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NEXT GENERATION SEQUENCING SHOULD BE PROPOSED TO EVERY WOMAN WITH “IDIOPATHIC” PREMATURE OVARIAN INSUFFICIENCY

S. Eskenazi^{1,2*}, A. Bachelot^{3,2*}, J. Hugon-Rodin^{4,5,6}, G. Plu-Bureau^{4,5,6}, A. Gompel^{4,5}, S. Catteau-Jonard⁷, D. Molina-Gomes⁸, D. Dewailly⁷, C. Dodé⁹, S. Christin-Maitre^{1,2,10**}, P. Touraine^{2,3**}

¹ Department of Reproductive Endocrinology, Saint-Antoine Hospital, AP-HP, Paris, France; Center for Rare Growth Disorders and Center for Developmental Disorders: CMERC

² Sorbonne University, Paris, France

³ Department of Endocrinology and Reproductive Medicine, Pitié-Salpêtrière Hospital, AP-HP, Paris, France; Center for Rare Endocrine Disorders and Center of Rare Gynecological Disorders: CMERC

⁴ Departments of Gynecology and Endocrinology, Cochin/Port-Royal Hospital, AP-HP, Paris, France

⁵ Paris Descartes University, Paris, France

⁶ INSERM UMR 1153, EPOPE group

⁷ Univ. Lille, CHU Lille, Department of Medical Gynaecology, F-59000 Lille, France

⁸ Department of Assisted Reproductive Technics, Poissy Saint-Germain-en-Laye Hospital, Poissy, France

⁹ Department of Genetics and Molecular Biology, Cochin/Port-Royal Hospital, AP-HP, Paris, France

¹⁰ INSERM UMR-S933, 75012 Paris, France

*Co-first authors

**Co-last authors

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Corresponding author:

Pr Philippe TOURAINE

Department of Endocrinology and Reproductive Medicine, Pitié-Salpêtrière Hospital, AP-HP, Paris,
France

47-83 Boulevard de l'Hopital 75013, Paris. France

Tel 0033 (0)1 42 16 02 54.

Fax 0033 (0) 1 42 16 02 55

Philippe.touraine@aphp.fr

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ABSTRACT

Introduction

Primary ovarian insufficiency (POI) affects 1% of women under 40 years old. POI is idiopathic in more than 70% of cases. Though many candidate genes have been identified in recent years, the prevalence and pathogenicity of abnormalities are still difficult to establish.

Objectives

Our primary objective was to evaluate the prevalence of gene variations in a large prospective multicentric POI cohort. Our secondary objective was to evaluate the correlation between phenotype and genotype.

Patients and Methods

Two hundred and sixty-nine well-phenotyped POI patients were screened for variants of 18 known POI genes (*BMP15*, *DMC1*, *EIF2S2*, *FIGLA*, *FOXL2*, *FSHR*, *GDF9*, *GPR3*, *HFM1*, *LHX8*, *MSH5*, *NOBOX*, *NR5A1*, *PGRMC1*, *STAG3*, *XPNPEP2*, *BHLB* and *FSHB*) by next generation sequencing (NGS). Abnormalities were classified as “variant” or “variant of unknown significance” (VUS) according to available functional tests or algorithms (SIFT, Polyphen-2, MutationTaster).

Results

One hundred and two patients (38%) were identified as having at least 1 genetic abnormality. Sixty-seven patients (25%) presented at least 1 variant. Forty eight patients presented at least 1 VUS (18%). Thirteen patients (5%) had combined abnormalities. *NOBOX* variants were the most common gene variants involved in POI (9%). Interestingly, we saw no significant differences in the previous

family history of POI, ethnic origin, age at onset of POI, primary amenorrhea or secondary menstrual disturbances between the different genotypes.

Conclusion

In our study, a high percentage of patients presented gene variants detected by NGS analysis (38%). Every POI patient should undergo NGS analysis to improve medical cares of the patients.

KEYWORDS: primary ovarian insufficiency; next generation sequencing; genetic results; phenotype

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INTRODUCTION

Primary (Premature) ovarian insufficiency (POI) is defined as a loss of ovarian activity before the age of 40, and is characterized by menstrual disturbances (amenorrhea or oligomenorrhea) with elevated gonadotropins (FSH \geq 25 IU/L) and low serum estradiol levels¹. The incidence of POI is around one per 100 women^{2,3} overall, and one per 1000 women under the age of 30 years⁴. POI leads to infertility and an increased risk of osteoporosis and cardiovascular disease^{5,6}. Different mechanisms are known to be involved in the pathogenesis of POI: decreased primordial follicular pool at birth, accelerated follicular atresia, or a dysfunction of follicular growth⁷. Several causes of POI have been identified, including autoimmunity or iatrogenic causes like chemotherapy or ovarian surgery⁸. Some authors have also suggested environmental causes^{9,10}. Genetic disorders involved include not only Turner syndrome (4-5% of cases of POI) and *FMR1* (Fragile X Mental Retardation type 1) gene premutation (3% to 15% of cases of POI)¹¹, but also monogenic disorders (syndromic or non-syndromic)^{12,13}. Around 70% of cases remain unexplained¹¹, though some of these cases of idiopathic POI may be linked to genetic abnormalities. In recent years, new genetic screening techniques have identified genetic alterations that may be linked to POI. Many familial studies have identified mutations involved in POI¹⁴⁻¹⁷, and a few cohort studies have described variants of candidate genes or copy number variants¹⁸⁻²³. Interestingly, a few studies have reported the use of next generation sequencing (NGS) to analyze a panel of candidate genes in patients with POI²⁴⁻²⁶. The prevalence of known genetic alterations is estimated at 20-25%⁷. The primary goal of our study was to describe the prevalence of genetic abnormalities of 18 candidate genes by NGS in a large cohort of 269 POI patients. The secondary goal was to evaluate the correlation between those abnormalities and the patients' phenotype.

MATERIALS AND METHODS

- Patients

From January 2015 to January 2017, all patients newly diagnosed with POI in five different Reproductive Medicine Centers in France (La Pitié-Salpêtrière Hospital, Saint-Antoine Hospital, Port-Royal Hospital in Paris, Poissy/Saint-Germain-en-Laye Hospital, and Jeanne de Flandre Hospital, Lille) were included in this study. POI was diagnosed based on amenorrhea or oligomenorrhea associated with FSH levels above 25 IU/l and low serum estradiol levels, before the age of 40. Every patient underwent karyotyping of at least 20 cells and a *FMR1* molecular analysis. We excluded patients found to have Turner syndrome based on the karyotype or any other karyotype abnormality, as well as patients with a *FMR1* premutation. Patients who previously underwent a gonadotoxic treatment (chemotherapy or pelvic radiation) or extensive ovarian surgery were also excluded from the study. All patients signed an informed consent form. The study was approved by the local ethics committee.

- Clinical data

Ethnic origin, family history of POI (at least one first-degree female relative with POI according to the patient), pubertal development, menstrual history (primary amenorrhea or secondary menstrual disturbance) as well as prior spontaneous pregnancies were recorded during the medical consultations at diagnosis. Clinical symptoms suggesting syndromic POI were evaluated and any personal history of autoimmune disorders was noted. Furthermore, tests were done for 21-hydroxylase antibodies, ovarian antibodies, thyroid peroxidase (TPO) antibodies, thyroglobulin (TG) antibodies, glutamate decarboxylase (GAD) antibodies, antibodies common in celiac disease and lupus antibodies.

- DNA sequencing

Genomic DNA was extracted either from the patient's blood cells or from a derived lymphoblastoid. The 18 genes studied were selected because of their potential implication in POI according to previous studies (Table 1). We tested for mutations in the coding exons and abutting splice sites of *BMP15* (NM_005448.2), *DMC1* (NM-007068.3), *EIF2S2* (NM_003908.2), *FIGLA* (NM_001004311.3), *FOXL2* (NM-023067.3), *FSHR* (NM_000145.3), *GDF9* (NM_005260.5), *GPR3* (NM_005281.3), *HFM1* (NM_001017975.4), *LHX8* (NM_00100933.1), *MSH5* (NM-172165.3), *NOBOX* (NM_001080413.3), *NR5A1* (NM_004959.4), *PGRMC1* (NM_006667.4), *STAG3* (NM_001282717.1), *BHLHB9* (NM_001142528), *FSHB* (NM_001018080) and *XPNPEP2* (NM_003399.5) using the Ion Torrent semiconductor sequencing technique. The primers were designed using the Ampliseq Designer software. The libraries were prepared from 50 ng of genomic DNA using the Ion Plus fragment library kit. Adapter ligation, nick repair, and amplification were performed according to the Ion Torrent protocol (Life Technologies). The Ion One Touch template kit was used for the emulsion PCR and enrichment steps. Sequencing of the amplicon libraries was done on the Ion Torrent PGM system with 316 chips, and the Ion Xpress barcode adapters kit was used for the barcoding. Version 2 of the Ion sequencing kit was used for all sequencing reactions, according to the recommended protocol. After sequencing, reads were mapped to the human genome 19 assembly with the Torrent mapping alignment program. Single-nucleotide variants and small insertions/deletions (indels) were identified using Torrent Variant Caller (Life Technologies) and Nextgene software ²⁷. All the mutations detected were confirmed by Sanger sequencing of new PCR products.

- Nomenclature and in silico analyses of the mutations

The effects of the missense variants were considered based on the results of functional tests described previously (when available). In the absence of a functional study, the effects of the missense variants were predicted using three different algorithms: Sorting Intolerant From Tolerant (SIFT) (sift.jcvi.org/www/SIFT_enst_submit.html), PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) and

MutationTaster (<http://www.mutationtaster.org/>). The SIFT and PolyPhen-2 algorithms give scores ranging from 0 to 1. A mutation is predicted as “deleterious” by SIFT if its score is below 0.05; otherwise it is predicted as “tolerated”. A mutation is predicted as “possibly damaging” by PolyPhen-2 if its score is greater than 0.15, and as “probably damaging” if it is greater than 0.85; otherwise it is predicted as “benign”. The Mutation Taster algorithm indicates the probability of an alteration being a polymorphism or a disease-causing alteration. The scores range from 0 to 1, with a score of 1 indicating a high security of prediction.

We considered an alteration as pathogenic or a polymorphism based on the results of functional tests described previously. When no such tests had been done previously, we considered a missense variant as pathogenic when two of the three algorithms (SIFT, PolyPhen-2 and MutationTaster) gave identical results.

Missense variants can also affect the splicing of the primary transcripts. These effects of each missense variant were predicted using the MaxEntScan scores (genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSplice (omictools.com/nnsplICE-tool), and Human Splicing Finder (rd-connect.eu/tools-resources/human-splicing-finder) algorithms.

- Statistical analysis

The statistical analyses were done using SAS software (SAS Institute, Inc., Cary, NC). The student test and χ^2 test were used to assess the differences in the characteristics of the women between two groups according to genotype. The data are presented as a percentage (for qualitative variables) or mean and standard deviation (SD) for quantitative variables. A p-value < 0.05 was considered as statistically significant.

RESULTS

DESCRIPTION OF THE POPULATION (Table 2)

We included 269 consecutive patients diagnosed with POI. As shown in Table 2, 126 patients (52%) were of Caucasian origin, 57 (23%) came from Sub-Saharan Africa, 50 (20%) were from North Africa and 13 (5%) came from Asia (missing data N=23). Forty-three patients (16%) had a family history of POI (missing data N=5). Primary amenorrhea (PA) was observed in 34 patients (13%) and 229 patients (87%) (missing data N=6) had a secondary menstrual disturbance (SMD) (amenorrhea or oligomenorrhea). Among the patients with PA, the prevalence of those with or without spontaneous pubertal development was 25% (N=8) and 19% (N=6) respectively. Eighteen patients (56%) had incomplete pubertal development (missing data N=2). An SMD occurred before the age of 20 years in 30 patients (15%), between the age of 20 and 29 years in 47 patients (23%) and between the age of 30 and 39 years in 127 patients (62%). Ninety-six patients (37%) had been pregnant before the diagnosis of POI (missing data N=25). Fifty-seven patients (23.5%) showed signs of autoimmunity (59% with thyroid antibodies).

NGS RESULTS

The NGS results are presented in Table 3. One hundred and two patients (38%) presented at least one abnormality, of one to five genes each. Forty-eight patients (18%) presented a VUS. Sixty-seven (25%) had at least one variant. Thirteen patients (5%) had at least one variant and one VUS.

Among the 18 genes tested, variants were identified for only 13 genes (Table 3) and 1 to 6 different variants were found for each. *NOBOX* variants were the most common autosomal gene variant (N=24; 9% of the patients). Twenty patients shared *DMC1* variants (7%). Nine patients presented *BMP15* (3%) variants, 6 for *HFM1* (2%), 5 for *NR5A1* (2%), 5 for *STAG3* (2%), 2 for *XPNPEP2* (0.7%), 4 for *GDF9* (1.5%), 3 for *MSH5* (1%), 2 for *FSHR* (0.7%) and 1 for *FOXL2* and *FIGLA*.

Fourteen patients (5.2%) had 2 to 4 variants, of which 2 had homozygous variants (*BMP15* and *NOBOX*) (Table 4).

PHENOTYPE ACCORDING TO GENOTYPE

In the cases of PA, 32% of the patients presented with variants and most of those concerned the *NOBOX* gene (45%). In the patients with an SMD, 24% had a variant. Among the entire POI cohort, for every variant identified, the clinical presentation of the POI most often involved an SMD, except for the *STAG3* variants that were associated with PA in 60% of the cases (Table 3). Fifty-six percent of the patients from Sub-Saharan Africa had a genetic variant (50% for *DMC1* and 42% for *NOBOX*) and 58% of the patients with *NOBOX* variants originated from Sub-Saharan Africa. Among the patients with a family history of POI, 21% presented with variants and most of them involved *DMC1* (40%), followed by *NOBOX* and *NR5A1* (20% each).

The phenotype/genotype analysis, as shown in Table 5, found no statistical difference between patients with or without variants regarding ethnic origin, familial history of POI, PA, age at the onset of an SMD and previous history of natural pregnancy. As *NOBOX* variants were the variants most commonly observed, the patients with only *NOBOX* variants (heterozygous N=17, homozygous N=1) were compared to those without the variant (Table 5). There was no statistically significant difference regarding phenotype between patients with *NOBOX* variants and patients without variant. Furthermore, phenotype was not different in patient presenting one variant compared to patients with combined variants (data not shown). The sole patient with a variant of the *FOXL2* gene presented with blepharophimosis, ptosis and epicanthus inversus syndrome.

In our cohort, 38% of patients with at least one variant had a spontaneous pregnancy before the diagnosis of POI. Among the 24 patients with a *NOBOX* variant, 8 had been pregnant. All were heterozygous for the *NOBOX* variant, but 3 had additional variants of other genes such as *DMC1* for patient 1, *DMC1* and *FIGLA* for patient 2 and *BMP15*, *XPNPEP2* and *DMC1* for patient 3. Among the 9

patients with *BMP15* variants, 5 had been pregnant. All were heterozygous for the *BMP15* variant but 2 had additional variants of other genes such as *DMC1* for one of them. The other patient has been described previously as having a *NOBOX* variant.

DISCUSSION

To our knowledge, this is the largest study to report NGS results and the correlation between genotype and phenotype in a POI cohort, after excluding other identified POI etiologies, such as iatrogenic POI, Turner syndrome and patients with *FMR1* premutation.

Only a few studies have tested POI patients using NGS technology to date. Fonseca *et al.* reported an NGS analysis combined with Sanger sequencing that found 4 variants of 3 genes in a small cohort of 12 POI patients²⁶. Bouilly *et al.* screened a cohort of 100 POI patients for 19 loci. Nineteen percent of the patients were identified with at least one variant²⁴. In a cohort including 69 patients, Patino *et al.* found 48% of patients with variants of 49 genes among the 420 candidate POI genes tested using NGS combined with Sanger sequencing¹⁹. None of these studies included a statistical genotype/phenotype analysis.

We used NGS to determine that 38% of our patients had at least one variant that may be involved in the onset of POI, though it is difficult to establish causality between genetic abnormalities found by NGS and POI. Interestingly, none of our POI patients presented variants of 5 genes that have previously been implicated in the pathophysiology of POI (*EIF2S2*, *GPR3*, *PGRMC1*, *BHLHB9*, *FSHB*)²⁸⁻³⁴. This suggests that these genetic variants are not a major cause of POI.

Upon analysis of our patients' clinical data with respect to the presence of variants, we did not find any statistically-significant difference in the phenotype according to the genotype.

With regard to the menstrual cycle, Bouilly *et al.* reported PA in 20% of mutated patients²⁴. In our study, 17% of patients with variants presented with PA. On the other hand, among all POI patients with variants, SMD occurred mainly after the age of 30 (49%). This result is quite unexpected, as deleterious variants could be linked to earlier ovarian deficiency, though this does match the results of Patiño *et al.* who reported that 62% of cases of SA occurred after the age of 30 in mutated patients¹⁹.

There does seem to be a relationship with the ethnic origin, as a higher prevalence of POI has been described in the African American population compared to Japanese women³. In our study, 52% of patients with a variant came from Sub-Saharan Africa and 58% of those patients presented a *NOBOX* variant, though this prevalence may be under-estimated because most of our patients were of Caucasian origin. Our *NOBOX* variant findings are similar to those of the study of Bouilly *et al.*, in which 58% of patients with *NOBOX* variants came from Sub-Saharan Africa³⁵. We did not find any *NOBOX* mutations among Asian patients, as reported previously in two cohorts including only Chinese patients^{36,37}.

Sixteen percent of our cohort mentioned at least one case of POI in the family. According to previous studies, 4 to 31% of cases of POI are familial forms⁸. It is noteworthy that familial cases of POI among our patients did not present more variants than non-familial cases, even when there were several variants. However, these data should be viewed with caution as we determined the family history based on questionnaires. Hormonal testing of family members was not available.

In our cohort, 37% of patients had been pregnant before the diagnosis of POI. Previous studies have described pregnancy before diagnosis in around 20% of patients¹⁸. Interestingly, the occurrence of a pregnancy before diagnosis was similar among patients with or without variants.

Furthermore, patients with *NOBOX* and *BMP15* mutations (genes known to be involved in folliculogenesis) had been spontaneously pregnant before the diagnosis of POI and some of those were found to have combined variants of different genes. Therefore, other mechanisms may account for the secondary amenorrhea after fertility.

Previous studies have suspected and even underscored the polygenic pathogenesis of POI^{19,24}. In our cohort, 5% of patients had more than one variant. This is in line with Patiño *et al.* who used whole exome sequencing to find 5% of patients with 2 mutations¹⁹. In their study, all genetic disorders were considered together, whereas, in our study, we were able to distinguish between variants and variants of unknown significance.

Bouilly *et al.* found that most patients presenting with PA had 2 genetic defects (3 of 5 patients). In the same study, the mean age at onset of POI was lower in patients with two combined variants compared to patients with only one (17 versus 27)²⁴. On the contrary, in our study, the age at onset of PA and the age at onset of a secondary menstrual disturbance did not differ between patients with combined variants or with only one single variant. Therefore, no gene “dose effect” was not found in our study.

There are several possible hypotheses explaining the absence of phenotype/genotype correlations. The first is that all the genes we analyzed have different pathogenicities, which may have influenced our results. Indeed, it is challenging to determine the pathogenicity of each individual genetic abnormality. For example, the *DMC1*-p.Met200Val variant is common among the African population and has been considered as a polymorphism by other groups, though biochemical analyses have shown that the variant has reduced stability, and is only moderately effective at catalyzing *in vitro* chromosomal recombination reactions³⁸. Moreover, it has been shown that different types of variants of a single gene may result in variable phenotypes³⁹. A second hypothesis is the influence of non-genetic factors in the development of POI. Few studies have focused on the possible environmental causes of POI and most of those were animal studies. A recent review of 19 studies (animal and human data) described phthalates, bisphenol A, pesticides and tobacco as the substances

most often reported to have a negative impact on ovarian function, with increased follicular depletion leading to earlier menopause⁹. In their work, Béranger *et al.* underscored the impact of exposure to 2-bromopropane, perfluorooctanoate and cadmium on ovarian reserve¹⁰. The study of Gallichio *et al.* also supports a toxic origin, as it reported a higher prevalence of POI among Caucasian hair dressers using hair dyes without gloves⁴⁰. Environmental causes could modulate the expression of certain genes involved in POI.

Our study is interesting for several reasons. To our knowledge this is the largest POI cohort tested with NGS. Our study focused on “idiopathic” POI as we excluded known causes of POI. Patients who presented auto-antibodies were not excluded of the analysis. Indeed, positive auto-antibodies were mostly represented by ant thyroid auto-antibodies which are positive in 15% of euthyroid women⁴¹. Moreover, the autoimmune nature of POI is not always clear since a positive antibody doesn’t mean that there are organic consequences and genetic disorders may be involved in these patients. We performed a thorough genetic analysis to classify the variants as deleterious variants or VUS. We are the first to report that there is no correlation between the patient’s phenotype and genetic results. Our study emphasizes that NGS should be proposed to every POI patient, regardless of their clinical presentation. Finding a mutation in a candidate gene may help to accept the diagnosis of POI, as depression is common in these patients⁴². Furthermore, accepting the diagnosis may improve the patient’s compliance with hormonal replacement therapy (HRT), as women with POI often stop their treatment⁶. Finally, identifying a mutation may be particularly relevant for female relatives, to whom fertility preservation could then be proposed.

Nevertheless, we should point out the multiple weaknesses of our study. Indeed, although more than 70 genes have been identified as candidate genes in the literature⁴³, we only tested 18. However, those genes were chosen as the genes most commonly identified previously in women with POI. Several human cases had been reported in the literature for each gene. As for the clinical data, we should remember that determining the age of onset of POI can be challenging because, in some patients, amenorrhea occurs when stopping oral contraceptives to become pregnant.

Furthermore, it would have been very useful to evaluate familial cases in greater detail. Studying the segregation of variants among our families with a history of POI could illustrate their involvement in the occurrence of POI. However, DNA samples could not be obtained from the patients' relatives in most cases.

In conclusion, genetic screening would improve the care of patients diagnosed with "idiopathic" POI. Furthermore, performing NGS on POI patients would help geneticists to better understand the pathogenicity of the various genes implicated. One major remaining challenge will be to predict the age of onset of POI in women according to their genetic defect.

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References

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Table 1: A panel of 18 candidate genes

Genes	location	Function(s)	Reference of human POI description & functional study
<i>BHLHB9</i> (NM_001142528)	Xq22.1		28
<i>BMP15</i> (NM_005448.2)	Xp11.2	Follicular activation-Development and maturation-Cell division	44
<i>DMC1</i> (NM-007068.3)	22q13.1	Meiosis	45
<i>EIF2S2</i> (NM_003908.2)	<i>EIF2B2</i> -14q24.3; <i>EIF2B4</i> - 2p23.3; <i>EIF2B5</i> - 3q27.1	Cell death-Damage-Autophagy	29
<i>FIGLA</i> (NM_001004311.3)	2p13.3	Oogenesis	46
<i>FOXL2</i> (NM-023067.3)	3q23	Gonadogenesis - Oogenesis	47
<i>FSHR</i> (NM_000145.3)	2p21-p16	Follicular activation-Development and maturation-Hormonal support	48
<i>GDF9</i> (NM_005260.5)	5q31.1	Follicular activation-Development and maturation-Cell division	49
<i>GPR3</i> (NM_005281.3)	1p36.1-p35	Meiosis	34 30
<i>HFM1</i> (NM_001017975.4)	1p22.2	DNA division and repair	50
<i>LHX8</i> (NM_00100933.1)	1p31.1	Oogenesis	24
<i>MSH5</i> (NM-172165.3)	6p21.3	Meiosis	45
<i>NOBOX</i> (NM_001080413.3)	7q35	Gonadogenesis – Oogenesis-Follicular activation-Development and maturation	51
<i>NR5A1</i> (NM_004959.4)	9q33	Gonadogenesis - Oogenesis	52
<i>PGRMC1</i> (NM_006667.4)	Xq24	Hormonal support	32
<i>STAG3</i> (NM_001282717.1)	7q22.1	Cell division	16
<i>FSHB</i> (NC_000011.10)	11p14.1	Follicular activation-Development and maturation-Hormonal support	31 33 53
<i>XPNPEP2</i> (NM_003399.5)	Xq25		54

Table 2: clinical data of the cohort (N=269 women)

	N (%)
Ethnic origin	
Caucasian	126/246 (52)
North Africa	50/246 (20)
Sub-Saharan Africa	57/246 (23)
Asia	13/246 (5)
Familial history of POI	43/264(16)
Primary amenorrhea	34/263 (13)
No pubertal development	6/32 (19)
Incomplete pubertal development	18/32 (56)
Complete pubertal development	8/32 (25)
Age at secondary menstrual disturbance (years old)	
<20	30/204 (15)
20-29	47/204 (23)
30-39	127/204(62)
Pregnancy before POI	96/259 (37)

POI: primary ovarian insufficiency

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Table 3: Variants found in the cohort

Gene	DNA mutation	Protein alteration	rs number	Polyphen-2 (score)	Sift (score)	Mutation taster	Functional test	Nb of patients	PA/SMD
NOBOX	c.131G>T	p.Arg44Leu	rs115206969	B(0)	NS	P(1)	No effect ^{24,55} . Protein instability ⁵⁶	3	0/3
	c.349C>T	p.Arg117Trp	rs7800847	B(0,35)	D(0,02)	DC(0)	↘ transcriptional activity ^{24,35,55}	7	1/6
	c.454G>A	p.Gly152Arg	rs201806397	B(0)	T(0,79)	P(1)	↘ transcriptional activity ⁵⁶	3	0/3
	c.1354G>A	p.Asp452Asn	rs112190116	B(0)	T(1)	P(1)	No effect ^{24,55} ↗ transcriptional activity ⁵⁶	4	1/3
	c.271G>T	p.Gly91Trp	rs77587352	D(0,98)	NS	DC(0)	↘ transcriptional activity ^{24,35,55}	5 (+/-) 1 (+/+)	2/3 1/0
	c.1064G>C	p.Arg355Pro	rs201947677	D(0,99)	D(0)	DC(1)	p.Arg355His disrupts DNA binding ⁵¹	1	0/1
BMP15	c.443T>C	p.Leu148Pro	rs114823607	D(0,983)	D(0,97)	P(0,89)	disrupted mature protein secretion ⁵⁷	8 (+/-) 1 (+/+)	0/7 0/1 *
HFM1	c.11C>A	p.Ser4*	rs376463557	NS	NS	NS		1	1/0
	c.1477A>C	p.Lys493Gln	rs113908392	D(0,95)	D(0)	DC(0,99)		3	1/2
	c.2308G>A	p.Asp770Asn	rs143399622	D(0,93)	T(0,08)	DC(1)		1	0/1
NR5A1	c.43G>A	p.Val115Met	rs104894124	D(0,99)	D(0)	DC(1)	↘DNA binding and transcriptional activity ⁵⁸	1	0/1
	c.386C>T	p.Pro129Leu	rs200749741	B(0,12)	D(0)	DC(0)	↘ transcriptional activity ⁵²	1	1/0
	c.772C>T	p.Gln258*	NOVEL	NS	NS	NS	truncated and/or unstable protein	1	0/1
	c.1093C>T	p.Arg365Trp	NOVEL	D(0,94)	D(0)	DC(1)		2	0/2
STAG3	c.659T>G	p.Leu220Arg	NOVEL	D(0,99)	D(0)	DC(1)		1	1/0
	c.938A>T	p.Tyr313Phe	NOVEL	D(0,93)	T(0,15)	DC(1)		1	1/0
	c.1999C>T	p.Arg667Cys	rs141693812	B(0,37)	D(0,01)	DC(1)		1	1/0
	c.2473C>G	p.Leu825Val	rs764688962	D(0,84)	T(0,4)	DC(1)		1	0/1
	c.2612G>A	p.Arg871His	NOVEL	D(0,98)	T(0,07)	DC(1)		1	0/1
GDF9	c.1275C>A	p.Ser425Arg	rs116926261	D(0,53)	T(0,5)	ND	Pathogenic ⁵⁹	1	0/1
	c.1360C>T	p.Arg454Cys	rs61754582	D(0,99)	D(0,01)	ND		3	0/3
MSH5	c.138- c.138G>A	p.Glu46dup	rs781096458	NS	NS	NS	potential splicing modification (HSF -8.7%)	1	0/1
	c.416-1G>A	p.?	NOVEL	-	-	-		1	0/1
	c.952-2A>G	p.?	rs143453834	-	-	-		1	0/1
FOXL2	c.384G>T	p.Trp128Cys	NOVEL	D(0,98)	D(0)	DC(1)		1	0/1
XPNEP2	c.754C>G	p.Arg252Gly	rs189381278	D(0,99)	D(1)	DC(0,98)		1	0/1
	c.1154A>G	p.Lys385Arg	rs145287846	D(0,99)	T(0,05)	DC(0,98)		1	0/1

FIGLA	c.274G>A	p.Val92Met	NOVEL	D(0,99)	D(0)	DC(1)		1	0/1
DMC1	c.449G>A	p.Gly150Asp	rs58396845	D(0,92)	D(0)	DC(1)		1	1/0
	c.598A>G	p.Met200Val	rs2227914	B(0)	D(0)	P(1)	unstable protein ³⁸	19	1/17*
FSHR	c.334A>C	p.Asn112His	rs201909194	D(0,90)	T(0,42)	P(0,85)		1	0/1
	c.1330G>A	p.Ala444Thr	rs202162496	B(0,07)	D(0)	DC(1)		1	0/1
LHX8	c.974C>T	p.Ala325Val	rs34889650	D(0,82)	T(0,15)	DC(1)		1	0/1

B : benign ; D : damaging ; T : tolerated ; NS: not scored; P: polymorphim; DC: disease causing; +/-: heterozygous; -/- : homozygous; PA: primary amenorrhea; SMD secondary menstrual disturbance; *: missing data N=1

Table 4: patients with combined variants. N= 14

Patients with combined variants	Genes	DNA mutation	Protein alteration	PA or SMD	Familial POI
1	<i>BMP15</i>	c.443T>C	p.Leu148Pro	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
2	<i>STAG3</i>	c.938A>T	p.Tyr313Phe	PA	No
		c.1999C>T	p.Arg667Cys		No
3	<i>BMP15</i>	c.443T>C	p.Leu148Pro	SMD	No
	<i>NOBOX</i>	c.349C>T	p.Arg117Trp		No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
	<i>XPNP2</i>	c.754C>G	p.Arg252Gly		No
4	<i>NR5A1</i>	c.386C>T	p.Pro129Leu	PA	No
	<i>HFM1</i>	c.11C>A	p.Ser4*		No
5	<i>NOBOX</i>	c.349C>T	p.Arg117Trp	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
6	<i>FSHR</i>	c.334A>C	p.Asn112His	SMD	No
	<i>HFM1</i>	c.1477A>C	p.Lys493Gln		No
7	<i>HFM1</i>	c.1477A>C	p.Lys493Gln	SMD	No
	<i>LHX8</i>	c.974C>T	p.Ala325Val		No
8	<i>NOBOX</i>	c.131G>T	p.Arg44Leu	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
	<i>FIGLA</i>	c.274G>A	p.Val92Met		No
9	<i>NOBOX</i>	c.349C>T	p.Arg117Trp	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
10	<i>NOBOX</i>	c.131G>T	p.Arg44Leu	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
11	<i>BMP15</i>	c.443T>C	p.Leu148Pro	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
12	<i>NOBOX</i>	c.131G>T	p.Arg44Leu	SMD	No
	<i>DMC1</i>	c.598A>G	p.Met200Val		No
13	<i>BMP15</i>	c.443T>C	p.Leu148Pro	SMD	No
	<i>BMP15</i>	c.443T>C	p.Leu148Pro		No
14	<i>NOBOX</i>	c.271G>T	p.Gly91Trp	PA	No
	<i>NOBOX</i>	c.271G>T	p.Gly91Trp		No

Table 5: Clinical characteristics of POI patients with or without variant found in NGS sequencing

	NO VARIANT N (%)	ALL VARIANTS N (%)	P*	NOBOX VARIANTS^α N (%)	P**
Total	202	67		18	
Ethnic origin					
Caucasian	99/183 (54)	27/63 (43)		9/17 (53)	
North Africa	48/183 (26)	2/63 (3)	NS	1/17 (6)	NS
Sub-Saharan Africa	25/183 (14)	32/63 (51)		7/17 (41)	
Asian	11/183 (6)	2/63 (3)		0	
Familial POI	33 /197 (17)	10/67 (15)	NS	1 /18 (6)	NS
Primary amenorrhea	23/201 (11)	11/65 (17)	NS	5/18 (28)	0.11
Age at secondary menstrual disturbance (years old)					
<20	24/155 (15)	6/49 (12)		1/13 (8)	
20-29	29/155 (19)	18/49 (37)	NS	5/13 (38)	NS
30-39	102/155(66)	25/49 (51)		7/13 (54)	
Spontaneous pregnancy before POI	71/194 (37)	25/65 (38)	NS	5 /18 (28)	NS

* p all variants versus no variant ** p *NOBOX variant* versus no variant

^α excluding patients with combined variant in other genes