OXFORD

human reproduction

Human Reproduction, 2024, **39(1)**, 177–189
https://doi.org/10.1093/humrep/dead233
Advance Access Publication Date: November 9, 2023
Original Article

Reproductive endocrinology

Improving diagnostic precision in primary ovarian insufficiency using comprehensive genetic and autoantibody testing

Elinor Chelsom Vogt [1,2,*], Eirik Bratland [1,3], Siren Berland[3], Ragnhild Berentsen[3], Agnethe Lund[4], Sigridur Björnsdottir[5,6], Eystein Husebye[1,2], and Marianne Øksnes[1,2]

[1]Department of Clinical Science, University of Bergen, Bergen, Norway
[2]Department of Medicine, Haukeland University Hospital, Bergen, Norway
[3]Department of Medical Genetics, Haukeland University Hospital, Bergen, Norway
[4]Department of Gynecology and Obstetrics, Haukeland University Hospital, Bergen, Norway
[5]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden
[6]Department of Endocrinology, Metabolism and Diabetes, Karolinska University Hospital, Stockholm, Sweden

*Correspondence address. Department of Medicine, Haukeland University Hospital, N-5021 Bergen, Norway. E-mail: elinor.vogt@uib.no https://orcid.org/0000-0001-9810-987X

STUDY QUESTION: Is it possible to find the cause of primary ovarian insufficiency (POI) in more women by extensive screening?

SUMMARY ANSWER: Adding next generation sequencing techniques including a POI-associated gene panel, extended whole exome sequencing data, as well as specific autoantibody assays to the recommended diagnostic investigations increased the determination of a potential etiological diagnosis of POI from 11% to 41%.

WHAT IS KNOWN ALREADY: POI affects ∼1% of women. Clinical presentations and pathogenic mechanisms are heterogeneous and include genetic, autoimmune, and environmental factors, but the underlying etiology remains unknown in the majority of cases.

STUDY DESIGN, SIZE, DURATION: Prospective cross-sectional study of 100 women with newly diagnosed POI of unknown cause consecutively referred to Haukeland University Hospital, Bergen, Norway, January 2019 to December 2021.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In addition to standard recommended diagnostic investigations including screening for chromosomal anomalies and premutations in the fragile X mental retardation 1 gene (FMR1) we used whole exome sequencing, including targeted analysis of 103 ovarian-related genes, and assays of autoantibodies against steroid cell antigens.

MAIN RESULTS AND THE ROLE OF CHANCE: We identified chromosomal aberrations in 8%, FMR1 premutations in 3%, genetic variants related to POI in 16%, and autoimmune POI in 3%. Furthermore in 11% we identified POI associated genetic Variants of unknown signifcance (VUS). A homozygous pathogenic variant in the ZSWIM7 gene (NM_001042697.2) was found in two women, corroborating this as a novel cause of monogenic POI. No associations between phenotypes and genotypes were found.

LIMITATIONS, REASONS FOR CAUTION: Use of candidate genetic and autoimmune markers limit the possibility to discover new markers. To further investigate the genetic variants, family studies would have been useful. We found a relatively high proportion of genetic variants in women from Africa and lack of genetic diversity in the genomic databases can impact diagnostic accuracy.

WIDER IMPLICATIONS OF THE FINDINGS: Since no specific clinical or biochemical markers predicted the underlying cause of POI discussion of which tests should be part of diagnostic screening in clinical practice remains open. New technology has altered the availability and effectiveness of genetic testing, and cost-effectiveness analyses are required to aid sustainable diagnostics.

STUDY FUNDING/COMPETING INTEREST(S): The study was supported by grants and fellowships from Stiftelsen Kristian Gerhard Jebsen, the Novonordisk Foundation, the Norwegian Research Council, University of Bergen, and the Regional Health Authorities of Western Norway. The authors declare no conflict of interest.

TRIAL REGISTRATION NUMBER: NCT04082169

Keywords: primary ovarian insufficiency (POI) / premature ovarian insufficiency / next generation sequencing / genetic / autoimmune

Received: September 23, 2022. **Revised:** July 31, 2023. **Editorial decision:** August 25, 2023.

Introduction

Primary ovarian insufficiency (POI) affects ~1% of women and is defined as premature loss of ovarian function due to follicle depletion or dysfunction (Golezar *et al.*, 2019; Panay *et al.*, 2020). The diagnosis is made in women under the age of 40 years with amenorrhea >4 months in association with serum FSH levels in the postmenopausal reference range, measured twice at least 4 weeks apart (Webber *et al.*, 2016; National Institute for Health and Care Excellence: Clinical Guidelines, 2019).

The diagnosis of POI has life-changing consequences, primarily due to reduced fertility and increased risk of complications related to premature estrogen deficiency. Clinical presentations are heterogeneous and involve multiple etiological factors, including genetic, autoimmune, infectious, radiation, and toxin-related. Most striking is the large proportion of idiopathic cases; in 70–90% of women with POI, the underlying cause remains unknown (Webber *et al.*, 2016; Panay *et al.*, 2020).

Ovarian development and function are complex, implicating several genetic pathways. Numerical and structural anomalies on the X chromosome are well-established causes of POI, explaining 10–15% of cases (Jiao *et al.*, 2017; Panay *et al.*, 2020). Premutations in the fragile X mental retardation 1 gene (*FMR1*) are found in 2–4% of POI women (Murray *et al.*, 2014). Recently, numerous additional genetic variants associated with POI have been identified (Tucker *et al.*, 2016; França and Mendonca, 2020; Yang *et al.*, 2021). A few studies have performed next generation sequencing (NGS) in women with sporadic POI attributing genetic causes to 10–75% of cases depending on the size of the applied gene panel and criteria for causality (Bouilly *et al.*, 2016; Patiño *et al.*, 2017; Jolly *et al.*, 2019; Yang *et al.*, 2019; França *et al.*, 2020; Bestetti *et al.*, 2021; Eskenazi *et al.*, 2021; Rossetti *et al.*, 2021).

Autoimmune disease often coexists with POI, especially hypothyroidism and primary adrenal insufficiency (Addison's disease) (Silva *et al.*, 2014; Kirshenbaum and Orvieto, 2019). The exact molecular mechanism behind this association is not fully known, but autoimmune oophoritis with an immune infiltrate selectively affecting the theca cell layer of the follicle has been demonstrated (Hoek *et al.*, 1997). A diagnostic ovarian biopsy is not recommended, instead serum autoantibodies against steroidogenic cell enzymes are used as surrogate markers, including autoantibodies against 21-hydroxylase (21OH), side chain cleavage (SCC) enzyme, 17alpha-hydroxylase (17OH), as well as NACHT leucine-rich-repeat protein 5 (NALP5), all of which are highly expressed in the ovaries (Winqvist *et al.*, 1993; Reato *et al.*, 2011; Brozzetti *et al.*, 2015). The reported prevalence of autoimmune POI varies considerably from 0% to 30%, depending on patient selection and assay methods (Bakalov *et al.*, 2005; Silva *et al.*, 2014; Jiao *et al.*, 2017; Kirshenbaum and Orvieto, 2019).

Recommended diagnostic evaluations to assess the cause of spontaneous POI include chromosome analysis and testing for *FMR1* premutation, while screening for autoantibodies against thyroid peroxidase (TPO), and 21OH or adrenocortical antibodies can be considered if an immune disorder is suspected (Yeganeh *et al.*, 2019). Currently, extended investigations with chromosomal microarray (CMA) and panel-based NGS, as well as other autoantibody assays, are not included in the routine assessment of POI (Webber *et al.*, 2016; Yeganeh *et al.*, 2019).

The discrepancy in the frequency of etiological diagnosis, and the large proportion of idiopathic POI cases, may reflect variation in study populations or insufficient investigation tools. Therefore, it is essential to improve diagnostic precision to predict outcomes and tailor future treatments for women with POI.

Here, we aimed to identify the etiology of as many POI women as possible by careful clinical phenotyping and use of the latest diagnostic tools including an NGS gene panel of 103 POI-related genes and specific autoantibody assays, with the ultimate aim to provide personalized diagnosis and follow-up.

Materials and methods

Patients

Women with newly diagnosed POI of unknown cause referred for evaluation to the Endocrinology or Gynecology outpatient clinics at Haukeland University Hospital, Bergen, Norway, between January 2019 and December 2021, were recruited and systemically evaluated in a prospective cross-sectional trial. Women <40 years were included if they had primary amenorrhea or secondary amenorrhea for at least 4 months, and serum FSH in the menopausal range (>21 IU/l), on at least two occasions more than one month apart. Patients who were <16 years or who had previously undergone gonadotoxic treatment (chemotherapy or radiation) or ovarian surgery were excluded from participation. Of the 105 consecutive patients identified with spontaneous POI, five patients did not meet the study eligibility criteria. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee 2018/1206/REK Midt.

Clinical assessment

Clinical, reproductive, and familial data as well as anthropometrics (weight in kilograms (kg), height in centimeters (cm), blood pressure (mmHg)) were recorded by the same examiner by standard recommended methods (Webber *et al.*, 2016; Panay *et al.*, 2020).

Bone mineral density assessment

Bone mineral density (BMD) was assessed using a Dual-Energy X-ray Absorptiometry (DXA) (General Electric Lunar iDXA). BMD results from the femoral neck and lumbar spine (L_2–L_4) were expressed as *Z* and *T* scores. Low bone mass was defined as a *Z*-score < −2 SD below the age-adjusted mean and osteoporosis was defined as a *T*-score < −2.5 SD (Kiriakova *et al.*, 2019; Panay *et al.*, 2020).

Laboratory assessment

FSH and LH, prolactin, adrenocorticotropin (ACTH), thyroid-stimulating hormone, free thyroxine (fT4), vitamin D, sex hormone-binding globulin, and anti-Müllerian hormone (AMH) were analyzed using electro-/chemiluminescent immune assays and the steroid hormones (progesterone, testosterone, androstenedione, dehydroepiandrosterone sulfate (DHEA-S), and cortisol 17β-estradiol) were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS (Hormone laboratory, Haukeland University Hospital, Bergen, Norway)).

Genetic investigations

The genetic evaluation included chromosomal karyotyping and CMA, *FMR1* analysis, and an NGS approach based on clinical whole exome sequencing (WES) (Fig. 1).

Karyotyping

G-banded chromosome analysis was performed according to standard protocols. At least 10 metaphases were screened for structural and numerical anomalies. In individuals with suspected mosaicism for structural anomalies on the X chromosome (monosomy X in 1/30 single metaphases), additional karyotyping was performed using cultured skin fibroblasts (Barch *et al.*, 1997).

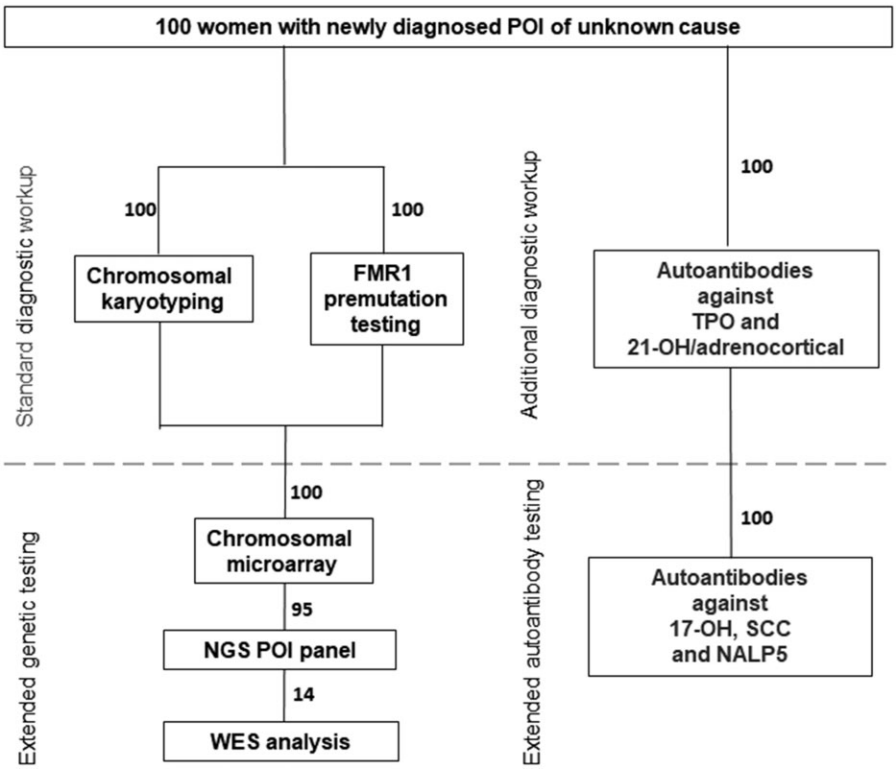

Figure 1. Flow chart of diagnostic workup in primary ovarian insufficiency. POI, primary ovarian insufficiency; NGS, next generation sequencing; WES, whole exome sequencing; TPO, thyroxide peroxidase; 21OH, 21-hydroxylase; 17OH, 17-hydroxylase; SCC, side-chain-cleavage enzyme; NALP5, NACHT leucine-rich-repeat protein 5.

Chromosomal microarray

Submicroscopic genomic copy number variations (CNVs), such as deletions and duplications, and long continuous stretches of homozygosity (LCSH) were investigated by the CytoscanHD array (Thermo Fisher Scientific) following the supplier's protocol and analysis software.

FMR1 premutation testing

CGG repeat numbers in the 5′ untranslated region of the *FMR1* gene were determined by the AmplideX® *FMR1* PCR kit from Asuragen, as recommended by the manufacturer. Alleles containing between 55 and 200 CGG repeats were defined as premutations.

NGS and variant interpretation

We used a two-tiered NGS-based strategy to identify genetic variants causative of POI. First, libraries for WES were prepared from genomic DNA and gene panel-based filtration of variants identified by WES were performed using the expert-curated POI (Version 1.60) panel from Genomics England PanelApp, and an in-house designed panel for general hypogonadism. In total, 103 genes were included based on ovarian biological studies and were categorized ontologically as follows: ovarian development, meiosis and DNA repair, follicle function, metabolism and immune regulation, hypothalamic–pituitary–ovarian axis (Martin *et al.*, 2019; Rossetti *et al.*, 2021; Ruth *et al.*, 2021).

Secondly, since LCSH regions are associated with increased risk of recessive disorders, in women with considerable genomic regions of LCSH (defined as at least two LCSH regions ≥5Mb on two or more chromosomes), we searched for potentially causal variants in POI candidate genes among the entire protein-encoding exome (Fan *et al.*, 2021).

Libraries for WES were prepared using SEQCap EZ HyperCap (v1.2) or KAPA HyperCap (v3.0) library preparation kits, with SeqCap EZ MedExome- or KAPA HyperExome target enrichment kits, respectively (all kits and reagents from Roche). Libraries were sequenced on Illumina NextSeq500 or Illumina NovaSeq 6000 instruments using paired-end 150 bp or 100 bp reads, respectively. Data processing, alignment to GRCh37 and variant calling were performed essentially as described (Bredrup *et al.*, 2021), except that GATK v.3.8.1 was used according to GATK's Best Practices guidelines (McKenna *et al.*, 2010; Van der Auwera *et al.*, 2013). Variant annotation and interpretation were performed using Alissa Interpret (Agilent Technologies) and Alamut Visual 2.15 (Sophia Genetics). As this study was performed in a clinical setting, only rare variants were assessed; defined as variants with allele frequencies ≤0.1% (autosomal dominant model) or ≤2.0% (autosomal recessive model) in the public database gnomAD (versions v2.1.1 and v3.1.2).

Formal clinical classification of the identified variants was based on the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards *et al.*, 2015). Based on 28 criteria, these guidelines classify variants according to a 5-tier system: Class 5 (Pathogenic), Class 4 (Likely Pathogenic), Class 3 (Variants of Unknown Significance (VUS)), Class 2 (Likely Benign), Class 1 (Benign). However, not all 28 classification criteria were available in the current study (e.g. family data to assess co-segregation of variants and disease). Furthermore, the ACMG guidelines are primarily intended for classifying highly penetrant variants in patients with rare, monogenic diseases. Modified disease-specific guidelines have been developed, but not yet for POI (Loong *et al.*, 2022). Hence, most of the variants identified in the current study were placed in the VUS category. Based on the strength of available evidence, and according to guidelines published from the Association of Clinical Genomic Science in the

UK (https://www.acgs.uk.com/media/11631/uk-practice-guidelines-for-variant-classification-v4-01-2020.pdf), we differentiate between hot/warm VUS'es and cool/cold/ice-cold VUS'es. Hot/warm VUS'es are variants with a high level of supporting evidence of causality and where additional evidence might be obtained to allow re-classification as likely pathogenic. In the following a hot/warm VUS will be referred to as possibly pathogenic or VUS+, to discriminate from cold VUS'es less likely to be pathogenic. Summaries of all variant interpretations are also included in Supplementary Data File S1.

Autoimmune antibody assays

Assays for autoantibodies against 21OH, SCC, 17OH, and NALP-5 were performed by radio-binding ligand assays as described previously (Oftedal *et al.*, 2008). Positive cut-offs were calculated using positive and negative controls with index thresholds of 2 SD above the mean of healthy subjects (>57, >200, >102, and >65 for 21OH, SCC, 17OH, and NALP-5, respectively) (Falorni *et al.*, 2015). Autoantibodies against TPO were analyzed by electrochemiluminescence immunoassay (Roche Cobas). Individuals with positive 21OH and SCC autoantibodies were classified as autoimmune POI.

Statistical analysis

Continuous data in normality distribution were expressed as mean and SD; otherwise, data were presented as median within the quartile range [IQR]. Categorical data was expressed in percentage and compared by Chi-square test with Yates Continuity Correction.

Correlations between continuous variables such as age at menarche, timing of POI, years since amenorrhea, hormone levels and BMD, were investigated using linear regression analysis and Spearman rank correlation.

Differences between groups related to timing POI, family history of POI, fertility, symptoms of estrogen deficiency and POI etiology, were investigated using Student's *t*-test, one-way analysis of variance or Mann–Whitney *U* test.

Results

Population characteristics

One hundred women with POI of unknown cause were included in the study. Demographic, clinical, and reproductive characteristics are presented in Table 1. Fifteen women (15%) had primary amenorrhea, and 85% had secondary amenorrhea with a median age of 33 [29–37] years (Fig. 2). No significant association was found between age at menarche and timing of POI diagnosis.

A family history of POI among first-degree relatives was reported in 20% (mothers 13%, sisters 10%, mother and sister 3%), including two sisters in the study. In addition, 17% reported second-degree relatives with POI or early menopause (grandmothers, aunts, or cousins). Women with a family history of POI developed POI 3 years later than those without ($P = 0.019$).

Half of the women (51%) had been pregnant, and 33% were multiparous. Three women had given birth after egg donations. Previous pregnancy was associated with POI diagnosis 5 years later compared to those who had never been pregnant ($P < 0.03$).

Most women reported symptoms of estrogen deficiency (81%) with a great degree of overlap between symptoms (Fig. 3). Vasomotor symptoms were more common in women with secondary amenorrhea than primary amenorrhea (71% versus 40%). Symptoms were not related to time since amenorrhea, use of hormone replacement therapy (HRT) or levels of 17β-estradiol,

Table 1. Clinical characteristics of women with premature ovarian insufficiency (POI).

	(n 100)
Age at menarche (years)	13 [12–14]
Primary amenorrhea (%)	15
Age secondary amenorrhea (years)	32 [23–37]
<20 (%)	4
20–29 (%)	20
30–39 (%)	61
Family history of POI[1] (%)	20
Ever pregnant[2] (%)	51
Age first pregnancy (years) (n = 51)	26 [24–30]
Age last pregnancy (years) (n = 32)	32 [30–34]
In a relationship/partner (%)	64
BMI (Kg/m^2)	24.2 [21.7–27.1]
Height (cm)	167 (162–170)
Smoke (%)	7
Ethnicity	
European (%)	75
African (%)	17
Asian (%)	8
Other disease	
Osteoporosis (n = 76) (%)	8
Osteopenia (n = 76) (%)	10
Hypothyroidism (%)	17
Type 1 diabetes (%)	4
Multiple sclerosis (%)	5
Laboratory[3]	
FSH IU/l (<21[4])	70.4 [50.1–104.0]
17β-estradiol pmol/l (74–1940[4])	43.0 [13.5–85.0]

Median [Quartile 1–Quartile 3] or frequency/percent (%).
[1] Mother and/or sister with POI.
[2] Egg donation in three women.
[3] Before starting hormone replacement therapy (HRT).
[4] Normal range.

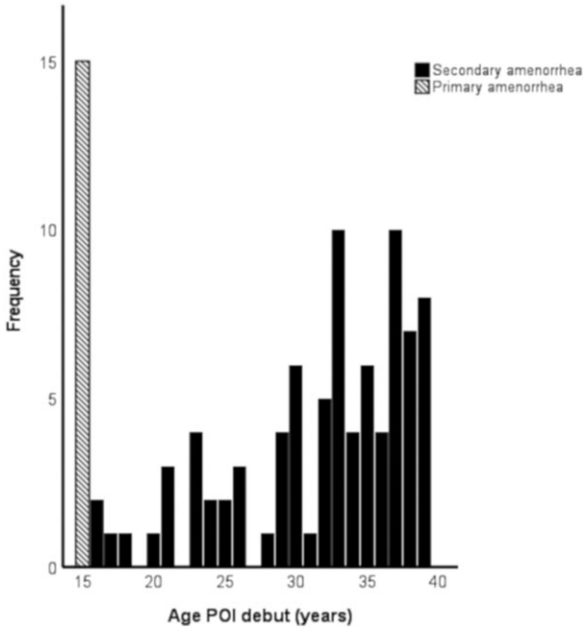

Figure 2. Age at primary ovarian insufficiency. Primary amenorrhea: 15%. Secondary amenorrhea: 85%.

However, there was a border-line association between higher FSH levels and vasomotor symptoms ($P = 0.057$).

DXA scans were available from 76 women. Nearly one in four women (23%) had reduced bone mass. Low bone mass (osteopenia) was found in 12% and osteoporosis in 11%. Women with primary amenorrhea (n 12) had lower BMD compared with those

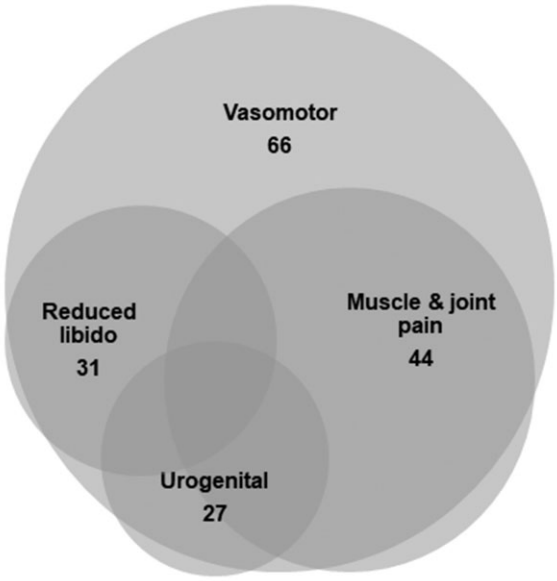

Figure 3. Reported symptoms in women with primary ovarian insufficiency. Venn diagram showing overlap of symptoms.

with secondary amenorrhea (n 64) (lumbar spine *Z* score −1.50 (−3.90 to −0.20) versus −0.50 (−4.5 to 3.20), $P = 0.023$, and *T* score −3.0 (−4.7 to 3.2) versus −1.35 (−3.90 to 0.0), $P = 0.014$, respectively). Younger age at POI diagnosis was associated with lower BMD (Spearman Rank Order $r = -0.300$, $P = 0.012$), also after adjusting for duration of POI, BMI, and HRT use ($P < 0.01$).

Women who had not yet started HRT (45%) exhibited typical postmenopausal endocrine profiles with FSH levels 70.4 [50.1–104.0] IU/l (premenopausal reference <21 IU/l) and 17β-estradiol 43.0 [13.5–85] pmol/l (premenopausal reference 74–1940 pmol/l). S-AMH was not detectable or below the age-specific range (<1.0 pmol/l) in 93% of women, and in the lower range (median 2.3, minimum 1.1 to maximum 10.3 pmol/l) in 7%. S-AMH values did not correlate with age, previous pregnancy status, family history of POI, or other hormone values.

Etiology

Chromosome analysis

Chromosomal anomalies were found in eight women (8%) (Table 2 and Fig. 4). Standard chromosome karyotyping analysis revealed X chromosome anomalies in five women (5%). Of these, three involved deletions and/or duplications, while two women had numerical anomalies (one had low-grade mosaicism for monosomy X, and one had trisomy X (47, XXX)).

CMA mapped the breakpoints of the larger structural X chromosome rearrangements (Table 2). Furthermore, CMA detected smaller copy number variants in three women with normal karyotypes: a 1.9 Mb deletion on Chromosome 8 involving several POI candidate genes (*KIF13B, LEPROTL1*, *RBPMS*, and *TEX15*), an intragenic duplication on 8p23.3p23.2 involving Exons 27–70 of the gene *CSMD1* (involved in ovarian development), and an intergenic duplication on 22q13.2 involving *MEI1* (involved in meiosis) and 13 other protein-coding genes (Supplementary Data File S1).

In 14 women, CMA revealed at least two regions of homozygosity on different chromosomes (Supplementary Data File S1). As LCSH regions are associated with increased risk of recessive disorders, we searched for rare homozygous variants in novel POI candidate genes across the entire protein-coding exome in these women (see below).

Skin biopsy and chromosome karyotyping in cultured fibroblasts was done on suspicion of mosaicism (45, X) from karyotyping of blood in four women, but were all normal.

FMR1 premutation analysis

FMR1 premutations were detected in three women (3%) (Table 3). Two had a family history of POI on the paternal side of the family, indicating paternal inheritance of the *FMR1* premutation. There were no family members with known fragile X-syndrome in any of the three families.

Gene panel-based NGS and extended WES analysis

Altogether, panel-based NGS and WES revealed variants in POI associated genes in 38% of the women (Fig. 5).

In 16 women (16%), we identified genetic variants classified as pathogenic and likely or possibly pathogenic (the latter also referred to as warm/hot VUS'es). These included variants in genes associated with ovarian development (*EIF4ENIF1, NANOS3, SOHLH2, SOX8*), or meiosis and DNA repair (*BUB1B, MCM8, MCM9*) (Table 4). Furthermore, we identified two patients with ultra-rare homozygous variants in *MTOR* and *SMC3*, two genes not previously associated with POI, but with highly important roles in ovarian development and meiosis and DNA repair, respectively. In one patient, we detected a rare missense variant in the *TP63* gene, encoding the transcriptional regulator p63 that is critical for maintenance of the oocyte genome integrity. In two female relatives with primary amenorrhea, we found a two base pair deletion in the *ZSWIM7* gene (NM_001042697.2: c.231_232del, p.(Cys78Phefs*21)), introducing a frameshift and premature stop codon expected to lead to complete loss-of-function.

Furthermore, in 11 (11%) other women we identified monoallelic variants in genes primarily associated with autosomal recessive inheritance (Table 4). These genes were also involved in ovarian development (*SOHLH1*), or meiosis and DNA repair (*C14ORF39, HFM1, PSMC3IP, SGO2, STAG3*), but variants in genes involved in follicle function (*FSHR*), and metabolism and immune regulation (*AARS2*) were identified as well. Among the detected variants were both hot and cold VUS'es and likely pathogenic variants. Since the presence of POI in these women may be caused by these variants along with an unidentified gene variant on the other allele, we searched extensively for potential second variants in these genes in the NGS and CMA raw data, without finding any.

Finally, in 11 (11%) additional women we also detected monoallelic variants in genes associated with autosomal recessive disease not consistent with the clinical phenotype (Table 4). We therefore consider these variants incidental potential carrier findings.

Autoantibodies

A total of 23% of women presented with known autoimmune disorders, including hypothyroidism (17%), Type 1 diabetes (T1D, 4%), and multiple sclerosis (MS, 5%). Two women had MS and hypothyroidism, and one woman had MS and T1D.

In total, 3% of POI individuals were considered to have a likely autoimmune etiology based on the presence of autoantibodies against 21OH and SCC (Table 5). All had AMH levels above the detection threshold (AMH, 1.0, 0.2, and 10.3 pmol/l, respectively). None had clinical characteristics or hormone levels consistent with Addison's disease, as all had normal ACTH and cortisol levels.

Apart from the patients with autoantibodies described above, isolated autoantibodies against 17OH were found in three and NALP5 were found in four additional women without other

Table 2. Chromosomal anomalies detected by karyotyping and chromosomal microarray.

ID	Karyotype (ISCN)	Chromosomal microarray	Phenotype						
			POI age (years)	Menarche age (years)	Fertility	Ethnicity	Height (cm)	Familial POI	Additional phenotype
P25	46, X, del(X)(q21.2)	arr[GRCh37] Xq21.2q28(85917017_154542526)x1	25	13	G1/P1[#]	European	166	–	Hypothyroidism Vitiligo
P34	46, X, der(X) (pter-q26.3::p11.3-pter)	arr[GRCh37] Xp22.33p11.3(287730_44199601)x3, arr[GRCh37] Xq26.3q28(136980232_155233731)x1	33	12	G0	African	165	–	Dysmorphic (asymmetric) facial features
P39	46, X, del(X)(q21)	Not performed	30	12	G0	European	150	–	Vitiligo
P41	46, XX [26], 47, XXX [2], 45, X[2]	Not performed	31	11	G0	Asian	158	–	Hypothyroidism
P69	46, XX	arr[GRCh37] 22q13.2(42154189_42578609)x3	37	12	G2/P2	European	165	Sister	–
P75	46, XX	arr[GRCh37] 8p23.3p23.2(2179649_3142724)x3	30	14	G0	African	162	–	Osteoporosis
P93	46, XX	arr[GRCh37] 8p12(28933652_30847526)x1	37	16	G4/P4	European	160	–	–
P96	47, XXX	arr[GRCh37] Xp22.33q28(168547_155233731)x3	29	12	G0	European	164	–	Hypogammaglobulinemia. Alopecia. Nail dystrophy. WPW syndrome. ADHD.

[#] Pregnant after egg donation.

ADHD, attention deficit hyperactive disorder; G, Gravida; ISCN, International System for Human Cytogenetic Nomenclature; P, Para; WPW syndrome, Wolf–Parkinson–White syndrome.

See Supplementary Data File S1 for interpretation of findings.

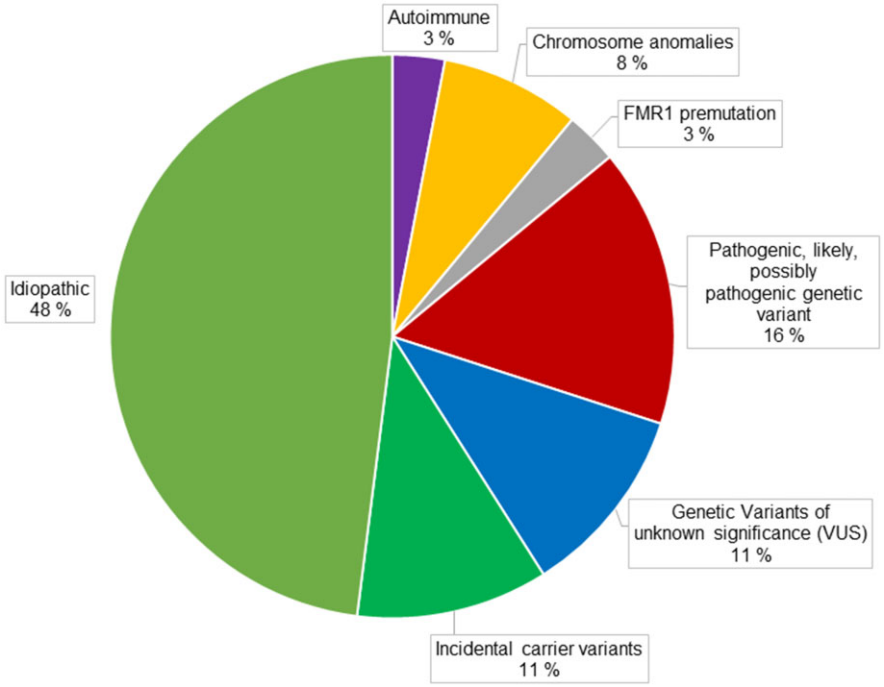

Figure 4. Primary ovarian insufficiency associated gene panel and ovarian function. Genetic variants found and classified: Red: pathogenic, likely or possibly pathogenic variant. Blue: variants of unknown significance (VUS). Black: no variants found in these genes. The figure was generated using Servier Medical Art.

Table 3. FMR1 premutations.

ID		Phenotype						
	CGG repeat number	POI age (years)	Menarche age (years)	Fertility	Ethnicity	Height (cm)	Familial POI	Additional phenotype
P1	30/85	29	11	G0	European	170	Pat.gr.mother	–
P63	26/78	32	12	G1/P1	European	163	–	–
P74	23/78	28	12	G0	European	167	Pat.gr.mother	–

POI, premature ovarian insufficiency; *FMR1*, Fragile X mental retardation 1 gene; G, Gravida; P, para; Pat. gr.mother, paternal grandmother.

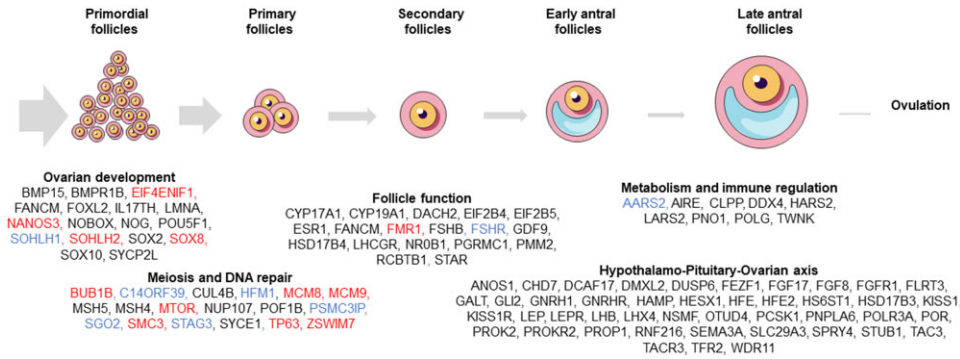

Figure 5. Etiology in women with primary ovarian insufficiency.

biochemical or clinical markers of autoimmunity. Positive TPO autoantibodies were detected in 21% of all the women.

Phenotype–genotype correlations

No significant between-group differences related to the cause of POI were found regarding the timing of menarche or POI debut, frequency of complications and symptoms of estrogen deprivation, family history, or hormone levels. Women with X chromosomal anomalies were slightly shorter than idiopathic POI (height: 161 cm versus 168 cm, $P = 0.042$). More POI women with sub-Saharan African ethnicity had a POI associated genetic variant compared to POI women with European ethnicity

Table 4. Primary ovarian insufficiency (POI) associated genetic variants detected by panel-based next generation sequencing (NGS) and extended whole exome sequencing (WES) analysis.

ID	Gene (REFSEQ)	cDNA	Protein	Zygosity	ACMG class	Phenotype						
						POI age (years)	Menarche age (years)	Fertility	Ethnicity	Height (cm)	Familial POI	Additional phenotype
Pathogenic, likely or possibly pathogenic genetic variants												
P5	*SOX8(NM_014587.4)*	c.1163C>T	p.(Ala388Val)	Het	VUS+	PA	–	G0	European	170	–	No pubarche Osteoporosis
P12	*SOHLH2(NM_017826.3)*	c.298_301del	p.(Val100LeufsTer2)	Het	VUS+	31	15	G1/P1	African	173	–	–
P33	*MCM9(NM_017696.2)*	c.3031C>A	p.(Pro1011Thr)	Het	VUS+	32	13	G3/P1	African	172	Sister	Sickle cell anemia
P35	*NANOS3(NM_001098622.2)*	c.292G>A	p.(Glu98Lys)	Het	VUS+	38	14	G1/SpA1	European	167	Aunt	–
P37	*SOX8(NM_014587.4)*	c.968C>T	p.(Ser323Leu)	Het	VUS+	33	15	G1/SpA1	African	155	–	Osteopenia
	SOX8(NM_014587.4)	c.1064C>T	p.(Ser355Leu)	Het	VUS+							
P50	*MTOR(NM_004958.4)*	c.259A>G	p.(Ile87Val)	Hom	UC	PA	–	G0	Asian	176	–	Migraine
P51	*MCM8(NM_032485.5)*	c.832C>T	p.(Arg278Cys)	Hom	VUS+	36	15	G1/SpA1	Asian	163	–	–
P62	*BUB1B(NM_001211.5)*	c.2995C>T	p.(Arg999Trp)	Het	VUS+	35	11	G0	European	162	Mother	–
P71*	*ZSWIM7(NM_001042697.2)*	c.231_232del	p.(Cys78PhefsTer28)	Hom	P	PA	–					–
P72*	*ZSWIM7(NM_001042697.2)*	c.231_232del	p.(Cys78PhefsTer28)	Hom	P	PA	–					
P78	*SMC3(NM_005445.4)*	c.969 + 4A>C	p.?	Hom	UC	35	13	G0	Asian	154	–	–
P85	*EIF4ENIF1(NM_019843.4)*	c.5A>G	p.(Asp2Gly)	Het	VUS+	33	11	G5/P3/ProvAb2	African	165	–	–
P86	*BUB1B(NM_001211.5)*	c.3029C>T	p.(Ser1010Phe)	Het	VUS+	34	15	G4/P3/SpA1	African	166	–	Epilepsy
P90	*EIF4ENIF1(NM_019843.4)*	c.490C>T	p.(Arg164Trp)	Het	VUS+	23	11	G1/P1	European	166	–	–
P97	*TP63(NM_003722.5)*	c.290G>T	p.(Arg97Leu)	Het	LP	37	14	G1/P1	European	184		–
P102	*SOX8(NM_014587.4)*	c.443A>G	p.(Lys148Arg)	Het	VUS+	35	14	G4/P2/SpA2	European	160	Cousin	Osteopenia
Monoallelic variants in genes associated with autosomal recessive inheritance												
P9	*SOHLH1(NM_001012415.2)*	c.743C>T	p.(Ser248Leu)	Het	VUS	34	13	G3/P2/SpA1	European	160	Sister	–
P21	*AARS2(NM_020745.3)*	c.1534G>C	p.(Asp512His)	Het	VUS	39	11	G3/P3	European	167	–	–
P22	*STAG3(NM_001282716.1)*	c.2351G>C	p.(Cys784Ser)	Het	VUS+	35	13	G1/P1	European	172	Pat.gr. mother	Pollen allergy
P24	*FSHR(NM_000145.3)*	c.1330G>A	p.(Ala444Thr)	Het	VUS	21	12	G0	European	162	Pat.gr. mother	Osteoporosis Vitiligo
P26	*STAG3(NM_001282716.1)*	c.3305_3306insGCC	p.Ile1102delinsMetPro	Het	VUS	38	12	G0	European	169	–	Hypothyroidism
P27	*FSHR(NM_000145.3)*	c.910A>G	p.(Met304Val)	Het	VUS	23	12	G0	African	167	–	–
	PSMC3IP(NM_013290.6)	c.13C>G	p.(Arg5Gly)	Het	VUS							
P30	*C14ORF39(NM_174978.3)*	c.748A>G	p.(Arg250Gly)	Het	VUS	39	12	G3/P3	European	172	–	Type 1 diabetes
P45	*STAG3(NM_001282716.1)*	c.3515 + 5G>A	p.?	Het	P	30	14	G3/P1/SpA2	European	168	–	Psoriasis
P47	*C14ORF39(NM_174978.3)*	c.748A>G	p.(Arg250Gly)	Het	VUS	23	12	G2/P1/ProvA1[#]	European	180	–	–
P62	*SGO2(NM_001160033.1)*	c.217C>T	p.(Arg73Ter)	Het	P	35	11	G0	European	162	Mother	–
P106	*HFM1(NM_001017975.4)*	c.2449A>G	p.(Lys817Glu)	Het	VUS	15	12	G0	European	180	–	–
Incidental carrier findings												
P4	*LMNA(NM_170707.2)*	c.1487C>T	p.(Thr496Met)	Het	VUS	32	9	G1/P1	African	164	–	–
P14	*EIF2B5(NM_003907.3)*	c.190C>G	p.(Pro64Ala)	Het	VUS+	33	13	G1/ProvA1	African	161	–	–
P36	*EIF2B4(NM_001318966.1)*	c.29G>T	p.(Gly10Val)	Het	VUS	18	16	G0	African	166	–	Osteopenia
P46	*POLG(NM_002693.3)*	c.1639G>T	p.(Ala547Ser)	Het	VUS	38	17	G2/P2	European	172	–	Epilepsy
P62	*GALT(NM_000155.4)*	c.563A>G	p.(Gln188Arg)	Het	P	35	11	G0	European	162	Mother	–
P64	*HARS2(NM_012208.4)*	c.591T>G	p.(Cys197Trp)	Het	VUS	37	12	G0	European	153	–	MS. Osteopenia. Hypothyroidism
P66	*PMM2(NM_000303.3)*	c.422G>A	p.(Arg141His)	Het	P	PA	–	G0	European	177	–	–
P75	*AIRE(NM_000383.2)*	c.1367G>A	p.(Arg456His)	Het	VUS	30	14	G0/P0	African	162	–	Osteoporosis
P94	*AIRE(NM_000383.2)*	c.769C>T	p.(Arg257Ter)	Het	P	37	12	G2/P2	European	168	–	Ulcerative colitis
P99	*GALT(NM_000155.4)*	c.197C>T	p.(Pro66Leu)	Het	VUS	31	13	G1/P1	European	159	–	Hypothyroidism
P104	*POLG(NM_002693.3)*	c.2243G>C	p.(Trp748Ser)	Het	P	38	12	G5/P2/SpA2/ProvA1	European	160	Sister	Hypothyroidism

[#] Pregnant after egg donation.
* P71 and P72 did not consent to publishing ethnic, anthropometric, family or clinical information.
ACMG, American College of Medical Genetics and Genomics; G, Gravida; Het, heterozygous; Hom, homozygous; P, pathogenic; MS, multiple sclerosis; P, para; Pat. gr.mother, paternal grandmother; PA, primary amenorrhea; ProvA, provoked abortion; VUS+, possibly pathogenic; REFSEQ, reference sequence gene annotation; SpA, spontaneous miscarriage; UC, unclassified; VUS, variants of unknown significance.
See Supplementary Data File S1 for interpretation of findings.

Table 5. Autoimmune premature ovarian insufficiency (POI).

Patient ID	Autoantibodies (index titer)				Phenotype						
	21OH (Pos > 57)	SCC (Pos > 200)	17OH (Pos > 102)	TPO (Pos > 33)	POI age (years)	Menarche age (years)	Fertility	Ethnicity	Height (cm)	Familial POI	Additional phenotype
P17	211	433	111	111	39	13	G1/P1	European	162	–	Multiple Sclerosis. Hypothyroidism.
P80	135	458	167	29	14	12	G0	European	154	–	Osteoporosis
P99	809	765	88	37	31	13	G1/P1	European	159	–	Hypothyroidism. Cystic ovaries.

21OH, 21-hydroxylase; 17OH, 17-hydroxylase; SCC, side-chain-cleavage enzyme; TPO, thyroxide peroxidase.

(59% versus 33%, $P = 0.043$). Women with autoimmune POI experienced a trend toward later amenorrhea compared to non-autoimmune POI (median 34 versus 32 years). AMH levels were also higher in these women (2.6 versus 0.1 pmol/l).

Discussion

This extensive clinical study identified a likely underlying cause of POI in approximately one-third of the women. Adding a POI-related NGS gene panel and exome sequencing to the recommended diagnostic investigations increased the frequency of an etiological diagnosis. No specific clinical or biochemical markers predicted the underlying cause of POI, actualizing the discussion of which tests should be included as diagnostic screening in clinical practice.

We found X-chromosomal anomalies in the lower range of what has previously been reported (Panay *et al.*, 2020). As chromosomal anomalies may result in more extreme phenotypes, including syndromic features, short stature, and primary amenorrhea, the frequency in our study is likely underestimated as many of these individuals are diagnosed already in childhood (Jiao *et al.*, 2017). Karyotyping is suitable for detecting large chromosomal aberrations, such as deletions, duplications, balanced, and unbalanced rearrangements. Here, we identified structural anomalies that included Xq13–Xq21 and Xq23–27, underlining the importance of this region for the POI phenotype (Rizzolio *et al.*, 2006). Apart from confirming the findings on the X-chromosome and estimating the breakpoints, CMA revealed three potentially clinically relevant CNVs involving genes involved in ovarian biology and meiosis and DNA repair. The 1.9 Mb deletion on Chromosome 8 was intriguing, as it contained at least four genes implicated in different ovarian processes: *KIF13B, LEPROTL1*, *RBPMS*, and *TEX15*. *RBPMS* encodes a transcriptional regulator demonstrated to be important for oocyte development (Aguero *et al.*, 2016; Kaufman *et al.*, 2018). Based on the finding of this deletion on Chromosome 8, we therefore suggest *RBPMS* as a novel POI-associated gene. Others have also found CMA useful in identifying candidate POI genes (Tšuiko *et al.*, 2016; Bestetti *et al.*, 2021). In our hands, CMA was valuable in mapping LCSH regions, and thus for selecting patients for whole exome analysis. Still, chromosomal karyotyping during the diagnostic workup of POI was the most useful test for detecting chromosomal anomalies or mosaicism.

The prevalence of *FMR1* premutation in our cohort (3%) was in accordance with previous reports (Murray *et al.*, 2014). As none of the three families had family members with known fragile X syndrome, a negative family history of fragile X should not be used to guide who will be offered *FMR1* testing or not. POI occurs in 20% of *FMR1* premutation allele carriers, and the risk of developing POI increases at CGG repeat numbers above 80. In addition to explaining POI, these females are at high risk of giving birth to children with fragile X syndrome, and prenatal diagnostics and preimplantation genetic testing should be discussed. Screening for *FMR1* is therefore considered both useful and important in POI (Hunter *et al.* 1993–2023; Monaghan *et al.*, 2013).

Using an NGS POI-related panel and additional WES, we found that 27% had at least one variant that may be involved in POI. Hundreds of genes have been suggested to be associated with POI based on their involvement in processes related to different aspects of ovarian function (França and Mendonca, 2022). In our study, it is striking that the vast majority of genetic variants were found in genes involved in ovarian and follicular development, and in meiosis and DNA repair. This is consistent with other recent studies employing NGS-based analyses to identify genetic causes of POI (Bestetti *et al.*, 2021; Huang *et al.*, 2021; Heddar *et al.*, 2022).

We identified variants in *EIF4ENIF1*, *NANOS3*, *SOHLH2*, and *SOX8* encoding transcription factors, transcriptional regulators, and RNA-binding proteins playing key roles in ovarian/gonadal development (Panula *et al.*, 2016; Shin *et al.*, 2017; Portnoi *et al.*, 2018; Shang *et al.*, 2022).

Further, we also found variants in *BUB1B*, *MCM8*, *MCM9*, MTOR, SMC3, *TP63*, and *ZSWIM7*; all involved in chromosomal stability, homologous recombination during meiosis, and repair of DNA breaks (Park *et al.*, 2013; Touati *et al.*, 2015; Prakash *et al.*, 2021). The *TP63* gene encodes a transcriptional regulator p63 that is critical for maintenance of the oocyte genome integrity (Suh *et al.*, 2006). Pathogenic variants in the *TP63* gene have lately been established as a cause of isolated POI, through a gain-of-function mechanism (Tucker *et al.*, 2022; Huang *et al.*, 2023). Recently, pathogenic variants in the *ZSWIM7* gene have been reported in both males and females with infertility (Hussain *et al.*, 2022; McGlacken-Byrne *et al.*, 2022). *ZSWIM7* encodes Zinc finger SWIM domain-containing protein 7 (ZSWIM7), also known as SWIM domain-containing SRS2-interacting protein 1 (SWS1), which is critical for meiotic homologous recombination (Abreu *et al.*, 2018). Both female and male *ZSWIM7* knockout mice are infertile due to impaired meiotic DNA recombination (Li *et al.*, 2021). The same deletion that we report in two relatives with POI was initially described in unrelated male patients with azoospermia but has also been seen in a Turkish woman with POI (Alhathal *et al.*, 2020; Li *et al.*, 2021; Yatsenko *et al.*, 2022).

In pace with increasing knowledge of the underlying molecular mechanisms of POI, custom-built gene panels and variant classification must be dynamic. In particular, we suggest that *ZSWIM7* should be included into POI-related gene panels based on our findings of a homozygous loss-of-function variant in ZSWIM7 in two relatives with primary amenorrhea.

However, it can be challenging to establish definite causality, as family investigations and functional characterizations are

needed to corroborate the pathogenicity of variants. The inheritance pattern can be autosomal dominant or recessive. In 11 women, we detected monoallelic variants (ranging from VUS's to pathogenic) in genes associated with autosomal recessive POI. No second variant in any of those genes could be detected, but cannot be ruled out, and should be followed up in the future with more specialized methods such as whole genome- and/or long-range sequencing. Recently, there has also been increased focus on oligogenic inheritance with possible synergistic effects of variants in several genes explaining the variance in the observed phenotype (Rossetti *et al.*, 2021). Thus, precise classifying of the genetic variants is complex.

The prevalence of autoimmune POI in our study (3%) is in the lower range of what has earlier been reported, possibly because we used more specific autoantibody assays than earlier studies (Novosad *et al.*, 2003; Jiao *et al.*, 2017; Kirshenbaum and Orvieto, 2019; Panay *et al.*, 2020; Vogt *et al.*, 2022). All three women had autoantibodies against both 21OH and SCC, and both therefore appear to be the markers with the highest diagnostic sensitivity for autoimmune POI (Winqvist *et al.*, 1995; Hoek *et al.*, 1997; Bakalov *et al.*, 2005; Falorni *et al.*, 2012). Autoantibodies against 17OH and NALP5 have previously been shown to be present in women with autoimmune POI, but we could not replicate these findings (Dal Pra *et al.*, 2003; Brozzetti *et al.*, 2015). Although autoantibodies against TPO were prevalent, these are common among postmenopausal women in the background population (15%) and therefore not useful as markers of ovary-specific autoimmunity (McLeod and Cooper, 2012).

After thyroid disease, adrenal autoimmunity is the most common autoimmune condition associated with POI (Kirshenbaum and Orvieto, 2019; Panay *et al.*, 2020). While ~10% of women with Addison's disease have POI, 2–3% of women with POI develop adrenal autoimmunity (Bakalov *et al.*, 2005; Reato *et al.*, 2011; Kirshenbaum and Orvieto, 2019). Even though none of the women with autoantibodies against 21OH and SCC reported here had Addison's disease, they are at risk of developing adrenal autoimmunity and must be followed up with functional adrenal testing (Webber *et al.*, 2016; Vogt *et al.*, 2021).

Some limitations apply to our study. First, there will always be a question of whether this cohort of POI women is representative. To our knowledge, we included all women with suspected POI who were referred to our hospital in the study period. It is, however, possible that women with extreme phenotypes were not included because they had received the POI diagnosis in childhood. The use of candidate genetic and autoimmune markers allows us only to identify already known POI markers of interest, limiting the possibility to discover new markers. The number of genes used in our NGS panel is therefore limited by current knowledge of genes of clinical relevance. Indeed, increasing the number of genes in gene panels, or ultimately expanding the analyses to WES strategies, may help identify previously undetected pathogenic variants, including those in genes not previously surveyed in smaller gene panels. However, expanding to larger gene panels or WES will also increase the number of variants of uncertain significance and incidental findings will place a considerable burden on medical genetic laboratories in analyzing and interpreting variants within a clinical setting (Molina-Ramírez *et al.*, 2022). The workload of interpretation is an important consideration when implementing high-throughput sequencing in the clinic. We therefore argue that the limitations regarding the use of targeted NGS panels do not necessarily include the number of genes per se. From our perspective, the challenge is to focus the analyses on the highest possible number of genes consistent with the clinical phenotype of each individual patient. Furthermore, it would have been useful to evaluate familial cases of POI in order to further investigate any co-segregation with the genetic variants found in these women. We found a relatively high proportion POI-associated genetic variants in women from sub-Saharan Africa, and although previous studies have observed a higher prevalence of POI in non-European populations, lack of genetic diversity in the public genomic databases such as gnomAD can impact diagnostic accuracy (Luborsky *et al.* 2003; Kessler *et al.* 2016; Mishra *et al.* 2019; Eskenazi *et al.* 2021; Marwaha *et al.*, 2022).

The strength of this study is that it encompasses a relatively large and well-characterized cohort. We also used a large gene panel and focused on rare variants using strict criteria that are useful in a clinical setting. Another strength is the use of specific autoantibodies instead of indirect immunofluorescence.

The introduction of NGS technology has dramatically altered the availability and effectiveness of genetic testing (Tucker *et al.*, 2016; França and Mendonca, 2022). However, as no specific clinical or biochemical markers predicted the underlying cause of POI, the question of *Who should be offered genetic testing* remains controversial, and cost-effectiveness analyses are required to aid sustainable diagnostics in clinical guidelines. In this study, adding an NGS POI-related gene panel increased the determination of an etiological diagnosis. More research is needed to aid interpretative approaches and classification of genetic variants.

In conclusion, we suggest that women with newly diagnosed POI go through standard recommended diagnostic investigations including screening for chromosomal anomalies, *FMR1* premutations and testing for 21OH autoantibodies. Adding NGS gene panels will increase the diagnostic yield.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgements

We thank Dr Kristin Viste and the Hormone Laboratory at Haukeland University Hospital for analyzing the blood tests as well as help in distributing information about the study. We also thank the National Registry of Organ-Specific Autoimmune Diseases (ROAS), Department of Medicine, Haukeland University Hospital, Bergen, Norway, for analyzing autoantibodies.

Authors' roles

The study was designed and directed by M.Ø., E.H., A.L., S.B., and E.V. Clinical work and statistical analysis were performed by E.V. The genetic analysis and classifications were performed by E.B., S.B., and R.B. All authors contributed to interpretation of findings. The manuscript was drafted by E.V. with contributions to revision and final version by all authors.

Funding

The study was supported by grants and fellowships from Stiftelsen Kristian Gerhard Jebsen, the Novonordisk Foundation,

the Norwegian Research Council, University of Bergen, and the Regional Health Authorities of Western Norway.

Conflict of interest

The authors decleare no conflict of interest.

References

Abreu CM, Prakash R, Romanienko PJ, Roig I, Keeney S, Jasin M. Shu complex SWS1-SWSAP1 promotes early steps in mouse meiotic recombination. *Nat Commun* 2018;**9**:3961.

Aguero T, Zhou Y, Kloc M, Chang P, Houliston E, King ML. Hermes (Rbpms) is a critical component of RNP complexes that sequester germline RNAs during oogenesis. *J Dev Biol* 2016;**4**:2.

Alhathal N, Maddirevula S, Coskun S, Alali H, Assoum M, Morris T, Deek HA, Hamed SA, Alsuhaibani S, Mirdawi A *et al.* A genomics approach to male infertility. *Genet Med* 2020;**22**:1967–1975.

Bakalov VK, Anasti JN, Calis KA, Vanderhoof VH, Premkumar A, Chen S, Furmaniak J, Smith BR, Merino MJ, Nelson LM. Autoimmune oophoritis as a mechanism of follicular dysfunction in women with 46,XX spontaneous premature ovarian failure. *Fertil Steril* 2005;**84**:958–965.

Barch MJ, Knutsen T, Spurbeck JL. *Association of Genetic Technologists. The AGT Cytogenetics Laboratory Manual*. Philadelphia: Lippincott-Raven Publisher, 1997.

Bestetti I, Barbieri C, Sironi A, Specchia V, Yatsenko SA, De Donno MD, Caslini C, Gentilini D, Crippa M, Larizza L *et al.* Targeted whole exome sequencing and Drosophila modelling to unveil the molecular basis of primary ovarian insufficiency. *Hum Reprod* 2021;**36**:2975–2991.

Bouilly J, Beau I, Barraud S, Bernard V, Azibi K, Fagart J, Fèvre A, Todeschini AL, Veitia RA, Beldjord C *et al.* Identification of multiple gene mutations accounts for a new genetic architecture of primary ovarian insufficiency. *J Clin Endocrinol Metab* 2016;**101**:4541–4550.

Bredrup C, Cristea I, Safieh LA, Di Maria E, Gjertsen BT, Tveit KS, Thu F, Bull N, Edward DP, Hennekam RCM *et al.* Temperature-dependent autoactivation associated with clinical variability of PDGFRB Asn666 substitutions. *Hum Mol Genet* 2021;**30**:72–77.

Brozzetti A, Alimohammadi M, Morelli S, Minarelli V, Hallgren A, Giordano R, De Bellis A, Perniola R, Kampe O, Falorni A; Italian Addison Network. Autoantibody response against NALP5/MATER in primary ovarian insufficiency and in autoimmune Addison's disease. *J Clin Endocrinol Metab* 2015;**100**:1941–1948.

Dal Pra C, Chen S, Furmaniak J, Smith BR, Pedini B, Moscon A, Zanchetta R, Betterle C. Autoantibodies to steroidogenic enzymes in patients with premature ovarian failure with and without Addison's disease. *Eur J Endocrinol* 2003;**148**:565–570.

Eskenazi S, Bachelot A, Hugon-Rodin J, Plu-Bureau G, Gompel A, Catteau-Jonard S, Molina-Gomes D, Dewailly D, Dodé C, Christin-Maitre S *et al.* Next generation sequencing should be proposed to every woman with "idiopathic" primary ovarian insufficiency. *J Endocr Soc* 2021;**5**:bvab032.

Falorni A, Bini V, Betterle C, Brozzetti A, Castano L, Fichna M, Kampe O, Mellgren G, Peterson P, Chen S. Determination of 21-hydroxylase autoantibodies: inter-laboratory concordance in the euradrenal international serum exchange program. *Clin Chem Lab Med* 2015;**53**:1761–1770.

Falorni A, Brozzetti A, Aglietti MC, Esposito R, Minarelli V, Morelli S, Sbroma Tomaro E, Marzotti S. Progressive decline of residual follicle pool after clinical diagnosis of autoimmune ovarian insufficiency. *Clin Endocrinol (Oxf)* 2012;**77**:453–458.

Fan Y, Wang L, Sun Y, Xu T, Gong Z, Zhao Q, Qiu W, Liang L, Xiao B, Zhang H *et al.* Diagnostic yield of additional exome sequencing after the detection of long continuous stretches of homozygosity (LCSH) in SNP arrays. *J Hum Genet* 2021;**66**:409–417.

França MM, Funari MFA, Lerario AM, Santos MG, Nishi MY, Domenice S, Moraes DR, Costalonga EF, Maciel GAR, Maciel-Guerra AT *et al.* Screening of targeted panel genes in Brazilian patients with primary ovarian insufficiency. *PLoS One* 2020;**15**:e0240795.

França MM, Mendonca BB. Genetics of primary ovarian insufficiency in the next-generation sequencing era. *J Endocr Soc* 2020;**4**:bvz037.

França MM, Mendonca BB. Genetics of ovarian insufficiency and defects of folliculogenesis. *Best Pract Res Clin Endocrinol Metab* 2022;**36**:101594.

Golezar S, Ramezani Tehrani F, Khazaei S, Ebadi A, Keshavarz Z. The global prevalence of primary ovarian insufficiency and early menopause: a meta-analysis. *Climacteric* 2019;**22**:403–411.

Heddar A, Ogur C, Da Costa S, Braham I, Billaud-Rist L, Findikli N, Beneteau C, Reynaud R, Mahmoud K, Legrand S *et al.* Genetic landscape of a large cohort of primary ovarian insufficiency: new genes and pathways and implications for personalized medicine. *EBioMedicine* 2022;**84**:104246.

Hoek A, Schoemaker J, Drexhage HA. Premature ovarian failure and ovarian autoimmunity. *Endocr Rev* 1997;**18**:107–134.

Huang C, Guo T, Qin Y. Meiotic recombination defects and premature ovarian insufficiency. *Front Cell Dev Biol* 2021;**9**:652407.

Huang C, Zhao S, Yang Y, Guo T, Ke H, Mi X, Qin Y, Chen ZJ, Zhao S. TP63 gain-of-function mutations cause premature ovarian insufficiency by inducing oocyte apoptosis. *J Clin Invest* 2023;**133**:162315.

Hunter JE, Berry-Kravis E, Hipp H, Todd PK. FMR1 sisorders. In: Adam MP, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A (eds). *GeneReviews®*. Seattle (WA): University of Washington, 1993–2023. https://www.ncbi.nlm.nih.gov/books/NBK1384/.

Hussain S, Nawaz S, Khan I, Khan N, Hussain S, Ullah I, Fakhro KA, Ahmad W. A novel homozygous variant in homologous recombination repair gene ZSWIM7 causes azoospermia in males and primary ovarian insufficiency in females. *Eur J Med Genet* 2022;**65**:104629.

Jiao X, Zhang H, Ke H, Zhang J, Cheng L, Liu Y, Qin Y, Chen ZJ. Premature ovarian insufficiency: phenotypic characterization within different etiologies. *J Clin Endocrinol Metab* 2017;**102**:2281–2290.

Jolly A, Bayram Y, Turan S, Aycan Z, Tos T, Abali ZY, Hacihamdioglu B, Coban Akdemir ZH, Hijazi H, Bas S *et al.* Exome sequencing of a primary ovarian insufficiency cohort reveals common molecular etiologies for a spectrum of disease. *J Clin Endocrinol Metab* 2019;**104**:3049–3067.

Kaufman OH, Lee K, Martin M, Rothhämel S, Marlow FL. Correction: rbpms2 functions in Balbiani body architecture and ovary fate. *PLoS Genet* 2018;**14**:e1007768.

Kessler MD, Yerges-Armstrong L, Taub MA, Shetty AC, Maloney K, Jeng LJB, Ruczinski I, Levin AM, Williams LK, Beaty TH *et al.*; Consortium on Asthma among African-ancestry Populations in the Americas (CAAPA). Challenges and disparities in the application of personalized genomic medicine to populations with African ancestry. *Nat Commun* 2016;**7**:12521.

Kiriakova V, Cooray SD, Yeganeh L, Somarajah G, Milat F, Vincent AJ. Management of bone health in women with premature ovarian insufficiency: systematic appraisal of clinical practice guidelines and algorithm development. *Maturitas* 2019;**128**:70–80.

Kirshenbaum M, Orvieto R. Premature ovarian insufficiency (POI) and autoimmunity-an update appraisal. *J Assist Reprod Genet* 2019;**36**:2207–2215.

Li Y, Wu Y, Zhou J, Zhang H, Zhang Y, Ma H, Jiang X, Shi Q. A recurrent ZSWIM7 mutation causes male infertility resulting from decreased meiotic recombination. *Hum Reprod* 2021;**36**:1436–1445.

Loong L, Garrett A, Allen S, Choi S, Durkie M, Callaway A, Drummond J, Burghel GJ, Robinson R, Torr B *et al.*; CanVIG-UK. Reclassification of clinically-detected sequence variants: Framework for genetic clinicians and clinical scientists by CanVIG-UK (Cancer Variant Interpretation Group UK). *Genet Med* 2022;**24**:1867–1877.

Luborsky JL, Meyer P, Sowers MF, Gold EB, Santoro N. Premature menopause in a multi-ethnic population study of the menopause transition. *Hum Reprod* 2003;**18**:199–206.

Martin AR, Williams E, Foulger RE, Leigh S, Daugherty LC, Niblock O, Leong IUS, Smith KR, Gerasimenko O, Haraldsdottir E *et al.* PanelApp crowdsources expert knowledge to establish consensus diagnostic gene panels. *Nat Genet* 2019;**51**:1560–1565.

Marwaha S, Knowles JW, Ashley EA. A guide for the diagnosis of rare and undiagnosed disease: beyond the exome. *Genome Med* 2022;**14**:23.

McGlacken-Byrne SM, Le Quesne Stabej P, Del Valle I, Ocaka L, Gagunashvili A, Crespo B, Moreno N, James C, Bacchelli C, Dattani MT *et al.* ZSWIM7 is associated with human female meiosis and familial primary ovarian insufficiency. *J Clin Endocrinol Metab* 2022;**107**:e254–e263.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M *et al.* The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 2010;**20**:1297–1303.

McLeod DS, Cooper DS. The incidence and prevalence of thyroid autoimmunity. *Endocrine* 2012;**42**:252–265.

Mishra GD, Chung HF, Cano A, Chedraui P, Goulis DG, Lopes P, Mueck A, Rees M, Senturk LM, Simoncini T *et al.* EMAS position statement: predictors of premature and early natural menopause. *Maturitas* 2019;**123**:82–88.

Molina-Ramírez LP, Kyle C, Ellingford JM, Wright R, Taylor A, Bhaskar SS, Campbell C, Jackson H, Fairclough A, Rousseau A *et al.* Personalised virtual gene panels reduce interpretation workload and maintain diagnostic rates of proband-only clinical exome sequencing for rare disorders. *J Med Genet* 2022;**59**:393–398.

Monaghan KG, Lyon E, Spector EB; American College of Medical Genetics and Genomics. ACMG standards and guidelines for fragile X testing: a revision to the disease-specific supplements to the standards and guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics and Genomics. *Genet Med* 2013;**15**:575–586.

Murray A, Schoemaker MJ, Bennett CE, Ennis S, Macpherson JN, Jones M, Morris DH, Orr N, Ashworth A, Jacobs PA *et al.* Population-based estimates of the prevalence of FMR1 expansion mutations in women with early menopause and primary ovarian insufficiency. *Genet Med* 2014;**16**:19–24.

National Institute for Health and Care Excellence: Clinical Guidelines. *Menopause: Diagnosis and Management, 2019*. London: Copyright © NICE, 2019.

Novosad JA, Kalantaridou SN, Tong ZB, Nelson LM. Ovarian antibodies as detected by indirect immunofluorescence are unreliable in the diagnosis of autoimmune premature ovarian failure: a controlled evaluation. *BMC Womens Health* 2003;**3**:2.

Oftedal BE, Wolff AS, Bratland E, Kampe O, Perheentupa J, Myhre AG, Meager A, Purushothaman R, Ten S, Husebye ES. Radioimmunoassay for autoantibodies against interferon omega; its use in the diagnosis of autoimmune polyendocrine syndrome type I. *Clin Immunol* 2008;**129**:163–169.

Panay N, Anderson RA, Nappi RE, Vincent AJ, Vujovic S, Webber L, Wolfman W. Premature ovarian insufficiency: an international menopause society white paper. *Climacteric* 2020;**23**:426–446.

Panula S, Reda A, Stukenborg JB, Ramathal C, Sukhwani M, Albalushi H, Edsgärd D, Nakamura M, Söder O, Orwig KE *et al.* Over expression of NANOS3 and DAZL in human embryonic stem cells. *PloS One* 2016;**11**:e0165268.

Park J, Long DT, Lee KY, Abbas T, Shibata E, Negishi M, Luo Y, Schimenti JC, Gambus A, Walter JC *et al.* The MCM8-MCM9 complex promotes RAD51 recruitment at DNA damage sites to facilitate homologous recombination. *Mol Cell Biol* 2013;**33**:1632–1644.

Patiño LC, Beau I, Carlosama C, Buitrago JC, González R, Suárez CF, Patarroyo MA, Delemer B, Young J, Binart N *et al.* New mutations in non-syndromic primary ovarian insufficiency patients identified via whole-exome sequencing. *Hum Reprod* 2017;**32**:1512–1520.

Portnoi MF, Dumargne MC, Rojo S, Witchel SF, Duncan AJ, Eozenou C, Bignon-Topalovic J, Yatsenko SA, Rajkovic A, Reyes-Mugica M *et al.* Mutations involving the SRY-related gene SOX8 are associated with a spectrum of human reproductive anomalies. *Hum Mol Genet* 2018;**27**:1228–1240.

Prakash R, Sandoval T, Morati F, Zagelbaum JA, Lim PX, White T, Taylor B, Wang R, Desclos ECB, Sullivan MR *et al.* Distinct pathways of homologous recombination controlled by the SWS1-SWSAP1-SPIDR complex. *Nat Commun* 2021;**12**:4255.

Reato G, Morlin L, Chen S, Furmaniak J, Smith BR, Masiero S, Albergoni MP, Cervato S, Zanchetta R, Betterle C. Premature ovarian failure in patients with autoimmune Addison's disease: clinical, genetic, and immunological evaluation. *J Clin Endocrinol Metab* 2011;**96**:E1255–1261.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E *et al.*; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;**17**:405–424.

Rizzolio F, Bione S, Sala C, Goegan M, Gentile M, Gregato G, Rossi E, Pramparo T, Zuffardi O, Toniolo D. Chromosomal rearrangements in Xq and premature ovarian failure: mapping of 25 new cases and review of the literature. *Hum Reprod* 2006;**21**:1477–1483.

Rossetti R, Moleri S, Guizzardi F, Gentilini D, Libera L, Marozzi A, Moretti C, Brancati F, Bonomi M, Persani L. Targeted next-generation sequencing indicates a frequent oligogenic involvement in primary ovarian insufficiency onset. *Front Endocrinol (Lausanne)* 2021;**12**:664645.

Ruth KS, Day FR, Hussain J, Martinez-Marchal A, Aiken CE, Azad A, Thompson DJ, Knoblochova L, Abe H, Tarry-Adkins JL *et al.* Genetic insights into biological mechanisms governing human ovarian ageing. *Nature* 2021;**596**:393–397.

Shang L, Ren S, Yang X, Zhang F, Jin L, Zhang X, Wu Y. EIF4ENIF1 variants in two patients with non-syndromic premature ovarian insufficiency. *Eur J Med Genet* 2022;**65**:104597.

Shin YH, Ren Y, Suzuki H, Golnoski KJ, Ahn HW, Mico V, Rajkovic A. Transcription factors SOHLH1 and SOHLH2 coordinate oocyte differentiation without affecting meiosis I. *J Clin Invest* 2017;**127**:2106–2117.

Silva CA, Yamakami LY, Aikawa NE, Araujo DB, Carvalho JF, Bonfa E. Autoimmune primary ovarian insufficiency. *Autoimmun Rev* 2014;**13**:427–430.

Suh EK, Yang A, Kettenbach A, Bamberger C, Michaelis AH, Zhu Z, Elvin JA, Bronson RT, Crum CP, McKeon F. p63 protects the female germ line during meiotic arrest. *Nature* 2006;**444**:624–628.

Touati SA, Buffin E, Cladière D, Hached K, Rachez C, van Deursen JM, Wassmann K. Mouse oocytes depend on BubR1 for proper chromosome segregation but not for prophase I arrest. *Nat Commun* 2015;**6**:6946.

Tšuiko O, Nõukas M, Žilina O, Hensen K, Tapanainen JS, Mägi R, Kals M, Kivistik PA, Haller-Kikkatalo K, Salumets A *et al.* Copy number variation analysis detects novel candidate genes involved in follicular growth and oocyte maturation in a cohort of premature ovarian failure cases. *Hum Reprod* 2016;**31**:1913–1925.

Tucker EJ, Grover SR, Bachelot A, Touraine P, Sinclair AH. Premature ovarian insufficiency: new perspectives on genetic cause and phenotypic spectrum. *Endocr Rev* 2016;**37**:609–635.

Tucker EJ, Gutfreund N, Belaud-Rotureau MA, Gilot D, Brun T, Kline BL, Bell KM, Domin-Bernhard M, Théard C, Touraine P *et al.* Dominant TP63 missense variants lead to constitutive activation and premature ovarian insufficiency. *Hum Mutat* 2022;**43**:1443–1453.

Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, Jordan T, Shakir K, Roazen D, Thibault J *et al.* From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics* 2013;**43**:11–10.11-11.10.33.

Vogt EC, Breivik L, Røyrvik EC, Grytaas M, Husebye ES, Øksnes M. Primary ovarian insufficiency in women with Addison's disease. *J Clin Endocrinol Metab* 2021;**106**:e2656–e2663.

Vogt EC, Real FG, Husebye ES, Björnsdottir S, Benediktsdottir B, Bertelsen RJ, Demoly P, Franklin KA, de Aja Gallastegui LS, González FJC *et al.* Premature menopause and autoimmune primary ovarian insufficiency in two international multi-center cohorts. *Endocr Connect* 2022;**11**:e220024.

Webber L, Davies M, Anderson R, Bartlett J, Braat D, Cartwright B, Cifkova R, de Muinck Keizer-Schrama S, Hogervorst E, Janse F *et al.* ESHRE guideline: management of women with premature ovarian insufficiency. *Hum Reprod* 2016;**31**:926–937.

Winqvist O, Gebre-Medhin G, Gustafsson J, Ritzen EM, Lundkvist O, Karlsson FA, Kampe O. Identification of the main gonadal autoantigens in patients with adrenal insufficiency and associated ovarian failure. *J Clin Endocrinol Metab* 1995;**80**:1717–1723.

Winqvist O, Gustafsson J, Rorsman F, Karlsson FA, Kampe O. Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. *J Clin Invest* 1993;**92**:2377–2385.

Yang Q, Mumusoglu S, Qin Y, Sun Y, Hsueh AJ. A kaleidoscopic view of ovarian genes associated with premature ovarian insufficiency and senescence. *FASEB J* 2021;**35**:e21753.

Yang X, Touraine P, Desai S, Humphreys G, Jiang H, Yatsenko A, Rajkovic A. Gene variants identified by whole-exome sequencing in 33 French women with premature ovarian insufficiency. *J Assist Reprod Genet* 2019;**36**:39–45.

Yatsenko SA, Gurbuz F, Topaloglu AK, Berman AJ, Martin PM, Rodrigue-Escribà M, Qin Y, Rajkovic A. Pathogenic variants in ZSWIM7 cause primary ovarian insufficiency. *J Clin Endocrinol Metab* 2022;**107**:e2359–e2364.

Yeganeh L, Boyle JA, Wood A, Teede H, Vincent AJ. Menopause guideline appraisal and algorithm development for premature ovarian insufficiency. *Maturitas* 2019;**130**:21–31.