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

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1 Title: Non-classic cytochrome P450 oxidoreductase deficiency strongly
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4
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12 Running title: Non-classic POR deficiency as cause of infertility

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35 **ABSTRACT**

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

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

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

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

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

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

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

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

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

75 Study funding/competing interest(s): None.

76

77 **KEYWORDS**

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

80 **INTRODUCTION**

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

91 PORD presents with a broad and heterogeneous clinical spectrum depending on the severity
92 of the causative bi-allelic *POR* mutations and the subsequent relative impairment of *POR*-
93 dependent enzymes (Krone et al. 2012). Diagnosis is typically made at birth by a
94 pathognomonic combination of disordered sex development and skeletal defects resembling
95 Antley-Bixler syndrome [ABS] (Huang et al. 2005; Krone et al. 2012). The latter is a rare
96 craniosynostosis syndrome, which may be caused by disrupted sterol synthesis by lanosterol
97 14 α -demethylase (CYP51A1) and squalene epoxidase, two *POR*-dependent enzymes (Kelley
98 et al. 2002; Schmidt et al. 2009). Less frequent syndromic forms with anorectal or urinary
99 malformations have been attributed to altered retinoic acid metabolism because of CYP26A1-
100 C1 deficiency (Fukami et al. 2010). Cases with minimal or absent bone involvement have also
101 been reported (Fluck et al. 2004; Williamson et al. 2006).

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

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

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

120

121 **PATIENTS AND METHODS**

122 *Setting*

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

129 *Clinical summaries before referral*

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

133 patient took an oral contraceptive pill [OCP], which was discontinued at age 27 in order to get
134 pregnant. Because of recurrence of irregular cycles, she had a first ovarian assessment at day
135 2 after spontaneous menses, which showed a normal Anti-Müllerian hormone [AMH] level
136 (15.3 pmol/L) despite a slightly elevated serum Follicle-stimulating hormone [FSH] (11 IU/L,
137 normal range: 4.3-9.0). The ovarian ultrasound revealed multiple macrocysts, the largest of
138 which measured 28 mm at maximum diameter. Basal serum 17-OHP was increased at 16.3
139 nmol/l (5.4 ng/ml) (normal range for follicular phase: < 3 nmol/l), raising at that time the
140 suspicion of NC-CAH.

141 **Patient 2 [P2]** was referred at age 36 for infertility. Family history was remarkable for
142 suspected classic 21-OHD in her younger sister, who presented with mild genital virilization
143 (labial fusion) at birth but had not been genetically explored. Despite normal onset of puberty
144 (thelarche and pubic hair at age 12), she had no menses at age 16 (primary amenorrhea), and
145 voluminous ovarian cysts (> 5 cm) were observed on ultrasound. One cyst was resected and
146 was considered to contain part of a corpus luteum at histopathology. Hormonal status at
147 presentation showed low-normal circulating estradiol (E2) at 140 pmol/l (normal range 130-
148 310 and 340-810 pmol/l for early follicular and luteal phase respectively), normal serum FSH
149 (6.8 IU/L) and prolactin levels. High serum P level at 9.5 nmol/l (3 ng/ml) was considered as
150 suggestive of luteal phase. The patient was started on OCP, allowing pill-induced regular
151 withdrawal bleeding. Oligomenorrhea and recurrence of ovarian macrocysts were observed
152 following OCP interruption at age 20. At age 34, a novel endocrine evaluation revealed an
153 increase in 17-OHP level at 8.3 nmol/l (normal range for follicular phase: < 4.5 nmol/l), further
154 rising after ACTH-stimulation at 21.4 nmol/l but no definitive diagnosis was drawn at that time.
155 The patient discontinued the OCP at age 35 in order to get pregnant. Due to high levels of
156 FSH in conjunction with low E2 and decreased serum AMH levels, premature ovarian
157 insufficiency was suspected.

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

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

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

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

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

202 *Clinical and hormonal evaluation*

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

214 mg) (Dorin, Qualls, and Crapo 2003). Androgens and adrenal precursors were measured by
215 liquid chromatography-mass spectrometry [LC-MS] as previously described (Kamenicky et al.
216 2019), while commercial immunoassays were used for the remaining hormonal
217 measurements. Pelvic ultrasound and/or MRI was used to assess endometrial thickness and
218 to measure antral follicle count [AFC] when possible.

219 *Genetic studies and ethical approval*

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

231 *Fertility induction*

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

240

241 **RESULTS**242 *Clinical phenotype*

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

255 *Hormonal evaluation and ultrasonographic findings*

256 All five patients with PORD exhibited a similar hormonal pattern (Table II). Serum E2 and TT
257 levels were low normal while serum FSH and LH levels were in the high normal to moderately
258 elevated range. A marked increase in serum P levels was observed independently of the
259 presumed phase of menstrual cycle. Basal 17-OHP levels were also increased. ACTH
260 stimulation test performed in the four probands revealed that serum cortisol increased to levels
261 below (P2, P5) or in the low-normal range (P1, P4). An excessive stimulation of P, 17-OHP,
262 21-deoxycortisol (4.8 to 31.5-fold, 3.6 to 11.5-fold and 5 to 70-fold, respectively – Supplemental
263 Table S2) was also induced by ACTH stimulation. Conversely, serum androstenedione levels
264 were low with virtually no response to ACTH (Figure 2). Pelvic imaging revealed several
265 ovarian macrocysts in three of the five women (Table I). In the remaining two patients (P2, P3)

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

268 *Genetic studies*

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

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

293 *Assisted reproductive treatment and fertility outcomes*

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

306 P1 had a normal twin pregnancy with an uneventful course. She delivered two healthy girls at
307 36 weeks of gestation. P2 had a single pregnancy complicated by hypertension and
308 preeclampsia, requiring emergency caesarian section at 39 weeks of gestation. A healthy son
309 was born. All three newborns had normal genitalia and lacked skeletal malformations
310 suggestive of ABS. Given the insufficient response of cortisol to ACTH stimulation in both
311 patients (Table II), they were considered to be at risk of adrenal insufficiency. Therefore, stress
312 doses of hydrocortisone (100 mg intravenously) were administered during delivery.

313

314 **DISCUSSION**

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

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

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

329 In particular, all affected women exhibited a concomitant increase of serum P and 17-OHP that
330 misleadingly suggested NC-CAH/21-OHD. Interestingly, P levels were higher than 17-OHP in
331 3 cases whereas the opposite is usually seen in NC-CAH/21-OHD (Kamenicky et al. 2019).
332 More importantly, our patients with non-classic PORD displayed low circulating androgen
333 levels, in contrast with women having PCOS or NC-CAH (Bidet et al. 2009). This may be
334 related to a partial inhibition of 17-20 lyase enzymatic activity induced by mutated POR proteins
335 (Huang et al. 2005). Our patients also exhibited low-normal circulating E2 levels with
336 insufficient increase of this ovarian steroid during ovarian hyperstimulation. The decrease in
337 ovarian E2 production might be attributed to two mechanisms that are not mutually exclusive.
338 First, the ovarian decrease of androgens biosynthesis by internal theca cells (defective 17-20
339 lyase activity) (Sahakitrungruang et al. 2009) could decrease the amount of substrate available
340 for aromatase in adjacent granulosa cells. Secondly, some POR mutations have been shown
341 to inhibit aromatase activity *in vitro* (Pandey et al. 2007). The relative decrease of E2 in turn
342 could explain the observed increase in FSH, which may falsely raise the suspicion of primary
343 ovarian insufficiency especially if AMH levels are low. The normal AFC in the two of the patients
344 argued against this hypothesis. Low-normal for age AMH levels were observed in some
345 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 glucocorticoid 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,

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

375 The cause of infertility in women with POR is likely multifactorial (Reichman et al. 2014; Robin
376 et al. 2014). The main contributing factor is presumably the excessive non-cyclic P levels,
377 which could exert a hypothalamic and/or pituitary anti-gonadotropic effect (Bry-Gauillard et al.
378 2008). Gonadotropin levels in our patients were in the high-normal range, but cyclical
379 gonadotropin changes during the menstrual cycles were not evaluated although in the
380 presence of oligomenorrhea, ovulation seldom occurs. Such a mechanism could result from
381 the lack of physiological preovulatory estradiol rise and positive feedback on ovulatory LH.
382 Anovulation in POR women could also result from other mechanisms such as defective
383 follicular maturation due to androgen deficiency (Dewailly et al. 2016) and/or the presence of
384 macrocysts. In addition, high P exerts a direct anti-proliferative effect on the endometrium,
385 limiting the chance of successful implantation (Holmes-Walker et al. 1995) and also alters the
386 cervical mucus contributing to the infertility of POR women.

387 Unassisted conception has not been reported in female POR patients. In our series, ovarian
388 stimulation was remarkable for disproportionately low E2 levels, despite maturation of several
389 dominant follicles and normal oocyte retrieval (Bry-Gauillard et al. 2017). This constellation
390 was described in the only previous report of fertility *via* IVF-ET in a woman with POR (Song
391 et al. 2018). Prior to frozen embryo transfer, both P1 and P2 received glucocorticoid therapy
392 to suppress serum P based on clinical practice in women with CYP21A2 deficiency (Witchel
393 2012; Boscolo et al. 2015) and some data in women undergoing IVF for common infertility
394 (Bosch et al. 2010). The latter study analyzed IVF cycles from more than 4,000 women and
395 revealed that serum P levels < 4.8 nmol/l (< 1.5 ng/ml) during ovulation induction were
396 associated with higher live pregnancy rates. This difference was no longer present when the
397 fertilized oocytes were implanted to other recipients, thus arguing against a defect in oocyte
398 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

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

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

424 FC: collection and analysis of genetic data, critical discussion

425 AR: collection and analysis of clinical data, critical discussion

426 SCJ: collection and analysis of clinical data, critical discussion

427 OBB: collection and analysis of genetic data, critical discussion

428 NP: study design and critical discussion

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

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

431

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440

CONFLICT OF INTEREST

442 The authors have no potential conflicts of interest to declare.

443

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- 590

591 **FIGURE LEGENDS**

592

593 Figure 1:

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

601

602 Figure 2:

603 Hormonal status of four probands (P1, P2, P4, P5) diagnosed with non-classic PORD, before
604 (T0) and after (T60) standard cosyntropin (ACTH) stimulation (0.25 mg intravenously). All
605 measurements were performed using LC-MS. Grey rectangles represent normal range (5th to
606 95th percentiles) suggested in a previous study that evaluated with LC-MS 44 controls aged 8-
607 58 years (Kulle et al. 2015). Dotted blue lines correspond to the normal median value for such
608 hormone. To more clearly visualize the contrast between our patients and the controls, the
609 normal range for progesterone (panel C) is shown maximized (rectangle with red borders). The
610 four probands reduced or low-normal elevations of cortisol are consistent with partial adrenal
611 insufficiency (panel A). Characteristic exaggerated increase of 17-hydroxyprogesterone (17-
612 OHP) (panel B) and progesterone (panel C) was observed in all patients, coupled with a flat
613 response of androstenedione (panel D).

614

615 Figure 3:

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

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

631

632 Figure 4:

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