Protocol

Polycystic ovary syndrome mouse model by prenatal exposure to high anti-Müllerian hormone

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Highlights

Protocol to induce
PCOS traits in
offspring using
prenatal exposure to
high AMH

Procedure describing
key steps for reliable
assessment of PCOS-
like reproductive
traits

Applicable to other
established animal
models of PCOS

Here, we describe a protocol that provides the steps required for the generation of a mouse model of polycystic ovary syndrome (PCOS) by exposing dams to elevated levels of anti-Müllerian hormone during late gestation. This protocol also describes the steps required to assess the PCOS-like equivalents of the Rotterdam PCOS diagnostic criteria in mice.

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https://doi.org/10.1016/j.xpro.2021.100684

SUMMARY

Here, we describe a protocol that provides the steps required for the generation of a mouse model of polycystic ovary syndrome (PCOS) by exposing dams to elevated levels of anti-Müllerian hormone during late gestation. This protocol also describes the steps required to assess the PCOS-like equivalents of the Rotterdam PCOS diagnostic criteria in mice.

For complete details on the use and execution of this protocol, please refer to Tata et al. (2018) and Mimouni et al. (2021).

BEFORE YOU BEGIN

> *Note:* All animal work presented here was performed in accordance with and approved by the Institutional Ethics Committees of Care and Use of Experimental Animals of the University of Lille (France; Ethical protocol number: APAFIS#2617-2015110517317420 v5 and APAFIS#13387 2017122712209790 v9). All experiments were performed in accordance with the guidelines for animal use specified by the European Council Directive of 22 September 2010 (2010/63/EU).

Recently published International Guidelines for the Assessment and Management of PCOS (Teede et al., 2018) recommend a clinical diagnosis requiring at least 2 out of the following 3 Rotterdam criteria: (i) the evidence of either biological or clinical hyperandrogenism; (ii) irregular ovulatory function (oligomenorrhea or amenorrhea); (iii) and polycystic ovarian morphology on ultrasound. Relevant animal models of PCOS must have comparability to women with PCOS by exhibiting 2 or more PCOS-like equivalents of the Rotterdam criteria and no other PCOS-excluding endocrinopathies.

To date, considerable efforts have been made to generate several animal models as translatable alternatives to dissect the causes of PCOS and enable optimization of potential therapeutics. You can refer to (Stener-Victorin et al., 2020) to review all PCOS-like animal models generated to date, together with a full description of the models that best or poorly reflect clinical PCOS.

The prenatal AMH treated (PAMH) animals are excellent preclinical models to mimic the human PCOS condition. At the best of our knowledge, the PAMH is the only rodent model that recapitulates in full the mouse equivalents of the PCOS Rotterdam criteria which are associated with metabolic disturbances, typical of the human PCOS condition (hyperglycemia, glucose-intolerance, hyperinsulinemia and type-2 diabetes), without additional environmental challenges (i.e.,: high fat diet) (Tata et al., 2018; Mimouni et al., 2021). The other existing PCOS rodent models, prenatally or postnatally

androgenized by testosterone (T) or dihydrotestosterone (DHT) treatment, or postnatally treated with Letrozole or with dehydroepiandrosterone (DHEA), show either only reproductive PCOS-like traits or metabolic disturbances associated with mild neuroendocrine and reproductive defects (Stener-Victorin et al., 2020).

Moreover, since PCOS-like reproductive and metabolic traits are transmitted for at least three generations (Mimouni et al., 2021), the PAMH model is also amenable to study the mechanisms underlying epigenetic inheritance of PCOS and susceptibility to the disease.

Mechanistically, exposing the dams to high AMH at the end of the gestational period, drives a 3-fold rise in aromatizable T levels in the dams, via inhibition of placental aromatase and increase in GnRH/LH release in the gestating animals (Tata et al., 2018). Organizational effects driven by gonadal steroid hormones (e.g., estradiol and testosterone) promote the molecular reprograming of reproductive circuits that influence the activation of the hypothalamic-pituitary-gonadal axis of the offspring (Tata et al., 2018), leading in adult PAMH females to enhanced excitatory inputs onto GnRH neurons and persistent GnRH neuronal activity, which finally accounts for increased LH-driven steroidogenesis (Tata et al., 2018).

Here, we provide a protocol to generate PAMH mice, together with the detailed experimental paradigms to assess whether the neuroendocrine and reproductive traits qualify their inclusion as PCOS-like. We must emphasize that PAMH female mice develop by the age of 6 months the major metabolic derangements typical of the human PCOS condition (Mimouni et al., 2021).

For space limitations, we do not provide the protocols for measurement of energy metabolism in mouse models. For details on designing and executing studies of mouse energy metabolism, please refer to (Tschöp et al., 2011).

Generation of the prenatally AMH exposed mice

⏱ Timing: 30 days

This protocol was developed specifically to induce the emergence of PCOS-like traits in female offspring by exposing pregnant mice to high levels of AMH during late gestation. The next steps describe the generation of timed pregnant mice and the administration of treatments to generate PAMH and control (CNTR) animals.

1. Purchase C57BL/6J (B6) female (2–3 months-old) and male mice (2–3 months-old) (Charles River, USA).
2. Allow the animals to acclimate for 1 week upon arrival to the animal facility. To our experience, the chosen period does not affect the physiological neuroendocrine response of mice.

   Note: Even though one week acclimation period upon arrival was used in our studies, a 48–72 hours is the minimal period recommended for the acclimation in rodents. This period allows the animals to adapt to their new environment i.e. change of bedding, food and temperature. Transport/shipping induces physiological changes in the cardiovascular, endocrine, immune and reproductive systems in rodents, but these alterations are restored after the acclimation period (Obernier and Baldwin, 2006).

3. Group female mice in Tecniplast cages GM500 (Cage dimension: 391 × 199 × 160 mm) and isolate males in individual cages, under specific pathogen-free conditions in a temperature-controlled room (21°C–22°C) with a 12-h light/dark cycle and ad libitum access to food and water.
4. Use standard diet RM3 (9.5 mm Pelleted RM3, Special Diets Services, France) with the following nutritional profile: Protein 22.45%, Fat 4.2%, Fiber 4.42%, Ash 8%, Moisture 10%, Nitrogen free extract 50.4%; Calories: 3.6 kcal/gr.
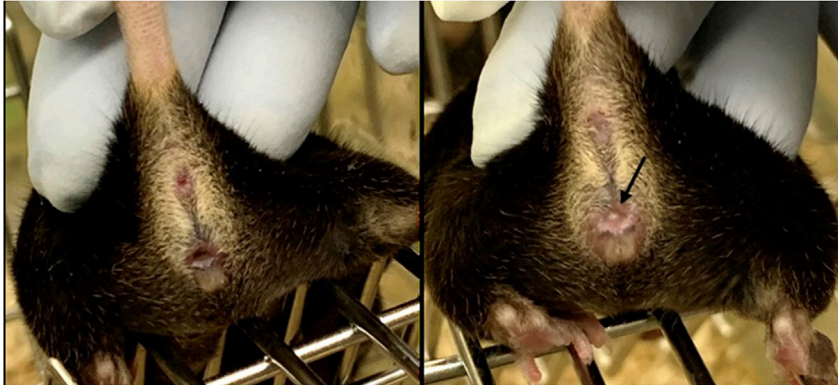
Figure 1. Comparison between females with or without a vaginal plug
Arrow indicates a vaginal plug.

5. Ensure daily follow-up of health issues by qualified personnel.
6. Randomly assign 1–2 females into each individually housed male's cage one hour before lights off. Breeding more than 2 females per male (housed in trio) is not recommended since female mice can be sometimes more territorial than males and putting a male into a crowded female's cage can cause them to become aggressive and/or refuse to mate (Hoffmann, 2018).
7. Check for vaginal plugs (also called copulation plugs) in the following morning (between 7.30 a.m. and 9.30 a.m.; Figure 1) and separate males from females.

   Note: since the presence of a plug is not a definitive indicator of true pregnancy, it is always recommended to confirm pregnancy by palpation or visual inspection two weeks after the putative mating (embryonic day 14).

The term "embryo" is used to define all prenatal stages of murine development between fertilization and birth, with the stage of development indicated by the gestational age (with conception, occurring approximately 0.5 days after mating and defined as E0.5) (Kaufman, 1999).

8. Once you identify the potentially pregnant females, transfer them into individual cages.

   ⚠ CRITICAL: Appropriate regulations and guidelines for mouse experiments must be followed.

Prenatal anti-Müllerian hormone (PAMH) treatment

   ⏱ Timing: 3 days: from E16.5 to E18.5

9. Freshly prepare the injectable solutions as described in Materials and Equipment and keep them on ice (4°C) until use.
10. Inject intraperitoneally (i.p.) the dams for 3 consecutive days between 8.00–9.00 a.m, with 200 μL of phosphate buffered saline (PBS, pH 7.4) to generate the prenatal control-treated female offspring: CNTR group; or with 200 μL of the recombinant human AMH (0.12 mg/kg/day) to generate the prenatal AMH-treated female offspring, PAMH group (Figure 2).

    Note: Recombinant human AMH is highly conserved across species and the AMHc (the bioactive form), used to generate the PAMH model, is almost identical between mice and humans (94% homology).

Prenatal injections with the AMH precursor recombinant protein (proAMH, 140 kDa) or with the bioactive AMH form (AMHc, 23.4 kDa) give comparable results (Tata et al., 2018).
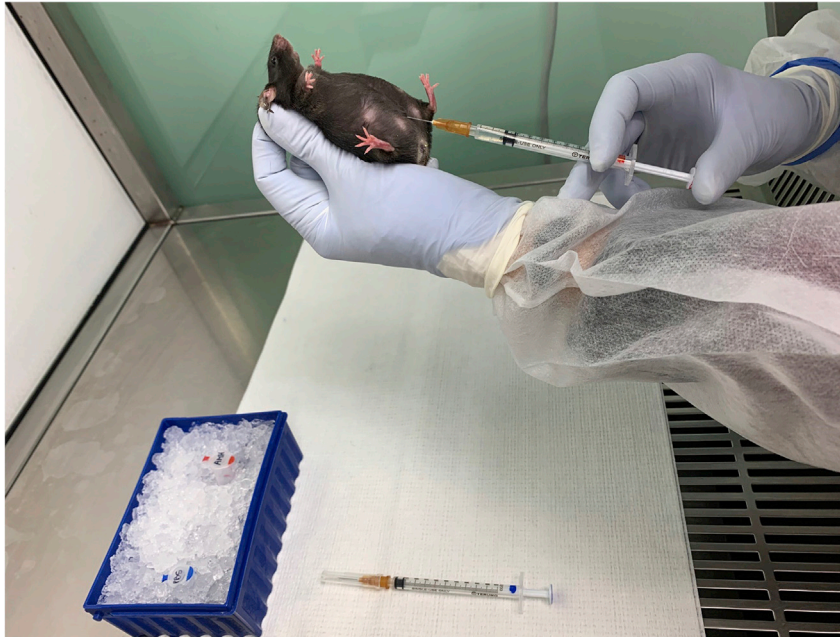
Figure 2. Intraperitoneal administration in pregnant dams

The (E16.5-E18.5) temporal window for the treatment was chosen because it lies beyond the developmental stages during which gonadal and genital tract differentiation takes place in mice (E12.5-E14.5), therefore excluding any morphogenetic effects that exogenous AMH could have. It is the same temporal window used to generate prenatal androgen-treated mice, PNA (Sullivan and Moenter, 2004, Moore et al., 2013, 2015).

The survival rate to the pregnant mice receiving AMH is 100%, however, it is important to take into account when planning the generation of PAMH animals, that AMH-treated animals have significantly smaller litters (approximately 50% less pups/litter) as compared with control animals (Tata et al., 2018), probably as the result of the AMH-dependent placental insufficiency and subsequent increased abortions' rate detected in these animals (Tata et al., 2018).

11. Monitor births and wean female offspring at postnatal day 21 (P21).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal antibody, anti-bovine LH beta subunit	University of California	Cat#518B7
Polyclonal antibody, rabbit LH antiserum.	Provided by Dr. A.F. Parlow and NIDDK-NHPP	Cat#AFP240580Rb
Polyclonal goat anti-rabbit IgG/HRP	Dako	Cat#P0448
Chemicals, peptides, and recombinant proteins		
Recombinant human anti-Müllerian hormone (AMH)	R&D Systems	Cat#1737-MS-10
Hematoxylin	Sigma-Aldrich	Cat#GHS132
Eosin Y	Sigma-Aldrich	Cat#HT1103128

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phosphate-Buffered Saline (PBS)	Gibco	Cat#14190144
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#9888
Paraformaledehyde (PFA)	Sigma-Aldrich	Cat#158127
di-Sodium hydrogen phosphate (Na_2HPO_4).	Sigma-Aldrich	Cat#1065660100
Sodium phosphate monobasic (NaH_2PO_4) solution	Sigma-Aldrich	Cat#567545
Sodium bicarbonate ($NaHCo_3$)	Sigma-Aldrich	Cat#222321
Sodium carbonate (Na_2Co^3)	Sigma-Aldrich	Cat#S6014
Bovine Serum Albumin (BSA)	VWR	Cat#1005-70
Tween 20	Thermo Fischer Scientific	Cat#003005
1-Step Ultra TMB-ELISA	Thermo Fisher Scientific	Cat#34028
3M hydrochloric acid (HCL)	Thermo Fisher Scientific	Cat#10053023
Critical commercial assays		
Mouse Testosterone ELISA Kit	Demeditec Diagnostics	Cat#DEV9911
Experimental models: Organisms/strains		
C57BL/6J Mice	Charles River	N/A
Software and algorithms		
Prism 8 Version 8.3.1	GraphPad, LLC	https://www.graphpad.com/scientific-software/prism/
Other		
Standard diet, 9.5 mm Pelleted RM3	Special Diets Services (SDS)	Cat#801030
Skim milk powder	N/A	N/A
1.5 mL Anti-coagulant microcentrifuge tubes	VWR	Cat#20901-757
26 Gauge X 0.5 AGANI Needle (0.45 X 13mm)	Terumo	Cat#AN1326R1
1mL Syringe	Terumo	Cat#CE019
Corning® 96-Well Clear Flat Bottom Polystyrene High Bind Microplate	Thermo Fisher Scientific	Cat#9018
Mouse LH used to generate the standard curve	National Institute of Diabetes and Digestive and Kidney Diseases — National Hormone and Pituitary Program (NIDDK-NHPP)	Cat#AFP-5306A
Lightfield Microscope	Leica Microsystems	Leica ICC50
Black-coated, 8 numbered circles, glass slide	Polysciences	Cat#18357-1
Black-coated, 8 numbered circles, glass slide	Polysciences	Cat#18357-1
Microtome	Leica Microsystems	Cat#Leica RM2255
Centrifuge	Eppendorf	Cat#5424R
Microplate Orbital Shaker	VWR®	Cat#12620-928
Microplate Absorbance Reader	Molecular Devices	Spectramax

MATERIALS AND EQUIPMENT

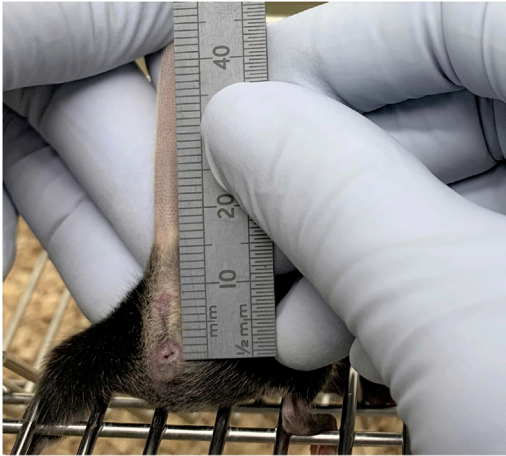| Preparation of the injectable solutions to generate CNTR and PAMH mice		
Reagent	Final concentration	Volume (µL)/mouse
Recombinant human anti-Müllerian hormone (AMH) – (1737-MS-10)	0.12 mg/kg	600 µL for 3 days (200 µL/day)
Phosphate-Buffered Saline (PBS)	0.1 M	600 µL for 3 days (200 µL/day)
Store lyophilized AMH at −20°C for three months		

Figure 3. Anogenital distance measurement in female mice

STEP-BY-STEP METHOD DETAILS
Assessment of hyperandrogenism

⏱ Timing: 30 days

Assessment of hyperandrogenism via measurement of anogenital distance

1. Position the females on a horizontal surface and gently lift their tail to have a clear access to the genitalia.
2. Measure the distance from the top of anus to the bottom of the vagina using an adapted ruler (millimeters), from post-natal day P30 and throughout post-natal development (P35, 40, 50 and 60) (Figure 3).
3. Record each measurement for subsequent statistical analysis.

   Note: Anogenital distance measurement is a reliable metric tool to appreciate prenatal testosterone impregnation in humans (Barrett et al., 2018; Dean and Sharpe, 2013) and in rodents (Mitchell et al., 2015).

Assessment of hyperandrogenism via testosterone ELISA assay

⏱ Timing: 4–5 h

   Note: The testosterone mouse Kit (Cat#DEV9911) is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding (www.demeditec.com).

Material needed for the next steps: ice, anti-coagulant collection tubes (Cat# 20901-757), 1.5 mL collection tubes (Cat#2150N), a refrigerated centrifuge (Cat#5175R), a microplate shaker (Cat#12620-928), a microplate absorbance reader (Spectramax).

   Optional: Microplate automated washer (Cat#8.1000).

4. Collect mouse trunk blood between 10.00 a.m and 12.00 a.m from females in diestrous stage (when GnRH secretion into the portal circulation is low and therefore mean luteinizing hormone (LH) concentration is at basal levels) of each experimental group in 1.5 mL anti-coagulant collection tubes.

   ⚠ CRITICAL: Habituate animals to handling at least one week (preferably two weeks) before the experiments.

5. Set the temperature of the centrifuge at 4°C before use.
6. Centrifuge the blood samples for at least 15 min at 500–1000 gravitation force (g), pipette the plasma (upper phase) into a 1.5 mL collection tube and discard the remaining sample.
7. Immediately store at −80°C until use.
8. Prepare your plate (96 microplate).
9. Start by diluting the *wash solution* (provided in the kit) by mixing 50 mL of 10× concentrated with 450 mL deionized water (ddH20).
10. Gradually thaw on ice the plasma samples previously stored at −80°C.
11. Add 10 μL of each Calibrator (0 ng/mL, 0.1 ng/mL, 0.4 ng/mL, 1.5 ng/mL, 6.0 ng/mL, 25 ng/mL) and sample in duplicates into appropriate wells.

> ⚠ CRITICAL: Steroid hormones are sensitive to temperature variations which could eventually lead to their degradation. Therefore, to ensure an accurate hormonal measurement always keep the samples on ice and pipette samples and reagents as quickly as possible.

12. Add 100 μL of Incubation Buffer into each well (provided in the kit).
13. Add 50 μL of Enzyme Conjugate into each well (provided in the kit).
14. Incubate for 60 min at 18°C–25°C on a microplate orbital shaker (Cat#12620-928), at the speed of 320 rpm.
15. Discard the content of the wells and rinse the wells 4 times with 300 μL of Wash Solution (provided in the kit). Eliminate as much wash solution as possible by tapping your plate on absorbent lab paper to remove the residue.
16. Add 200 μL of substrate solution to each well (provided in the kit).
17. Incubate for 30 min (use aluminum foil to protect the wells from light).
18. Add 50 μL of stop solution to each well.
19. Read the plate within 15 min by measuring the absorbance of each well at 450 nm using a microplate absorbance reader (Spectramax).

> ⚠ CRITICAL: Each run must include its own standard curve as well as internal quality control to validate the reproducibility.

20. Calculate the average absorbance values for calibrators, quality controls and samples.
21. Using a semi logarithmic graph, build a standard curve by plotting the mean absorbance obtained from each standard against its concentration as following: plot absorbances values on the vertical (Y) axis and concentrations on the horizontal (X) axis.
22. Determine the corresponding concentration from the calibration curve.
23. To ensure reproducibility, always calculate the inter (between replicates) and intra (between runs) assays variations.

> *Note:* In this study, Intra-assay coefficient of variation for testosterone was 7% and inter-assay coefficient of variation was 11%.

> *Alternatives:* Calculated automatically using a 4 Parameter Logistics (4PL) curve fit.

Luteinizing hormone ELISA assay

> ⊙ Timing: 2 days

> *Note:* This protocol has been adapted from (Steyn et al., 2013).

24. Follow the same steps as detailed above (steps 4–7).

⚠ CRITICAL: Habituate animals to handling at least one week (preferably two weeks) before the experiments.

25. Prepare your plate design and appropriate number the microplate wells to accommodate standards and samples in duplicates.
26. Coat the 96 Well Clear Flat Bottom Polystyrene High Bind Microplate (Cat#9018) with 50 μL bovine LH-beta 51887 monoclonal antibody diluted at 1:1000 into each well at 4.00 p.m.
27. Incubate at 4°C for 12–16 h.
28. Decant the capture antibody and tap the plate on paper towel to remove the residue.
29. Wash each well with PBS-Tween (PBST).
30. Pipette 200 μL of blocking buffer per well.
31. Cover the plate with parafilm and incubate for 2 h on an orbital shaker (speed +/- 320 rpm) at 18°C–21°C.
32. Discard buffer and wash with PBST (200 μL per well) 4 times.
33. Gradually thaw on ice the plasma samples previously stored at −80°C.
34. Prepare duplicates of the plasma samples in new Eppendorf tubes at 1:5 dilution as follows: for each duplicate, dilute 10 μL of plasma in 40 μL of PBST.
35. Thaw the mLH stock solution (2500 ng/mL) and prepare the series of standards at the following concentrations: 25 ng/mL, 5 ng/mL, 2.5 ng/mL, 1 ng/mL, 0.5 ng/mL, 0.1 ng/mL, 0.05 ng/mL, 0.005 ng/mL and 0 ng/mL (PBST-BSA).

Note: Standards should be loaded pure and at a dilution of 1:5; samples should only be loaded at a dilution of 1:5.

36. Pipette 25 μL of the standard or sample per well in duplicates.
37. Cover with parafilm and incubate for 2 h on orbital shaker at 18°C–21°C.
38. Wash with PBST 4 times (200 μL per well).
39. Add 50 μL of detection primary antibody in each well. Once covered with parafilm, incubate for 90 min on an orbital shaker at 18°C–21°C.
40. Discard the antibody solution from the wells and wash with PBST 4 times (200 μL per well).
41. Add 100 μL of TMB solution in each well. This time cover the plate with aluminum foil to protect it from light and incubate while shaking manually for 10 min.
42. Stop the reaction by adding 50 μL of HCl 3M in each well.
43. Read the plate at 490 nm via a standard absorbance microplate reading (Spectramax).
44. Calculate and interpolate the unknown values from the standard curve equation to obtain the LH concentrations.
45. Calculate the inter (between replicates) and intra (between runs using a quality control) assays variations.

Step/Solution	Preparation	Final volume	Time / T°/ pH
Phosphate Buffered Saline (0.1M)	Di-Sodium hydrogen phosphate (Na2HPO4) + Sodium Phosphate, Monobasic (NaH₂PO₄)+ NaCl + ddH20	N/A	pH = 7.4
Phosphate Buffered Saline (0.1M)- (0.05%) Tween20 (PBST)	Add 250 μL of Tween20 to 500mL of PBS.	500 mL	pH = 7.4
(0.1M) Phosphate Buffered Saline-(0.05%) Tween20- (0.25%) Bovine Serum Albumin (PBST-BSA)	Add 1g of BSA to 100 mL of PBST	100 mL	pH = 7.4
Coating buffer	Add 0.8g of Na_2Co_3 (0.015M) in 500mL ddH20 Add 1.47g of $NaHCo_3$ in 500mL ddH20.	Mix 250 mL of Na_2Co_3 with $NaHCo_3$ until pH is 9.6	pH = 9.6
Coating with the capture antibody (Cat#9018)	Dilution: 1:1000 Mix 5μL of capture antibody in 4.995 mL of Na_2HCO_3/ $NaHCO_3$	5 mL per 96 wells plate 50 μL per well	12–16 h at 4°C

(Continued on next page)

Continued

Step/Solution	Preparation	Final volume	Time / T°/ pH
Blocking buffer (5% Milk- PBST-BSA)	Add 5g of skim milk powder to 100mL of PBST-BSA solution	100 mL 200 μL per well	120 min
Incubation with the detection primary antibody (#AFP240580Rb)	Dilution: 1:1000 Mix 20 μL of the stock of detection primary antibody (which is already diluted to 1:40) in 4.980 mL of blocking buffer.	5 mL per 96 wells plate 50 μL per well	90 min at 18°C–21°C
Incubation with conjugated secondary antibody (#P0448)	Dilution: 1:1000 Mix 50 μL of conjugated secondary antibody in 4.995 mL of blocking buffer.	5 mL per 96 wells plate 50 μL per well	90 min at 18°C–21°C
Incubation with TMB solution	Ready to use	100 μL	10–30 min at 18°C–21°C
Stop Solution	3M HCL	50 μL	N/A

Preparation of the solutions used in the LH ELISA assay (for a single 96 multiwell microplate)

Assessment of estrous cyclicity and fertility
Assessment of ovulatory cycle

© Timing: 14 days: ~4–5 min per mouse

Before you start:

The following materials are required for this step: a glass slide (Cat# 18357-1), 0.09% saline solution (Cat#9888), a pipette and a bright field microscope (Leica ICC50).

> *Note:* On cytological evaluation, the four stages of the estrous cycle are defined by the absence, presence, or proportion of 4 basic cell types (epithelial cells, cornified cells, and leukocytes) as well as by the cell density and arrangement of the cells on the slide (Byers et al., 2012, McLean et al., 2012). The full estrous cycle in mice, as well as in rats, occurs over 4–5 days.

46. Perform vaginal smears for 14 consecutive days: gently insert a pipette tip filled with 10–20 μL of the saline solution into the vaginal orifice of the females (depth 1–2 mm maximum) and flush back and forward 2–3 times.
47. Place a small drop of the solution on a glass slide and spread it into a thin layer (cover slip not required for the observation).
48. Analyze cell morphology and proportion of cell types under a bright field microscope (Leica ICC50) using a 10× or 20× objective.
49. Identify the specific stage of the estrous cycle cell types as followed:

Proestrus: characterized by nucleated epithelial cells (Figure 4A). These cells may appear in clusters or individually. This stage corresponds to the pre-ovulatory day, Estradiol (E2) increases, followed by luteinizing hormone (LH) and Follicle-stimulating hormone (FSH) surges, when ovulation occurs (McLean et al., 2012).

Estrus: predominance of cornified epithelial cells (Figure 4B). These cells are characterized by the absence of nucleus, a granular cytoplasm and an irregular shape (McLean et al., 2012).

Metestrus: characterized by the presence of both of leukocytes and nucleated epithelial cells (Figure 4C) (McLean et al., 2012)
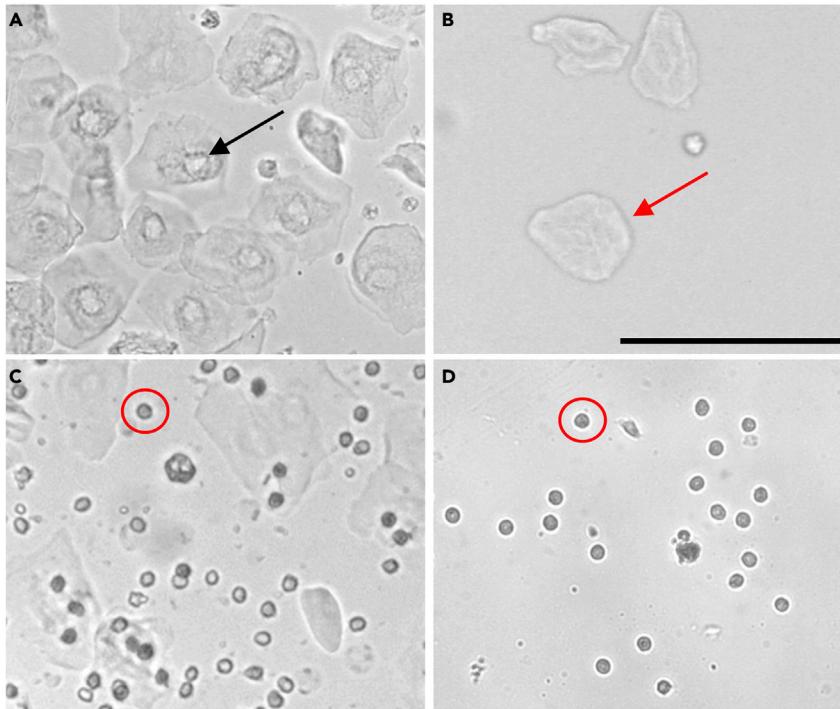
Figure 4. Representative images of the four estrous cycles in a female mouse (20× objective)
(A) Proestrus; (B) Estrus; (C) Metestrus; (D) Diestrus. With Leucocytes (circle), cornified epithelial cells (Black arrow), nucleated epithelial cells (Red arrow). Scale bar: 100 μm.

Diestrus: characterized by the presence of only leukocytes (Figure 4D). (McLean et al., 2012) * During estrus (ii), metestrus(iii) and diestrus (iv), circulating levels of LH and FSH remain low.

50. Calculate the percentage of time spent in every cycle.

> △ CRITICAL: For accurate analysis, monitor estrous cyclicity for at least two weeks.

Assessment of reproductive competence

> ⏱ Timing: 90 days

Evaluate the reproductive competence of female offspring using a 90-days mating protocol as described below.

51. Prepare a clean cage with water, food, bedding and nesting for each breeding pair.
52. Select unexperienced males (8–12 weeks old) and primiparous females (8–12 weeks old) from at least three different litters.
53. Put one female and one male per cage as indicated: CNTR female with naïve males, PAMH females with naïve males. Use at least 3 breeding cages per group for a period of 3 months.
54. Daily check each breeding cage and keep detailed records of the date of new litters and of the number of pups .
55. Remove the pups from each new litter at P0 (right after parturition).
56. Do not separate the breeding pair until the end of the fertility study.
57. Using the recorded data, evaluate the main 3 fertility parameters:
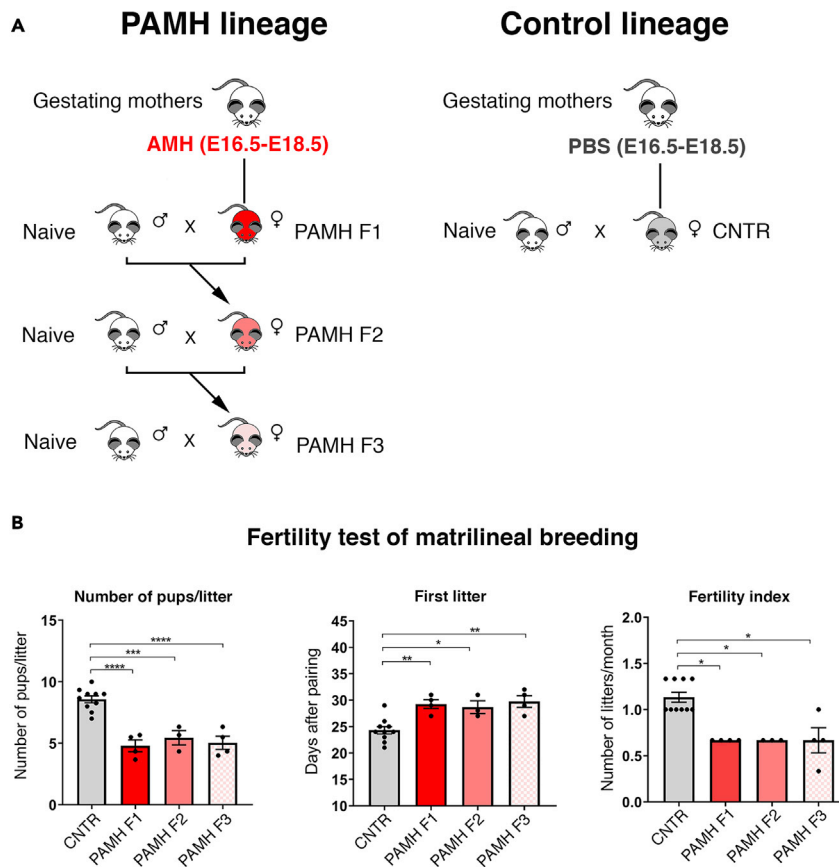   a. Number of pups/litter: average number of pups per litter of each experimental group over 90 days.

Figure 5. Female mice prenatally exposed to excess AMH show diminished fertility and fecundity for at least three generations

(A) Schematic illustration of matrilineal and control breeding scheme.

(B) Fertility was assessed in control and PAMH lineage for 90 days. Fertility tests included the analysis of the number of pups per litter, the delay in days after pairing to give the first litter and the fertility index (number of litters produced per female during 90 days mating protocol). Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison *post hoc* test: Number of pups/litter, $F_{3,17} = 23.90$ *** $p < 0.0001$; Time to first litter, $F_{3,17} = 9.774$, ***$p = 0.0006$; Analysis of fertility index between groups was assessed using Kruskal Wallis followed by Dunn's multiple comparisons *post hoc* test: **$p = 0.0016$. Data in B are represented as mean $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$. Adapted with permission from (Tata et al., 2018, Mimouni et al., 2021).

    b. Fertility index: average number of litters per females over of each experimental group over 90 days.

    c. Time to first litter: number of days between the beginning of the breeding and the day of the first delivery.

Note: A 90-day mating protocol is common practice in the field (Hoffmann, 2018) and it is advised to assess precisely reproductive competence.

PAMH lineage obtained by breeding PAMH female offspring with control naïve males in a matriline breeding scheme show diminished fertility and fecundity for at least three generations, from F1 to F3, as indicated by fewer pups per litter produced over a 3-month period, by a significant delay in their first litter and by fewer litters produced during the 90 days mating protocol (Figure 5) (Mimouni et al., 2021).

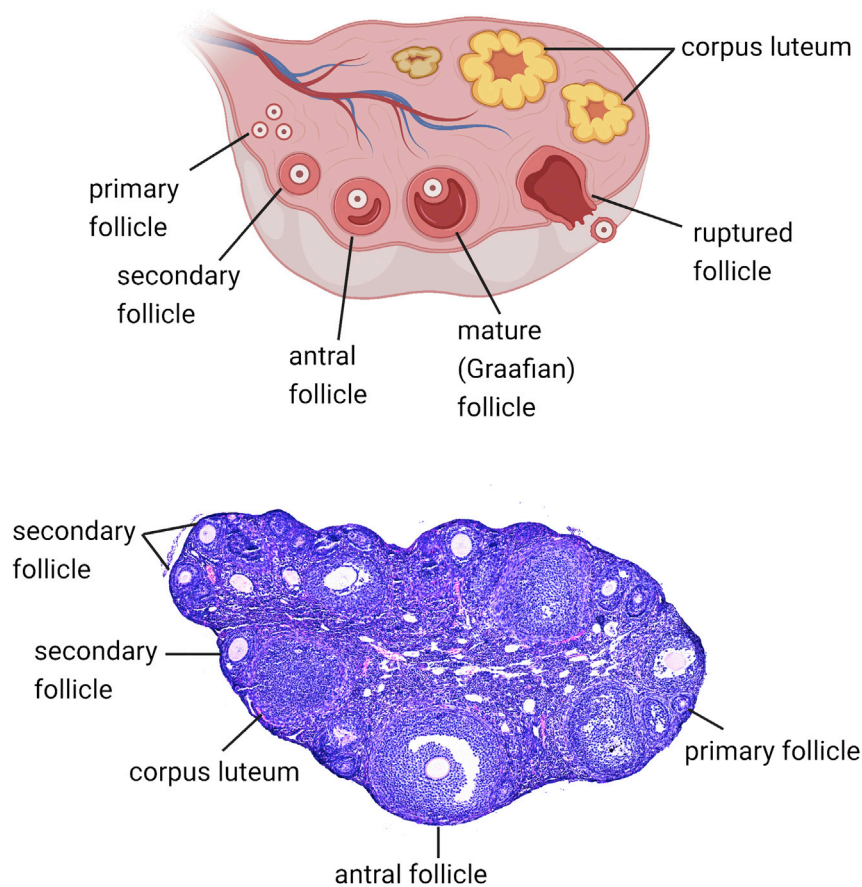### Histological analysis of the ovaries

⏱ Timing: 3–5 days

Figure 6. Illustration and photomicrograph of a healthy mouse ovary section (P60–P90) stained with hematoxylin–eosin

58. Collect ovaries from 3-month-old females in diestrus and immediately fix them in 4% paraformaldehyde solution (PFA 4%, pH 7.4) in PBS at 4°C for 12–24 h.
59. Transfer the ovaries to 70% ethanol.
60. Dehydrate the ovaries through a series of graded ethanol baths and include them in paraffin.
61. Section the paraffin-embedded ovaries at a thickness of 5 μm, using a microtome (Leica RM2255).
62. Deparaffinize your tissues with xylene and then rehydrate them in graded ethanol baths (100%, 90%, 80%, 70% and 50%) followed by rinses in distilled water.
63. Stain with hematoxylin-eosin (Sigma Aldrich; Cat#GHS132, Cat#HT1103128).

Note: The mouse ovary is composed of : *Corpora Lutea (CL)*: characterized by a central cavity, filled with blood and follicular fluid remnants or by prominent polyhedral to round luteal cells; *Primordial follicles*: characterized by an oocyte surrounded by a partial or complete layer of squamous granulosa cells; *Primary follicles*: have a single layer of cuboidal granulosa cells; *Secondary follicles*: possess more than one layer of granulosa cells with no visible antrum; *Early antral follicles*: are recognized if they often contain only one or two small antrum; *Antral follicles*: can be distinguished if they possess a single large antral space; *Preovulatory follicles*: are characterized by an oocyte surrounded by a rim of cumulus cells; *Atretic follicles*: a follicle is considered to be atretic if it displays 2 pyknotic nuclei or/and granulosa cells within the antral cavity, or/and uneven layers of granulosa cells (Figure 6).

64. Meticulously examine the ovarian sections of CNTR and PAMH mice, during diestrous stage, corresponding to the same stage at which mean LH and T levels have been assessed.
65. Count the number of ovarian follicles and CL (Caldwell et al., 2017).

⚠ CRITICAL: Perform this step under the microscope using an ocular scale to classify follicles as a function of their diamater into preantral, large growing (200–400 μm) and cystic/atretic follicles (> 400 μm). To avoid repetitive counting, count CL every 100 μm.

EXPECTED OUTCOMES

Based on our published results (Tata et al., 2018), the intraperitoneal delivery of AMH, at the indicated concentration, to C57BL/6J (B6) dams between E16.5 and E19.5 is highly effective in generating offspring (PAMH) manifesting at adulthood the major diagnostic PCOS-like features: hyperandrogenism, oligo-anovulation, disrupted fertility, altered ovarian function and morphology (Figure 7). Most patients with PCOS also exhibit high serum AMH and LH levels (Teede et al., 2019). In keeping with the elevation of these hormones observed in women with PCOS, we do also find a significant increase in AMH (data not shown) and LH serum levels in adult PAMH females as compared to control females (Figure 7D). Finally, PCOS is strongly associated with a number of metabolic disorders, including obesity, type 2 diabetes, hypertension, and cardiovascular disease (Boyle and Teede, 2016, Dokras et al., 2017). Similar to the human condition, PAMH female mice develop metabolic alterations as a function of age. By the age of 6 months, PAMH females show higher body weight and fat mass, lower glucose tolerance and insulin sensitivity, higher fasting glucose levels and hypertrophy of pancreatic islets of Langerhans as compared with controls. For more details please refer to (Mimouni et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 8.3.1 software (https://www.graphpad.com) is used to calculate normality (Shapiro–Wilk test and/or D'Agostino & Pearson test) and statistical significance.

All comparisons between groups, whose distribution is not normal, are performed using Mann-Whitney U test (comparison between two experimental groups), followed by a Dunn's post hoc analysis. For analyses of populations normally distributed, data are compared using an unpaired two-tailed Student's t-test. A probability level of $p < 0.05$ is adopted as the level of statistical significance for all analyses.

LIMITATIONS

This protocol was optimized to generate PCOS-like mice using the strain of inbred mice C57BL/6J (B6). We ignore whether using different mouse strains or rodent models, such as rats, could yield to similar phenotypic outcome.

The method used to measure testosterone levels in this study is not the most sensitive one. Liquid chromatography–isotopic dilution tandem mass spectrometry can be used to quantify the total testosterone as this method provides consistently high accuracy and excellent precision for testosterone measurement.
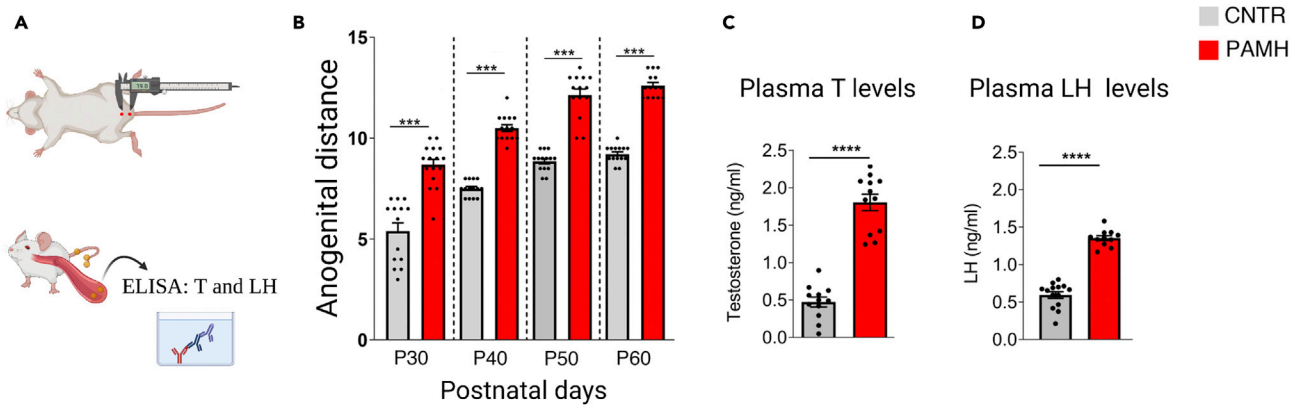
TROUBLESHOOTING

Problem 1

Unsuccessful breeding (in Generation of prenatally AMH exposed mice, step 6).

Potential solution

To maximize pregnancy rate, cycle your females the day of the breeding and preferably use females in proestrus for breeding.

Measurement anogenital distance, T levels and LH levels

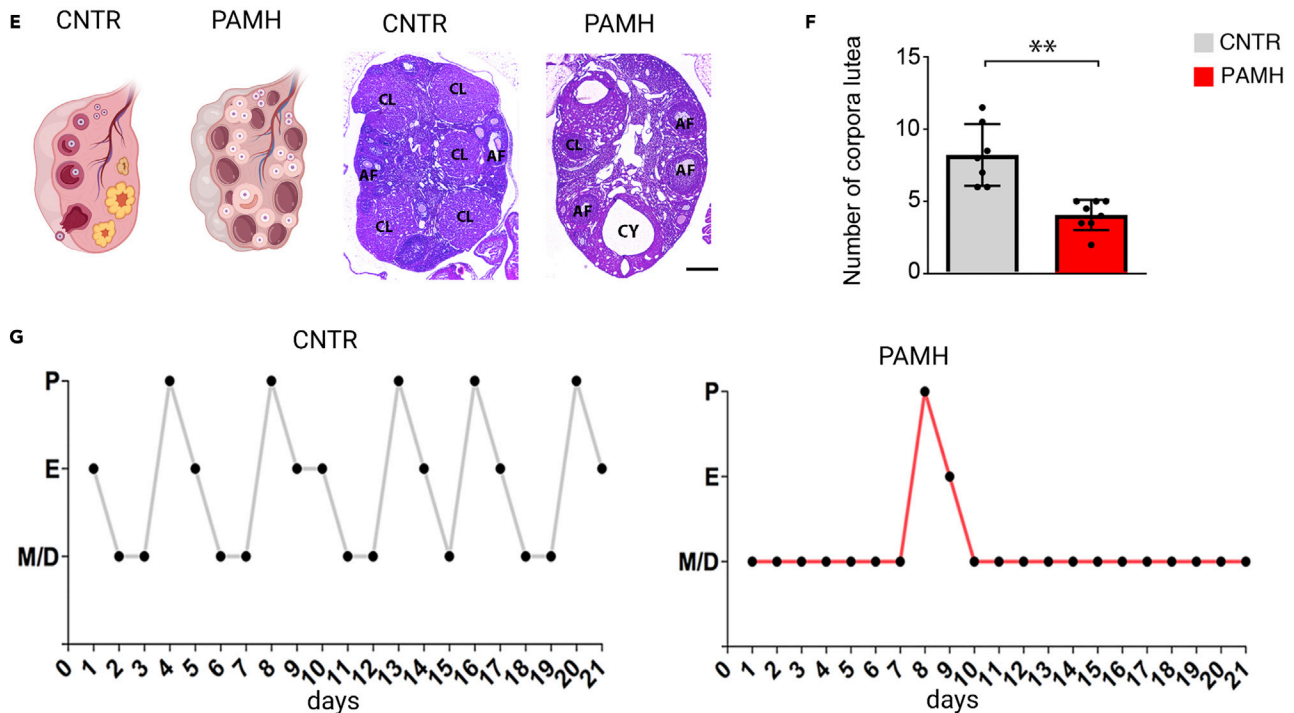Assessment of ovarian morphology and estrous cyclicity

Figure 7. Female mice prenatally exposed to excess AMH exhibit the cardinal neuroendocrine and ovulatory defects typical of PCOS

(A) Schematic illustration of experimental design.

(B) Anogenital distance measurement over post-natal days (P) 30, 40, 50 and 60 in adult control females (*n* = 14), PAMH (*n* = 13–16) mice.

(C and D) Plasma testosterone and LH concentrations in adult females (P60-P90) in diestrus (CNTR, *n* = 12–14; PAMH, *n* =11–12).

(E) Representative photomicrographs of ovaries stained with hematoxylin–eosin from adult (P60–P90) diestrous control (CNTR) and PAMH mice. Scale bar, 200 μm.

(F) Quantitative analyses of the mean number of corpora lutea (CL) in control and PAMH mice. Data in (B–D) and (F) are represented as mean ± s.e.m. For statistical analysis, p values were calculated by an unpaired two-tailed Student's t test. *p < 0.05; **p < 0.005; ***p < 0.0005.

(G) Representative estrous cyclicity of CNTR and PAMH mice (P60-P90) during 21 consecutive days. M/D: Metestrus/Diestrus phase, P: Proestrus, E: Estrus. Adapted with permission from Mimouni et al., (2021); Tata et al., (2018).

Isolating stud males 1 week prior to mating will maximize their fertility and increase the probability of breeding.

In addition, if none of the above significantly improved the rate of pregnancies, we suggest to expose females to a sexually-experienced male's bedding to synchronize their estrous cycles. Approximately 50% of exposed females will be in proestrus and receptive to mating on the third night following exposure (Gangrade and Dominic, 1984).

Problem 2
Cannibalism after parturition (in prenatal anti-Müllerian hormone (PAMH) treatment, step 11)

Potential solution
This problem usually arises when the animals are stressed in concomitance with their delivery. Minimizing noise and human presence at the time of parturition and adding enrichment items in the cage (nesting material, shelters and deeper bedding) usually helps solving this problem.

Problem 3
Values of T and LH are highly heterogenous within the experimental group (in assessment of hyperandrogenism via Testosterone ELISA assay, step 4 and luteinizing hormone ELISA assay, step 24).

Potential solution
Habituate females to handling for at least 1 week before starting the experiments (2 weeks of habituation is preferrable when performing LH repeated measurements).

Problem 4
Unsure classification of the estrous cycle stages (in assessment of estrous cyclicity and fertility, step 48).

Potential solution
Monitor estrous cyclicity of mice with a consistent time schedule (i.e., early in the morning or late in the evening) and for a minimum of 7 days to be able to follow at least one complete ovulatory cycle. To avoid any difficulties in the subsequent cell morphological analysis, clean the area around the genitalia to avoid contaminating the vaginal secretion collected in the pipette.

Problem 5
Variability in hormonal levels between experiments (in assessment of hyperandrogenism via Testosterone ELISA assay, step 4 and luteinizing hormone ELISA assay, step 24).

Potential solution
To minimize the variations in hormonal levels and ensure the accuracy and reproducibility of the measurements, please follow the indications listed below:

Use the same sampling and storage conditions in all the experiments. Avoid thawing cycles of plasma samples and prepare aliquots beforehand. Use the same quality controls when performing hormonal measurements. If quality controls are not provided in the ELISA kits, generate your own quality controls. Calculate the inter assay coefficient of variation (between replicates) and intra assay coefficient of variations (between runs).

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paolo Giacobini (paolo.giacobini@inserm.fr).

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