High-resolution array-CGH analysis on 46,XX patients affected by early onset primary ovarian insufficiency discloses new genes involved in ovarian function

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STUDY QUESTION: Can high resolution array-CGH analysis on a cohort of women showing a primary ovarian insufficiency (POI) phenotype in young age identify copy number variants (CNVs) with a deleterious effect on ovarian function?

SUMMARY ANSWER: This approach has proved effective to clarify the role of CNVs in POI pathogenesis and to better unveil both novel candidate genes and pathogenic mechanisms.

WHAT IS KNOWN ALREADY: POI describes the progression toward the cessation of ovarian function before the age of 40 years. Genetic causes are highly heterogeneous and despite several genes being associated with ovarian failure, most of genetic basis of POI still needs to be elucidated.

STUDY DESIGN, SIZE, DURATION: The current study included 67 46,XX patients with early onset POI (<19 years) and 134 control females recruited between 2012 and 2016 at the Medical Cytogenetics and Molecular Genetics Lab, IRCCS Istituto Auxologico Italiano.

PARTICIPANTS/MATERIALS, SETTING, METHODS: High resolution array-CGH analysis was carried out on POI patients’ DNA. Results of patients and female controls were analyzed to search for rare CNVs. All variants were validated and subjected to a gene content analysis and disease gene prioritization based on the present literature to find out new ovary candidate genes. Case-control study with statistical analysis was carried out to validate our approach and evaluate any ovary CNVs/gene enrichment. Characterization of particular CNVs with molecular and functional studies was performed to assess their pathogenic involvement in POI.

MAIN RESULTS AND THE ROLE OF CHANCE: We identified 37 ovary-related CNVs involving 44 genes with a role in ovary in 32 patients. All except one of the selected CNVs were not observed in the control group. Possible involvement of the CNVs in POI pathogenesis was further corroborated by a case-control analysis that showed a significant enrichment of ovary-related CNVs/genes in patients (P = 0.0132; P = 0.0126). Disease gene prioritization identified both previously reported POI genes (e.g. BMP15, DIAH2, CPEB1, BNC1) and new candidates supported by transcript and functional studies, such as TP63 with a role in oocyte genomic integrity and VLDLR which is involved in steroidogenesis.

Introduction

Primary ovarian insufficiency (POI) is a heterogeneous group of disorders whose incidence increases with increasing age (1:10,000 women by age 20, and 1:100 by age 40) (Goswami and Conway, 2005). POI describes the progression toward the cessation of ovarian function and can occur as either primary (PA) or secondary amenorrhea (SA), in syndromic or non-syndromic phenotypes (Rossetti et al., 2017). While PA represents the most severe manifestation of the disorder, characterized by absent pubertal development and/or ovarian dysgenesis (OD), SA is generally associated with milder phenotypes with a post-pubertal onset (Beck-Peccoz and Persani, 2006; Cox and Liu, 2014). Genetic causes are highly heterogeneous and might explain at least some of the sporadic idiopathic cases, which comprise 50–90% of cases (Chapman et al., 2015). Both numerical and structural chromosomal anomalies of which Turner’s syndrome (45,X) is the most frequent, as well as 46,XX non-syndromic patients’ genomic alterations have been so far reported as causative of POI (Fonseca et al., 2015; Laissue, 2015; Huhtaniemi et al., 2018).

Recently, thanks to genome-wide investigation based on both single-nucleotide polymorphism array and array comparative genomic hybridization (array-CGH) technologies, several X-linked and autosomal copy number variants (CNVs) affecting loci with a possible role in female fertility have been identified (Aboura et al., 2009; Dudding et al., 2010; Ledig et al., 2010; Quilter et al., 2010; Knauff et al., 2011; Zhen et al., 2013; Castronovo et al., 2014). A few high resolution studies have analyzed small groups of 46,XX non-syndromic POI patients, either phenotypically mixed (i.e., PA, SA and infertile patients) (McGuire et al., 2011; Norling et al., 2014; Jaillard et al., 2016) or homogeneously affected by early menopause (Tsuiko et al., 2016). Besides genes already known to be involved in POI pathogenesis, other novel genes with an important role in ovarian development have been identified and proposed as candidates. However, the low detection rate (<50%) suggests that most of the genetic basis of POI still needs to be elucidated, possibly by a complementary high throughput genome-wide approach such as whole exome sequencing (Tsuiko et al., 2016). In addition, the extreme degree of genetic heterogeneity as well as the presence of multiple rare rearrangements/single nucleotide variants carried by single individuals (McGuire et al., 2011; Norling et al., 2014; Tsuiko et al., 2016) seem to be constant features of POI genetic architecture.

Materials and Methods

Study design

A cohort of 46,XX patients affected by severe primary ovarian insufficiency was collected and screened by high resolution array-CGH analysis to identify novel ovary-related genes involved in the POI phenotype, which were then analyzed by disease gene prioritization to highlight the most likely candidates. To validate our approach, a case-control study using a cohort of fertile females was also carried out and the pathogenic effect of some identified CNVs containing candidate genes was further investigated at the genomic and transcript level (Fig. 1).

Here, we performed the first high resolution array-CGH-based case-control investigation on a cohort of young 46,XX patients (POI onset <19 years) homogeneously affected by PA and/or OD to identify genetic defects caused by highly penetrant variants with an earlier deleterious effect on ovarian function. This approach has proved effective to clarify the role of CNVs in POI pathogenesis and to better unveil both novel candidate genes and pathogenic mechanisms.

**Key words:** primary ovarian insufficiency / array-CGH / ovarian dysgenesis / TP63 / VLDLR
Ethical approval
Written informed consent to the research investigation, approved by the Ethical Clinical Research Committees of IRCCS Istituto Auxologico Italiano and of San Raffaele Research Institute, was obtained from either the adult patients or parents of underage patients.

Patients and controls
There were 67 Caucasian patients affected by POI, mean age 19.2 ± 7.3 years (median: 16 years), were enrolled from July 2011 to December 2014. The patients were referred and gave their informed consent to the study at the Division of Endocrine and Metabolic Diseases of Istituto Auxologico Italiano and at the Division of Genetics and Cell Biology of San Raffaele Research Institute in Milan. The inclusion criteria were 46,XX karyotype, primary amenorrhea (61 patients) or very early secondary amenorrhea (6 patients) occurring immediately after menarche. Mean age at diagnosis was 15.1 ± 1.7 years and the hormone levels in the analyzed patients were in the postmenopausal range (FSH > 40 UI/l and estradiol <30 pg/ml). All included patients were phenotypically normal and considered idiopathic because they did not have any POI-related conditions such as ovarian surgery or previous chemo- or radio-therapy (Supplementary Table SI). Patients cohort included 35 sporadic (52.23%), and 25 familial (37.31%) POI cases; clinical details are reported in Supplementary Table SI.

The control cohort, selected from our in-house array-CGH data of healthy individuals, consisted of 134 healthy Caucasian women with a normal karyotype, who had given birth to at least one child, and with a mean age of 46.5 ± 10.4 years.

Array-CGH analysis, CNV classification and validation
The genome scan was performed using the Human Genome CGH Microarray Kits 1 × 244 K (9 patients) and 2 × 400 K (58 patients), according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA, USA). Data were extracted and analyzed for copy number changes using Agilent CytoGenomics v.3.0 (Agilent Technologies). CNV classification was performed according to the Database of Genomic Variants (DGV) (http://projects.tcg.ca/variation/, release March 2016) to exclude common polymorphic CNVs with a frequency >1% in healthy controls. To confirm the actual rarity of the CNVs identified in POI patients, in-house array-CGH results were drawn for the female controls previously analyzed with 244 K (n = 71) and 400 K (n = 63) platforms. CNVs detected by ≤5 probes or with an unknown inheritance were validated by means of real-time quantitative PCR (qPCR) or Long-Range PCR (LR-PCR). Specifically, qPCR was performed on gDNA using SYBR Green methodology (Thermo Fisher Scientific, Waltham, MA, USA) and data were analyzed through the 2−ΔΔCt method (Livak and Schmittgen, 2001). LR-PCR with specific primers able to amplify the aberrations breakpoints was carried out using TaKaRa LA Taq™ (TaKaRa, JP). PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Electropherograms obtained were analyzed using ChromasPro 1.5 software (Technelysium Pty Ltd., Tewantin QLD, Australia). A full list of primers is available upon request.

Ovarian CNVs selection and classification
In order to pick out CNVs with a putative role in POI etiology, the rare CNVs identified in patients and controls were assessed for gene content and compared with similar already reported CNVs using public databases such as ISCA (https://www.clinicalgenome.org/, update November 2012), and DECIPHER v9.10 (http://www.sanger.ac.uk/PostGenomics/decipher/, last accessed March 2017). Genes involved in rare CNVs were analyzed in both patients and controls according to the following features: (i) expression and function in ovary, (ii) association with POI in humans and (iii) presence of animal models. A positive feedback in at least one out of three evaluated features classified the genes and relative CNVs as ovary-related. Ovarian Kaleidoscope Database (OKdb, http://okdb.appliedbioinfo.net, last accessed July 2017) was consulted for expression, function, and reported variants, and Mouse Genome Informatics database (MGI, http://www.informatics.jax.org/), last accessed July 2017 was investigated to evaluate the presence of fertile or subfertile animal models.

Prioritization strategy
To identify the top POI candidates, we applied a prioritization strategy by scoring the genes as reported in Table I. Based on final ranking [1–8], the genes were classified as ‘low score’ [1–3], or ‘high score’ [4–8] genes. This approach was applied to both patients and controls.

Statistical analyses
Statistical analyses for the case-control study were performed by online 2 × 2 contingency table (www.vassarstats.net/odds2x2.html) for Pearson’s Chi-square test (Chi-q) and R.3.1.0 software for Wilcoxon rank sum test. Chi-q was used to investigate significant differences between patients and controls in the number of individuals having either rare CNVs or ovary-related rare CNVs. Wilcoxon rank sum test was used to evaluate a relation between CNVs and POI disease. In detail, the total number of: (i) rare CNVs, (ii) ovary-related rare CNVs (total number, duplications and deletions) and (iii) common susceptibility variants were compared between patients and controls. Wilcoxon rank sum test was used to match the number of: (i) ovary-related genes [score 1–8], (ii) high score genes [4–8] and (iii) low score genes [1–3]. Data used for statistical analyses are reported in Supplementary Table SII. Identical rearrangements found in

<table>
<thead>
<tr>
<th>Table I Criteria used for gene POI association score assessment.</th>
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<tr>
<td><strong>Total score [0–8]</strong></td>
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<tr>
<td><strong>Expression and Function in ovary</strong> [score 0–3]</td>
</tr>
<tr>
<td>0 nothing reported related to ovary</td>
</tr>
<tr>
<td>1 gene expressed during oocyte/follicle development in animals</td>
</tr>
<tr>
<td>2 gene with a known ovarian function</td>
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<tr>
<td>3 gene with more clarified ovarian function</td>
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<tr>
<td><strong>Association to POI in humans</strong> [0–3]</td>
</tr>
<tr>
<td>0 never associated to POI</td>
</tr>
<tr>
<td>1 Reported in 1 or 2 patients with fertility defects or POI</td>
</tr>
<tr>
<td>2 Reported in more than 2 patients with POI</td>
</tr>
<tr>
<td>3 OMMIE disease gene for POI</td>
</tr>
<tr>
<td><strong>Animal model</strong> [0–1]</td>
</tr>
<tr>
<td>0 No infertile or subfertile animal models</td>
</tr>
<tr>
<td>1 Presence of infertile or subfertile animal models</td>
</tr>
<tr>
<td><strong>Literature ovary-related</strong> [0–1]</td>
</tr>
<tr>
<td>0 Less than 5 articles related to ovarian function/POI</td>
</tr>
<tr>
<td>1 More than 5 articles related to ovarian function/POI</td>
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*Expression evaluated based on published articles or expression data from Ovarian Kaleidoscope Database (http://okdb.appliedbioinfo.net).
familial cases were counted as one. A P-value <0.05 assigned a statistically significant test.

**Gene expression analysis, CNVs molecular characterizations, and luciferase assay**

Transcriptional expression of ovary-related genes was evaluated on RNA from (i) adult ovary (Human Ovary Poly A+ RNA, Clontech), (ii) fetus (Human Fetus, Whole Poly A+ RNA, Clontech) and (iii) peripheral blood. cDNAs from each tissue were obtained with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) using 500 ng of RNAs. PCR reactions were performed using the AmpliTaq Gold® kit (Thermo Fisher Scientific).

For genomic characterizations of CNVs involving TP63 and VLDLR, specific primers to amplify duplication/deletion breakpoints were designed for LR-PCR reactions (TaqKaRa LA Taq™) and sequencing; qPCR on gDNA was carried out with SYBR-Green technology (Thermo Fisher Scientific) for SYCE1 rearrangements. RT-qPCR reactions for TP63, VLDLR and DIAPH2 were performed using the TaqMan method with three housekeeping genes as normalizers (TBP, GUSB, and RPLPO) (Supplementary Table SIII). The 2$^{-\Delta\Delta C_{t}}$ method was applied.

For CNVs position effect assessment using luciferase assays, we selected both CNVs adjacent to genes with a high POI association score and CNVs present in patients whose mRNA was available for further validations. The only variant that met these conditions was the 9p24.2 CNV (chr3:189,424,338-189,592,155 (hg19). RT-PCR and sequencing of duplication breakpoints conformed a direct tandem orientation and the two OMIM POI genes, and five CNVs overlap with variants previously reported in POI patients (Supplementary Fig. S1).

Among common rearrangements counted as susceptibility loci for POI (i.e. NAIP at 3q13.2, DUSP22 at 6p25.2, and SYCE1 at 10q26.3), 26 aberrations were found in 25 patients and 41 common aberrations were found in 39 controls (Supplementary Table SIV). All rare and selected common CNVs identified in patients were submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/, July 24, 2018, date last accessed; accession numbers SCV000787656 to SCV000787743).

**Gene content analysis of CNVs and disease gene prioritization indicate potentially relevant regions for POI**

Among the 44 ovary-related genes, we identified two OMIM POI genes (DIAPH2, POF2A, OMIM#300511; BMP15, PO4, OMIM#300510), seven genes already associated with POI in human (BNC1, CPEB1, PSMB1, SH3GL3, STS, TBP and ZFR2) and 35 genes either not well or never associated with POI (Supplementary Table SV). The expression in the ovary was confirmed by RT-PCR on all selected ovary-related genes (data not shown) and detailed information about their function are reported in Supplementary Table SIV. The disease prioritization strategy attributing to each gene a POI association score [1–8] (Table 1, Supplementary Table SIVI) identified the most promising candidates (Fig. 2). Similarly, 48 ovary-related genes were identified and prioritized in controls (Supplementary Tables SV and SVI).

**Statistical analysis revealed in patients a significant enrichment in ovary-related CNVs and genes**

In order to validate our methodological approach and results obtained with prioritization, a case-control analysis was performed. Chi-q test showed that the number of patients carrying either rare CNVs (n = 49) or ovary-related CNVs (n = 32) is statistically significantly higher compared to the number of controls (P = 0.0386, P = 0.0065, respectively) (Fig. 3A, C). Wilcoxon rank sum test did not reveal any differences in the number of rare CNVs between patients and controls (P = 0.1852); conversely the number of rare ovary-related CNVs was statistically significant (P = 0.0132), in particular for duplications (P = 0.0128). Similar results were obtained for ovary-related genes (P = 0.0126), particularly for the genes classified with the highest POI association score (P = 0.0192) (Fig. 3B and D). Even though the Wilcoxon test did not reveal any difference regarding the number of previously associated common CNVs (P = 0.2494; Fig. 3B and D), no SYCE1 deletions were detected in controls.

**Pathogenic effect evaluation of particular CNVs**

The identified 161 kb paternal intragenic duplication at 3q28 in POI-8 and POI-9 (Fig. 4A) involves exons 2–9 of the longest TP63 transcript variant (NM_003722) (Fig. 4B and C). LR-PCR and sequencing of duplication breakpoints confirmed a direct tandem orientation and the inclusion of exon 10 (Fig. 4D), refining the duplication at chr3:189,424,338-189,592,155 (hg19). RT-PCR and sequencing
reactions specific for exon junction 10-2 confirmed that 3q28 duplication produces an aberrant transcript without disruption of the reading frame (Fig. 4E). RT-qPCRs carried out on junction 7–8, 11–12 indicated that TP63 transcript levels in the patient are comparable to that in controls, and only a small amount of the duplicated aberrant mRNA is present (about 30% of the total level) (Fig. 4E). Since the
reading frame is unaffected and that wild-type Tp63 protein is composed of 680 aa, the hypothetical aberrant protein might be formed by 1109 aa (Fig. 4F).

Although 11 CNVs might be perturbed by position effect, we focused our attention on deletion at 9p24.2 in POI-44 because it involves the high score gene VLDLR and patient mRNA was available.

Figure 4 Molecular characterization of TP63 gene duplication. (A) array-CGH profile output. (B) Duplication view according to UCSC genome browser; intragenic duplication is represented by a blue bar from IVS1 to IVS9. (C) TP63 isoforms are shown with the respective duplicated portion of 161 kb based on array-CGH results. (D) Duplication breakpoint characterization on gDNA performed by LR-PCR and sequencing with specific primers for the duplicated allele is shown (direct tandem orientation is considered); an overlapping sequence of 11 bp between the breakpoint junctions in IVS11 and IVS1 is shown. (E) Wild-type mRNA and mutated transcript with the relative sequences are shown. RT-qPCR analysis revealed a statistically significant TP63 overexpression in patients compared to controls which is indicative of the amount of the aberrant transcript. (F) Wild-type and hypothetical aberrant Tp63 proteins are shown.
Array-CGH analysis revealed a four-probe deletion of about 14.6 kb (chr9:2654,203-2668,811, hg19), involving VLDLR 3'UTR (NM_003383) and predicted regulatory elements (Fig. 5A and B). LR-PCR and sequencing revealed that the VLDLR gene was preserved confirming a deletion of 12.4 kb (chr9:2656,920-2669,483, hg19) (Fig. 5B). Through luciferase assays, the region containing the predicted regulatory elements showed a dramatic downregulation of VLDLR mRNA compared to the average of controls (Fig. 5C). The heterozygosity assay specific for rs6148 in VLDLR exon 14 showed a mono-allelic expression (Fig. 5E).
insulator and the 3000 bp downstream (INS-BgIII) showed a strong specific enhancer effect in COV434, and particularly on the VLDLR promoter (Fig. 5C). Indeed, RT-qPCR performed on patient peripheral blood cDNA revealed a significant transcript downregulation (P < 0.001; Fig. 5D). Accordingly, the identification on the patients’ DNA of a heterozygous SNP (rs6148) in the coding region, highlighted mono-allelic mRNA expression (Fig. 5D).

Regarding the intronic duplication at Xq21.33 involving DIAPH2 IVS26 and the entire long non-coding RNA (DIAPH2-antisense), RT-qPCR was performed on POI-6 blood mRNA to evaluate a possible DIAPH2 alteration, but no mRNA level changes were detected in the analyzed tissue (data not shown).

CNVs at 10q26.3 involving CYP2E1 and SYCE1 were identified in patients and controls. Namely, a deletion in POI-60 and a duplication in POI-9, POI-11, POI-17, POI-62, CTR_F003, CTR_F008, CTR_F021, CTR_F053, CTR_F084, and CTR_F086. To evaluate any differences between patients and controls SYCE1 duplication CNVs, which might explain a different variant effect reflecting a diverse phenotype, qPCR experiments were carried out to clarify SYCE1 5’UTR complete or partial involvement. The analyses revealed no differences between POI patients and controls rearrangements (Supplementary Fig. S2).

Discussion

We report an array-CGH study, carried out on a cohort of 67 46,XX young patients with PA or OD, which identified both old and novel (i.e. TP63 and VLDLR) potentially deleterious CNVs for ovarian function with a detection rate of 47.8%. The study carried out on a control cohort confirmed the rarity of identified variants and their possible involvement in POI pathogenesis: it revealed a significant enrichment in the number of patients presenting ovary-related variants compared to controls, and similarly in the number of ovary-related CNVs, genes and high score genes (Fig. 3). Despite ovary-related rare CNVs being identified also in controls, most of them likely do not exert any effect on the gene content expression, thus being less deleterious rearrangements compared to those of patients. In contrast, as previously reported (Tsuiko et al., 2016), no differences in the number of selected common variants were found between patients and controls suggesting that common CNVs might enhance the POI burden in combination with other variants (Fig. 3).

Besides the identification of CNVs involving POI genes, such as DIAPH2 and BMP15, we detected variants previously reported on POI cohorts, supporting their contribution in disease pathogenesis (Supplementary Fig. S1). Among them, the 15q25.2 deletion (POI 54), involving the ovary-related genes CPEB1, BNC1, WHAMM, SH3GL3, and BTBD1, was previously identified in five POI unrelated patients (McGuire et al., 2011; Hyon et al., 2016; Tsuiko et al., 2016). Based on the severe phenotype of most patients (PA or early SA), it can be hypothesized that such a variant might contribute to an early onset of POI and that CPEB1 can be considered a leading candidate for ovarian failure because it is involved in synaptonemal complex formation during oocyte maturation (Tay and Richter, 2001). The additional identification of a homozygous microdeletion involving CPEB1 IVS1 (POI-46) further support its role in POI pathogenesis. The 15q25.2 region seems a POI-specific locus as other ovary-related genes are included, such as the high prioritized BNC1 which plays a role in mouse oocytes differentiation and causes its knock-out mouse to be sub-fertile (Tian et al., 2001).

The duplications at 6q27 (POI-53) and at 19p13.3 (POI-63) involving TBP and ZFR2, respectively, had been previously reported in single cases (Norling et al., 2014; Tsuiko et al., 2016). TBP is essential for mouse oocyte maturation (Gazdagh et al., 2007; Sun et al., 2013), whereas only expression in follicles is known for ZFR2, whose role in the ovary is still unknown (Fig. 2).

Another reported CNV, of uncertain significance because it was shared between patients and controls (Supplementary Fig. S1), was the duplication encompassing the STS gene which is involved in the steroidogenesis (Otsuka et al., 2005). Besides its previous identification in two SA patients (Quilter et al., 2010; Tsuiko et al., 2016), the evidence of the same CNV in controls highlights its unclear role in POI pathogenesis.

Deletions or duplications at 10q26.3 encompassing SYCE1 gene (Supplementary Fig. S2) had been previously reported in several POI cohorts (McGuire et al., 2011; Zhen et al., 2013; de Vries et al., 2014; Jaillard et al., 2016; Tsuiko et al., 2016). SYCE1 plays a role in the synaptonemal complex stability during meiosis (Costa et al., 2005) and its haploinsufficiency in mouse causes oocytes apoptosis and infertility (Bolcun-Filas et al., 2009). In humans, a nonsense homozygous mutation in the SYCE1 gene, observed in two POI sisters might have a similar effect (de Vries et al., 2014). Supporting its pathogenic role, we found the SYCE1 deletion only in patients (POI-60) but not among controls, confirming a frequency (1.49%) similar to that previously reported (Tsuiko et al., 2016). Conversely, SYCE1 duplication was found with a similar frequency among patients and controls (5.97% and 4.48%, respectively) and should not have any POI susceptibility role.

Our array-CGH analysis and the prioritization method uncovered ‘high score genes’ such as TP63 (3q28) and VLDLR (9p24.2). TP63 encodes a protein belonging to the p53 family and point mutations are associated in autosomal dominant manner with a wide phenotypic spectrum (OMIM#603273) including ADULT and Rapp-Hodgkin syndromes. In sisters, POI-8 and POI-9 no TP63-phenotypes were observed probably due to a different genetic defect. What is consistent with their POI disorder, is that TP63 turns out to be a main regulator of female germ cells genomic integrity during meiotic arrest (Suh et al., 2006) and its knockout mice are infertile because oocytes do not undergo apoptosis after DNA damage (Deutsch et al., 2011; Kim and Suh, 2014). Indeed, TP63 intragenic duplications, affecting the main functional domain of the protein (p-53 DNA binding domain), give rise to a stable aberrant mRNA in patients, and consequently a reduction ofTp63 activity might have deleterious effects on the oogenesis through an impaired function on genome integrity maintenance. Interestingly POI-8 and POI-9 are also carriers of a predicted probably damaging BMP15 heterozygous mutation (p.Y235C) (Di Pasquale et al., 2004), and we propose that the combination of TP63 and BMP15 alterations contributes to the ovarian dygenesis seen in both sisters, consistent with the recently reported oligogenic architecture of early onset POI (Bouilly et al., 2016).

VLDLR in humans is relevant for steroidogenesis as its expression in granulosa cells of pre-ovulatory follicles retains a relevant role in lipoprotein endocytosis during follicular growth (Murata et al., 1998). Moreover, in hens with VLDLR point mutations, the oocytes fail to grow and therefore undergo atresia causing anovulation and sterility.
gene, and consistent with its ovarian function, we propose that TP63 leading to an impaired folliculogenesis.

Although further functional studies are needed to clarify and prove TP63 and VLDLR involvement on POI phenotype, the whole of these array-CGH data identified novel pathogenic mechanisms for POI and supported the extremely wide genetic heterogeneity of this disease. The combination of extremely and moderately rare variants in an individual patients confirms that the oligogenic architecture can frequently underlie POI with PA or an early age at onset. In such a context, the combination of array-CGH with next generation sequencing represents the most suitable approach to obtain a comprehensive genetic characterization of POI patients.

**Supplementary data**

Supplementary data are available at *Human Reproduction* online.

**Acknowledgments**

The authors thank the patients and their families for cooperation.

**Authors’ roles**

IB: study design, analysis and interpretation of clinical and experimental data, CNV characterizations, statistical analyses, manuscript preparation; CCast: study design, analysis and interpretation of control array-CGH data; AS: qPCR analyses for CNVs characterization; CA: Luciferase analysis, RT-qPCR; CS: clinical data collection, statistical analysis; RR, IF: clinical data collection; MC: interpretation of experimental data; AP: Luciferase analysis; DT, LP: clinical data collection, critical revision of the manuscript; AM: clinical data collection, Luciferase analysis, critical revision of the manuscript; PF: study design, analysis and interpretation of clinical and experimental data, critical revision of the manuscript. All authors read and approved the final manuscript.

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**Conflict of interest**

The authors declare that they have no conflict of interests.

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