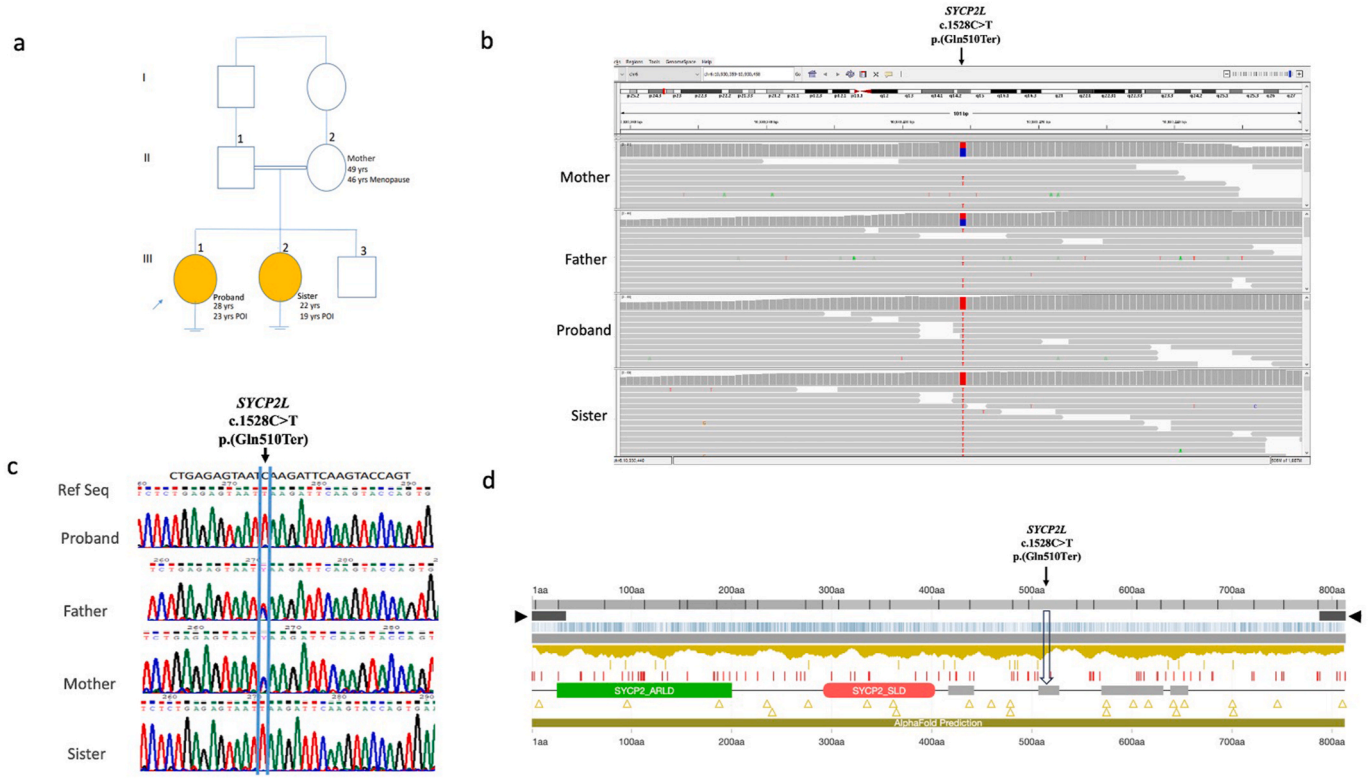

Fig. 1. A homozygous nonsense *SYCP2L* variant in Patient 1 and Patient 2. **a.** Familial pedigree depicts the proband by an arrow, yellow circles represent POI (III1 and III12), III1: Proband, III2: Sister, II1: Father and II2: Mother **b.** IGV visualisation of the *SYCP2L* (c.1528C > T) variant in the family, confirming homozygosity in the proband and her sister, and heterozygosity in the parents. **c.** Sanger sequencing demonstrating the segregation of the *SYCP2L* variant with disease. **d.** Decipher database view demonstrating the variant location with respect to the protein (Black arrow). The transparent arrow shows the location of the premature stop codon falling within a region of compositional bias (grey bars) of the protein. Arrow heads show the NMD escape regions, which are distant from the variant.

binding domain of the protein, which is essential for DNA repair in response to genotoxic stress (Tao et al., 2012). The *GNRHR* variant is located in the first exon of 3 and interrupts the 7tm_GPCRs (seven--transmembrane G protein-coupled receptor superfamily) conserved domain. This domain is essential for activation of intracellular signalling pathways mediated by G proteins and play a crucial role in regulating the synthesis and release of gonadotropins (Naor 2009; Kinoshita and Okada 2015). Both *FANCM* and *GNRHR* variants, however, are distant from the NMD escape region and are likely to induce NMD, resulting in loss of the proteins. (Figs. 2d and 3c).

4.1.3. A homozygous missense HHAT variant: c.1270C > T p. (Arg424Cys)

Patient 5, of Iranian descent and born to consanguineous parents, received a primary amenorrhea diagnosis at age 19 without undergoing FSH assessment. Pelvic ultrasound showed atrophic ovaries and a thin myometrium. The patient had a history of nephrotic syndrome and received a kidney transplant at age 16. Although recruited as a female patient, WES analysis revealed that the individual was in fact 46, XY after detecting Y chromosome sequence, including the SRY gene. Coverage across the X chromosome was ~50% compared to XX control individuals, consistent with a likely 46, XY karyotype (Fig. 4). Additionally, WES identified a novel homozygous missense variant in *HHAT*: Chr1:210,623,550 (hg38), NM_018194.6 c.1270C > T p. (Arg424Cys) (Fig. 5a). Sanger sequencing validated the *HHAT* variant in the patient (Fig. 5b). Parental DNA was not available to confirm parental genotype, however, microarray did not reveal a copy number variation at this site suggesting it is unlikely the patient harbours a deletion of one allele causing apparent homozygosity. Identity by descent is also in keeping with the consanguinity of the pedigree. This variant was detected in heterozygous state in 24 individuals in gnomAD v4 (allele frequency 0.00001487) and predicted damaging by some online algorithms (Table 5). The identified missense variant affects p.Arg424 which is conserved in mammals (Fig. 5c). The HHAT variant residue is situated within a critical membrane-bound Oacyltransferase (MBOAT) domain of the protein, which is highly conserved and essential for the protein's activity. Therefore, the variant has the potential to disrupt the function of HHAT protein (Fig. 5d). Given the 3D structure of HHAT is known, we used the HOPE modeling tool (http://www.cmbi.ru.nl/hope/) (Venselaar et al., 2010) to simulate the effects of the missense variant. The variant residue is smaller, neutral and more hydrophobic than the original wild-type residue, which was positively charged. These changes can potentially lead to the loss of critical interactions of the residue with other molecules or residues, including hydrogen bonds, and disrupt proper protein folding, thereby affecting the function of HHAT protein (Fig. 5e). Using the American College of Medical Genetics (ACMG) and Genomics variant classification criteria, this variant is considered a Class 3 variant of uncertain significance. The WES data was also analysed for variants potentially responsible for kidney disease given the patient's history of nephrotic syndrome. A hemizygous c.2500G > A, p. (Glu834Lys) in *TBC1D8B* was detected. Missense variants in this gene have previously been reported in X-linked nephrotic syndrome without sex reversal (Kampf et al., 2019), suggesting the potential for dual diagnosis in this individual.

4.1.3.1. HHAT enzymatic activity. We measured the enzymatic activity of HHAT wildtype and variant (HHAT R424C) using polarization-based Acyl-cLIP assays to investigate the influence of this missense mutation. The kinetics studies showed that HHAT R424C exhibits a Michaelis constant (K_M) of 125 (95% confidence interval, CI:114–136) nM, which is slightly but significantly higher than the K_M of the wt 98 (95% CI: 89–108) nM. Furthermore, the maximum rate (Vmax) is 0.397 (95% CI,

Table 5
Summary of variants identified in Patients 1–5.

Patient	Gene	RefSeq	Status	gDNA variant (hg38)	cDNA variant	Protein variant	Quality metrics	Polyphen	Mutation Taster	CAAD	eigen	Phylop100	SpliceAI	Pangolin	References for other gene variants
1 and 2	*SYCP2L*	NM_001040274.3	Homozygous-Nonsense	Chr6:10,930,409 C > T	c.1528C > T	p. (Gln510Ter)	Depth:56 Balance:1.0 Quality:99	NA	Disease causing	35	2.322	−0.377	0.12	Splice loss: 0.16 Splice gain: 0.01	(He et al., 2021)
3	*FANCM*	NM_020937.4	Homozygous-Frameshift	Chr14:45,173,106 CTG > C	c.2215_2216delTG	p. (Trp739AlafsTer11)	Depth:114 Balance:1.0 Quality:99	NA	Disease causing	NA		9.043	0.04	Splice loss: 0.05 Splice gain: 0.05	(Fouquet et al., 2017; Jaillard et al., 2020; Heddar et al., 2022)
4	*GNRHR*	NM_000406.3	Homozygous-Frameshift	Chr4:67,754,087 CA > C	c.248delT	p. (Leu83ArgfsTer3)	Depth:78 Balance:1.0 Quality:99	NA	Disease causing	NA		9.24	0.01	Splice loss: 0.05 Splice gain: 0.12	(Cioppi et al., 2019)
5	*HHAT*	NM_018194.6	Homozygous-Missense	Chr1:210,623,550 C > T	c.1270C > T	p. (Arg424Cys)	Depth: 107 Balance:1.0 Quality:99	Possibly damaging	Benign	23.4	3.965	2.239	0.00	Splice loss: 0.01 Splice gain: 0.00	(Callier et al., 2014; Abdel-Salam et al., 2019; Baz-Redon et al., 2022; Mazen et al., 2022; Pande et al., 2022; Saini et al., 2023)

0.387–0.407) for the mutant, which is slightly but significantly lower than 0.438 (95% CI, 0.428–0.449) for the wild type (Fig. 6). These results demonstrate that there is minimal difference observed on the enzymatic activity between HHAT R424C and wt in the Acyl-cLIP assays.

4.1.4. Mosaic turners with a ring X chromosome

Patient 6, who comes from a non-consanguineous Iranian family, was diagnosed with secondary amenorrhea at the age of 32. Her hormonal assessment showed elevated FSH (71 IU/l) and low AMH (0.01 ng/ml). WES revealed that the individual is likely to have mosaic Turners with the allele balance of variants on the X chromosome around ~85% and ~15% in the patient in comparison to the expected ~50% in 46XX controls. Furthermore, WES coverage across the X chromosome was comparable to the coverage in 46, XY individuals (Fig. 7a, b and c). Microarray confirmed the WES data interpretation, indicating a mosaic Turners variant. In particular, there was a 45, XO cell line in 90% of cells and a ring chromosome X, with a 46,X,r(X) (p22.13q22.3) karyotype, in 10% of cells (Fig. 7d).

5. Discussion

In the present study we have used WES to investigate the genetic cause of amenorrhea in six patients from a small cohort of Iranian descent. Interestingly, WES revealed five different genetic causes of amenorrhea including novel gene variants (*SYCP2L*, *FANCM*, *GNRHR*) and cytogenetic causes (46, XY sex reversal, Turners syndrome).

5.1. A novel homozygous nonsense variant in SYCP2L consolidates the role of this gene in isolated POI

SYCP2L (Synaptonemal complex protein 2 like) encodes one of the proteins participating in assembly of synaptonemal complex (SC) during meiotic prophase (Cahoon and Hawley 2016). *SYCP2L* is expresses exclusively in ovaries and testes and is required for male and female fertility (He et al., 2021). In particular, *SYCP2L* regulates the survival of primordial oocytes and lacking this component from SC structure leads to meiotic arrest and ultimately atretic oocytes. Additionally, *Sycp2l*-deficient female mice are subfertile (Zhou et al., 2015). The association of the genes that have key roles in meiosis and DNA repair with POI has been previously reported (Veitia 2020, Tucker et al., 2022). *SYCP2L* has recently been identified as a POI gene with one report describing two POI patients with biallelic loss-of-function alleles (He et al., 2021). Similar to the variants outlined in the prior report, the *SYCP2L* homozygous nonsense variant (c.1528C > T, p. (Gln510Ter)) identified in the first family (Patient 1 and Patient 2) of our study is also a predicted loss-of-function allele. According to ACMG criteria, the variant is classified as likely pathogenic. Its status could be elevated to pathogenic if loss-of-function could be demonstrated experimentally via the analysis of patient cells, however further biological sample from the patient is not available. In vitro expression of truncated protein is also unlikely to model the patient situation given the location of the premature termination codon (PTC) prior to the last 50bp of the penultimate exon. PTCs introduced at this position usually trigger nonsense mediated decay (Kurosaki and Maquat 2016) and ACMG recommendation is to treat these as likely loss of function, allowing the application of very strong (VS1) pathogenicity criterion (Abou Tayoun et al., 2018). Our research expands the spectrum of *SYC2PL* variants associated with POI and adds new evidence to support the association of this recently reported POI gene with female infertility.

5.2. Two independent genetic causes for amenorrhea in a single pedigree

The second family of this study had a mother and daughter who presented with amenorrhea. The mother had causative variants in *FANCM* and the daughter had causative variants in *GNRHR*. The Fanconi

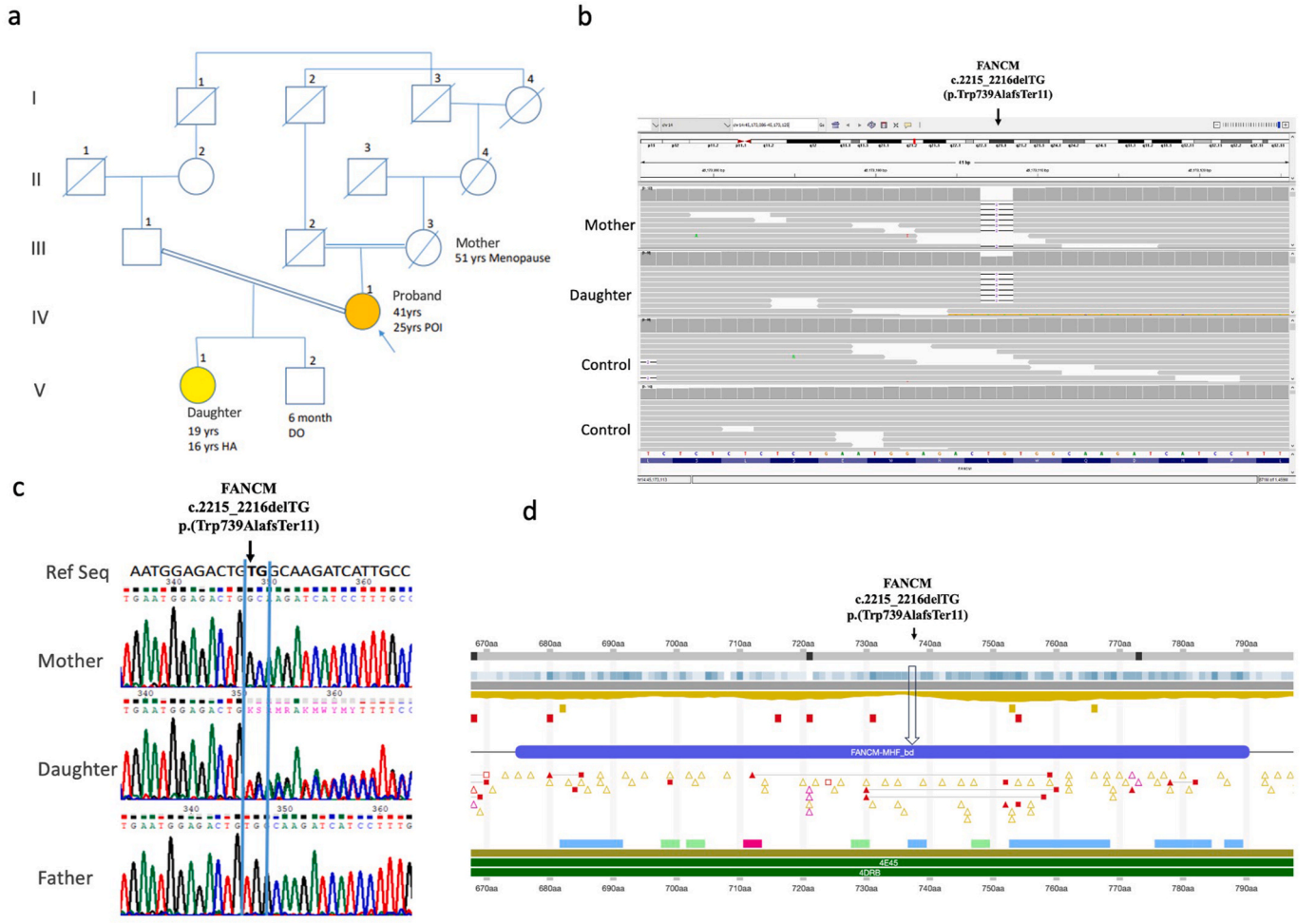

Fig. 2. A frameshift *FANCM* variant in patient 3. **a.** Familial pedigree of Patient 3 and Patient 4 depicts the proband (Patient 3) by an arrow, orange circle represents POI (IV1) and yellow circle refers to hypothalamic amenorrhea in the daughter (V1/pPtient 4). **b.** IGV view of *FANCM* (c.2215_2216delTG) variant in the family confirmed the proband was homozygous for the *FANCM* deletion and her daughter was heterozygous for the variant. **c.** Sanger sequencing of the family revealed the segregation of *FANCM* variant with disease. **d.** The Decipher database view shows the variant's location within a central exon of the gene (Black arrow). Transparent arrow shows a conserved FANCM-MHF binding domain, which is interrupted by the *FANCM* variant.

anaemia complementation group M (*FANCM*) encodes a tumour suppressive DNA translocase involved in chromosomal stability and DNA repair. *Fancm*-deficient mice have meiotic defects leading to disrupted follicular development and depleted follicular supply (Fouquet et al., 2017). *FANCM* biallelic variants in human have been shown to be associated with early menopause (Catucci et al., 2018), non-syndromic POI (Fouquet et al., 2017; Heddar et al., 2022) and male infertility (Kasak et al., 2018; Yin et al., 2019). The identified *FANCM* variant in our study is a novel predicted loss-of-function allele. This is in accordance with previously reported pathogenic *FANCM* variants in patients with non-syndromic POI that are also loss-of-function variants (Fouquet et al., 2017; Jaillard et al., 2020). Importantly, heterozygous variants in this gene are associated with ovarian and breast cancer predisposition (Basbous and Constantinou 2019, Schubert et al., 2019) and there is some evidence suggesting biallelic *FANCM* variants are associated with cancer predisposition, especially early-onset breast cancer in women (Bogliolo et al., 2018; Catucci et al., 2018). Accordingly, this highlights the necessity to offer genetic counselling and breast screening to patients with POI due to underlying *FANCM* variants. Further biological sample for research was not accessible from this patient, however, she could benefit from clinical cytogenetic analysis to determine sensitivity to DNA damage. We have previously demonstrated chromosomal instability in an individual harbouring pathogenic *FANCM* variants (Jaillard et al., 2020). This analysis could determine the relative risk of cancer development. Future studies in larger cohorts of patients with long term follow-up will provide further insight into the risk of cancers in these patients. Interestingly, the cause of amenorrhea in the proband's daughter (Patient 4) was independent – she experienced hypothalamic amenorrhea due to variants in *GNRHR*. *GNRHR* encodes gonadotropin releasing hormone (GnRH) receptor that mediates gonadotropic signalling and stimulates the secretion of gonadotropins (LH and FSH). *GNRHR* variants associated with congenital hypogonadotropic hypogonadism (cHH) are typically loss-of-function (LOF) variants and occur across the coding sequence of the *GNRHR* gene (Cioppi et al., 2019). The identified frameshift *GNRHR* variant in our study lies within the first exon of the gene which is far from the NMD escape region and is therefore predicted to induce NMD and likely the loss of GNRHR protein. If additional biological sample could be obtained, this could be confirmed by RNA analysis or Western blot. The clinical effect of different *GNRHR* variants is highly variable based on the type of variant and the subsequent level of functional protein expression. Typically, patients harbouring homozygous loss-of-function *GNRHR* variants have cHH presenting with undetectable LH and FSH in serum and absence of spontaneous puberty characterised by underdeveloped/absent breast development and amenorrhea in females and cryptorchidism or micropenis in males (Gianetti et al., 2012; Correa-Silva, Fausto et al., 2018, Cioppi et al., 2019; Hussain et al., 2019). It is likely that the loss-of-function *GNRHR* variant in Patient 4 is responsible for her

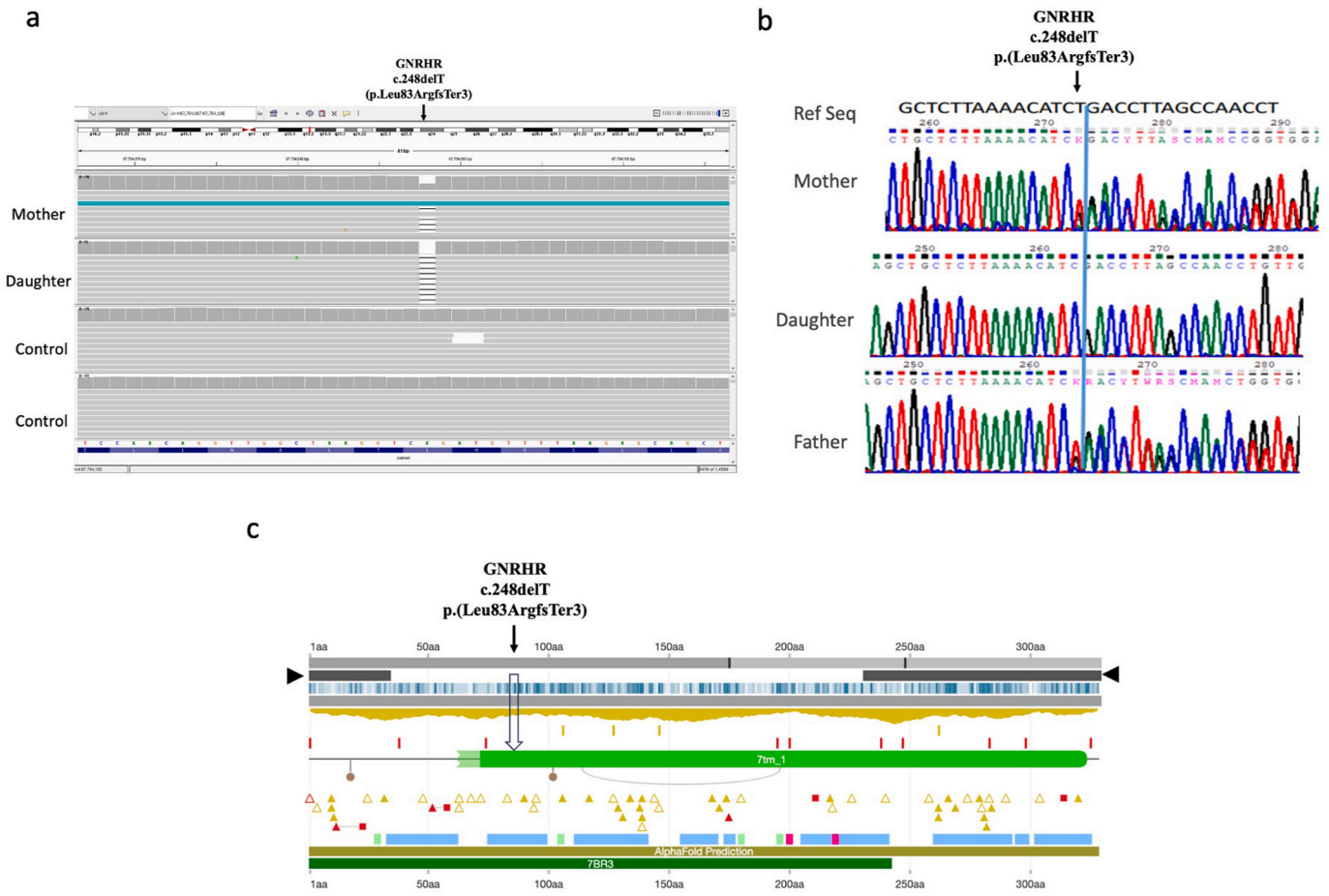

Fig. 3. A frameshift *GNRHR* variant in patient 4. a. IGV view of *GNRHR* (c.248delT) variant in the family confirmed the daughter being a homozygous carrier of the deletion and her mother being heterozygous. b. Sanger sequencing of the family was consistent with the WES results and indicated the segregation of *GNRHR* variant with disease. c. Decipher view of the variant that affects the first exon of *GNRHR* (Black arrow). The transparent arrow shows 7tm_GPCRs conserved protein domain that is interrupted by the *GNRHR* variant. Arrow heads show the NMD escape regions, which are distant from the variant.

hypogonadotropic hypogonadism. The fact that this family presented with two independent causes of amenorrhea highlights the diverse nature of the condition, and that a genetic cause in one family member does not always indicate cause in relatives, particularly in consanguineous pedigrees for which there is an excess of homozygosity.

5.3. A homozygous missense variant of uncertain significance in HHAT is identified in an individual with complete sex reversal

WES analysis of Patient 5 revealed a 46, XY chromosomal complement. Despite an initial diagnosis of amenorrhea with atrophic ovaries, WES coverage across the Y chromosome, including the SRY gene, was consistent with a 46, XY status. WES also identified a novel homozygous missense variant in *HHAT* (c.1270C > T (p. (Arg424Cys)) in this patient. *HHAT* encodes a palmitoyl acyltransferase that belongs to the membrane-bound Oacyltransferase (MBOAT) superfamily and is a key enzyme in the Hedgehog signalling pathway. HHAT is broadly expressed, including in the testes and ovaries, at the time of sexual differentiation in human embryonic development (Callier et al., 2014). The study of *Hhat* knock-out mice revealed that a deficiency in Hhat can lead to significant neuronal and growth abnormalities and osteochondrogenic and skeletal defects such as holoprosencephaly, acrania, agnathia and dwarfism. Moreover, the lack of functional Hhat in mice led to impaired testicular organogenesis, including disrupted testis cord formation and fetal Leydig cell differentiation (Dennis et al., 2012; Callier et al., 2014). The clinical presentation associated with human *HHAT* variants has considerable variation, however, there have been multiple reports describing 46, XY sex reversed females with homozygous missense *HHAT* variants (Callier et al., 2014; Pande et al., 2021; Mazen et al., 2022; Rjiba et al., 2023).

Genetic variants in *HHAT* are associated with Nivelon-Nivelon-Mabille syndrome, which was first reported in a family with an affected female having a karyotype of 46, XY (NNMS; OMIM 600092). The patient carried a homozygous *HHAT* missense variant (p. Gly287Val) and presented with microcephaly, cerebellar vermis hypoplasia, short stature, skeletal dysplasia and sex reversal with testicular dysgenesis (Nivelon et al., 1992; Thauvin-Robinet et al., 2005; Callier et al., 2014). Similarly, the patient reported in our study who carried a homozygous missense variant in *HHAT* had 46, XY sex reversal and presented as a female with primary amenorrhea and atrophic ovaries.

Additional cases described in the literature have expanded the phenotypic spectrum associated with variants in *HHAT* with gonadal dysgenesis being a common feature in 46XY individuals. Other features include microcephaly and cerebellar vermis hypoplasia, muscle hypertrophy, muscle spasms, facial dysmorphism and eye defects (Abdel-Salam et al., 2019; Baz-Redón et al., 2022; Mazen et al., 2022; Pande et al., 2022; Saini et al., 2023). All but one previously reported pathogenic *HHAT* variant lies in the highly conserved functional MBOAT domain. Consistent with these previously reported variants, the homozygous missense variant identified in the present study (p.Arg424Cys) was also found to be located in the MBOAT domain. These variants may impair the ability of HHAT to palmitoylate Hedgehog proteins like SHH and DHH, leading to disrupted gonadal development. Patient 5 of this study also had nephrotic syndrome requiring kidney transplantation at age 16.

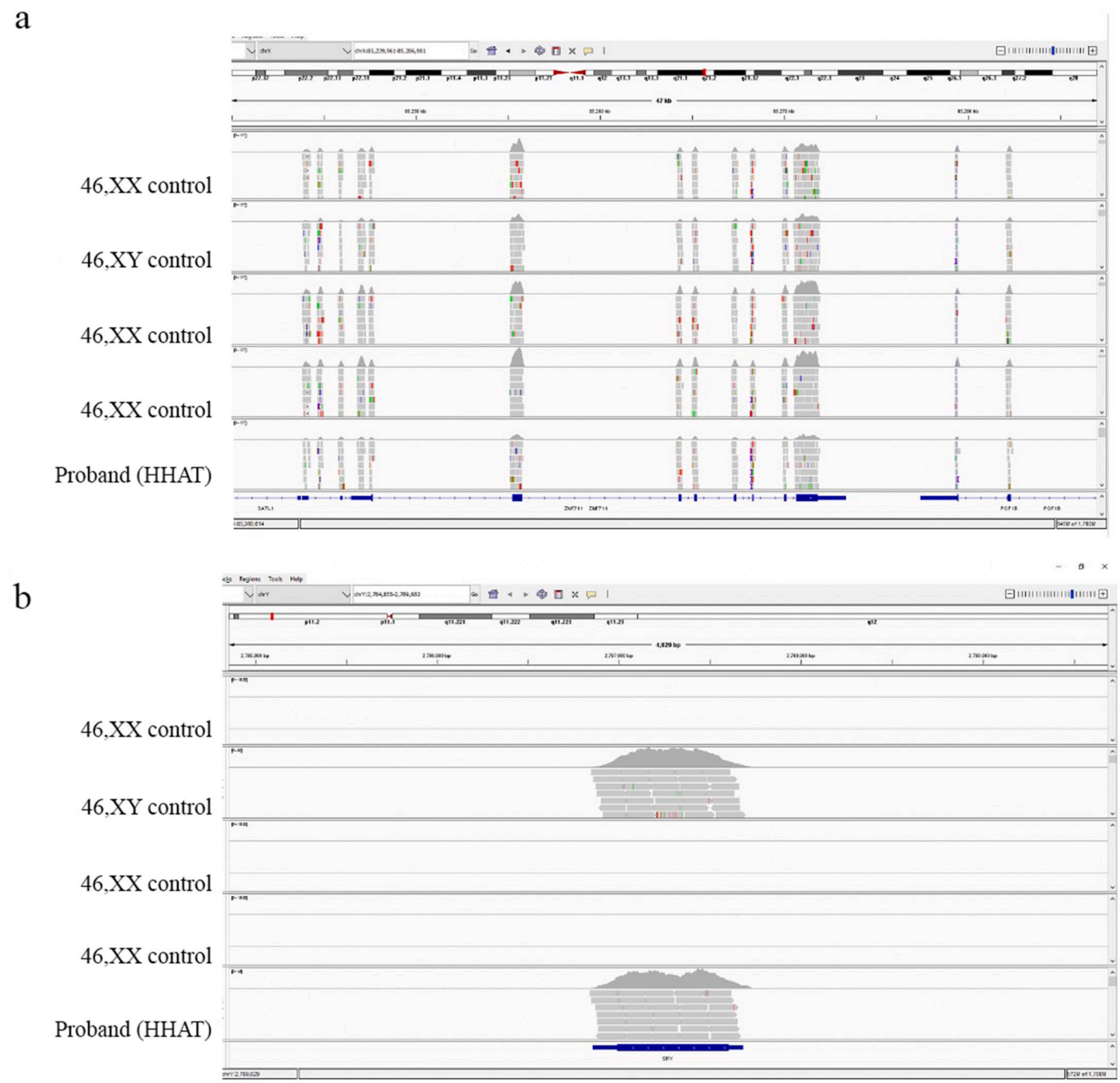

Fig. 4. WES coverage data showing reads across the X and Y chromosome in Patient 5 compared to controls. a. WES coverage across the X chromosome was comparable to that in a 46, XY control b. WES coverage across the Y chromosome including *SRY* in Patient 5 was comparable to that in 46, XY control. There was no sequence across Y chromosome in 46, XX female individuals.

Whether kidney pathology is due to *HHAT* deficiency is uncertain, however, there has been one sex-reversed 46, XY female individual described previously who displayed bilateral renal pyelectasis during fetal development and high levels of creatinine phosphokinase (CPK, 922.0 U/L) during adulthood. She presented with additional clinical manifestations of HHAT deficiency such as short stature, microcephaly and severe microphthalmia (Pande et al., 2022). Importantly, we identified a hemizygous variant of uncertain significance in an X-linked nephrotic syndrome gene, *TBC1D8B,* which requires functional validation. This indicates that the patient could have a dual diagnosis with sex reversal and nephrotic syndrome having independent genetic causes. *In silico* analysis tools such as Polyphen and Mutation Taster had conflicting predictions with regards to the likely pathogenicity of the *HHAT* variant identified in Patient 5 and conservation of this residue was restricted to mammals (Fig. 5c). According to ACMG variant classification, this variant (NM_018194.6: c.1273C > T) was classified as a variant of uncertain significance (VUS). Two *HHAT* variants reported in recent studies including a homozygous missense variant (c.1001 T > A (p.Met334Lys)) and a homozygous in frame deletion (c.365_367del (p.Thr122del)) also were classified as VUSs but were subsequently found to be damaging to protein function (Baz-Redón et al., 2022; Pande et al., 2022). In our study, the assessment of HHAT enzymatic activity using purified HHAT and labelled SHH peptide did not provide evidence supporting the pathogenicity of the HHAT variant (p.Arg424Cys). However, we could not exclude the possibility of changed protein stability or PTM pattern at cellular context caused by this mutation (Konitsiotis et al., 2015). Our previous studies revealed that HHAT is palmitoylated on multiple cytosolic cysteines, including Cys188,

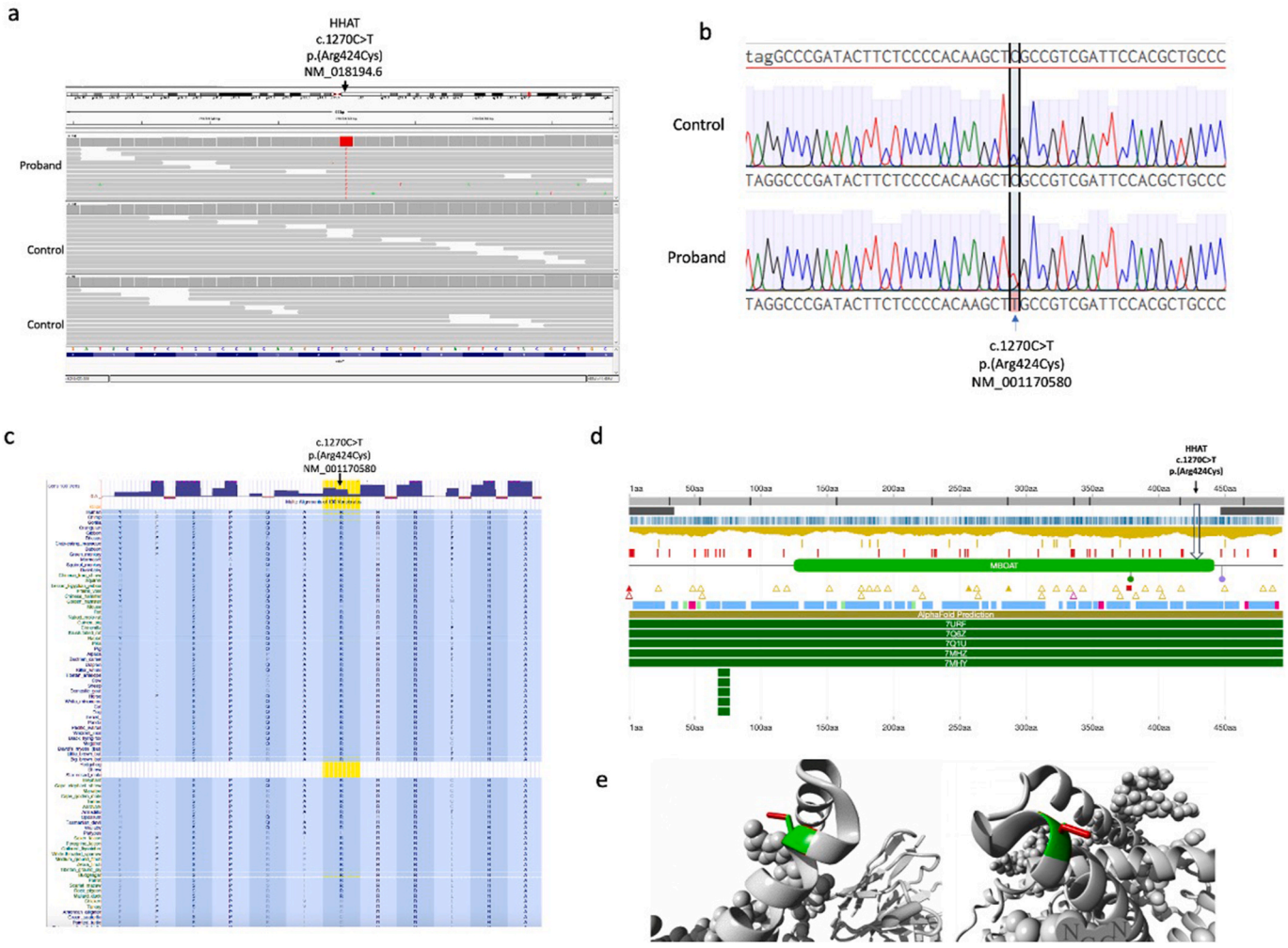

Fig. 5. A homozygous missense *HHAT* variant in patient 5: **a.** IGV view of the variant shows the patient is harbouring a homozygous *HHAT* variant. **b.** Sanger sequencing confirmed the presence of a homozygous variant in *HHAT* in the patient when compared to the control. **c.** Multiz alignment of the protein indicating conservation of the affected residue in mammals. **d.** View of the variant in Decipher demonstrates the *HHAT* variant is located within the MBOAT domain (Arrows). **e.** The 3D-structure of HHAT and the effect of missense variant were visualized using HOPE database. Substitution of Arg424 (coloured green) with Cys residue (coloured red) shows in two different positions of the 3D-structure of HHAT.

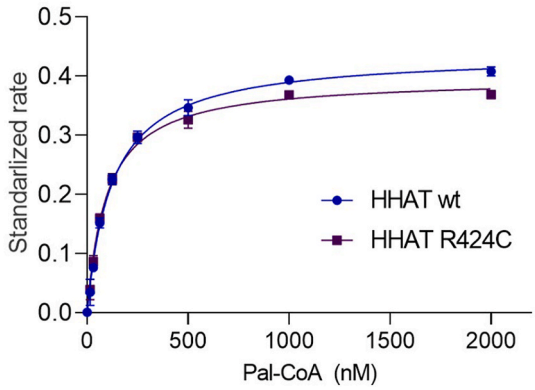

Fig. 6. Michaelis–Menten kinetics plots of HHAT wt and HHAT R424C mutant over Pal-CoA. WT shows a Vmax of 0.438 (95% CI, 0.428–0.449) and $K_M = 125$ nM (95% CI, 114–136); R424C shows a Vmax of 0.397 (95% CI, 0.387–0.407) and $K_M = 98$ nM (95% CI, 89–108). Error bars: standard error of the mean, n = 4 replicates.

Cys242, Cys324, and Cys410, which we proposed helps to regulate protein structure in the membrane (Konitsiotis et al., 2015). Arg424 is in close proximity to Cys410, which is located on the loop formed by α-helix α11 and α12 on the cytoplasmic face (Coupland et al., 2021). p. Arg424Cys might interfere with cellular HHAT palmitoylation or even introduce a site for de novo palmitoylation, subsequently leading to an increase or decrease in activity. Additionally, deletion of HHAT residues 417–426 resulted in decreased stability and loss of function, indicating this region may play an important role (Buglino and Resh 2010). Consequently, this variant remains of uncertain significance. The identification of 46, XY karyotype via WES analysis had clinical consequence for the patient, highlighting potential of dysgenic gonads that may pose a cancer risk. Further investigation via ultrasound and gonadal biopsy may be recommended with removal of gonads with malignant tissue.

5.4. *WES data analysis detected mosaic turners in patient 6*

WES also identified a cytogenetic cause of amenorrhea in Patient 6, who presented with secondary amenorrhea and elevated FSH. Atypical allelic balance of X chromosome variants indicated a likely mosaic 45, XO Turners karyotype, that was subsequently confirmed by microarray. Individuals with Turner syndrome typically present as female, but experience POI, short stature, short neck, and/or cardiovascular disease.

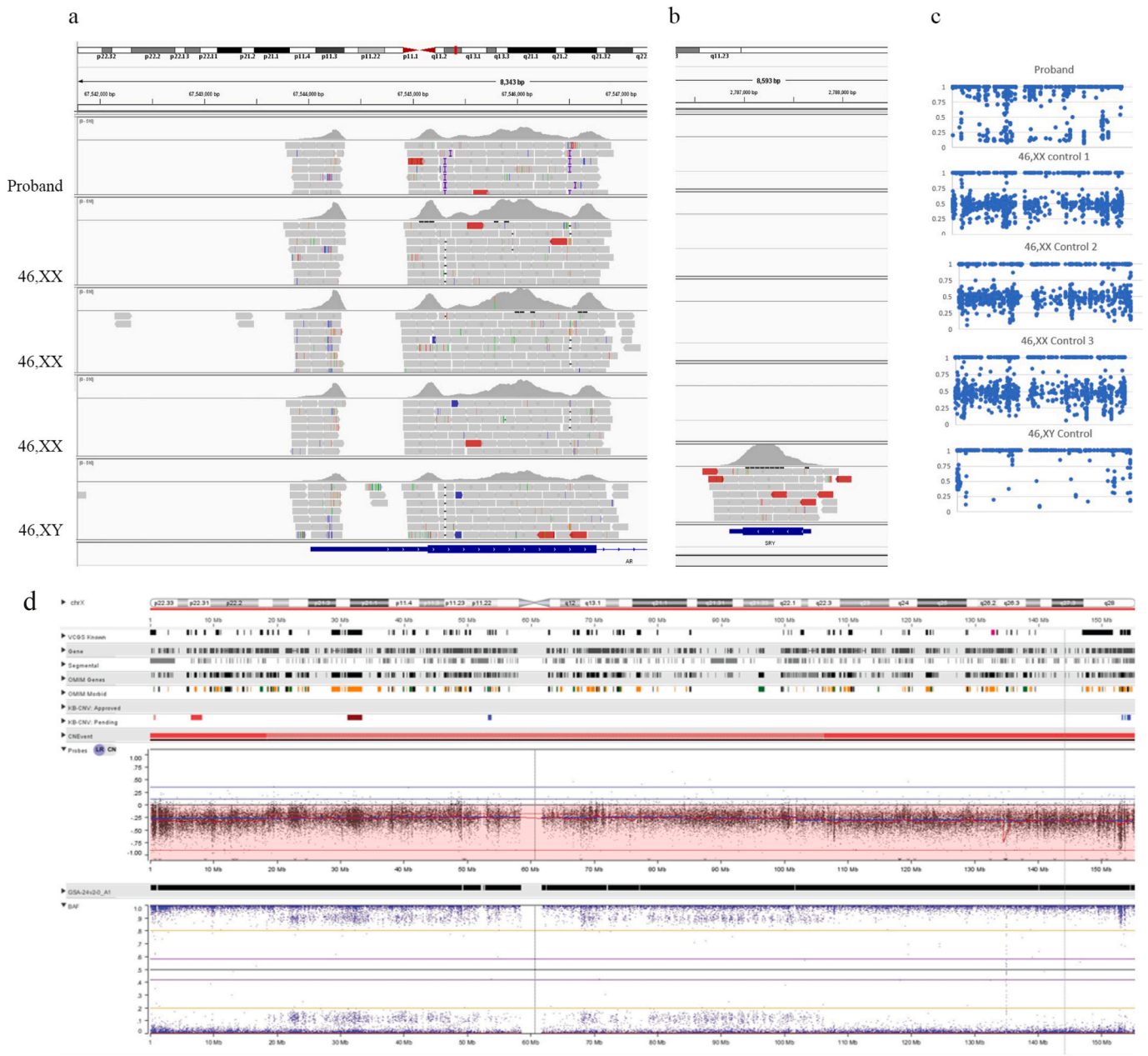

Fig. 7. WES coverage data in Patient 6 indicating mosaic Turners syndrome. **a.** Representative WES coverage across X chromosome (comparable to that in 46, XY individuals and ~50% coverage in 46, XX individuals), **b.** No sequence across Y chromosome, including *SRY* gene, **c.** Allele balance of variants on the X chromosome clusters around ~15% and 85% in the patient vs. 50% in the 46XX controls. **d.** Microarray analysis showed a mosaic Turners karyotype with one copy of X chromosome in 90% of cells and a ring X chromosome in about 10% of cells. The size of the ring X chromosome is approximately 88 Mb with genomic coordinates of chrX:18,387,356–106,251,294 and chromosome band location of Xp22.13q22.3.

For the past three decades, studies of chromosomes have revealed that short stature and primary amenorrhea are present in at least 95% of all people diagnosed with Turner syndrome (Moka et al., 2013). The most prevalent cytogenetic cause of primary amenorrhea is believed to be a complete absence of an X chromosome (45, XO), while secondary amenorrhea is more frequently associated with mosaic Turners 45, XO/46, XX (Fechner et al., 2006; Moka et al., 2013; Barros et al., 2020). Females with Turner Syndrome often present with ovarian dysgenesis and a decreased ovarian reserve before puberty due to the accelerated follicular atresia (Qin et al., 2015). The majority of women who have a ring chromosome X, known as r(X), present with Turners syndrome due to somatic loss of the unstable X chromosome and a mosaic 45, XO karyotype (Dennis et al., 2000). The clinical symptoms of Turners syndrome tend to correlate with the ratio of normal (46,XX) cells and 45, XO cells. The variable clinical presentation can also be attributed to undetected mosaicisms, which may be the explanation for infrequent instances of normal ovarian function and even more uncommon occurrences of pregnancy among women with Turners syndrome (Moka et al., 2013; Barros et al., 2020). In the present study, Turners syndrome had not been identified clinically until WES revealed mosaic Turners with a karyotype of 90% 45, XO and 10% 46, X,r(X).

Studies have shown that a considerable number of females with TS receive a delayed diagnosis, with 22% of cases diagnosed after the age of 12 years and up to 10% diagnosed in adulthood (Massa et al., 2005; Murdock et al., 2017). The delayed diagnosis of TS is a well-recognized issue as it may prevent affected individuals from receiving timely and essential medical interventions and comorbidity screening (Savendahl and Davenport 2000, Stochholm et al., 2006). Early diagnosis enables

prompt treatment options, including hormone therapy to induce age-appropriate puberty (Bondy and Group 2007), growth hormone therapy starting at 4 years of age (Davenport et al., 2007; Linglart et al., 2011), fertility preservation options in young girls (such as ovarian tissue freezing and oocyte cryopreservation (Birgit et al., 2009) and assessment for congenital heart disease (Bondy and Group 2007). In the present study, there was a delay in diagnosing Patient 6, as she was only diagnosed with POI at the age of 32. However, using WES, we were able to identify TS mosaicism in this individual. Similarly, in a previous study, WES was used to detect isochromosome Xq, cryptic Y-chromosome material, and low-level mosaicism (as low as 5%) in a cohort of female individuals with Turner syndrome (Murdock et al., 2017). This highlights WES as a sensitive and specific screening test for TS and other chromosomal abnormalities, which were conventionally only achievable through karyotype or microarray analysis.

6. Conclusion

Technological advances have made WES more accessible and affordable while detecting a wider range of genetic aberrations. Despite the challenges associated with next-generation sequencing, including the identification of variants with unknown clinical significance, the potential benefits of this powerful tool for cytogenetic and molecular diagnoses of POI are evident. This study highlights the various factors contributing to amenorrhea within a limited sample of individuals from a single ethnic cohort. Our findings highlight that when a genetic cause is identified in one individual, it may not account for similar cases within the same family. Additionally, our study demonstrated that WES can effectively identify both cytogenetic and sequence variants that underly POI pathogenesis and highlight the potential for WES as a first-tier diagnostic test to accelerate diagnosis of POI, leading to timely personalised treatment and improved patient management.

Ethics approval

All procedures were in accordance with the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

CRediT authorship contribution statement

Shabnam Bakhshalizadeh: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Fateme Afkhami:** Writing – review & editing, Resources, Methodology, Investigation. **Katrina M. Bell:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Gorjana Robevska:** Writing – review & editing, Project administration, Methodology. **Jocelyn van den Bergen:** Writing – review & editing, Project administration, Methodology. **Sara Cronin:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sylvie Jaillard:** Writing – review & editing, Validation, Investigation. **Katie L. Ayers:** Writing – review & editing, Supervision, Project administration. **Pramod Kumar:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Christian Siebold:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Zhangping Xiao:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Edward W. Tate:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Shahla Danaei:** Writing – review & editing, Resources, Investigation. **Laya Farzadi:** Writing – review & editing, Resources, Investigation. **Shirin Shahbazi:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Andrew H. Sinclair:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Elena J. Tucker:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2024.112212.

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