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

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

3

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32

**33 Abstract**

34

**35 Research Question**

36 Premature ovarian insufficiency (POI) is characterised by amenorrhea associated with  
37 elevated follicle stimulating hormone (FSH) under the age of 40 years and affects 1-3.7%  
38 women. Genetic factors explain 20-30% of POI cases, but most causes remain unknown  
39 despite genomic advancements.

**40 Design**

41 We used whole exome sequencing (WES) in four Iranian families, validated variants via  
42 Sanger sequencing, and conducted the Acyl-cLIP assay to measure HHAT enzyme activity.

**43 Results**

44 Despite ethnic homogeneity, WES revealed diverse genetic causes, including a novel  
45 homozygous nonsense variant in SYCP2L, impacting synaptonemal complex (SC) assembly,  
46 in the first family. Interestingly, the second family had two independent causes for  
47 amenorrhea – the mother had POI due to a novel homozygous loss-of-function variant in  
48 FANCM (required for chromosomal stability) and her daughter had primary amenorrhea due  
49 to a novel homozygous GNRHR (required for gonadotropic signalling) frameshift variant.  
50 WES analysis also provided cytogenetic insights. WES revealed one individual was in fact  
51 46,XY and had a novel homozygous missense variant of uncertain significance in HHAT,  
52 potentially responsible for complete sex reversal although functional assays did not support  
53 impaired HHAT activity. In the remaining individual, WES indicated likely mosaic Turners  
54 with the majority of X chromosome variants having an allelic balance of ~85% or ~15%.  
55 Microarray validated the individual had 90% 45,XO.

**56 Conclusions**

57 This study demonstrates the diverse causes of amenorrhea in a small, isolated ethnic cohort  
58 highlighting how a genetic cause in one individual may not clarify familial cases. We propose  
59 that, in time, genomic sequencing may become a single universal test required for the  
60 diagnosis of infertility conditions such as POI.

61

## 62 **Keywords**

63 Premature ovarian insufficiency, Amenorrhea, Infertility, Whole exome sequencing

64

## 65 **Key Message**

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

71

72

## 73 **Introduction**

74

75 Premature ovarian insufficiency is a leading cause of infertility in women associated with  
76 elevated gonadotropins reflecting impairment of ovarian development or function (European  
77 Society for Human, Embryology Guideline Group on et al. 2016). Global prevalence of POI  
78 is 3.7% as reported by a recent meta-analysis (Golezar, Ramezani Tehrani et al. 2019).  
79 Based on current American and European established guidelines, the diagnosis of POI is  
80 confirmed in women < 40 years through elevated serum gonadotropin measurement on two  
81 sequential occasions at least 4 weeks apart (follicle stimulating hormone (FSH) > 25 IU/ml),

82 with concomitant low oestradiol (E2) levels (<50 pg/mL), and amenorrhea or oligomenorrhea  
83 for 4 to 6 months (European Society for Human, Embryology Guideline Group on et al.  
84 2016, Hewitt and Gerancher 2018).

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

91 The most frequent genetic cause of POI is arguably premutation of the CAG triplet repeat in  
92 the 5' untranslated region of the *fragile-X messenger ribonucleoprotein (FMR1)* gene  
93 (Wittenberger, Hagerman et al. 2007, Sullivan, Welt et al. 2011). Given that the incidence of  
94 *FMR1* premutation is 2-5% in sporadic POI cases and 11.5-16% in familial POI (Murray,  
95 Schoemaker et al. 2014, Turkyilmaz, Alavanda et al. 2022), *FMR1* premutation testing is  
96 indicated in POI women according to European Society of Human Reproduction and  
97 Embryology (ESHRE) Guidelines (European Society for Human, Embryology Guideline  
98 Group on et al. 2016).

99 Abnormalities in the X chromosome are another entrenched genetic cause of POI, and their  
100 frequency is approximately 12-14% (Janse, Knauff et al. 2010, Qin, Zhao et al. 2012),  
101 however, they are more frequent in patients with primary amenorrhea or familial POI (Rebar,  
102 Erickson et al. 1982). Turner syndrome (TS), with incidence of 1 in 2500-3000 females  
103 (Sybert and McCauley 2004), is due to complete or partial loss of one X chromosome (Ford,  
104 Jones et al. 1959) and typically manifests as short stature, short neck, cardiovascular  
105 disorders, kidney malformations as well as ovarian dysgenesis and/or loss of ovarian reserve  
106 before puberty (Zinn, Page et al. 1993). While 45, X is the most common cytogenetic cause

107 of primary amenorrhea, individuals with mosaic 45, X/46, XX often have a milder phenotype  
108 of secondary amenorrhea (Fechner, Davenport et al. 2006, Barros, Carvalho et al. 2020).  
109 Given chromosomal aberrations are a well-established cause of POI, cytogenetic analysis is  
110 recommended in patient diagnosis (European Society for Human, Embryology Guideline  
111 Group on et al. 2016). Traditionally this was done via high-resolution banding, but  
112 subsequently by fluorescent in situ hybridisation (FISH). More recently, comparative  
113 genomic hybridisation (CGH) array is the preferred approach for cytogenetic analysis due to  
114 its higher resolution and cost-effectiveness (Barros, Carvalho et al. 2020). CGH arrays  
115 currently identify a genetic aetiology in ~10% of POI patients (Qin, Jiao et al. 2015, Jaillard,  
116 Akloul et al. 2016, Franca, Moraes et al. 2022).

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

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

127 To date, there have been over 100 genes in which variants have been described in association  
128 with POI and amenorrhea (Supplementary File S1). These genes are implicated in diverse  
129 processes such as ovarian development, chromosome stability (for example, *FANCM*,  
130 required for DNA damage repair), meiosis (including *SYCP2L* required for synaptonemal  
131 complex formation for alignment of chromosomes during cell division), and hormone

132 signalling (such as *GNHRH* required for regulating the reproductive axis) (Chapman, Cree et  
133 al. 2015, Qin, Jiao et al. 2015, Tucker, Grover et al. 2016). Although NGS has accelerated the  
134 rate of identifying causative genes associated with disease, the analysis of NGS data can be  
135 challenging, and functional validation is imperative to accurate variant curation.

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

147

148

## 149 **Materials and Methods**

150

### 151 **Participants and ethical adherence**

152 Six amenorrheic patients from four Iranian families were included in this study – five  
153 diagnosed with POI with elevated FSH (>20 mIU/mL) and one diagnosed with  
154 hypogonadotropic hypogonadism. Table 3 includes the available family and medical history.  
155 No patient reported a history of autoimmune disease, although ovarian autoantibodies were  
156 not assessed. All procedures were approved by the Human Research Ethics Committee of the

157 Royal Children's Hospital, Melbourne (HREC# 22073). Written informed consent was  
158 obtained from all participants.

159

### 160 **General molecular techniques**

161

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

167

### 168 **Sanger sequencing**

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

172

### 173 **Clinical microarray**

174

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

180

### 181 **Whole-exome sequencing (WES)**



182 Whole-exome sequencing of DNA from the patients was performed at VCGS on the  
183 NovaSeq 6000 (Illumina) with SureSelect Human All Exon V6 (Agilent) exome capture.  
184 Variant detection was performed using Cpipe (Sadedin, Dashnow et al. 2015). WES data  
185 were analysed using SeqR software (<https://seqr.broadinstitute.org/>) through two phases of  
186 analysis (Table 4) including gene-centric and variant-centric approaches as reported  
187 previously (Tucker, Jaillard et al. 2019). Gene priority using POI candidate genes (Tucker,  
188 Jaillard et al. 2023) (Supplementary File S1), differences of sex development candidate gene  
189 list from Eggers et al. (Eggers, Sadedin et al. 2016), Differences of Sex Development gene  
190 list and Kidneyome SuperPanel gene list from PanelApp-AUS  
191 (<https://panelapp.gha.umccr.org/>) have been used for gene-centric analysis. For gene-centric  
192 analysis we filtered for variants with  $MAF < 0.005$  and high quality score ( $Q > 20$ , allele  
193 balance  $> 25$ ). Variant-centric analysis focused on high quality ( $Q > 20$ , allele balance  $> 25$ )  
194 high-priority variants (likely leading to loss of function: nonsense, splice site, frameshift)  
195 with minor allele frequency ( $MAF < 0.0001$ ) in any gene and with any inheritance or high  
196 quality ( $Q > 20$ , allele balance  $> 25$ ) biallelic variants with predicted moderate-high impact  
197 (missense, in-frame indels, frameshift, nonsense or splice site) and  $MAF < 0.005$ . We chose  
198 to exclude variants with  $> 0.005$  MAF because POI affects  $\sim 1\%$  of the general population and  
199 is caused by variants in  $> 100$  genes. Each genetic cause of POI identified to date is rare. The  
200 rationale for MAF filtering thresholds for the different phases of analyses reflects the  
201 tolerance of recessive variants in the population (in heterozygous state) in contrast to the  
202 intolerance of dominant variants (that therefore must be rarer in the general population). We  
203 chose a higher MAF for gene-centric analysis to ensure we didn't overlook variants that were  
204 in clinically-relevant genes. The public database gnomAD v4  
205 (<https://gnomad.broadinstitute.org/>) was used to assess MAF and the tolerance of genes to  
206 missense and/or loss-of-function variation. Heterozygous "high" priority variants were

207 discounted if they affected 1) genes tolerant of LoF based on gnomAD data, 2) genes not  
208 expressed in gonadal tissue as per GTEx data, 3) transcripts with minimal expression that  
209 have not been validated or 4) genes that are not related to ovarian biology or development.  
210 We predicted missense variant pathogenicity in silico using several online tools including  
211 Mutation Taster (<http://www.mutationtaster.org/>), Polyphen-2  
212 (<http://genetics.bwh.harvard.edu/pph2/>), CADD (Combined Annotation-Dependent  
213 Depletion) (<https://cadd.gs.washington.edu/snv>), SIFT/Provean (<http://provean.jcvi.org/>) and  
214 Decipher database (<https://www.deciphergenomics.org/>). We assessed the conservation of  
215 affected residues by using Multiz Alignments of 100 vertebrates (UCSC Genome Browser  
216 <https://genome.ucsc.edu/>).

217

#### 218 **Data availability**

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

222

#### 223 **Acyl-cLIP assay**

224 The Acyl-cLIP assay was applied to measure the enzymatic activity of HHAT wild type (wt)  
225 or R424C mutant. HHAT wildtype and mutant proteins for the enzymatic assay were  
226 produced following an established procedure described in (Coupland, Andrei et al. 2021).  
227 The Acyl-cLIP assay has been described before (Lanyon-Hogg, Ritzefeld et al. 2019, Andrei,  
228 Tate et al. 2022). Briefly, a 7.5  $\mu$ M palmitoyl-CoA (Sigma Aldrich) was prepared in reaction  
229 buffer (100 mM MES, 20 mM NaCl, 1 mM DTT, 1 mM TCEP, 0.1% BSA, pH 6.5). Next,  
230 FAM-labelled SHH-peptide was diluted in reaction buffer to a concentration of 2  $\mu$ M.

231 Recombinant HHAT (about 0.1 mg/mL, obtained by following the published protocol  
232 (Coupland, Andrei et al. 2021) was diluted 30 times in storage buffer (20 mM HEPES, 350  
233 mM NaCl, 1% DDM, 5% glycerol, pH 7.3). Diluted HHAT (220  $\mu$ L) and SHH peptide (2  
234 mL) were mixed to prepare enzyme-substrate mixture. To start the reaction, SHH and HHAT  
235 mixture (12  $\mu$ L) was mixed with palmitoyl-CoA (4  $\mu$ L) and reaction buffer (4  $\mu$ L) in to each  
236 well of a 384 well plate, and the fluorescence polarization (Excitation 480 nm, emission 535  
237 nm) measured for 1 hour at two-minute intervals on an EnVision Xcite 2104 (PerkinElmer).  
238 The slope of the resulting curves was calculated for each well using Prism 9.1.2 (GraphPad)  
239 and each well was background subtracted by subtracting the average of values from wells  
240 without palmitoyl-CoA. The background subtracted data were then normalized to plot  
241 Michaelis–Menten kinetics using Prism with 95% confidence intervals (n=4 replicates).

242

243

## 244 **Results**

245

### 246 **Novel variants associated with amenorrhea in four families**

247

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

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

254 WES of the two affected siblings and parents identified a homozygous stop-gain *SYCP2L*  
255 variant: Chr6:10930409 (hg38), NM\_001040274.3(*SYCP2L*):c.1528C>T p.(Gln510Ter)

256 shared by both sisters and heterozygous in both parents (Fig. 1b). This variant is detected  
257 only once in gnomAD v4 (allele frequency 0.0000012) and predicted to be likely deleterious  
258 by online algorithms (Table 5). Sanger sequencing confirmed segregation of the *SYCP2L*  
259 variant with disease in the family (Fig. 1c). Since the variant is located in a central exon  
260 (exon 19 out of 30) and causes a premature stop codon, it is likely that the transcript is  
261 degraded by nonsense-mediated decay (NMD) resulting in loss of SYCP2L protein (Fig. 1d).  
262 If residual mRNA persisted, any truncated protein is likely to be non-functional. The variant  
263 falls within a region encoding compositional bias (polar residues **507-527**). Regions of  
264 polarity often have crucial roles in protein structure, stability, and function, often  
265 participating in interactions with other polar or charged molecules (Ayuso-Tejedor, Abián et  
266 al. 2011, Illergård, Kauko et al. 2011). Protein truncation at this site would also remove three  
267 downstream regions of compositional bias that are highly conserved and likely critical for  
268 protein structure/function (570-614 polar, 615-631 basic/acidic, 639-656 basic/acidic) (Fig  
269 1d).

270

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

273 Patient 3, also from a consanguineous Iranian pedigree was diagnosed with POI at the age of  
274 31 due to secondary amenorrhea and high FSH (65 IU/I), with pelvic ultrasound showing  
275 small ovaries (Fig. 2a). Her daughter (Patient 4) was diagnosed with primary amenorrhea at  
276 the age of 16, with low FSH (0.1 IU/I), indicating hypothalamic amenorrhea. Pelvic  
277 ultrasound could not detect a uterus or ovaries.

278 WES was performed for the mother and her daughter. Given both mother and daughter were  
279 affected by amenorrhea, dominant inheritance was considered, but no likely causative  
280 variants were identified. Data analysis instead identified two different genetic causes of

281 amenorrhea; variants in *FANCM* in the mother and variants in *GNRHR* in the daughter. These  
282 genes are in keeping with the disparate hormonal status of each affected individual – the  
283 mother having elevated FSH and the daughter having low FSH. Patient 3 (mother) had a  
284 novel homozygous loss-of-function *FANCM* variant: Chr14:45173106 (hg38),  
285 NM\_020937.4(*FANCM*):c.2215\_2216delTG p.(Trp739AlafsTer11), whereas Patient 4  
286 (daughter) had a novel homozygous frameshift *GNRHR* variant: Chr4:67754087 (hg38),  
287 NM\_000406.3(*GNRHR*):c.248delT p.(Leu83ArgfsTer3). (Fig. 2b and Fig. 3a). Sanger  
288 sequencing confirmed the WES findings, demonstrating that the mother was homozygous for  
289 the deletion in *FANCM*, and the daughter was heterozygous for the same variant (Fig. 2c).  
290 The *GNRHR* variant segregated with the disorder in the family, with the daughter being  
291 homozygous and her parents being heterozygous (Fig. 3b). Both variants were absent in  
292 public databases, such as gnomAD v.4 (Table 5) and were predicted to be likely damaging by  
293 online tools. The *FANCM* variant falls within exon 13 out of 23 and the region encoding the  
294 highly conserved *FANCM*-MHF binding domain of the protein, which is essential for DNA  
295 repair in response to genotoxic stress (Tao, Jin et al. 2012). The *GNRHR* variant is located in  
296 the first exon of 3 and interrupts the 7tm\_GPCRs (seven-transmembrane G protein-coupled  
297 receptor superfamily) conserved domain. This domain is essential for activation of  
298 intracellular signalling pathways mediated by G proteins and play a crucial role in regulating  
299 the synthesis and release of gonadotropins (Naor 2009, Kinoshita and Okada 2015). Both  
300 *FANCM* and *GNRHR* variants, however, are distant from the NMD escape region and are  
301 likely to induce NMD, resulting in loss of the proteins. (Fig. 2d and Fig. 3c).

302

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

304 Patient 5, of Iranian descent and born to consanguineous parents, received a primary

305 amenorrhea diagnosis at age 19 without undergoing FSH assessment. Pelvic ultrasound

306 showed atrophic ovaries and a thin myometrium. The patient had a history of nephrotic  
307 syndrome and received a kidney transplant at age 16. Although recruited as a female patient,  
308 WES analysis revealed that the individual was in fact 46,XY after detecting Y chromosome  
309 sequence, including the SRY gene. Coverage across the X chromosome was ~50% compared  
310 to XX control individuals, consistent with a likely 46,XY karyotype (Fig. 4). Additionally,  
311 WES identified a novel homozygous missense variant in *HHAT*: Chr1:210623550 (hg38),  
312 NM\_018194.6 c.1270C>T p.(Arg424Cys) (Fig. 5a). Sanger sequencing validated the *HHAT*  
313 variant in the patient (Fig 5b). Parental DNA was not available to confirm parental genotype,  
314 however, microarray did not reveal a copy number variation at this site suggesting it is  
315 unlikely the patient harbours a deletion of one allele causing apparent homozygosity. Identity  
316 by descent is also in keeping with the consanguinity of the pedigree. This variant was  
317 detected in heterozygous state in 24 individuals in gnomAD v4 (allele frequency 0.00001487)  
318 and predicted damaging by some online algorithms (Table 5). The identified missense variant  
319 affects p.Arg424 which is conserved in mammals (Fig. 5c). The *HHAT* variant residue is  
320 situated within a critical membrane-bound Oacyltransferase (MBOAT) domain of the protein,  
321 which is highly conserved and essential for the protein's activity. Therefore, the variant has  
322 the potential to disrupt the function of *HHAT* protein (Fig. 5d). Given the 3D structure of  
323 *HHAT* is known, we used the HOPE modeling tool (<http://www.cmbi.ru.nl/hope/>)  
324 (Venselaar, Te Beek et al. 2010) to simulate the effects of the missense variant. The variant  
325 residue is smaller, neutral and more hydrophobic than the original wild-type residue, which  
326 was positively charged. These changes can potentially lead to the loss of critical interactions  
327 of the residue with other molecules or residues, including hydrogen bonds, and disrupt proper  
328 protein folding, thereby affecting the function of *HHAT* protein (Fig. 5e). Using the  
329 American College of Medical Genetics (ACMG) and Genomics variant classification criteria,  
330 this variant is considered a Class 3 variant of uncertain significance. The WES data was also

331 analysed for variants potentially responsible for kidney disease given the patient's history of  
332 nephrotic syndrome. A hemizygous c.2500G>A, p.(Glu834Lys) in *TBC1D8B* was detected.  
333 Missense variants in this gene have previously been reported in X-linked nephrotic syndrome  
334 without sex reversal (Kampf, Schneider et al. 2019), suggesting the potential for dual  
335 diagnosis in this individual.

336

### 337 *HHAT enzymatic activity*

338 We measured the enzymatic activity of HHAT wildtype and variant (HHAT R424C) using  
339 polarisation-based Acyl-cLIP assays to investigate the influence of this missense mutation.  
340 The kinetics studies showed that HHAT R424C exhibits a Michaelis constant ( $K_M$ ) of 125  
341 (95% confidence interval, CI:114-136) nM, which is slightly but significantly higher than the  
342  $K_M$  of the wt 98 (95% CI: 89-108) nM. Furthermore, the maximum rate ( $V_{max}$ ) is 0.397  
343 (95% CI, 0.387-0.407) for the mutant, which is slightly but significantly lower than 0.438  
344 (95% CI, 0.428-0.449) for the wild type (Fig. 6). These results demonstrate that there is  
345 minimal difference observed on the enzymatic activity between HHAT R424C and wt in the  
346 Acyl-cLIP assays.

347

### 348 *Mosaic Turners with a ring X chromosome*

349 Patient 6, who comes from a non-consanguineous Iranian family, was diagnosed with  
350 secondary amenorrhea at the age of 32. Her hormonal assessment showed elevated FSH (71  
351 IU/I) and low AMH (0.01 ng/ml). WES revealed that the individual is likely to have mosaic  
352 Turners with the allele balance of variants on the X chromosome around ~85% and ~15% in  
353 the patient in comparison to the expected ~50% in 46XX controls. Furthermore, WES  
354 coverage across the X chromosome was comparable to the coverage in 46, XY individuals  
355 (Fig. 7a, b and c). Microarray confirmed the WES data interpretation, indicating a mosaic

356 Turners variant. In particular, there was a 45,XO cell line in 90% of cells and a ring  
357 chromosome X, with a 46,X,r(X)(p22.13q22.3) karyotype, in 10% of cells (Fig. 7d).

358

359

## 360 **Discussion**

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

365

### 366 **A novel homozygous nonsense variant in *SYCP2L* consolidates the role of this gene in** 367 **isolated POI**

368 *SYCP2L* (*Synaptonemal complex protein 2 like*) encodes one of the proteins participating in  
369 assembly of synaptonemal complex (SC) during meiotic prophase (Cahoon and Hawley  
370 2016). *SYCP2L* is expressed exclusively in ovaries and testes and is required for male and  
371 female fertility (He, Tan et al. 2021). In particular, *SYCP2L* regulates the survival of  
372 primordial oocytes and lacking this component from SC structure leads to meiotic arrest and  
373 ultimately atretic oocytes. Additionally, *Sycp2l*-deficient female mice are subfertile (Zhou,  
374 Stein et al. 2015). The association of the genes that have key roles in meiosis and DNA  
375 repair with POI has been previously reported (Veitia 2020, Tucker, Bell et al. 2022). *SYCP2L*  
376 has recently been identified as a POI gene with one report describing two POI patients with  
377 biallelic loss-of-function alleles (He, Tan et al. 2021). Similar to the variants outlined in the  
378 prior report, the *SYCP2L* homozygous nonsense variant (c.1528C>T, p.(Gln510Ter))  
379 identified in the first family (Patient 1 and Patient 2) of our study is also a predicted loss-of-  
380 function allele. According to ACMG criteria, the variant is classified as likely pathogenic. Its



381 status could be elevated to pathogenic if loss-of-function could be demonstrated  
382 experimentally via the analysis of patient cells, however further biological sample from the  
383 patient is not available. In vitro expression of truncated protein is also unlikely to model the  
384 patient situation given the location of the premature termination codon (PTC) prior to the last  
385 50bp of the penultimate exon. PTCs introduced at this position usually trigger nonsense  
386 mediated decay (Kurosaki and Maquat 2016) and ACMG recommendation is to treat these as  
387 likely loss of function, allowing the application of very strong (VS1) pathogenicity criterion  
388 (Abou Tayoun, Pesaran et al. 2018). Our research expands the spectrum of *SYC2PL* variants  
389 associated with POI and adds new evidence to support the association of this recently  
390 reported POI gene with female infertility.

391

### 392 **Two independent genetic causes for amenorrhea in a single pedigree**

393 The second family of this study had a mother and daughter who presented with amenorrhea.  
394 The mother had causative variants in *FANCM* and the daughter had causative variants in  
395 *GNRHR*. The Fanconi anaemia complementation group M (*FANCM*) encodes a tumour  
396 suppressive DNA translocase involved in chromosomal stability and DNA repair. *Fancm*-  
397 deficient mice have meiotic defects leading to disrupted follicular development and depleted  
398 follicular supply (Fouquet, Pawlikowska et al. 2017). *FANCM* biallelic variants in human  
399 have been shown to be associated with early menopause (Catucci, Osorio et al. 2018), non-  
400 syndromic POI (Fouquet, Pawlikowska et al. 2017, Heddar, Ogur et al. 2022) and male  
401 infertility (Kasak, Punab et al. 2018, Yin, Ma et al. 2019). The identified *FANCM* variant in  
402 our study is a novel predicted loss-of-function allele. This is in accordance with previously  
403 reported pathogenic *FANCM* variants in patients with non-syndromic POI that are also loss-  
404 of-function variants (Fouquet, Pawlikowska et al. 2017, Jaillard, Bell et al. 2020).  
405 Importantly, heterozygous variants in this gene are associated with ovarian and breast cancer

406 predisposition (Basbous and Constantinou 2019, Schubert, van Luttikhuizen et al. 2019) and  
407 there is some evidence suggesting biallelic *FANCM* variants are associated with cancer  
408 predisposition, especially early-onset breast cancer in women (Bogliolo, Bluteau et al. 2018,  
409 Catucci, Osorio et al. 2018). Accordingly, this highlights the necessity to offer genetic  
410 counselling and breast screening to patients with POI due to underlying *FANCM* variants.  
411 Further biological sample for research was not accessible from this patient, however, she  
412 could benefit from clinical cytogenetic analysis to determine sensitivity to DNA damage. We  
413 have previously demonstrated chromosomal instability in an individual harbouring  
414 pathogenic *FANCM* variants (Jaillard, Bell et al. 2020). This analysis could determine the  
415 relative risk of cancer development. Future studies in larger cohorts of patients with long  
416 term follow-up will provide further insight into the risk of cancers in these patients.  
417 Interestingly, the cause of amenorrhea in the proband's daughter (Patient 4) was independent  
418 – she experienced hypothalamic amenorrhea due to variants in *GNRHR*. *GNRHR* encodes  
419 gonadotropin releasing hormone (GnRH) receptor that mediates gonadotropic signalling and  
420 stimulates the secretion of gonadotropins (LH and FSH). *GNRHR* variants associated with  
421 congenital hypogonadotropic hypogonadism (cHH) are typically loss-of-function (LOF)  
422 variants and occur across the coding sequence of the *GNRHR* gene (Cioppi, Riera-Escamilla  
423 et al. 2019). The identified frameshift *GNRHR* variant in our study lies within the first exon  
424 of the gene which is far from the NMD escape region and is therefore predicted to induce  
425 NMD and likely the loss of GNRHR protein. If additional biological sample could be  
426 obtained, this could be confirmed by RNA analysis or western blot. The clinical effect of  
427 different *GNRHR* variants is highly variable based on the type of variant and the subsequent  
428 level of functional protein expression. Typically, patients harbouring homozygous loss-of-  
429 function *GNRHR* variants have cHH presenting with undetectable LH and FSH in serum and  
430 absence of spontaneous puberty characterised by underdeveloped/absent breast development

431 and amenorrhea in females and cryptorchidism or micropenis in males (Gianetti, Hall et al.  
432 2012, Correa-Silva, Fausto et al. 2018, Cioppi, Riera-Escamilla et al. 2019, Hussain, Murtaza  
433 et al. 2019). It is likely that the loss-of-function *GNRHR* variant in Patient 4 is responsible for  
434 her hypogonadotropic hypogonadism. The fact that this family presented with two  
435 independent causes of amenorrhea highlights the diverse nature of the condition, and that a  
436 genetic cause in one family member does not always indicate cause in relatives, particularly  
437 in consanguineous pedigrees for which there is an excess of homozygosity.

438

439 **A homozygous missense variant of uncertain significance in *HHAT* is identified in an**  
440 **individual with complete sex reversal**

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

456 missense *HHAT* variants (Callier, Calvel et al. 2014, Pande, Radhakrishnan et al. 2021,  
457 Mazen, Kamel et al. 2022, Rjiba, Mougou-Zerelli et al. 2023).

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

466 Additional cases described in the literature have expanded the phenotypic spectrum  
467 associated with variants in *HHAT* with gonadal dysgenesis being a common feature in 46XY  
468 individuals. Other features include microcephaly and cerebellar vermis hypoplasia, muscle  
469 hypertrophy, muscle spasms, facial dysmorphism and eye defects (Abdel-Salam, Mazen et al.  
470 2019, Baz-Redón, Soler-Colomer et al. 2022, Mazen, Kamel et al. 2022, Pande,  
471 Radhakrishnan et al. 2022, Saini, Das Bhowmik et al. 2023). All but one previously reported  
472 pathogenic *HHAT* variant lies in the highly conserved functional MBOAT domain.

473 Consistent with these previously reported variants, the homozygous missense variant  
474 identified in the present study (p.Arg424Cys) was also found to be located in the MBOAT  
475 domain. These variants may impair the ability of *HHAT* to palmitoylate Hedgehog proteins  
476 like SHH and DHH, leading to disrupted gonadal development. Patient 5 of this study also  
477 had nephrotic syndrome requiring kidney transplantation at age 16. Whether kidney  
478 pathology is due to *HHAT* deficiency is uncertain, however, there has been one sex-reversed  
479 46,XY female individual described previously who displayed bilateral renal pyelectasis  
480 during fetal development and high levels of creatinine phosphokinase (CPK, 922.0 U/L)

481 during adulthood. She presented with additional clinical manifestations of HHAT deficiency  
482 such as short stature, microcephaly and severe microphthalmia (Pande, Radhakrishnan et al.  
483 2022). Importantly, we identified a hemizygous variant of uncertain significance in an X-  
484 linked nephrotic syndrome gene, *TBC1D8B*, which requires functional validation. This  
485 indicates that the patient could have a dual diagnosis with sex reversal and nephrotic  
486 syndrome having independent genetic causes. *In silico* analysis tools such as Polyphen and  
487 Mutation Taster had conflicting predictions with regards to the likely pathogenicity of the  
488 *HHAT* variant identified in Patient 5 and conservation of this residue was restricted to  
489 mammals (Fig. 5c). According to ACMG variant classification, this variant (NM\_018194.6:  
490 c.1273C>T) was classified as a variant of uncertain significance (VUS). Two *HHAT* variants  
491 reported in recent studies including a homozygous missense variant (c.1001T>A  
492 (p.Met334Lys)) and a homozygous in frame deletion (c.365\_367del (p.Thr122del)) also were  
493 classified as VUSs but were subsequently found to be damaging to protein function (Baz-  
494 Redón, Soler-Colomer et al. 2022, Pande, Radhakrishnan et al. 2022). In our study, the  
495 assessment of HHAT enzymatic activity using purified HHAT and labelled SHH peptide did  
496 not provide evidence supporting the pathogenicity of the HHAT variant (p.Arg424Cys).  
497 However, we could not exclude the possibility of changed protein stability or PTM pattern at  
498 cellular context caused by this mutation (Konitsiotis, Jovanović et al. 2015). Our previous  
499 studies revealed that HHAT is palmitoylated on multiple cytosolic cysteines, including  
500 Cys188, Cys242, Cys324, and Cys410, which we proposed helps to regulate protein structure  
501 in the membrane (Konitsiotis, Jovanović et al. 2015). Arg424 is in close proximity to  
502 Cys410, which is located on the loop formed by  $\alpha$ -helix  $\alpha$ 11 and  $\alpha$ 12 on the cytoplasmic face  
503 (Coupland, Andrei et al. 2021). p.Arg424Cys might interfere with cellular HHAT  
504 palmitoylation or even introduce a site for de novo palmitoylation, subsequently leading to an  
505 increase or decrease in activity. Additionally, deletion of HHAT residues 417-426 resulted in

506 decreased stability and loss of function, indicating this region may play an important role  
507 (Buglino and Resh 2010). Consequently, this variant remains of uncertain significance. The  
508 identification of 46,XY karyotype via WES analysis had clinical consequence for the patient,  
509 highlighting potential of dysgenic gonads that may pose a cancer risk. Further investigation  
510 via ultrasound and gonadal biopsy may be recommended with removal of gonads with  
511 malignant tissue.

512

### 513 **WES data analysis detected mosaic Turners in Patient 6**

514 WES also identified a cytogenetic cause of amenorrhea in Patient 6, who presented with  
515 secondary amenorrhea and elevated FSH. Atypical allelic balance of X chromosome variants  
516 indicated a likely mosaic 45,XO Turners karyotype, that was subsequently confirmed by  
517 microarray. Individuals with Turner syndrome typically present as female, but experience  
518 POI, short stature, short neck, and/or cardiovascular disease. For the past three decades,  
519 studies of chromosomes have revealed that short stature and primary amenorrhea are present  
520 in at least 95% of all people diagnosed with Turner syndrome (Moka, Sreelakshmi et al.  
521 2013). The most prevalent cytogenetic cause of primary amenorrhea is believed to be a  
522 complete absence of an X chromosome (45, XO), while secondary amenorrhea is more  
523 frequently associated with mosaic Turners 45, XO/46, XX (Fechner, Davenport et al. 2006,  
524 Moka, Sreelakshmi et al. 2013, Barros, Carvalho et al. 2020). Females with Turner Syndrome  
525 often present with ovarian dysgenesis and a decreased ovarian reserve before puberty due to  
526 the accelerated follicular atresia (Qin, Jiao et al. 2015). The majority of women who have a  
527 ring chromosome X, known as r(X), present with Turners syndrome due to somatic loss of  
528 the unstable X chromosome and a mosaic 45,XO karyotype (Dennis, Coppin et al. 2000). The  
529 clinical symptoms of Turners syndrome tend to correlate with the ratio of normal (46,XX)  
530 cells and 45,XO cells. The variable clinical presentation can also be attributed to undetected

531 mosaicisms, which may be the explanation for infrequent instances of normal ovarian  
532 function and even more uncommon occurrences of pregnancy among women with Turners  
533 syndrome (Moka, Sreelakshmi et al. 2013, Barros, Carvalho et al. 2020). In the present study,  
534 Turners syndrome had not been identified clinically until WES revealed mosaic Turners with  
535 a karyotype of 90% 45, XO and 10% 46, X,r(X).

536 Studies have shown that a considerable number of females with TS receive a delayed  
537 diagnosis, with 22% of cases diagnosed after the age of 12 years and up to 10% diagnosed in  
538 adulthood (Massa, Verlinde et al. 2005, Murdock, Donovan et al. 2017). The delayed  
539 diagnosis of TS is a well-recognized issue as it may prevent affected individuals from  
540 receiving timely and essential medical interventions and comorbidity screening (Savendahl  
541 and Davenport 2000, Stockholm, Juul et al. 2006). Early diagnosis enables prompt treatment  
542 options, including hormone therapy to induce age-appropriate puberty (Bondy and Group  
543 2007), growth hormone therapy starting at 4 years of age (Davenport, Crowe et al. 2007,  
544 Linglart, Cabrol et al. 2011), fertility preservation options in young girls (such as ovarian  
545 tissue freezing and oocyte cryopreservation (Birgit, Julius et al. 2009) and assessment for  
546 congenital heart disease (Bondy and Group 2007). In the present study, there was a delay in  
547 diagnosing Patient 6, as she was only diagnosed with POI at the age of 32. However, using  
548 WES, we were able to identify TS mosaicism in this individual. Similarly, in a previous  
549 study, WES was used to detect isochromosome Xq, cryptic Y-chromosome material, and  
550 low-level mosaicism (as low as 5%) in a cohort of female individuals with Turner syndrome  
551 (Murdock, Donovan et al. 2017). This highlights WES as a sensitive and specific screening  
552 test for TS and other chromosomal abnormalities, which were conventionally only achievable  
553 through karyotype or microarray analysis.

554

555 **Conclusion**

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

567

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581

**582 Contribution statement**

583 EJT and AHS conceived the study. SB, EJT, FA, GR, JvdB, CS, PK, ZX and EWT acquired  
584 the data. SB, FA, KMB, SC, SJ, KLA, CS, PK, ZX, EWT, SD, LF, SS, AHS and EJT  
585 analyzed and interpreted the data. SB, EJT, KMB performed the bioinformatic, genomic  
586 analyses and CS, PK, ZX and EWT performed HHAT enzyme activity analyses. FA, SD, LF  
587 and SS recruited patients, liaised with families and collected and reviewed clinical data. SB  
588 and EJT wrote the manuscript, with input from all authors. All authors reviewed and  
589 approved the manuscript.

590

**591 Data Availability**

592 Some or all datasets generated during and/or analysed during the current study are not  
593 publicly available but are available from the corresponding author on reasonable request.

594

**595 Ethics approval**

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

598

**599 Consent to participate**

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

601

602

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967 Table 1. Genetic causes of POI  
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<i>Genetic cause</i>	<i>Frequency</i>	<i>References</i>
<b>Chromosomal aberrations</b>	10-14%	(Janse, Knauff et al. 2010, Qin, Zhao et al. 2012, Qin, Jiao et al. 2015, Jaillard, Akloul et al. 2016)
<b>FMRI premutation</b>	2-5% sporadic POI 11.5-16% familial POI	(Murray, Schoemaker et al. 2014, Turkyilmaz, Alavanda et al. 2022)
<b>Monogenic causes*</b>	50% familial POI 16-29% POI	(Liu, Wei et al. 2020, Heddar and Misrahi 2022, Rouen, Rogers et al. 2022, Vogt, Bratland et al. 2022)

969 \* The varied percentage attributed to monogenic causes reflects the stringency of criteria  
970 used for assigning pathogenicity. Often ACMG criteria are inappropriately applied in a  
971 research context, leading to overstatement of diagnostic yields.  
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Table 2. Timeline of changing genomic technologies for identifying genetic causing of POI.

<b>Genomic technologies</b>	<b>Timeline</b>	<b>Gene discovery examples</b>	<b>Reference</b>
<b>Conventional karyotyping</b>	1959	X chromosome defects	(Ford, Jones et al. 1959)
<b>fluorescence in situ hybridization (FISH)</b>	1994, 1998		(Powell, Taggart et al. 1994, Devi, Metzger et al. 1998)
<b>Direct sequencing of PCR products</b>	1995, 1996, 1998, 1999, 2000	<i>FSHR, LHR</i>	(Aittomäki, Lucena et al. 1995, Latronico, Anasti et al. 1996, Beau, Touraine et al. 1998, Touraine, Beau et al. 1999, Shelling, Burton et al. 2000)
<b>Comparative genomic hybridization (CGH) array</b>	2008, 2009		(Tachdjian, Aboura et al. 2008, Aboura, Dupas et al. 2009)
<b>Genome-Wide Association Studies (GWAS)</b>	2008, 2009, 2012		(Kang, Lee et al. 2008, Knauff, Franke et al. 2009, Pyun, Cha et al. 2012)
<b>Whole-exome sequencing (WES) in syndromic POI</b>	2010, 2013, 2014	<i>HSB17B4, LARS2, CLPP, TWNK</i>	(Pierce, Walsh et al. 2010, Jenkinson, Rehman et al. 2013, Pierce, Gersak et al. 2013, Morino, Pierce et al. 2014)

<b>Whole-exome sequencing (WES) in non-syndromic POI</b>	2014	<i>HFMI, STAG3, MCM9, SYCE1</i>	(Caburet, Arboleda et al. 2014, de Vries, Behar et al. 2014, Wang, Zhang et al. 2014, Wood-Trageser, Gurbuz et al. 2014)
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979 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	-

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982 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

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

Patient	Gene	RefSeq	Status	gDNA variant (hg38)	cDNA variant	Protein variant	Quality metrics	Polypeptide	Mutation Taster	CAAD	eigen	PhyloP100	SpliceAI	Pangolin	References for other gene variants
1 and 2	<i>SYCP2L</i>	NM_001040274.3	Homozygous-Nonsense	Chr6:10930409 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, Tan et al. 2021)
3	<i>FANCM</i>	NM_020937.4	Homozygous-Frameshift	Chr14:45173106 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, Pawlikowska et al. 2017, Jaillard, Bell et al. 2022, Heddar, Ogur et al. 2022)
4	<i>GNRHR</i>	NM_000406.3	Homozygous-Frameshift	Chr4:67754087 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, Riera-Escamilla et al. 2019)
5	<i>HHAT</i>	NM_018194.6	Homozygous-Missense	Chr1:210623550 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, Calvel et al. 2014, Abdel-Salam, Mazen et al. 2022, Mazen, Kamel et al. 2022, Pande, Radhakrishnan et al. 2022, Saini, Das Bhowmik 2023)

**Figure 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 III2), 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.

**Figure 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/pPatient 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.

**Figure 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.

**Figure 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

**Figure 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.

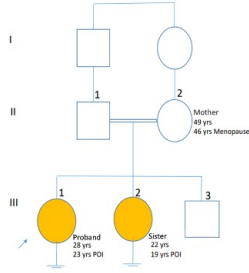
**Figure 6.** Michaelis–Menten kinetics plots of HHAT wt and HHAT R424C mutant over Pal-CoA. WT shows a  $V_{max}$  of 0.438 (95% CI, 0.428-0.449) and  $K_M=125$  nM (95% CI, 114-136); R424C shows a  $V_{max}$  of 0.397 (95% CI, 0.387-0.407) and  $K_M=98$  nM (95% CI, 89-108). Error bars: standard error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

**Figure 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:18387356-106251294 and chromosome band location of Xp22.13q22.3.

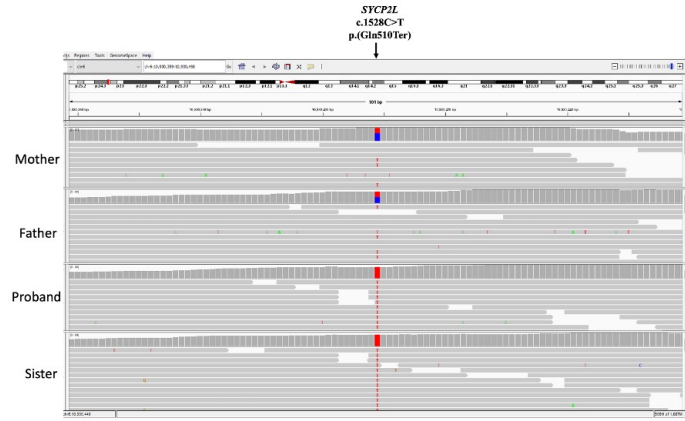
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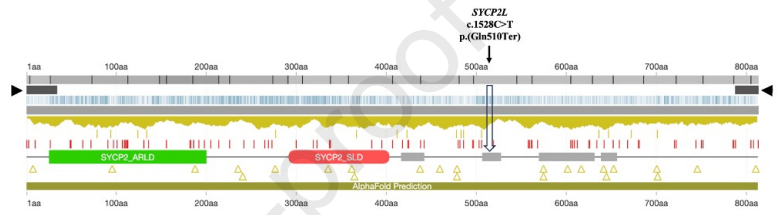
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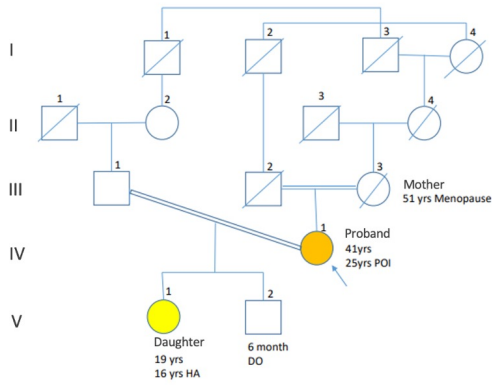
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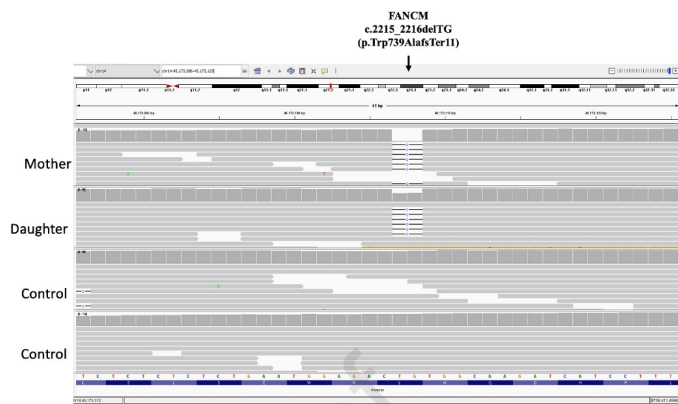
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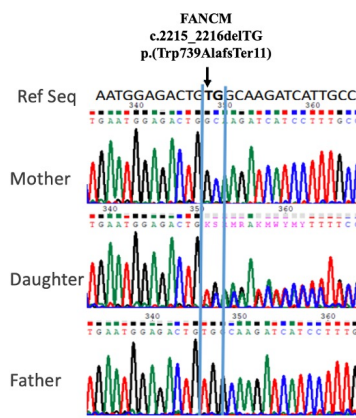
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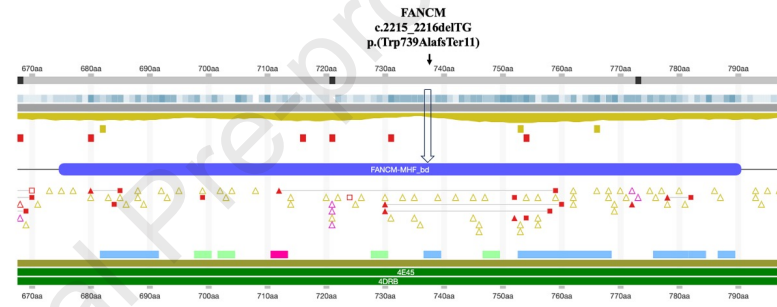
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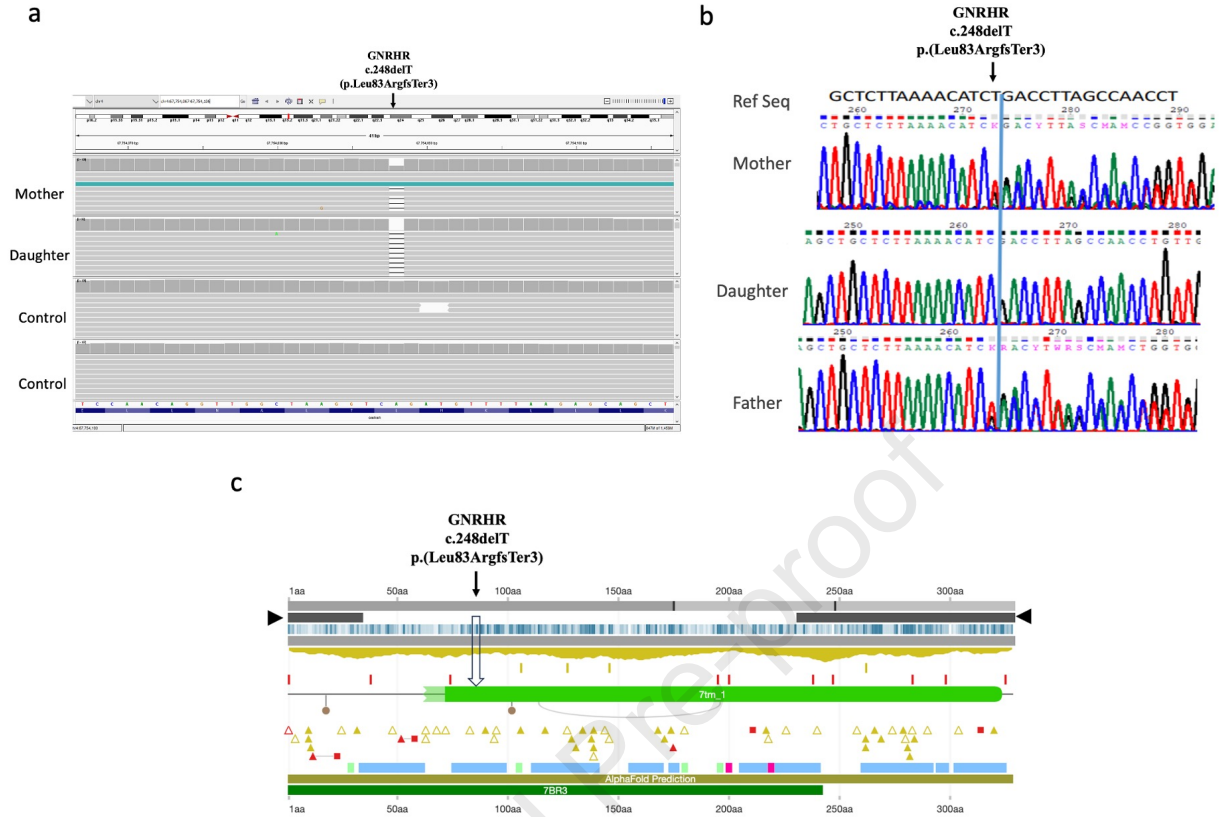


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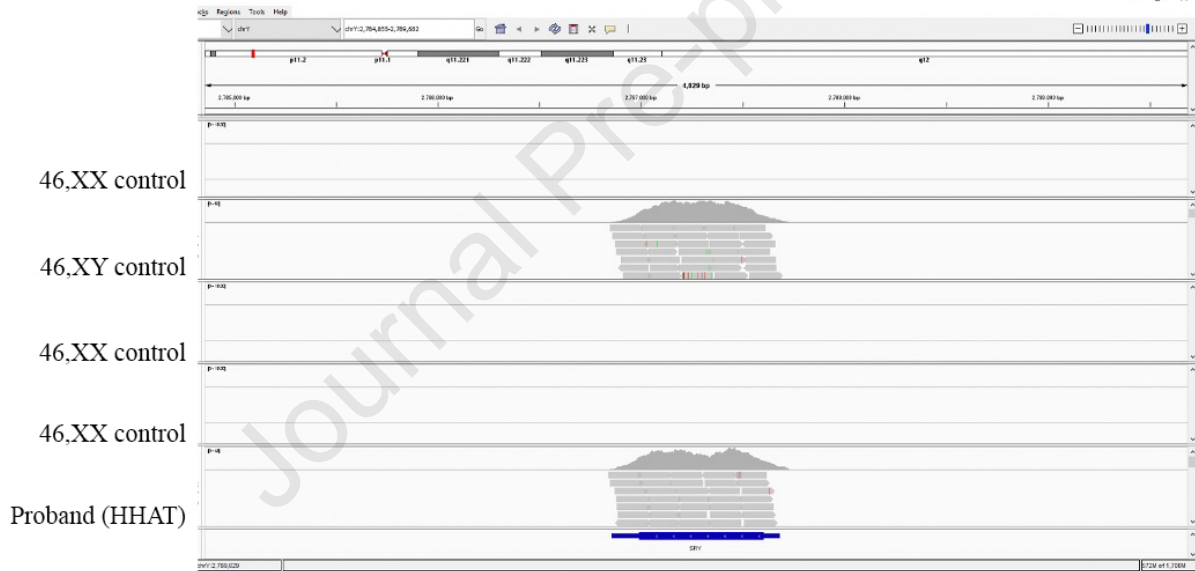


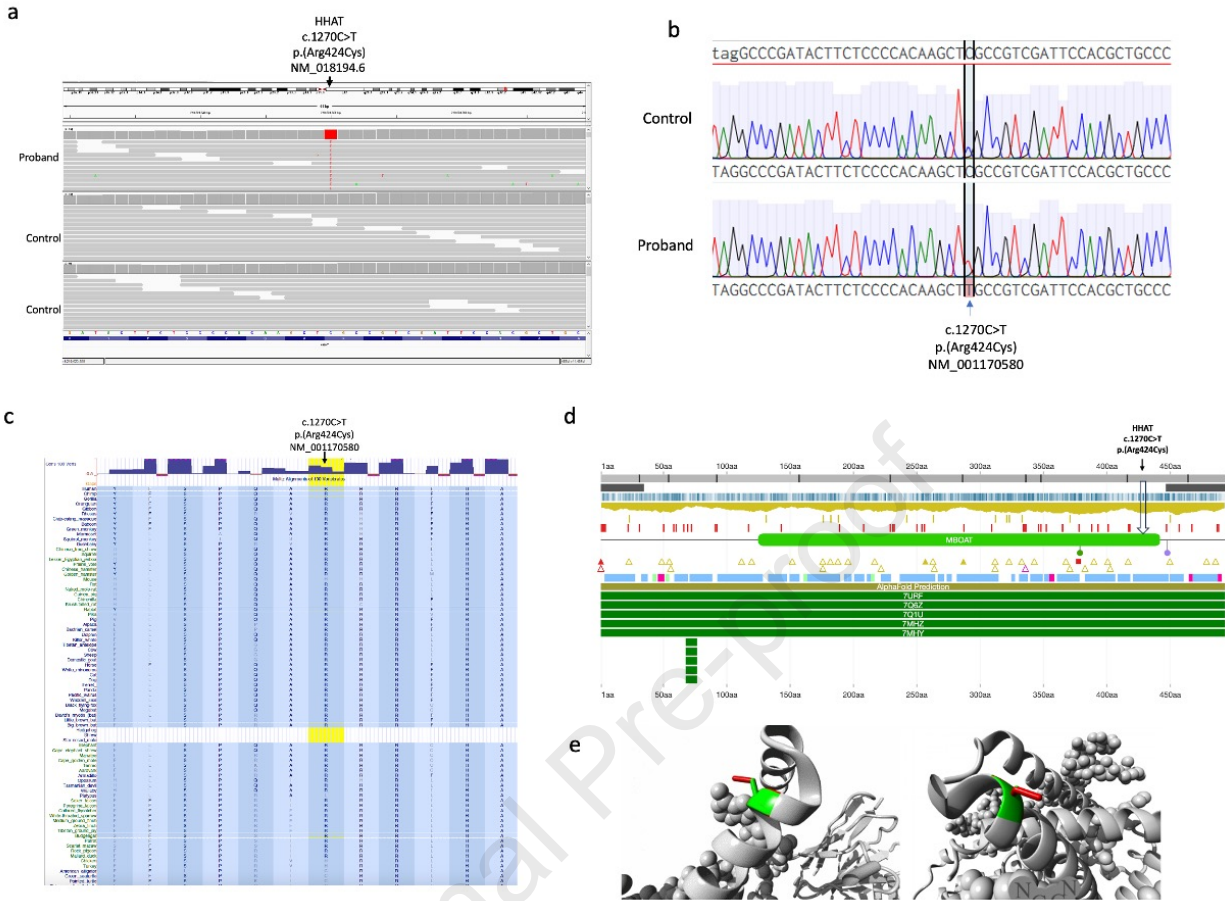


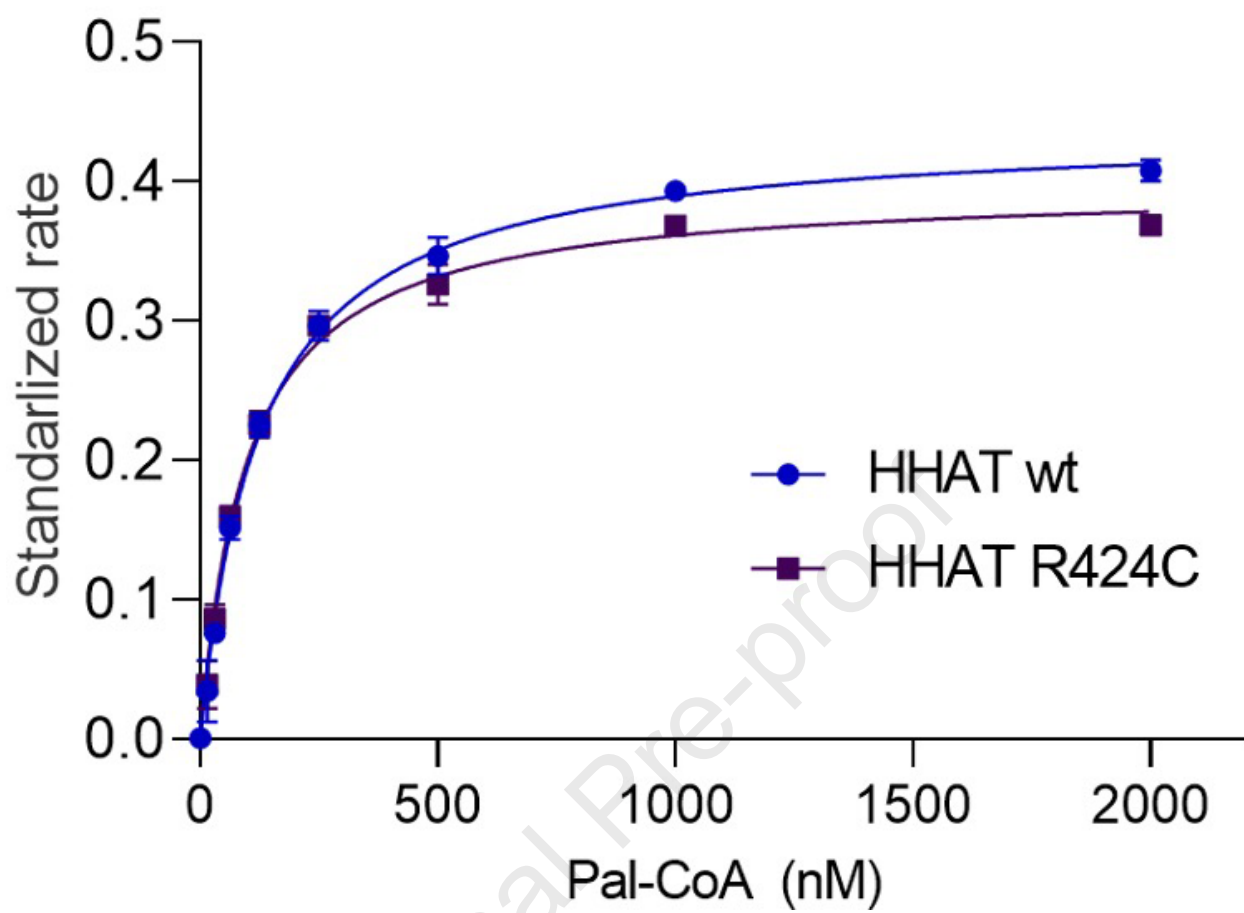
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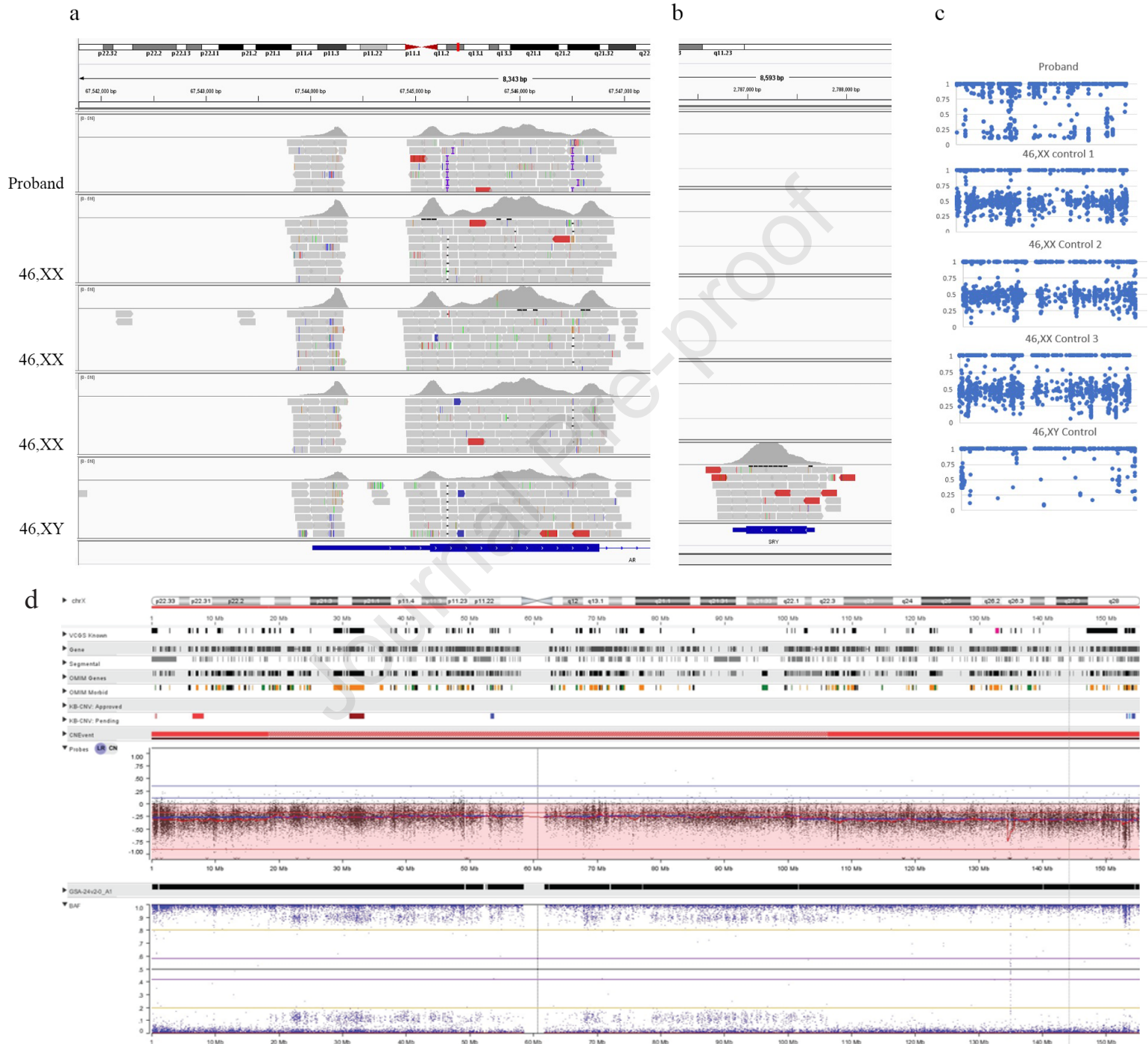


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Highlights

- Discovery of diverse causes of amenorrhea in an ethnically restricted cohort
- Expansion of the genotypic spectrum associated with amenorrhea
- Novel causal variants in *SYCP2L*, *FANCM* and *GNRHR*
- Importance of functional validation before assigning variant pathogenicity
- Potential of exome sequencing as a first-line diagnostic test for amenorrhea

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**Declaration of interests**

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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