Title: Non-classic cytochrome P450 oxidoreductase deficiency strongly linked with menstrual cycle disorders and female infertility as primary manifestations

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Running title: Non-classic POR deficiency as cause of infertility

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ABSTRACT

Study question: Can Cytochrome P450 oxidoreductase deficiency [PORD] be revealed lately in adult women through menstrual disorders and/or infertility?

Summary answer: PORD was biologically and genetically confirmed in five adult women with chronically elevated serum progesterone (P) who were referred for oligo-/amenorrhea and/or infertility.

What is known already: PORD is an autosomal recessive disease typically diagnosed in neonates and children with ambiguous genitalia and/or skeletal abnormalities. It is responsible for decreased activity of several P450 enzymes including CYP21A2, CYP17A1 and CYP19A1 that are involved in adrenal and/or gonadal steroidogenesis. Little is known about the optimal way to investigate and treat patients with adult-onset PORD.

Study design, size, duration: In this series, we report five adult females who were evaluated in three tertiary endocrine reproductive departments between March 2015 and September 2018.

Participants/materials, setting, methods: Five women aged 19-38 years were referred for unexplained oligo-/amenorrhea and/or infertility. Genetic testing excluded 21-hydroxylase deficiency (21OH-D), initially suspected due to increased 17-hydroxyprogesterone (17-OHP) levels. Extensive phenotyping, steroid profile by mass spectrometry, pelvic imaging and next-generation sequencing of 84 genes involved in gonadal and adrenal disorders were performed in all patients. In Vitro Fertilization [IVF] followed by frozen embryo transfer under glucocorticoid suppression therapy was performed in two patients.

Main results and the role of chance: All patients had oligomenorrhea or amenorrhea. None had hyperandrogenism. Low-normal serum estradiol (E2) and testosterone levels contrasted with chronically increased serum P and 17-OHP levels, which further increased after ACTH administration. Despite excessive P, 17OH-P and 21-deoxycortisol rises after ACTH stimulation suggesting non-classic 21-hydroxylase deficiency, *CYP21A2* sequencing did not support this hypothesis. Basal serum cortisol levels were low to normal, with inadequate response to ACTH in some women, suggesting partial adrenal insufficiency. All patients harbored rare bi-allelic *POR* mutations classified as pathogenic or likely pathogenic according

to American College of Medical Genetics standards. Pelvic imaging revealed bilateral ovarian macrocysts in all women. *In vitro* fertilization was performed in two women after retrieval of a normal oocyte number despite very low E2 levels during controlled ovarian hyperstimulation. Frozen embryo transfer under glucorticoid suppression therapy led to successful pregnancies.

Limitations, reasons for caution: The number of patients described here is limited and these data need to be confirmed on a larger number of women with non-classic PORD.

Wider implications of the findings: The diagnosis of PORD must be considered in infertile women with chronically elevated P and 17OH-P levels and ovarian macrocysts. Differentiation of this entity from non-classic 21-hydroxylase deficiency is important, as the multiple enzyme deficiency requires a specific management. Successful fertility induction is possible by IVF, providing that P levels be sufficiently suppressed by glucocorticoid therapy prior to implantation.

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KEYWORDS

P450-oxidoreductase deficiency, congenital adrenal hyperplasia, infertility, ovarian macrocysts, high progesterone

INTRODUCTION

Cytochrome P450 oxidoreductase deficiency [PORD] is a rare autosomal recessive form of congenital adrenal hyperplasia [CAH] caused by bi-allelic mutations of *POR* (OMIM #124015) (Fluck et al. 2004; Arlt et al. 2004). *POR* encodes for P450 oxidoreductase [POR], a flavoprotein that serves as an electron donor to all microsomal cytochrome P450 [CYP] enzymes (Miller 1986), including the key steroidogenic enzymes CYP17A1 (17α-hydroxylase and 17, 20 lyase), CYP21A2 (21-hydroxylase) and CYP19A1 (aromatase) (Fluck et al. 2004). The first case of PORD as a clinical syndrome with apparent combined CYP17A1 and CYP21A2 deficiency was reported in 1985 (Peterson et al. 1985). Nevertheless, *POR* was not recognized as the causative gene until 2004 (Fluck et al. 2004). The exact prevalence of the disorder is currently unknown.

PORD presents with a broad and heterogeneous clinical spectrum depending on the severity of the causative bi-allelic POR mutations and the subsequent relative impairment of POR-dependent enzymes (Krone et al. 2012). Diagnosis is typically made at birth by a pathognomonic combination of disordered sex development and skeletal defects resembling Antley-Bixler syndrome [ABS] (Huang et al. 2005; Krone et al. 2012). The latter is a rare craniosynostosis syndrome, which may be caused by disrupted sterol synthesis by lanosterol 14α-demethylase (CYP51A1) and squalene epoxidase, two POR-dependent enzymes (Kelley et al. 2002; Schmidt et al. 2009). Less frequent syndromic forms with anorectal or urinary malformations have been attributed to altered retinoic acid metabolism because of CYP26A1-C1 deficiency (Fukami et al. 2010). Cases with minimal or absent bone involvement have also been reported (Fluck et al. 2004; Williamson et al. 2006).

A unique characteristic of PORD compared with other forms of CAH is that ambiguous genitalia can occur in both 46, XX and 46, XY subjects. Interestingly, virilized genitalia are observed in affected females despite post-natal low serum androgen levels, indicating intrauterine androgen excess, which resolves postnatally (Arlt et al. 2004). Maternal virilization during pregnancy may occur as well (Reisch et al. 2013). Both phenomena are thought to be

secondary to reduced activity of placental aromatase (Fukami and Ogata 2014). Suboptimal cortisol response to adrenocorticotropic hormone [ACTH] stimulation is common, while symptomatic adrenal crisis are less frequently observed (Fukami et al. 2009). The majority of PORD patients are diagnosed during infancy or childhood making this condition a primarily pediatric disease (Fukami and Ogata 2014). Diagnosis of PORD without skeletal and genital involvement has been only been reported in a couple of phenotypically normal women (Fluck et al. 2004; Bai, Li, and Wang 2017).

Herein, we report a series of five adult females diagnosed with non-classic PORD and exhibiting menstrual cycle abnormalities, infertility and ovarian macrocysts. Two of the affected women successfully underwent *in vitro* fertilization and embryo transfer [IVF–ET], leading to the birth of three healthy infants. Our work expands the clinical spectrum of PORD to include females without ambiguous genitalia that are diagnosed at adult life due to anovulation, abnormal progesterone secretion and ovarian macrocysts.

PATIENTS AND METHODS

Setting

We explored five women aged 19-38 years presenting for unexplained oligo-/amenorrhea and/or infertility as well as elevated serum 17-hydroxyprogesterone [17-OHP] and progesterone [P] levels. All five were initially suspected to have non-classic CAH [NC-CAH] due to 21-hydroxylase deficiency (21-OHD) or polycystic ovary syndrome [PCOS] despite the absence of hyperandrogenism. Subsequently, they were referred to tertiary endocrine reproductive units for further investigation and fertility induction in two of the women.

Clinical summaries before referral

Patient 1 [P1] was referred at age 30 for a two-years lasting primary infertility. She had no relevant family history. First menses occurred at age 13 but cycles were irregular between 45 and 60 days (oligomenorrhea). There was no clinical sign of hyperandrogenism. At age 25, the

patient took an oral contraceptive pill [OCP], which was discontinued at age 27 in order to get pregnant. Because of recurrence of irregular cycles, she had a first ovarian assessment at day 2 after spontaneous menses, which showed a normal Anti-Müllerian hormone [AMH] level (15.3 pmol/L) despite a slightly elevated serum Follicle-stimulating hormone [FSH] (11 IU/L, normal range: 4.3-9.0). The ovarian ultrasound revealed multiple macrocysts, the largest of which measured 28 mm at maximum diameter. Basal serum 17-OHP was increased at 16.3 nmol/l (5.4 ng/ml) (normal range for follicular phase: < 3 nmol/l), raising at that time the suspicion of NC-CAH.

Patient 2 [P2] was referred at age 36 for infertility. Family history was remarkable for suspected classic 21-OHD in her younger sister, who presented with mild genital virilization (labial fusion) at birth but had not been genetically explored. Despite normal onset of puberty (thelarche and pubic hair at age 12), she had no menses at age 16 (primary amenorrhea), and voluminous ovarian cysts (> 5 cm) were observed on ultrasound. One cyst was resected and was considered to contain part of a corpus luteum at histopathology. Hormonal status at presentation showed low-normal circulating estradiol (E2) at 140 pmol/l (normal range 130-310 and 340-810 pmol/l for early follicular and luteal phase respectively), normal serum FSH (6.8 IU/L) and prolactin levels. High serum P level at 9.5 nmol/l (3 ng/ml) was considered as suggestive of luteal phase. The patient was started on OCP, allowing pill-induced regular withdrawal bleeding. Oligomenorrhea and recurrence of ovarian macrocysts were observed following OCP interruption at age 20. At age 34, a novel endocrine evaluation revealed an increase in 17-OHP level at 8.3 nmol/l (normal range for follicular phase: < 4.5 nmol/l), further rising after ACTH-stimulation at 21.4 nmol/l but no definitive diagnosis was drawn at that time. The patient discontinued the OCP at age 35 in order to get pregnant. Due to high levels of FSH in conjunction with low E2 and decreased serum AMH levels, premature ovarian insufficiency was suspected.

Patient 3 [P3], **the sister of P2**, was referred at age 33 following the diagnosis of non-classic PORD in her sister. Mild genital virilization with labial fusion was noted at birth, requiring

vaginoplasty. Diagnostic work-up at that moment was considered to be in favor of 21-hydroxylase deficiency (data not available). The patient underwent spontaneous puberty (breast development and pubic hair at age 12) but first menses were delayed and occurred at age 16. A pelvic ultrasound visualized bilateral macrocysts. Due to irregular menses, she was started on an OCP at age 18 allowing for regular withdrawal bleeding. Oligomenorrhea recurred after temporary interruption of OCP at age 24. The patient initially declined genetic testing to explore the hypothesis of NC-CAH/21-OHD. All contraception measures were interrupted at age 33 in order to pursue fertility. Menses resumed every 30-40 days with some intermediary spotting. At that time, hormonal assessment showed a low-normal serum E2, high luteinizing hormone (LH) and FSH and decreased AMH (2 pmol/l, normal range: 4.8-53.9 pmol/l). Both P and 17-OHP were elevated at 12.1 and 16.1 nmol/l respectively. Interestingly, serum total testosterone (TT) was unexpectedly in the low-normal range (0.4 nmol/l for normal range: 0.3-1.7 nmol/l).

Patient 4 [P4] was referred at age 38 for secondary amenorrhea. There was no family history of pubertal delay or infertility. She reported normal breast development, followed by spontaneous menarche at age 12. Because of oligomenorrhea, she started OCP at age 14. She had no acne nor hirsutism. Withdrawal of the OCP at age 23 was accompanied by recurrence of oligomenorrhea. No additional investigation was performed at that time and the OCP was resumed until age 29. Thereafter, the patient remained amenorrheic. She reported decreased libido associated with vaginal dryness but no vasomotor symptoms. Because of absent response to a progestin withdrawal challenge in the setting of prolonged amenorrhea, an exploratory laparoscopy was performed at age 34. A cystic ovarian lesion was sampled and the pathological result suggested a cyst of the corpus luteum. The first available hormonal studies were performed at age 36, showing a low-normal serum E2 and TT levels associated with a high-normal serum LH and FSH levels. Despite an atrophic endometrium at the pelvic ultrasound, serum P was high (13.7 nmol/l, normal range for follicular phase < 3 nmol/l). The patient underwent ovarian stimulation with human menopausal gonadotropin (75 U per day).

The endometrium remained atrophic (thickness of 3 mm) and serum E2 increased only slightly. A pelvic magnetic resonance imaging showed several large bilateral cysts (maximal diameter of 7 cm), prompting cessation of the stimulation.

Patient 5 [P5] was referred at age 19 for secondary amenorrhea. Medical background was notable for multiple surgeries for craniostenosis (between 11 months and 8 years old). She had persistent facio-stenosis with a crooked palate, arthrogryposis, a convergent strabismus and a Marfan-like phenotype. A Shprintzen syndrome was initially suspected but genetic evaluation including sequencing of *FGFR2* and *SKI* genes did not confirm this hypothesis. She underwent spontaneous puberty (thelarche) at age 10 with menarche at age 15, followed by irregular menses in intervals ranging from 45 to 50 days. She became amenorrheic at age 18. The first hormonal assessment showed high serum FSH and LH (8.4 IU/L and 23.3 IU/L, normal range 4.3-9.0 and 2.0-7.6 respectively), undetectable E2 levels (measured at that time by a routine assay with low sensitivity) and a normal serum AMH level (27 pmol/l). 17-OHP was elevated at 11.6 nmol/l (normal range for follicular phase: < 2.5 nmol/l). A transabdominal pelvic ultrasound visualized two large cysts on each ovary.

Clinical and hormonal evaluation

Detailed medical records were reviewed, focusing specifically on pubertal timing (menarche, breast development, pubic hair), pattern of menses during adolescence/adulthood and history of ambiguous genitalia at birth. In addition to anthropometric measurements and standard physical examination, particular attention was given to bone deformities, stigmata of genital virilization (clitoromegaly) and hyperandrogenic signs (acne, hirsutism). A detailed family history focused on ambiguous genitalia, skeletal malformations resembling ABS or other reproductive defects (menstrual cycle abnormalities, ovarian cysts, infertility). Biochemical assessment included measurement of serum reproductive hormones (E2, LH, FSH, P, AMH), androgen levels (TT, androstenedione, dehydroepiandrosterone sulfate [DHEA-S]), as well as adrenal precursors (17-OHP, 21-deoxycortisol, corticosterone) before and after ACTH stimulation according to standard procedures (intravenous administration of cosyntropin 0.25

mg) (Dorin, Qualls, and Crapo 2003). Androgens and adrenal precursors were measured by liquid chromatography-mass spectrometry [LC-MS] as previously described (Kamenicky et al. 2019), while commercial immunoassays were used for the remaining hormonal measurements. Pelvic ultrasound and/or MRI was used to assess endometrial thickness and to measure antral follicle count [AFC] when possible.

Genetic studies and ethical approval

Genetic testing was performed after obtaining informed consent according to local institutional guidelines. Given the presence of elevated serum 17-OHP levels, *CYP21A2* (NM_000500.6) molecular analysis was initially performed as previously reported (Bouvattier et al. 2015). Patients were subsequently analyzed by targeted next-generation sequencing (NGS) as detailed in the Supplementary Appendix. A panel of genes involved in disorders of steroidogenesis and reproductive function, including *POR* (NM_000941.3), was sequenced. The full list of the 84 genes is described in Supplementary Table S1. All variants were classified according to American College of Medical Genetics and Genomics (ACMG) 2015 classification (Richards et al. 2015) : benign, likely benign, uncertain significance, likely pathogenic and pathogenic. This classification was conducted using Varsome (https://varsome.com/) and InterVar (http://wintervar.wglab.org).

Fertility induction

Patients P1 and P2 received treatment for infertility at Lille, France and Olten, Switzerland respectively. Both patients underwent controlled ovarian stimulation, followed by IVF-ET under hormonal replacement (estradiol, progesterone). Both patients undergone conventional IVF protocols with some differences between the two centers. In particular, a long GnRH-agonist protocol (triptorelin), followed by recombinant FSH (300 IU per day) was implemented in P1, whereas P2 received a GnRH-antagonist protocol (ganirelix) and stimulation with hMG (375 IU per day). Ovulation was triggered in both patients using subcutaneous injection of 250 µg of recombinant hCG (choriogonadotropine alfa, Ovitrelle®, Merck Serono, Lyon France).

RESULTS

Clinical phenotype

The five affected females shared several clinical features (Table I). Following spontaneous puberty as suggested by normal breast development, they were all referred for chronically disrupted menstrual cycles, ranging from irregular menses to secondary or even primary amenorrhea. In contrast with the cardinal feature of women with classic PORD, the four probands (P1, P2, P4, P5) did not exhibit any degree of *in utero* virilization. Only P3 (the sister of P2) underwent vaginoplasty at birth for labial fusion. Another common finding was the presence of large ovarian cysts. Two patients (P1, P4) requiring targeted therapy with a GnRH analogue (triptorelin), which was largely successful at reducing both the number and the size of macrocysts (Figure 1). None of the patients had clinical hyperandrogenism at adolescence and adulthood. In addition, four out of five lacked any apparent skeletal malformations. Only P5 had skeletal defects though not fitting the classic ABS (presence of craniostenosis but not craniosynostosis).

Hormonal evaluation and ultrasonographic findings

All five patients with PORD exhibited a similar hormonal pattern (Table II). Serum E2 and TT levels were low normal while serum FSH and LH levels were in the high normal to moderately elevated range. A marked increase in serum P levels was observed independently of the presumed phase of menstrual cycle. Basal 17-OHP levels were also increased. ACTH stimulation test performed in the four probands revealed that serum cortisol increased to levels below (P2, P5) or in the low-normal range (P1, P4). An excessive stimulation of P, 17-OHP, 21-deoxycortisol (4.8 to 31.5-fold, 3.6 to 11.5-fold and 5 to 70-fold, respectively – Supplemental Table S2) was also induced by ACTH stimulation. Conversely, serum androstenedione levels were low with virtually no response to ACTH (Figure 2). Pelvic imaging revealed several ovarian macrocysts in three of the five women (Table I). In the remaining two patients (P2, P3)

in whom macrocysts had regressed following long-term OCP, AFC was as expected for age (n=8-10). Serum AMH levels were low-normal in all patients except in P4.

Genetic studies

Targeted exome sequencing showed that all affected females harbored bi-allelic rare *POR* variants (minor allele frequency [MAF] < 0.1 % in Genome Aggregation Database [gnomAD]) (Table III, Figure 3). The bi-allelic character of POR mutants was asserted thanks to parental DNA analysis, except for P1. The two mutations of the latter are very closely located (at 77 base pairs), while NGS reads are of 150 base pairs length. Based on the alignment (Supplemental Figure S1), we were able to conclude that the two mutations are located on two chromosomes of different parental origin. P5 was homozygous for a pathogenic missense mutation, (c.859G>C, p.Ala287Pro), which maps to the flavin adenine dinucleotide domain (Fluck et al. 2004). This mutation has been previously shown to be a loss-of-function (Fluck et al. 2004) and is the most frequently reported *POR* mutation in Caucasians, accounting for approximately 40% of pathogenic variants in this ethnic group (Krone et al. 2012). All other patients harbored pathogenic or likely pathogenic compound heterozygous *POR* mutations (Table III, Figure 3A). Among those, the p.Arg550Trp substitution was previously reported to be deleterious (Parween et al. 2019) (Table III). Most of the mutations map to the nicotinamide-adenine dinucleotide phosphate [NADPH]-binding domain (Table III, Figure 3B). As expected the two affected sisters, P2 and P3, carried the same bi-allelic mutations (Figure 3B), inherited by their parents who were heterozygous and thus asymptomatic.

P2, P3, P4 and P5 did not harbor any mutation in *CYP21A2*. Only P1 carried, similarly to her asymptomatic father (attested by targeted sequencing of *CYP21A2* prior to the inclusion of P1 in the current study), an incidental heterozygous duplication of *CYP21A2*, associated with a heterozygous non-sense variant (c.952C>T, p.Gln318*) in the same allele (NM_000500.6). Next-generation sequencing results on the panel of 83 genes were available for three probands (P1, P2, P4) and did not detect any clinically relevant variants (pathogenic, likely pathogenic or of uncertain significance) (Supplementary Table S2).

Assisted reproductive treatment and fertility outcomes

Ovarian responses to stimulation in PORD patients P1 and P2 are summarized in Figure 4. In both patients, E2 levels were only modestly increased during FSH stimulation and at ovulation triggering [330 and 110 pmol/l (90 and 30 pg/ml) in P1 and P2 respectively, compared to ten-fold higher levels in classic IVF cycles] despite a significant number of dominant follicles. The ovarian stimulation allowed for retrieval of 9 and 15 oocytes and subsequent *in-vitro* fertilization yielding 6 and 11 embryos in P1 and P2 respectively. Astonishingly, serum P levels were increased in the range of luteal phase before ovulation triggering: 19.4 and 7.2 nmol/l in P1 and P2 respectively (Figure 4). Embryo transfer was therefore withheld awaiting optimal P suppression by glucocorticoids (hydrocortisone 25-30 mg and dexamethasone 0.5 mg per day in P1 and P2 respectively). P levels subsequently decreased reaching a nadir of < 1 nmol/l. Clinical pregnancy was achieved in both patients after the second transfer of two and one embryo in P1 and P2, respectively.

P1 had a normal twin pregnancy with an uneventful course. She delivered two healthy girls at 36 weeks of gestation. P2 had a single pregnancy complicated by hypertension and preeclampsia, requiring emergency caesarian section at 39 weeks of gestation. A healthy son was born. All three newborns had normal genitalia and lacked skeletal malformations suggestive of ABS. Given the insufficient response of cortisol to ACTH stimulation in both patients (Table II), they were considered to be at risk of adrenal insufficiency. Therefore, stress doses of hydrocortisone (100 mg intravenously) were administered during delivery.

DISCUSSION

PORD can manifest with a broad spectrum of clinical phenotypes as the activity of multiple enzymes is affected in various combinations and degrees. The classic presentation combines abnormal genitalia, defects in adrenal and gonadal steroidogenesis and/or skeletal malformations leading to a diagnosis at birth or early infancy (Fukami and Ogata 2014).

Isolated cases presenting with delayed puberty or primary amenorrhea have been reported in a few 46,XX adolescents or young adults (Fluck et al. 2004; Sahakitrungruang et al. 2009; Idkowiak et al. 2011) yet their clinical characterization was incomplete. Thus, partial forms of PORD in post-pubertal females remain poorly characterized.

Our series of five adult females expands the clinical spectrum of non-classic PORD, highlighting milder menstrual disorders (oligomenorrhea), female infertility and ovarian macrocysts as the common features in this entity. Notably, four out of five women were diagnosed after the age of 30 years and lacked both genital and skeletal defects. After undergoing spontaneous puberty, all patients developed oligo- or amenorrhea and were initially misdiagnosed as NC-CAH or PCOS due to some overlapping features.

In particular, all affected women exhibited a concomitant increase of serum P and 17-OHP that misleadingly suggested NC-CAH/21-OHD. Interestingly, P levels were higher than 17-OHP in 3 cases whereas the opposite is usually seen in NC-CAH/21-OHD (Kamenicky et al. 2019). More importantly, our patients with non-classic PORD displayed low circulating androgen levels, in contrast with women having PCOS or NC-CAH (Bidet et al. 2009). This may be related to a partial inhibition of 17-20 lyase enzymatic activity induced by mutated POR proteins (Huang et al. 2005). Our patients also exhibited low-normal circulating E2 levels with insufficient increase of this ovarian steroid during ovarian hyperstimulation. The decrease in ovarian E2 production might be attributed to two mechanisms that are not mutually exclusive. First, the ovarian decrease of androgens biosynthesis by internal theca cells (defective 17-20 lyase activity) (Sahakitrungruang et al. 2009) could decrease the amount of substrate available for aromatase in adjacent granulosa cells. Secondly, some POR mutations have been shown to inhibit aromatase activity *in vitro* (Pandey et al. 2007). The relative decrease of E2 in turn could explain the observed increase in FSH, which may falsely raise the suspicion of primary ovarian insufficiency especially if AMH levels are low. The normal AFC in the two of the patients argued against this hypothesis. Low-normal for age AMH levels were observed in some patients (P1, P2 and P3). Rather than primordial follicle depletion, this could result from

346 androgen deficiency since androgens are suspected to be indirectly involved in AMH
347 production by growing follicles (Dewailly et al. 2016).

348 Our work highlights ovarian macrocysts as a common feature of non-classic PORD that has
349 been occasionally reported in classic PORD (Fukami et al. 2005; Bai, Li, and Wang 2017;
350 Idkowiak et al. 2011). The presence of these large cysts distinguishes PORD from PCOS that
351 is characterized by an excessive number of 2-9 mm follicles (Dewailly et al. 2014). Ovarian
352 macrocysts can be a source of significant morbidity ranging from mild abdominal discomfort to
353 acute abdominal pain due to a spontaneous cyst rupture requiring emergency ovariectomy
354 (Idkowiak et al. 2011; Fukami et al. 2009; Scott and Miller 2008). Excessive LH-mediated
355 ovarian stimulation combined with impaired ovarian steroidogenesis could be an underlying
356 mechanism (Salenave et al. 2015). Defective activity of CYP51A1, an enzyme that catalyzes
357 the conversion of lanosterol to meiosis-activating sterols, which are in turn important for oocyte
358 maturation, could be implicated as well (Grondahl et al. 2000). We used GnRH agonists to
359 reduce macrocysts size with impressive efficacy in two of our patients. This observation adds
360 to previous experience with oestro-progestative pills and glucorticoid suppression therapy
361 (Idkowiak et al. 2011; Fukami et al. 2009).

362 In line with the autosomal recessive PORD mode of inheritance, bi-allelic mutations in the *POR*
363 gene were detected in all patients (Xia et al. 2011). The flavin mononucleotide domain of POR
364 that interacts directly with P450 enzymes for the electron transfer was spared, while the
365 majority of mutations clustered in the NADPH-binding domain that serves as the electron donor
366 (Figure 3). Mutations mapping on this domain were more likely to produce a partial phenotype
367 in a previous study (Sahakitrungruang et al. 2009). Nevertheless, three large PORD cohorts
368 found poor genotype-phenotype correlation (Huang et al. 2005; Fukami et al. 2009; Krone et
369 al. 2012). To date, homozygosity for p.Ala287Pro has invariably led to ambiguous genitals in
370 affected 46,XX individuals (Krone et al. 2012; Burkhard et al. 2017), which contrasts with the
371 absence of genital virilization in P5. Notably, the two affected sisters (P2, P3) were discordant
372 regarding their genital phenotype despite harboring identical *POR* mutations. Taken together,

these results suggest that other genetic, epigenetic, or environmental factors could influence the phenotypic expression of *POR* mutants.

The cause of infertility in women with PORD is likely multifactorial (Reichman et al. 2014; Robin et al. 2014). The main contributing factor is presumably the excessive non-cyclic P levels, which could exert a hypothalamic and/or pituitary anti-gonadotropic effect (Bry-Gauillard et al. 2008). Gonadotropin levels in our patients were in the high-normal range, but cyclical gonadotropin changes during the menstrual cycles were not evaluated although in the presence of oligomenorrhea, ovulation seldom occurs. Such a mechanism could result from the lack of physiological preovulatory estradiol rise and positive feedback on ovulatory LH. Anovulation in PORD women could also result from other mechanisms such as defective follicular maturation due to androgen deficiency (Dewailly et al. 2016) and/or the presence of macrocysts. In addition, high P exerts a direct anti-proliferative effect on the endometrium, limiting the chance of successful implantation (Holmes-Walker et al. 1995) and also alters the cervical mucus contributing to the infertility of PORD women.

Unassisted conception has not been reported in female PORD patients. In our series, ovarian stimulation was remarkable for disproportionally low E2 levels, despite maturation of several dominant follicles and normal oocyte retrieval (Bry-Gauillard et al. 2017). This constellation was described in the only previous report of fertility *via* IVF-ET in a woman with PORD (Song et al. 2018). Prior to frozen embryo transfer, both P1 and P2 received glucocorticoid therapy to suppress serum P based on clinical practice in women with CYP21A2 deficiency (Witchel 2012; Boscolo et al. 2015) and some data in women undergoing IVF for common infertility (Bosch et al. 2010). The latter study analyzed IVF cycles from more than 4,000 women and revealed that serum P levels < 4.8 nmol/l (< 1.5 ng/ml) during ovulation induction were associated with higher live pregnancy rates. This difference was no longer present when the fertilized oocytes were implanted to other recipients, thus arguing against a defect in oocyte quality and suggesting a change in endometrial receptivity. Both of our patients achieved very

399 low serum P levels (< 1 nmol/l) using either supraphysiologic hydrocortisone doses or a long-
400 acting glucocorticoid (dexamethasone).

401 One limitation of our study is that the pathogenicity of a part of the identified mutations was not
402 evaluated by *in vitro* functional studies. Nevertheless, the finding of very rare and bi-allelic
403 mutations co-segregating with the phenotype and predicted to be deleterious (Richards et al.
404 2015) in combination with the typical steroid profile strongly supported the diagnosis of PORD
405 in our patients. The skeletal assessment in our cohort was based on physical examination.
406 Plain radiographies of the whole skeleton were not performed in all cases. Further, hormonal
407 and ultrasonographic evaluation was performed in three different institutions. On the contrary,
408 genetic testing was done in the same molecular unit. All the patients were clinically assessed
409 by experts in reproductive endocrinology.

410 In conclusion, our report establishes non-classic PORD revealed during adult life as a novel
411 cause of unexplained infertility in women who lack the genital and skeletal malformations of
412 the classic pediatric form. Though a rare entity, non-classic PORD may remain undiagnosed
413 in some women and prove to be more common than the classic form in the end. We have
414 characterized a typical serum steroid profile, the presence of ovarian macrocysts as well as a
415 specific pattern of response to ovarian stimulation. During IVF-ET, the discordance between
416 the normal number of derived oocytes and the low estradiol levels should lead reproductive
417 gynecologists and endocrinologists to consider the possibility of PORD. Our observations will
418 hopefully improve the timely diagnosis and effective treatment in this disorder.

419

AUTHORS' ROLES

GEP and AD: collection and analysis of clinical and biochemical data, execution of the study design, manuscript drafting, critical discussion

JB: collection and analysis of genetic data, manuscript drafting, critical discussion

FC: collection and analysis of genetic data, critical discussion

AR: collection and analysis of clinical data, critical discussion

SCJ: collection and analysis of clinical data, critical discussion

OBB: collection and analysis of genetic data, critical discussion

NP: study design and critical discussion

JY: study design, manuscript drafting, critical discussion, project coordination

DD: study design, manuscript drafting, critical discussion, project coordination

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CONFLICT OF INTEREST

The authors have no potential conflicts of interest to declare.

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FIGURE LEGENDS

Figure 1:

Pelvic magnetic resonance imaging (MRI) of Patient 4 illustrating the presence of ovarian macrocysts before (left column) and 2 months after treatment with a GnRH analogue (triptorelin, monthly intramuscular injection of 3.75 mg) (right column). Axial (panels A&D), coronal (panels B&E) and sagittal (panels C&F) cuts are shown. Large bilateral cysts exhibited characteristic enhanced signal at T2 MRI-sequences as indicated by red arrows at the pretreatment captures. Complete disappearance of cysts on the right ovary with only small remnants on the left ovary was achieved following administration of triptorelin.

Figure 2:

Hormonal status of four probands (P1, P2, P4, P5) diagnosed with non-classic PORD, before (T0) and after (T60) standard cosyntropin (ACTH) stimulation (0.25 mg intravenously). All measurements were performed using LC-MS. Grey rectangles represent normal range (5^{th} to 95^{th} percentiles) suggested in a previous study that evaluated with LC-MS 44 controls aged 8-58 years (Kulle et al. 2015). Dotted blue lines correspond to the normal median value for such hormone. To more clearly visualize the contrast between our patients and the controls, the normal range for progesterone (panel C) is shown maximized (rectangle with red borders). The four probands reduced or low-normal elevations of cortisol are consistent with partial adrenal insufficiency (panel A). Characteristic exaggerated increase of 17-hydroxyprogesterone (17-OHP) (panel B) and progesterone (panel C) was observed in all patients, coupled with a flat response of androstenedione (panel D).

Figure 3:

A: The pedigrees of the five affected patients are shown. Notably, Family #2 included two affected sisters, P2 and P3. Family DNA was available for all but Patient 1 (P1). Different

phenotypic reproductive traits are explained in the square at the lower left of the Figure. Mutants are shown with one-letter abbreviations for amino acids (P, proline; S, serine; Q, glutamine; W, Tryptophan; R, arginine; L, leucine). The genotype is consistent with the autosomal recessive transmission mode of PORD. Interestingly, the two affected sisters of Family #2 were discordant regarding their genital phenotype. B: Localization of POR mutations at the DNA and protein level. Schematic representation of POR gene and protein was based on previous literature (Krone et al. 2012) and UniProtKB data (entry P16435). Coding exons (CDS) are numbered (1-15). The first non-coding exon of transcript NM_00941.3 (16 exons) is not represented in this figure The three functional domains of the POR protein are shown: nicotinamide adenine dinucleotide phosphate (NADPH; light grey), flavin adenine dinucleotide (FAD; medium gray), and flavin mononucleotide (FMN; dark gray). All mutations in probands with late-onset P450 oxidoreductase deficiency (PORD) mapped to the NADPH or FAD domain. Previously reported and novel mutations are shown in blue and red respectively.

Figure 4:

A protocol with a GnRH agonist (triptorelin) and GnRH antagonist (ganirelix) was used in P1 and P2 respectively. P1 received recombinant FSH (rFSH) whereas P2 was put on human menopausal gonadotropin (hMG) (300-375 IU daily). Ovulation was triggered by recombinant human chorionic gonadotropin (rhCG) at Day 14 and 12 in P1 and P2 respectively and oocytes were retrieved in both 2 days later. Clinical outcomes are displayed in the upper panels: endometrial thickness (continuous simple line) and the number of developing follicles of different sizes (interrupted lines). Sex hormone levels evolution is shown in the lower panels. Despite a significant number of mature oocytes, serum estradiol remained particularly low. Interestingly, serum progesterone was already elevated at the beginning of each stimulation cycle, consistent with PORD.