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# The environment and female fertility : a study of the role of endocrine disruptors on the ovarian reserve

Maximilien Génard-Walton

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# THESE DE DOCTORAT DE

L'UNIVERSITE DE RENNES

ECOLE DOCTORALE N° 637

*Sciences de la Vie et de la Santé*

Spécialité : Epidémiologie, Analyse de Risque, Recherche Clinique

Par

**Maximilien GENARD-WALTON**

**Environnement et fertilité féminine : étude du rôle des perturbateurs endocriniens sur la réserve ovarienne**

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## **LIST OF SCIENTIFIC PUBLICATIONS NOT INCLUDED IN THE THESIS**

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## LIST OF ACRONYMS AND ABBREVIATIONS

<b>AFC</b>	Antral Follicle Count
<b>AMH</b>	Anti-Müllerian Hormone
<b>ART</b>	Assisted Reproductive Technology
<b>ATP</b>	Adenosine triphosphate
<b>BBzP</b>	Butylbenzyl phthalate
<b>BKMR</b>	Bayesian Kernel Machine Regression
<b>BMI</b>	Body Mass Index
<b>BMIM</b>	Bayesian Multiple Index Model
<b>BP</b>	Butylparaben
<b>BP-3</b>	Benzophenone-3
<b>BPA</b>	Bisphenol A
<b>BPF</b>	Bisphenol F
<b>BPS</b>	Bisphenol S
<b>CDC</b>	Center for Disease Control and Prevention
<b>CI</b>	Confidence Interval
<b>CI</b>	Credible Interval
<b>DAG</b>	Directed Acyclic Graph
<b>DDE</b>	Dichlorodiphenyldichloroethylene
<b>DDT</b>	Dichlorodiphenyltrichloroethane
<b>DEDuCT</b>	Database of Endocrine Disrupting Chemicals and their Toxicity Profiles
<b>DEHP</b>	Di(2-ethylhexyl)phthalate
<b>DEHTP</b>	Di-2-ethylhexyl terephthalate
<b>DEP</b>	Diethyl phthalate
<b>DES</b>	Diethylstilbestrol
<b>DiBP</b>	Di-iso-butyl phthalate
<b>DiDP</b>	Diisodecyl phthalate
<b>DINCH</b>	Di(isononyl)cyclohexane-1,2-dicarboxylate
<b>DiNP</b>	Di-iso-nonyl phthalate
<b>DMP</b>	Dimethyl phthalate
<b>DNA</b>	Deoxyribonucleic acid
<b>DnBP</b>	Di-n-butyl phthalate
<b>DOHAD</b>	Developmental Origins of Health and Disease

<b>DOR</b>	Diminished Ovarian Reserve
<b>EDC</b>	Endocrine Disrupting Chemical
<b>EHESP</b>	Ecole des Hautes Etudes en Santé Publique
<b>EP</b>	Ethylparaben
<b>EPA</b>	Environmental Protection Agency
<b>ESHRE</b>	European Society of Human Reproduction and Embryology
<b>EU</b>	European Union
<b>FC</b>	Free cholesterol
<b>FDA</b>	Food and Drug Administration
<b>FSH</b>	Follicle-Stimulating Hormone
<b>HBCD</b>	Hexabromocyclododecane
<b>HCB</b>	Hexachlorobenzene
<b>HCH</b>	Hexachlorocyclohexane
<b>ICC</b>	Intraclass Correlation Coefficient
<b>ICSI</b>	Intra-Cytoplasmic Sperm Injection
<b>INSEE</b>	Institut National de la Statistique et des Etudes Economiques
<b>INSERM</b>	Institut National de la Santé et de la Recherche Médicale
<b>IRSET</b>	Institut de Recherche en Santé, Environnement et Travail
<b>IVF</b>	In vitro fertilisation
<b>LERES</b>	Laboratoire d'Etude et de Recherche en Environnement et Santé
<b>LH</b>	Luteinizing Hormone
<b>LOD</b>	Limit of Detection
<b>MBP</b>	Mono-n-butyl phthalate
<b>MBzP</b>	Monobenzyl phthalate
<b>MCiOP</b>	Mono(carboxy-iso-octyl) phthalate
<b>MCMHP</b>	Mono(2-carboxy-hexyl) phthalate
<b>MDD</b>	Major Depressive Disorder
<b>MECPP</b>	Mono(2-ethyl-5-carboxypentyl) phthalate
<b>MEHHP</b>	Mono(2-ethyl-5-hydroxyhexyl) phthalate
<b>MEHP</b>	Mono(2-ethylhexyl) phthalate
<b>MEOHP</b>	Mono(2-ethyl-5-oxohexyl) phthalate
<b>MEP</b>	Monoethyl phthalate
<b>MGH</b>	Massachusetts General Hospital
<b>MHiNP</b>	Mono(hydroxy-iso-nonyl) phthalate



<b>MiBP</b>	Mono-iso-butyl phthalate
<b>MiNP</b>	Mono-iso-nonyl phthalate
<b>MMP</b>	Monomethyl phthalate
<b>MOiNP</b>	Mono(oxo-iso-nonyl) phthalate
<b>MP</b>	Methylparaben
<b>NCD</b>	Non-Communicable Disease
<b>NO<sub>2</sub></b>	Nitrogen dioxide
<b>OCP</b>	Organochlorine Pesticides
<b>OPFR</b>	Organophosphorus flame retardant
<b>OR</b>	Odds Ratio
<b>OSI</b>	Ovarian Sensitivity Index
<b>PAH</b>	Polycyclic Aromatic Hydrocarbon
<b>PBB</b>	Polybromobiphenyl
<b>PBDE</b>	Polybrominated Diphenyl Ethers
<b>PCB</b>	Polychlorinated Biphenyl
<b>PCOS</b>	Polycystic Ovary Syndrome
<b>PFAS</b>	Per-polyfluoroalkyl Substances
<b>PFBA</b>	Perfluorobutyric acid
<b>PFBS</b>	Perfluorobutanesulfonic acid
<b>PFDA</b>	Perfluorodecanoic acid
<b>PFHxA</b>	Perfluorohexanoic acid
<b>PFHxS</b>	Perfluorohexanesulfonic acid
<b>PFNA</b>	Perfluorononanoic acid
<b>PFOA</b>	Perfluorooctanoic acid
<b>PFOS</b>	Perfluorooctanesulfonic acid
<b>PFUDA</b>	Perfluoroundecanoic acid
<b>PIP</b>	Posterior Inclusion Probability
<b>PL</b>	Phospholipids
<b>PM</b>	Particulate Matter
<b>POI</b>	Poor Ovarian Insufficiency
<b>POP</b>	Persistent Organic Pollutant
<b>POR</b>	Poor Ovarian Response
<b>PP</b>	Propylparaben
<b>PVC</b>	Polyvinyl chloride

<b>QC</b>	Quality Control
<b>REACH</b>	Registration, Evaluation, Authorisation and Restriction of Chemicals
<b>RNA</b>	Ribonucleic acid
<b>SO<sub>2</sub></b>	Sulfur dioxide
<b>STI</b>	Sexually Transmitted Infection
<b>TBBPA</b>	Tetrabromobisphenol A
<b>TC</b>	Total Cholesterol
<b>TCDD</b>	2,3,7,8-Tetrachlorodibenzo-p-dioxin
<b>TG</b>	Triglycerides
<b>TTP</b>	Time-to-pregnancy
<b>UK</b>	United Kingdom
<b>UNEP</b>	United Nations Environment Program
<b>US</b>	United States
<b>UV</b>	Ultraviolet
<b>WHO</b>	World Health Organisation
<b>WQS</b>	Weighted Quantile Sum

## RESUME SUBSTANTIEL EN FRANÇAIS

### INTRODUCTION

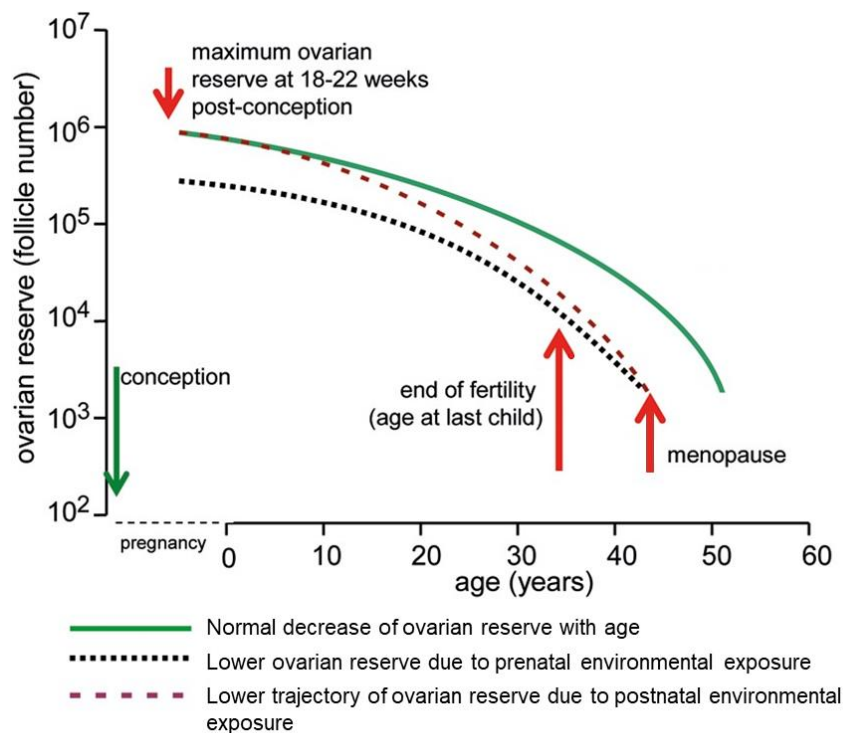
Les perturbateurs endocriniens (PEs) sont des substances ou des mélanges exogènes qui altèrent les fonctions du système endocrinien et provoquent par conséquent des effets indésirables sur un organisme intact, sa descendance ou des sous-populations, comme défini par l'Organisation Mondiale de la Santé (Damstra *et al.*, 2002). En raison de leur potentiel à interférer avec les récepteurs hormonaux, les PEs sont soupçonnés d'affecter les systèmes reproducteurs régulés par les hormones chez les femmes et les hommes (Sifakis *et al.*, 2017). Plusieurs de ces substances chimiques ont été mesurés dans diverses matrices humaines, dont le sang, l'urine et le liquide folliculaire (Hart, 2016). Des études ont montré des preuves de leurs impacts sur les troubles de la fertilité tels que le syndrome des ovaires polykystiques, l'endométriose, ou les paramètres spermatiques, mais aussi sur les anomalies du développement (Gore *et al.*, 2015). Cependant, peu d'attention a été accordée à leurs éventuelles associations avec la réserve ovarienne, composante clé de la fertilité féminine, et aucune à notre connaissance à la diminution de la réserve ovarienne (DOR), un indicateur clinique prédicteur de mauvaise réponse à une stimulation ovarienne. De plus, il existe peu de recherches portant sur les conséquences de l'exposition in utero à ces substances ou sur leur effet en tant que mélange sur la réserve ovarienne.

### CONTEXTE

La réserve ovarienne correspond au nombre total de follicules primordiaux dans les deux ovaires, bien que cette définition puisse parfois être étendue pour inclure les follicules intermédiaires et primaires (Hansen *et al.*, 2012). La réserve ovarienne est donc un stock fini qui ne peut pas se reconstituer et dont le système reproducteur puise tout au long de la vie reproductive pour produire des ovocytes (Gura et Freiman, 2018). La réserve ovarienne est ainsi directement liée à la capacité d'une femme à se reproduire.

La réserve ovarienne se constitue in utero, où elle peut atteindre jusqu'à sept millions de follicules (Figure 1), mais diminue ensuite à mesure que les femmes vieillissent jusqu'à la ménopause (Hansen *et al.*, 2008 ; Richardson *et al.*, 2014). À la naissance, elle est déjà réduite à un à deux millions. Cette diminution se poursuit jusqu'à la puberté (300 000 follicules), moment à partir duquel le déclin ralentit.

Le nombre de follicules diminue ensuite progressivement jusqu'à atteindre 1 000, déclenchant ainsi la ménopause, à l'âge de 50 ans en moyenne (Faddy et Gosden, 1996 ; Wallace et Kelsey, 2010).



**Figure 1. L'évolution de la réserve ovarienne avec l'âge (adapté de Richardson et al., 2014)**

Cependant, cette diminution peut survenir prématurément (courbe en tirets rouges sur la figure), mais elle peut également diminuer en raison de l'altération subie pendant des périodes de vulnérabilité (courbe en pointillés noirs sur la figure). Par exemple, si la réserve ovarienne ne se forme pas correctement in utero, elle atteindra un stock minimal critique plus tôt à l'âge adulte que pour une réserve normalement constituée. Ces deux scénarios peuvent ainsi aboutir à une DOR, un seuil sous lequel la réponse à la stimulation ovarienne ou la fécondité est réduite (Practice Committee of the American Society for Reproductive Medicine, 2015). C'est un état normal pour les femmes lorsqu'elles atteignent environ 45 ans, mais il est considéré comme pathologique à des âges plus jeunes. Bien que plusieurs facteurs de risque de la réserve ovarienne soient connus, le rôle des polluants environnementaux sur la réserve ovarienne, en particulier des PE, est beaucoup moins compris (Jurewicz *et al.*, 2020 ; Mínguez-Alarcón and Gaskins, 2017 ; Souter *et al.*, 2013).

Les PE sont des substances qui sont omniprésentes dans notre environnement et auxquelles nous sommes exposés à travers notre alimentation, dans l'eau et l'air, et dans certains produits de consommation comme les produits cosmétiques. Une fois libérés dans la circulation sanguine, les PE

peuvent atteindre différentes parties du système endocrinien. Celui-ci est composé d'une série de glandes réparties dans différentes parties du corps qui sécrètent des hormones amenées à réguler diverses fonctions de l'organisme (Organisation mondiale de la santé, 2012), notamment la reproduction mais aussi le métabolisme, la pression artérielle, la croissance, le développement, le stress, ainsi que plusieurs fonctions gastro-intestinales, cardiovasculaires et rénales (Damstra *et al.*, 2002). Les glandes endocrines comprennent l'hypophyse à la base du cerveau, la glande thyroïde dans le cou, l'utérus, les trompes utérines et les ovaires, qui sont essentiels au fonctionnement de la reproduction féminine, mais aussi les glandes mammaires, la glande surrénale et le pancréas.

Les PE peuvent perturber le système endocrinien à travers plusieurs mécanismes biologiques. Ceux-ci peuvent être résumés en cinq catégories principales : (1) l'imitation des hormones en activant (effet agoniste) ou en bloquant (effet antagoniste) les récepteurs hormonaux, (2) la perturbation de la synthèse des hormones et de leur transport, (4) l'altération de l'ADN, et (5) la dégradation cellulaire.

Les effets indésirables biologiques des PE sur le système reproducteur ont été largement étudiés dans la recherche animale (Sifakis *et al.*, 2017), cependant, la réserve ovarienne peut être plus difficile à évaluer chez les femmes sans avoir recours à des procédures invasives. En effet, les preuves de l'impact des PE sur la réserve ovarienne chez les femmes sont plus limitées.

Nous nous sommes intéressés spécifiquement à quatre familles de PE : les polluants organiques persistants (POPs), les métaux lourds, les phénols et les phtalates. Les POPs sont des substances principalement interdites dans la majorité des pays du monde qui peuvent persister dans l'environnement pendant de nombreuses années. Elles s'accumulent dans les tissus adipeux et remontent ainsi la chaîne alimentaire jusqu'à l'humain. Elles possèdent également une capacité de déplacement très importante dépendante du climat. Les métaux peuvent être toxiques à certaines doses pour les métaux dits 'essentiels' et à tout niveau pour les métaux dits 'non essentiels'. Nous y sommes exposés à travers la combustion et les activités minières entre autres. Les phénols, tels que le bisphénol A ou les parabens, et les phtalates, sont des substances qui ont des demi-vies dans l'organisme très courte mais qui par leur utilisation dans les produits quotidiens (plastiques, résines, cosmétiques, emballages alimentaires, etc) impliquent une exposition continue. Des effets biologiques indésirables des PE sur le système reproducteur ont été documentés dans la recherche animale (Sifakis *et al.*, 2017), cependant, les preuves de l'impact des PE sur la réserve ovarienne chez les femmes sont plutôt limitées, en particulier pour (mais pas exclusivement) nos familles d'intérêt.

L'objectif global de cette thèse était d'étudier l'impact des PE sur la réserve ovarienne. Plus précisément, nous avons examiné les effets de quatre familles de PE (53 POPs, 4 métaux lourds, 4 phénols et 8 phtalates), en tant qu'exposition individuelle et en tant que mélanges, sur différents indicateurs de la réserve ovarienne dont des marqueurs continus (hormones anti-Müllérienne (AMH), compte de follicules antraux (CFA) et hormones folliculo-stimulantes (FSH)) et un indicateur clinique (DOR). De plus, nous avons exploré si l'exposition in utero aux POPs était associée à l'AMH chez les adolescentes. Nous avons mené nos recherches auprès de deux populations de femmes consultant pour assistance à la reproduction dans deux pays (France et États-Unis), et au sein de la cohorte mère-enfant française PELAGIE.

Pour atteindre cet objectif, quatre études ont été menées au sein de ces trois populations d'étude :

1. Etude de la relation entre les polluants organiques persistants et la diminution de la réserve ovarienne dans l'étude cas-témoins AROPE.
2. Etude de la relation entre les métaux lourds et la diminution de la réserve ovarienne dans l'étude cas-témoins AROPE.
3. Etude de la relation entre un mélange de phénols et de phtalates, et la réserve ovarienne dans la cohorte EARTH.
4. Etude de la relation entre l'exposition in utero aux polluants organiques persistants et les niveaux d'hormone anti-Müllérienne chez les filles de 12 ans dans la cohorte PELAGIE.

## **METHODES**

### *Populations*

L'étude cas-témoins **AROPE** ("Altération de la réserve ovarienne : étude du rôle de l'exposition aux perturbateurs endocriniens persistants et aux solvants organiques") a inclus des femmes âgées de 18 à 40 ans parmi les couples consultant pour infertilité dans quatre centres d'aide médicale à la procréation de la région de Bretagne-Pays de la Loire, dans le nord-ouest de la France (Centre Hospitalier Universitaire de Rennes, Centre Hospitalier Universitaire de Nantes, Centre Hospitalier Universitaire de Brest et La Sagesse, une clinique semi-privée basée à Rennes) entre novembre 2016 et février 2020. Toutes les femmes consultant dans ces centres ont effectué un comptage standardisé

des follicules antraux par échographie endovaginale réalisé par un gynécologue formé entre les jours 2 et 4 d'un cycle menstruel spontané (Broekmans *et al.*, 2010), ainsi qu'une mesure de l'AMH dans le sang effectuée à l'inclusion (36.2% des cas et 31.8% des témoins) ou dans l'année précédant l'inclusion pour les femmes qui avaient déjà commencé à consulter dans les cliniques (63.8% des cas et 68.2% des témoins). Les cas de DOR ont été définis en utilisant les valeurs seuils pour le nombre de follicules antraux et les niveaux d'AMH proposés dans les critères de Bologne du consensus de la Société Européenne de Reproduction Humaine et d'Embryologie (ESHRE) (Ferraretti *et al.*, 2011). Ainsi, les cas étaient des femmes présentant au moins l'une des caractéristiques suivantes : un nombre de follicules antraux inférieur à 7 et/ou un taux d'AMH dans le sang inférieur ou égal à 1.1 ng/mL. Les témoins ont été sélectionnés par appariement de fréquence par centre et par groupe d'âge de cinq ans parmi les femmes dont l'évaluation de la fertilité était strictement normale (taux d'AMH entre 1.1 et 5 ng/mL et CFA  $\geq$  7, sans malformation génitale et avec un cycle menstruel de 26 à 35 jours). Des échantillons de sérum sanguin ont également été prélevés à l'inclusion pour mesurer les substances chimiques d'intérêt. Au total, 149 cas et 161 témoins ont été inclus dans l'étude AROPE. Les tailles de l'échantillon variaient très légèrement en fonction de la disponibilité des échantillons de sang utilisés pour mesurer l'exposition aux substances chimiques.

L'étude sur l'Environnement et la Santé de la Reproduction (**EARTH**) est une cohorte prospective (2004-2019) créée pour évaluer les déterminants environnementaux et alimentaires de la fertilité au Massachusetts General Hospital Fertility Center à Boston aux Etats-Unis (Messerlian *et al.*, 2018). Les femmes âgées de 18 à 45 ans et les hommes âgés de 18 à 55 ans qui n'avaient pas subi de vasectomie, qui ne prenaient pas d'hormones au moment de leur inscription et qui consultaient pour infertilité étaient éligibles pour participer, que cela soit individuellement ou en couple.

Les femmes ont été suivies depuis leur entrée dans l'étude, tout au long de leurs tentatives de conception, de leur grossesse, et jusqu'à l'accouchement. Le type d'infertilité primaire a été évalué en utilisant les définitions de la Société Américaine de Technologie de Reproduction Assistée (SART), et classé comme facteur féminin (DOR, endométriose, ovulatoire, tubaire, utérin et autre), facteur masculin, ou inexplicé. Les femmes ont fourni un (23%) ou deux (77%) échantillons d'urine ponctuels par cycle de FIV (Mínguez-Alarcón *et al.*, 2019), le premier étant collecté entre le jour 3 et le jour 9 de la phase de gonadotrophine, et le second étant collecté le jour du prélèvement des ovocytes (pour les cycles de fécondation in vitro (FIV) frais) ou le jour du transfert d'embryon (pour les cycles de FIV décongelés).

Les mesures de la réserve ovarienne comprenaient un CFA, des taux circulants dans le sang de FSH au jour 3, et la DOR. Un CFA bas a été défini en utilisant les critères de Bologne de CFA  $<$  7 (Ferraretti *et*

al., 2011) et la DOR a été définie comme CFA < 7, FSH > 10 UI/L et/ou un diagnostic d'infertilité primaire de DOR.

**PELAGIE** ("Perturbateurs endocriniens : étude longitudinale sur les anomalies de la grossesse, l'infertilité et l'enfance") est une cohorte mère-enfant qui a enrôlé 3 421 femmes enceintes dans trois départements de Bretagne de 2002 à 2006. Son objectif était d'étudier le rôle des polluants environnementaux sur la grossesse et sur la santé et le développement des enfants. Les gynécologues, obstétriciens et échographistes ont recruté les femmes lors de visites de soins prénatals de routine avant 19 semaines de gestation et les ont suivies jusqu'à l'accouchement. Au moment de la naissance, des échantillons de sang de cordon ombilical ont été prélevés chez 2 138 femmes (62%). Lorsque l'enfant a atteint l'âge de 12 ans, un questionnaire ("Votre enfant à 12 ans") a été envoyé à leurs parents pour enquêter sur la santé et l'environnement de leur enfant. À 12 ans, un examen clinique (comprenant une évaluation du stade pubertaire selon la méthode de Tanner et des mesures de poids et de taille) a également été proposé aux enfants pour lesquels un échantillon de sang de cordon ombilical avait été prélevé à la naissance. Au total, 420 filles avec des concentrations prénatales de POPs ont été incluses dans le suivi à 12 ans. Parmi elles, 239 filles avaient des concentrations d'AMH et ont été incluses dans les analyses.

#### *Analyses statistiques*

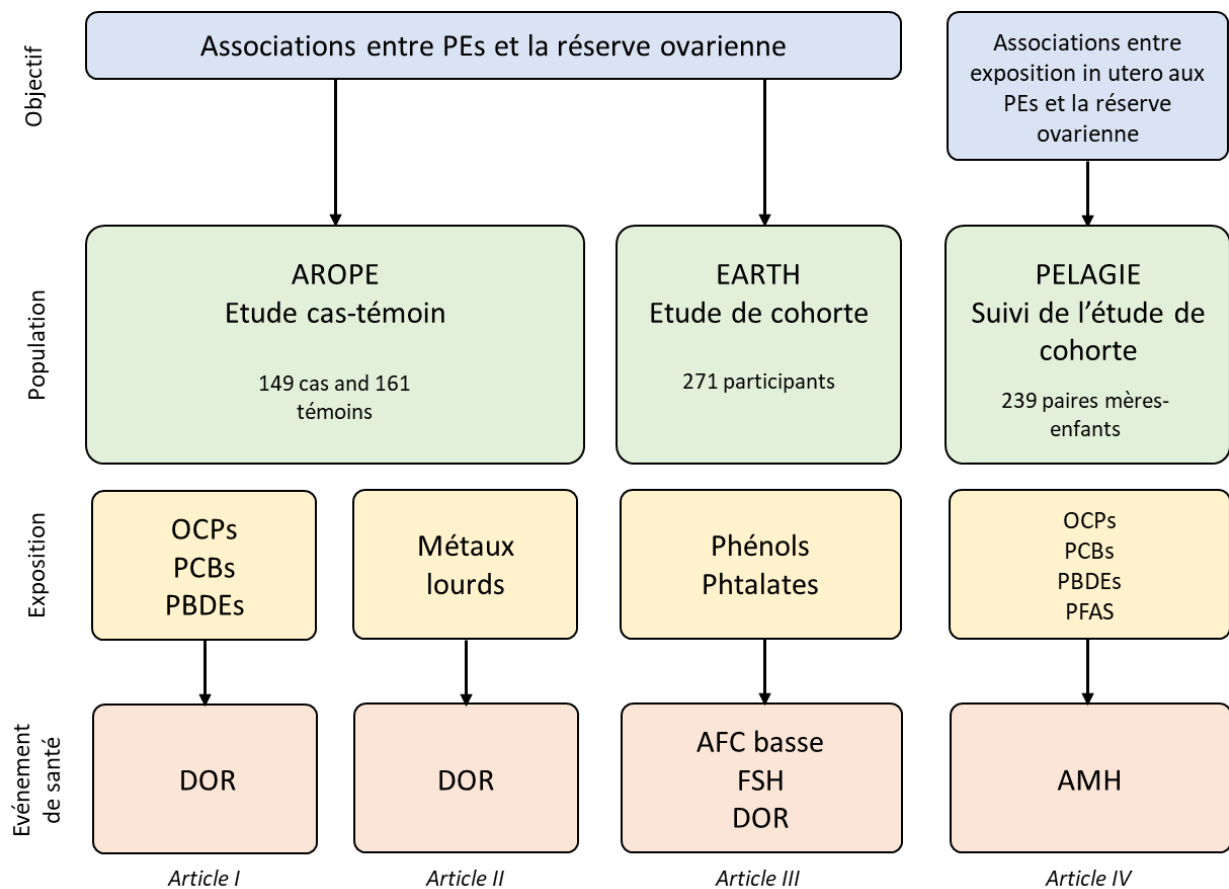
Les expositions en deçà des limites de détection des appareils de mesure des polluants ont été imputées de manière différente dans les études AROPE (pour les polluants détectés à plus de 50%) et dans les études EARTH et PELAGIE (pour les polluants détectés à plus de 70%). Dans AROPE, les polluants détectés dans moins de 20% des échantillons n'ont pas été inclus dans les analyses. Pour les polluants détectés dans entre 20% et 50% des échantillons (seulement le PCB 28), l'exposition a été dichotomisée en deux groupes (détectée versus non détectée). Pour les autres polluants (détectés dans plus de 50% des échantillons), les concentrations en dessous du seuil de détection (LOD) ont été remplacées par la méthode du maximum de vraisemblance et catégorisées en terciles. Pour ces polluants, des analyses ont également été effectuées sur l'exposition continue. Dans EARTH et PELAGIE, seulement les substances détectées dans plus de 70% des échantillons ont été analysées et imputées si nécessaire (moins de 100% de détection) par la méthode de censored likelihood multiple imputation.

Les associations entre les expositions individuelles et la réserve ovarienne ont été examinées à l'aide de régressions logistique (pour DOR) et linéaires (pour l'AMH) multivariées, en ajustant a priori pour les facteurs de confusion potentiels identifiés par des diagrammes acycliques causaux.



L'évaluation de l'exposition à un groupe de substances chimiques ou de mélanges permet une représentation plus précise de l'exposition humaine aux substances chimiques, car la population est généralement exposée à plusieurs polluants simultanément plutôt qu'un à la fois. Dans les études présentées ici, les effets des mélanges ont été étudiés à l'aide des méthodes de quantile g-computation et de Bayesian kernel machine regression (BKMR).

Nos objectifs, populations d'étude, expositions d'intérêt et résultats par étude sont présentés dans la Figure 2.



**Figure 2. Synthèse des objectifs de recherche, populations d'études, expositions et événements de santé**

## RESULTATS

### *1. Associations entre les polluants organiques persistants et la diminution de la réserve ovarienne dans l'étude cas-témoins AROPE*

Dix-sept POPs (mesurés en ng/L) ont été détectés dans plus de 20% des échantillons de sérum. Dans les régressions logistiques multivariées d'expositions individuelles ajustées sur l'âge (années, en continu), le niveau d'éducation (baccalauréat ou moins, baccalauréat + 2 ans, baccalauréat + 3 ans ou plus), l'indice de masse corporelle (IMC) (kg/m<sup>2</sup>, en continu), le centre (Nantes, Rennes et Brest, La Sagesse) et la saison d'inclusion (automne, hiver, printemps, été), le p,p'-DDE (médiane 165.0, IQR 161.0 ng/L chez les témoins) en tant qu'exposition continue était significativement associé à un risque accru de DOR (OR 1.39, IC 95% 1.10 – 1.77) et associé de manière non significative à un risque accru de DOR pour le deuxième et le troisième terciles (OR 1.46, IC 95% 0.74 – 2.87, et OR 1.72, IC 95% 0.88 – 3.37, respectivement). Le  $\beta$ -HCH (médiane 24.2, IQR 21.5 ng/L chez les témoins) était significativement associé à un risque réduit de DOR lorsque le  $\beta$ -HCH était traité comme une exposition continue (OR 0.63, IC 95% 0.44 – 0.89) et pour le troisième tercile d'exposition (OR 0.43, IC 95% 0.21 – 0.84), et associé de manière non significative à un risque réduit de DOR pour le deuxième tercile (OR 0.77, IC 95% 0.42 – 1.42). Toutes les analyses de sensibilité ont confirmé nos résultats. Le modèle BKMR a montré des associations similaires pour les expositions individuelles, mais n'a trouvé aucune association significative pour l'effet total de mélange. De plus, les résultats en utilisant un modèle BKMR n'ont suggéré aucune interaction entre les POPs. Les propriétés anti-androgéniques reconnues du p,p'-DDE et les propriétés œstrogéniques du  $\beta$ -HCH pourraient expliquer ces associations de directions opposées.

### *2. Associations entre les métaux lourds et la diminution de la réserve ovarienne dans l'étude cas-témoins AROPE*

Les taux de détection de Pb, Cd, Cr et Hg (mesurés en  $\mu$ g/L) étaient tous supérieurs à 88%. La corrélation la plus élevée entre les métaux concernait le Pb et le Cd ( $\rho = 0.15$ ). Dans les régressions ajustées sur l'âge (années, en continu), le niveau d'éducation (baccalauréat ou moins, baccalauréat + 2 ans, baccalauréat + 3 ans ou plus), l'indice de masse corporelle (IMC) (kg/m<sup>2</sup>, en continu), le centre (Nantes, Rennes et Brest, La Sagesse), l'éthnicité (origine Européenne et non-Européenne) et la saison d'inclusion (automne, hiver, printemps, été), nous avons observé des risques accrus non significatifs de DOR pour le cadmium (OR 1.89, IC 95% 0.98 - 3.67, pour le deuxième tercile d'exposition, et OR 1.18, IC 95% 0.55 - 2.52, pour le troisième tercile) et le chrome (OR 1.24, IC 95% 0.65 - 2.39, pour le

deuxième tercile d'exposition, et OR 1.77, IC 95% 0.84 - 3.70, pour le troisième tercile). Les méthodes quantile g-computation et BKMR ont toutes deux abouti à un risque augmenté non significatif de DOR pour le mélange de métaux, sans montrer d'indication d'interaction.

### *3. Associations entre un mélange de phénols et de phtalates, et la réserve ovarienne dans la cohorte EARTH*

Parmi les 271 femmes incluses dans cette analyse, 738 échantillons d'urine ont été collectés. Les phénols et phtalates étaient mesurés en µg/L. Dans les modèles de quantile g-computation ajustées sur l'âge (années, en continu), le niveau d'éducation (baccalauréat ou moins, baccalauréat + 2 ans, baccalauréat + 4 ans ou plus), l'IMC ( $\leq 20$ , entre 20 et 25, et entre 25 and 55 kg/m<sup>2</sup>), et la gravité spécifique, une augmentation d'un quartile dans le mélange de biomarqueurs d'exposition n'était pas significativement associée à un CFA inférieur (OR 1.10, IC 95% 0.52 - 2.30), à des niveaux de FSH du jour 3 (Beta 0.30, IC 95% -0.32 - 0.93) ou à une DOR (OR 1.02, IC 95% 0.52 - 2.05). De même, la BKMR n'a montré aucune association avec le mélange, ni d'interactions entre les substances chimiques. Malgré l'absence d'associations, ces résultats doivent être explorés chez les femmes dans d'autres études de cohortes pour les confirmer.

### *4. Associations entre l'exposition in utero aux polluants organiques persistants et les niveaux d'hormone anti-Müllerienne chez les filles de 12 ans dans la cohorte PELAGIE*

Au total, 18 POPs ont été détectés dans au moins 30% des échantillons. Aucune association significative n'a été trouvée dans les régressions linéaires multivariées ajustées sur l'âge maternelle à l'accouchement (années, en continu), niveau d'éducation maternelle à l'inclusion (inférieure au baccalauréat, baccalauréat, et éducation supérieure), et IMC maternel pré-conceptionnel ( $\leq 25$  versus  $> 25$  kg/m<sup>2</sup>), sauf pour le troisième tercile d'exposition au PCB 180, qui était significativement associé à une augmentation des niveaux d'AMH à 12 ans (Beta 0.51, IC 95% 0.02 - 0.99). Les résultats de quantile g-computation n'ont montré aucune association significative entre une augmentation d'un quartile des expositions en mélange et les niveaux d'AMH (Beta 0.04, IC 95% -0.33 - 0.41). Dans le modèle BKMR, la différence moyenne des niveaux d'AMH entre les concentrations d'exposition en mélange au 75e percentile par rapport au 25e percentile n'a pas changé de manière significative (Beta 0.04, IC 95% -0.37 - 0.46). Cette analyse BKMR n'a pas non plus montré d'effets d'interactions entre les POPs.

## SYNTHESE DES RESULTATS

Dans l'ensemble, nous avons trouvé peu d'associations entre les perturbateurs endocriniens et les marqueurs de la réserve ovarienne ou avec la DOR, que ce soit en analyse individuelle, ou en analyse par mélange. Dans l'étude AROPE, le p,p'-DDE était significativement associé à un risque accru de DOR, tandis que le  $\beta$ -HCH était significativement associé à un risque réduit de DOR. De plus, nous avons observé des risques de DOR non significativement accrus pour le cadmium et le chrome, avec une association proche de la signification pour le deuxième tercile du cadmium. Nous avons également constaté que l'augmentation du PCB 180 mesuré dans le sang de cordon ombilical augmentait significativement les taux d'AMH chez les adolescentes de la cohorte PELAGIE. Dans la cohorte américaine EARTH, les phénols et les phtalates étudiés sous forme de mélange n'étaient pas associés aux marqueurs de la réserve ovarienne (CFA, FSH ou DOR).

## CONSIDERATIONS METHODOLOGIQUES

### *Population d'étude*

Les études AROPE et EARTH ont recruté des patients dans des centres prise en charge de l'infertilité, tandis que la cohorte PELAGIE s'est concentrée sur des enfants issus d'une cohorte mère-enfant en population générale pour étudier l'impact de l'exposition prénatale sur la réserve ovarienne. Les taux de participation ont varié entre 60% pour PELAGIE et EARTH et 65-75% pour AROPE (65% pour les cas et 75% pour les témoins), ce qui correspond à la moyenne pour de telles études. Cependant, un éventuel biais de non-réponse pourrait encore exister, et l'accessibilité aux soins de fertilité différerait d'un pays à l'autre, la France offrant une couverture publique tandis que les États-Unis sont confrontés à des disparités d'accès dues à des raisons financières entre autres.

### *Variabilité*

La mesure et l'évaluation des PEs dans les études épidémiologiques sont difficiles en raison de leur variabilité et de leurs niveaux de persistance. Les POPs lipophiles dans le sérum sont considérés comme des marqueurs robustes de l'exposition à long terme, tandis que les métaux lourds avec des demi-vies plus courtes sont probablement moins certains pour mesurer l'exposition à long terme. Les substances chimiques non persistantes, avec des demi-vies très courtes, sont encore plus difficiles à évaluer, mais le regroupement des échantillons d'urine peut aider à estimer les moyennes

d'exposition. Dans les études mentionnées, les concentrations de PEs étaient généralement en accord avec les études nationales de biosurveillance.

### *Réserve ovarienne*

Dans l'étude AROPE, l'objectif était d'investiguer l'étude de la relation entre les POPs et la DOR plutôt que des indicateurs continus de la réserve ovarienne, ce qui permettait de recruter des femmes avec une réserve ovarienne plus faible. Cependant, ce choix impacte la puissance statistique avec une puissance moindre que celle avec des indicateurs continus. En revanche, la cohorte EARTH utilisait la DOR comme diagnostic mais également d'autres indicateurs continus pour évaluer les associations avec les substances chimiques non persistants, tandis que PELAGIE utilisait l'AMH comme résultat continu, car elle recrutait dans la population générale et que les seuils de DOR ne sont pas adaptés aux adolescentes.

AROPE et EARTH utilisaient tous deux des niveaux seuils pour le CFA et l'AMH basés sur le consensus de Bologne de l'ESHRE, couramment utilisé pour diagnostiquer la DOR dans les centres de fertilité. Cependant, certaines études suggèrent que ces seuils pourraient ne pas prédire avec précision la conception, avec des preuves pointant vers l'importance de la qualité des follicules plutôt que de leur quantité dans les résultats en matière de fertilité.

Quoi qu'il en soit, la DOR reste l'une des pathologies les plus fréquentes diagnostiquées chez les femmes consultant dans la prise en charge de l'infertilité (Pastore et al., 2018) et reste un enjeu majeur de santé publique en raison de son impact sur le pronostic de la FIV.

### *Mélanges*

Une force importante de toutes les études incluses dans cette thèse est l'évaluation de l'effet de mélange de plusieurs substances chimiques sur la réserve ovarienne à l'aide de deux méthodes complémentaires : la quantile g-computation et BKMR. La quantile g-computation estime comment les changements dans tous les biomarqueurs d'exposition d'un quantile affectent simultanément la réserve ovarienne, en supposant des associations linéaires sur l'échelle des quantiles et aucune interaction non additive. En revanche, la BKMR, bien qu'elle soit plus difficile à interpréter, permet à chaque exposition d'avoir sa propre relation potentiellement non linéaire avec les issues étudiées et permet d'investiguer des interactions synergiques ou antagonistes entre les expositions. BKMR peut soit corroborer les résultats de la quantile g-computation, soit mettre en évidence les cas où ses hypothèses pourraient ne pas être valables, comme la présence d'interactions ou de différentes relations fonctionnelles entre les expositions et les issues de santé étudiées.

## *Perspectives*

Afin de continuer à développer ce domaine de recherche, il serait intéressant d'explorer d'autres expositions chimiques (autres PEs, remplacements des PEs maintenant interdits, micro-nanoplastiques, solvants) en association avec la réserve ovarienne. Plusieurs facteurs pouvant impacter le système endocrinien et la réserve ovarienne pourraient aussi être pris en compte comme le stress, la dépression chronique, et l'activité physique. Ceux-ci pourraient notamment être inclus dans une étude exposomique, une méthode qui a pour but d'évaluer de manière exhaustive les effets complexes et cumulatifs des expositions environnementales sur des événements de santé. Il serait également pertinent d'utiliser une méthode 'meet-in-the-middle' en utilisant des données omiques afin d'élucider les mécanismes biologiques qui pourraient intervenir dans la relation entre PEs et la réserve ovarienne. Enfin, la mise en place d'une étude pré-conceptionnelle permettrait d'étudier des données de fertilité dans la population générale (plutôt qu'en centre d'aide à la procréation).

## **CONCLUSION**

Cette thèse avait pour objectif d'étudier l'impact des PEs sur la réserve ovarienne. Ce travail comprend les premières études sur les associations entre les POPs et les métaux lourds, et la survenue de DOR et a exploré les associations entre un mélange de phénols et phtalates et la réserve ovarienne ainsi qu'entre les POPs mesurés dans le sang de cordon et l'AMH à 12 ans. Les résultats ont suggéré la présence de certaines associations entre PEs et la diminution de la réserve ovarienne. D'autres études sont nécessaires pour valider nos résultats et explorer l'influence des PEs pendant différentes fenêtres de susceptibilité sur la réserve ovarienne à différentes étapes de la vie. Ces études devraient viser à inclure des populations plus importantes pour renforcer la puissance de l'étude, qui pourrait avoir été insuffisante dans nos analyses. Compte tenu de la nature ubiquitaire des PEs et de leurs effets potentiellement nocifs sur divers problèmes de santé, notamment la réserve ovarienne, la mise en place de mesures visant à réduire l'exposition est essentielle pour protéger la santé humaine et l'environnement.

# THE ENVIRONMENT AND FEMALE FERTILITY: A STUDY OF THE ROLE OF ENDOCRINE DISRUPTORS ON THE OVARIAN RESERVE

## INTRODUCTION

In France, the average age of first conception has risen significantly over the years. According to the Institut National de la Statistique et des Etudes Economiques (INSEE) (Institut National de la Statistique et des Etudes Economiques, 2023), it has increased from 26.5 years in 1977 to 31.0 years in 2022, and steadily continues to increase. This shift can largely be attributed to women's increasing empowerment in modern society, which includes greater access to higher education and longer studies, as well as careers with more responsibilities and leadership roles, contributing to improved socio-economic conditions for women and society as a whole. However, this trend is also supported by a lack of awareness among both men and women about the impact of age on fertility (Hammarberg *et al.*, 2017) and, for some, an overly optimistic belief that assisted reproduction treatment (ART) can resolve any issues of infertility (Hamamah and Berlioux, 2022).

Given these circumstances, it becomes crucial to address and prevent the risk of infertility at an early stage. Unfortunately, several factors associated with infertility have witnessed a surge in prevalence in recent decades, contributing to a potential increase in infertility rates over time. Notably, obesity (Branca *et al.*, 2007) and tobacco consumption in women (Janssen, 2019) appear to be on the rise. Directing public health policies on these risk factors could thus be an important lever for reducing infertility within society.

Increased exposure to human-made chemicals, especially endocrine disrupting chemicals (EDCs), has also been brought forward in recent years as a possible explanation for reduced fertility. Due to their ubiquitous nature and potential to interfere with hormones receptors, EDCs are suspected to affect the hormone-regulated reproductive systems of both women and men (Sifakis *et al.*, 2017).

Many of these chemicals have been measured in various human matrices, including blood, urine, and follicular fluid (Hart, 2016). Several studies have shown evidence of their impacts on infertility disorders such as Polycystic Ovary Syndrome (PCOS), endometriosis, or semen quality, but also on preterm birth and adverse birth outcomes (Gore *et al.*, 2015). Little attention has however been given to their potential associations with the ovarian reserve, a key component of female fertility and none, to our knowledge, to diminished ovarian reserve, a more clinically-focused indicator of the ovarian reserve. Furthermore, the research on the consequences of in utero exposure to these substances or of their effect as a mixture on the ovarian reserve is scarce.

To this end, this thesis will look at the effect of several families of pollutants considered as EDCs, as single-exposures and as a mixture, on the ovarian reserve, in different study populations. The first chapter will introduce current indicators and causes of female infertility as well as the different families of chemicals investigated. The following chapter will present the methods employed in the different studies that will be included in chapter three in the form of scientific articles. In the fourth and final chapter, we will discuss our results and explore possible future avenues of research in this field.



## CHAPTER 1: CONTEXT

### 1.1. INFERTILITY

#### 1.1.1. Definition and prevalence

Estimating global infertility rates has proven to be challenging, as estimates vary widely across studies and continents. The estimation is made especially difficult by the diverse infertility definitions used and the variable quality of population-based studies conducted in different countries. To promote consistency, the World Health Organization (WHO) has tried to standardise the definition of infertility using the following description: "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse". In contrast, fertility is not defined by a specific length of time but rather by the biological capacity to have a live birth. Fecundity is considered to be similar to fertility, though the fecundity rate refers to the average number of children per woman for a given population. Lastly, fecundability is the probability of a pregnancy, during a single menstrual cycle, in a woman with exposure to intercourse without contraception, to culminate in a live birth (Zegers-Hochschild *et al.*, 2017).

Global infertility rates estimates range from 1.9% and 16.7% (Boivin *et al.*, 2007; Mascarenhas *et al.*, 2012; Moghaddam *et al.*, 2016; Ombelet *et al.*, 2008), with some countries reaching up to 30% (more so in developing countries). A recent meta-analysis (Cox *et al.*, 2022) has attempted to pool estimates from 37 studies, actualising the number of datasets in the process compared to previous reviews. The pooled estimate of infertility prevalence was 17.5% and the pooled estimates of lifetime infertility prevalence was 12.6% within populations of reproductive age. As expected, heterogeneity coefficients were very high, but coefficients were consistent (overlapping confidence intervals (CI)) across sub-populations (region and gender) and in sensitivity analyses based on the quality of studies. However, the estimates were higher when restricting the analyses to prospective studies (21.8%) compared to retrospective studies (12.9%). Similar differences according to study designs were also found in a United States (US)-based study (Thoma *et al.*, 2013).

In France, the Observatory of Fecundity in France prospective 2007-2008 study (Slama *et al.*, 2012) has found that 24% of couples sampled from the general population (aged 18 to 44 years old) actively trying to conceive, experienced infertility. This proportion decreased to 8.3% when extending the period of observation from 12 to 24 months.

### 1.1.2. Causes of infertility

Data from clinical assessments of infertile couples also allows us to estimate the distribution of infertility causes amongst the population that has been able to pursue medical assistance. A French study (Thonneau *et al.*, 1991) reporting on the incidence and the main causes of infertility among 1686 couples evaluated that infertility was the consequence of medical disorders present in both the man and the woman in 39% of cases, in man alone in 20% of cases, in woman alone in 33% of cases, and was unexplained in 8% of cases. However, this study is potentially affected by a strong selection bias, even though the incidence of infertility found overall (15%) is in line with other estimations. It was also performed 35 years ago when the landscape of exposure to infertility risk factors was different and when some diagnostic tools were not as performant, notably for endometriosis (Pascoal *et al.*, 2022). Unfortunately, few other studies have been able to share contributions of identifiable causes of infertility (Carson and Kallen, 2021). One English study from 1984 reported comparable findings (Hull *et al.*, 1985) except for unexplained infertility (28%), whilst proportions of conditions such as tubal factors (between 11 and 67%) fluctuated widely in other studies (Audu *et al.*, 2009). Another study (Collins and Van Steirteghem, 2004) using data from 11 Canadian infertility clinics from 1984-87 also found higher unexplained infertility rates (30.7%).

#### 1.1.2.1. Male infertility

We provide here a synthesis of different causes of male infertility. These can be broadly divided into two categories: testicular infertility due to impaired semen production and post-testicular infertility due to genital tract lesions.

##### *Testicular causes*

Testicular infertility is the most common of male infertility and can be the result of congenital, acquired, or idiopathic testicular failure (Hamamah and Berlioux, 2022).

Common causes of testicular congenital defects include the Kallman syndrome, a genetic disorder causing hypogadism, and chromosomal anomalies (Klinefelter syndrome (47 XXY) and Y-chromosome microdeletions being the most frequent) (Agarwal *et al.*, 2021).

Among acquired factors, varicocele, the medical term for dilated, tortuous spermatic veins within the scrotum (Damsgaard *et al.*, 2016), is the most common cause of infertility (present in as much as 44% in men with primary infertility). Its aetiology cannot be attributed to a single factor but oxidative stress seems to play a central role. Varicocele does not necessarily need to be treated but it is more present

in men with abnormal semen (25.4%) compared to men with normal semen (11.7%) (Vander Borgh and Wyns, 2018), and can be corrected with surgery (Kirby *et al.*, 2016).

Orchitis, i.e. the swelling or inflammation of one or both testicles, is caused by viral or bacterial infections, such as mumps, sexually transmitted infections (STIs), or urinary tract infections, and can alter fertility (Agarwal *et al.*, 2021). However, medication can treat the responsible bacterial infections or ease some symptoms of viral origin.

Acquired testicular failure can also result from trauma, testicular torsion, medications, cancer (mainly leukaemia, Hodgkin's lymphoma, and testicular germ cell tumors) or surgery that may damage the testis (Sharma *et al.*, 2020).

#### *Post-testicular causes*

Post-testicular deficiency is due to either ejaculatory dysfunction or obstruction to sperm delivery but the obstructions located in the epididymis (inside the scrotum) are most common (Vander Borgh and Wyns, 2018). In addition, vas deferens, the tube that transports mature sperm from the epididymis to the urethra, can be absent due to congenital gene mutations, or be damaged during an infection, a vasectomy or a hernia repair (Agarwal *et al.*, 2021). Lastly, ejaculatory duct obstruction is found in 1–3% of post-testicular causes as a result of cystic obstructions or from an inflammatory reaction.

#### *Sperm quality*

About 30-50% of male infertility cases have no discernible cause and may be due to altered sperm quality (Agarwal *et al.*, 2021). In 2010, the WHO (Cooper *et al.*, 2010) established reference values to assess sperm quality. These include semen volume (1.5 mL), total sperm number (39 million per ejaculate), sperm concentration (15 million per ml), vitality (58% live), progressive motility (32%), total (progressive and nonprogressive) motility (40%), and morphologically normal forms (4.0%). They were established using semen samples from over 4,500 men based in 14 countries on four continents whose partners had a time-to-pregnancy (TTP) lower than 12 months.

Risk factors of altered semen parameters include oxidative stress, age, ethnicity, cigarette, alcohol, recreational drugs, obesity, psychological stress, as well as environmental or occupational exposure to toxic chemicals (Agarwal *et al.*, 2021).

#### 1.1.2.2. Female infertility

Female infertility conditions are usually separated into different sections including ovulatory disorders, tubal occlusion, uterine and cervical factors, and reduced ovarian function (Carson and Kallen, 2021) which we will synthetically describe here.

### *Ovulatory disorders and PCOS*

In 1973, the WHO provided a classification of infertile women with anovulation which is still in use today (Dhont, 2005). This scale ranges from primary or secondary amenorrhoea from central origin (Group 1), anovulation associated with a variety of menstrual disorders (including amenorrhoea) (Groupe 2), to primary or secondary amenorrhoea due to primary ovarian failure (Group 3). Group 2, which represents the largest group of patients, is further divided into different categories including one dedicated to PCOS patients.

PCOS is a heritable condition characterised by irregular (oligoovulation) or an absence of ovulation (anovulation), clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries. Two out of three of these criteria are considered sufficient by the European Society of Human Reproduction and Embryology (ESHRE, 2004) to establish a PCOS diagnosis. PCOS results in infertility 40% of the time and affects 4–8% of women of reproductive age (Sirmans and Pate, 2013), it is therefore considered a major contributor to the proportion of infertility due to ovulatory disorders. PCOS also increases the risks of developing diabetes, and possibly cardiovascular diseases, and endometrial cancer (ESHRE, 2004).

### *Tubal occlusion and endometriosis*

Tubal occlusion, the consequence of either blocked fallopian tubes or the inability of the tubes to pick up an oocyte from the ovary due to pelvic adhesions, can be the result of multiple conditions including STIs, endometriosis, peritubal adhesions, and hydrosalpinx, and can necessitate a surgical intervention (Carson and Kallen, 2021). Endometriosis affects up to 10% of women in the general population and between 25 and 50% of women with infertility (Becker *et al.*, 2022; Ozkan *et al.*, 2008). This condition, in which endometrium-like tissue and/or stroma (lesions) is present outside the uterine cavity, can not only impair the tubes, but also distort the uterine cavity resulting in uterine infertility. Endometriosis has a highly heterogeneous presentation of endometriotic lesions, multisystem symptom presentation (chronic pain, dysmenorrhoea, dyschezia, dyspareunia, dysuria, fatigue, depression), and comorbid conditions. Endometriosis exists as three non-exclusive subtypes: superficial peritoneal endometriosis (representing 80% of endometriosis cases), ovarian endometriosis, and deep endometriosis, but many other systems of classifications have been suggested (Horne and Missmer, 2022). Diagnosing endometriosis is a multi-step process that requires imaging technology and invasive procedures (laparoscopy) to have higher diagnostic accuracy (Becker *et al.*, 2022). The difficulty of diagnosing results in a situation where almost 60% of women will see three or more clinicians and will experience an average of seven years of symptoms before a diagnosis is made (Horne and Missmer, 2022).

### *Uterine and cervical factors*

Conditions that can distort the uterine cavity include endometrial polyps, fibroids, intrauterine synechiae, and congenital uterine malformations (Venetis *et al.*, 2014). However, surgery to correct uterine cavity defects is commonly performed to improve reproductive outcomes, though more research is needed to measure the effectiveness of the procedure. Cervical factor infertility, defined as an anatomical abnormality, post-surgical scarring, or decreased cervical mucous that interferes with the natural progression of sperm into the lower part of the uterus, can be caused by cervical stenosis (narrowing or closing of the passageway through the cervix) as a result of surgery or by conisation (a cancer-related biopsy of tissue from the mucous membrane of the cervix) (Carson and Kallen, 2021).

### *Reduced ovarian function*

Finally, reduced ovarian function result from the depletion of the ovarian reserve, which we will explore in a dedicated section along with a description of its modifiable factors.

#### 1.1.2.3. Unexplained infertility

Unexplained or idiopathic infertility is declared when all standard fertility test results are in normal ranges. These tests usually involve a clinical exam of both partners, semen analysis, tubal patency tests, and an assessment of ovulation and of the ovarian reserve (Gelbaya *et al.*, 2014), however, tests and their threshold values can vary depending on the country and occasionally on the fertility centre. Nonetheless, the two most important predictive factors remain the duration of infertility and the age of the woman trying to conceive.

Fortunately, several treatment procedures have been developed to help compensate for some of these pathologies and disorders and increase the likelihood of pregnancy.

#### 1.1.3. Assisted reproductive technology

##### 1.1.3.1. Definition and procedures

For women with anovulation, the initial treatment recommendation is usually an artificial ovulation induction with timed intercourse (Carson and Kallen, 2021). However, for many other fertility disorders, a lack of viable treatments often means couples need to recourse to ART.

ART includes fertility treatments in which eggs or embryos are handled in a laboratory (Sunderam *et al.*, 2022). These include in vitro fertilisation (IVF), gamete intrafallopian transfer, and zygote intrafallopian transfer, but more than 99% of procedures performed are IVF. The French Biomedicine

Agency's definition of ART also includes intrauterine insemination (Agence de la Biomédecine, 2016). Because an ART procedure consists of multiple steps over several weeks, a procedure is often referred to as a cycle.

Generally, for couples with unexplained infertility, women with endometriosis or men with mild infertility factors, an initial three to four cycles of ovarian stimulation with intrauterine insemination can be proposed. This method involves collecting and preparing sperm from the male partner or a donor to directly inject it into the uterus of the woman in synchronization with natural or induced ovulation. This practice accounts for approximately two thirds of medically assisted procreation attempts in France (Agence de la Biomédecine, 2016).

If these approaches do not result in pregnancy, IVF is considered. Immediate IVF can also be advised in cases of severe male factor infertility, DOR, or untreated bilateral tubal factor infertility (Carson and Kallen, 2021). An IVF cycle usually begins with a drug-induced ovarian stimulation which varies according to the protocol used. If oocytes are produced and reach a certain size, an intracytoplasmic sperm injection (ICSI) in which a single sperm is injected directly into the oocyte is performed. This method constitutes today the majority of IVF procedures (INSERM, 2012). However, if an ICSI is not used, suitable oocytes are retrieved from the ovaries and combined with sperm in a laboratory in vitro. If a successful fertilisation occurs, the most viable are selected for transfer back into the uterus. If an embryo implants in the uterus, a clinical pregnancy is diagnosed by the presence of a gestational sac detectable by ultrasound. When retrieving eggs, it has also become possible to freeze oocytes to use for future cycle attempts (Sunderam *et al.*, 2022).

In France, a frozen embryo can be donated to the couple seeking a pregnancy when all IVF attempts have failed, infertility is present in both partners, a genetic condition could be transmitted to the child, and/or if a woman with no male partner is infertile (Assurance Maladie, 2023a).

#### 1.1.3.2. The evolution of ART

Since the birth of the first IVF baby 35 years ago (Inhorn and Patrizio, 2015), IVF treatments have skyrocketed. The total number of assisted reproduction treatment (ART) cycles (4-to-6 week) have multiplied by close to five in Europe between 1997 (203 225 cycles) and 2017 (940 503 cycles) (Wyns *et al.*, 2021). The number of reported procedures in the US has tripled since 1996, and the number of infants born from ART procedures has almost quadrupled (Centers for Disease Control and Prevention, 2021). In France, where people are covered for up to four ART procedures by the national health insurance, at least one in 100 women, or more than 150 000 women, aged 20-49, are treated for infertility every year (Ben Messaoud *et al.*, 2020), with the mean age of treated women increasing from 33.0 years in 2008 to 33.7 years in 2017.

Even if many improvements have been made in the field of assisted reproductive technology over the years, success rates remain moderate. In European countries, pregnancy rates range from 18.4% to 42.3% and delivery rates from 10.4% to 40.9% in fresh cycles after IVF attempts (Wyns *et al.*, 2021). IVF rates also get considerably lower as a woman's age increase, with women between 38 and 40 having less than half the success of women under 35 (Centers for Disease Control and Prevention, 2021).

Moreover, the direct costs of one fresh ART treatment cycle fluctuate between 2,500 and over 11,000 euros depending on the country (Connolly *et al.*, 2010), which can represent a considerable weight on national health budgets when ART procedures are covered by the state or on a couple's personal budget if they are not. IVF can also be a considerable physiological and emotional burden for women (Boivin and Takefman, 1996; Verhaak *et al.*, 2007).

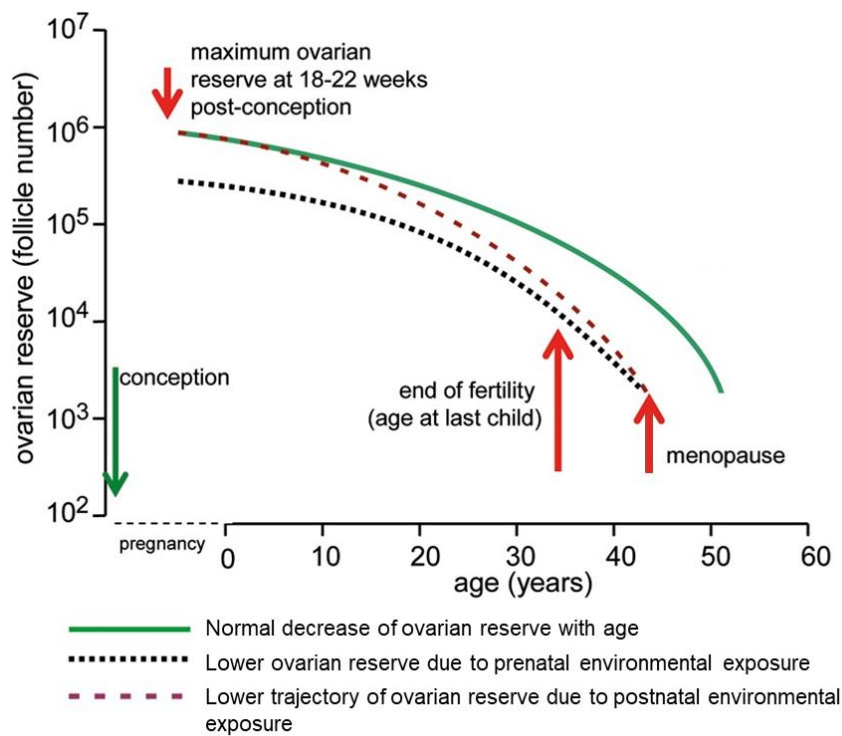
For these reasons, research efforts also need to be directed to preventing infertility causes such as a low ovarian reserve.

## **1.2. THE OVARIAN RESERVE**

### **1.2.1. The ovarian reserve and its importance in the female reproductive system**

The ovarian reserve is the total number of primordial follicles in both ovaries, though this definition is sometimes extended to include intermediate and primary follicles as well (Hansen *et al.*, 2012). These three types of follicles can all be referred to as unilaminar follicles. The ovarian reserve is a finite pool that cannot replenish itself and from which the reproductive system will draw from over the reproductive life (Gura and Freiman, 2018) to produce oocytes. The ovarian reserve is thus directly connected to a woman's ability to reproduce.

The ovarian reserve is constituted in utero where it can reach up to seven million follicles (Figure 1) but decreases thereafter as women age towards menopause (Hansen *et al.*, 2008; Richardson *et al.*, 2014). At birth it is already reduced to one to two million. This fall continues until puberty (300 000 follicles), a point from which the decline slows down. The number of follicles then progressively decreases to reach 1000, triggering menopause at the age of 50 on average (Faddy and Gosden, 1996; Wallace and Kelsey, 2010).

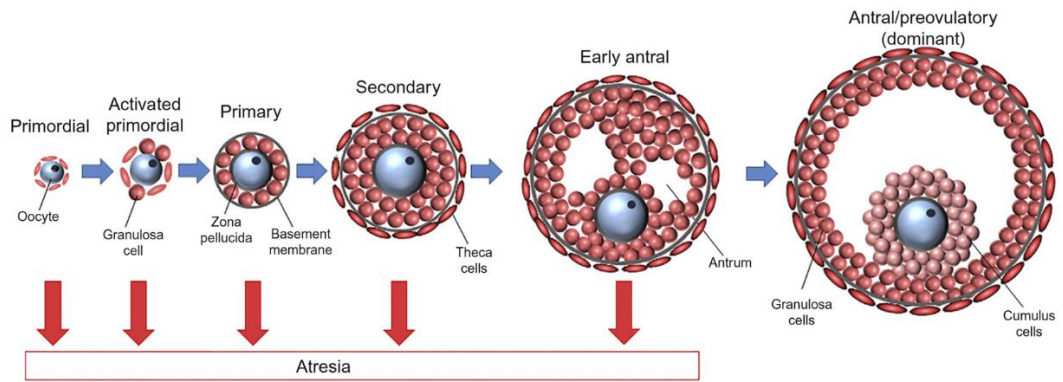


**Figure 1. The evolution of the ovarian reserve with age  
(adapted from Richardson et al., 2014)**

However, the ovarian reserve can deplete faster than the norm throughout life (red dashed line). It can also decrease as a result of damage suffered during different periods of vulnerability. For example, if the ovarian reserve does not constitute itself properly in utero, it will reach a minimal critical level in adult life than for regularly constituted ovarian reserves (black dotted line).

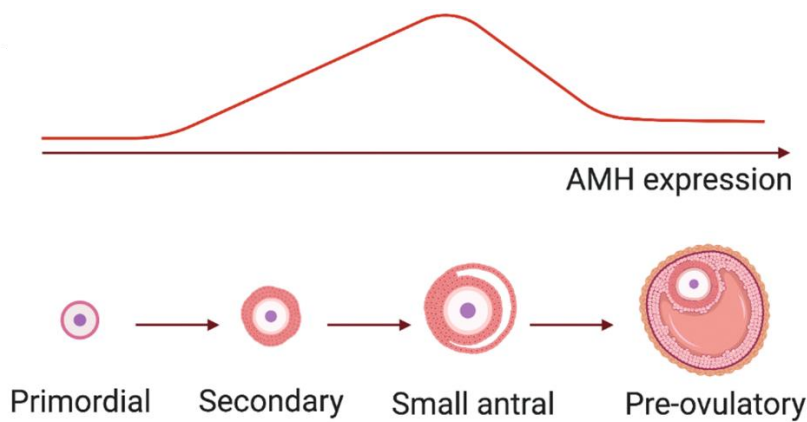
Each ovarian follicle contains an oocyte (Figure 2). At the primordial stage, this oocyte is surrounded by scaled granulosa cells which are responsible for producing hormones including estrogen and progesterone. Follicles become primary follicles when these granulosa cells transform into cuboidal cells, a process that is controlled by locally produced growth factors (Williams and Erickson, 2000). The evolution into secondary follicles is then characterised by the appearance of a follicular antrum within the granulosa layer and of more layers of granulosa cells surrounded by a basement membrane. Throughout these stages, several mechanisms occur, including the enlargement and proliferation of granulosa cells, the enlargement of the oocyte, the formation of a basement membrane surrounding the granulosa cells, and the formation of the zona pellucida which is necessary for the future penetration by the sperm cell around the membrane of the oocyte.





**Figure 2. Representation of the different stages of folliculogenesis (adapted from Findlay et al., 2019)**

Follicles then move into the antral phase, when the antrum is formed and filled by follicular fluid to grow the oocyte (Brinca *et al.*, 2022). Only some of these follicles will be selected to enter the final growth phase to become dominant (Graafian) follicles. This selection is part of a process of recruitment inhibition made possible by the anti-Müllerian hormones (AMH) produced in the follicles (Pellatt *et al.*, 2010). It intervenes mostly in the preantral and antral stages (Figure 3) to select some follicles which will become dominant.



**Figure 3. Levels of anti-müllerian hormones in relation to different stages of folliculogenesis (adapted from Moolhuijsen and Visser, 2020)**

This process is key to prevent all follicles to grow at one time and maintain a large enough stock of follicles to last several decades. When AMH is too active, as it can be the case in PCOS, follicles are not recruited to become dominant, prompting an absence of ovulation (Tal and Seifer, 2017). The mechanism by which AMH inhibits the recruitment of follicles is mostly unknown (Hannon and Curry, 2018) but could involve lowering their sensitivity to the follicle-stimulating hormone (FSH) to levels too low to sustain continued growth (Grynnerup *et al.*, 2012). Once they reach the dominant stage, follicles

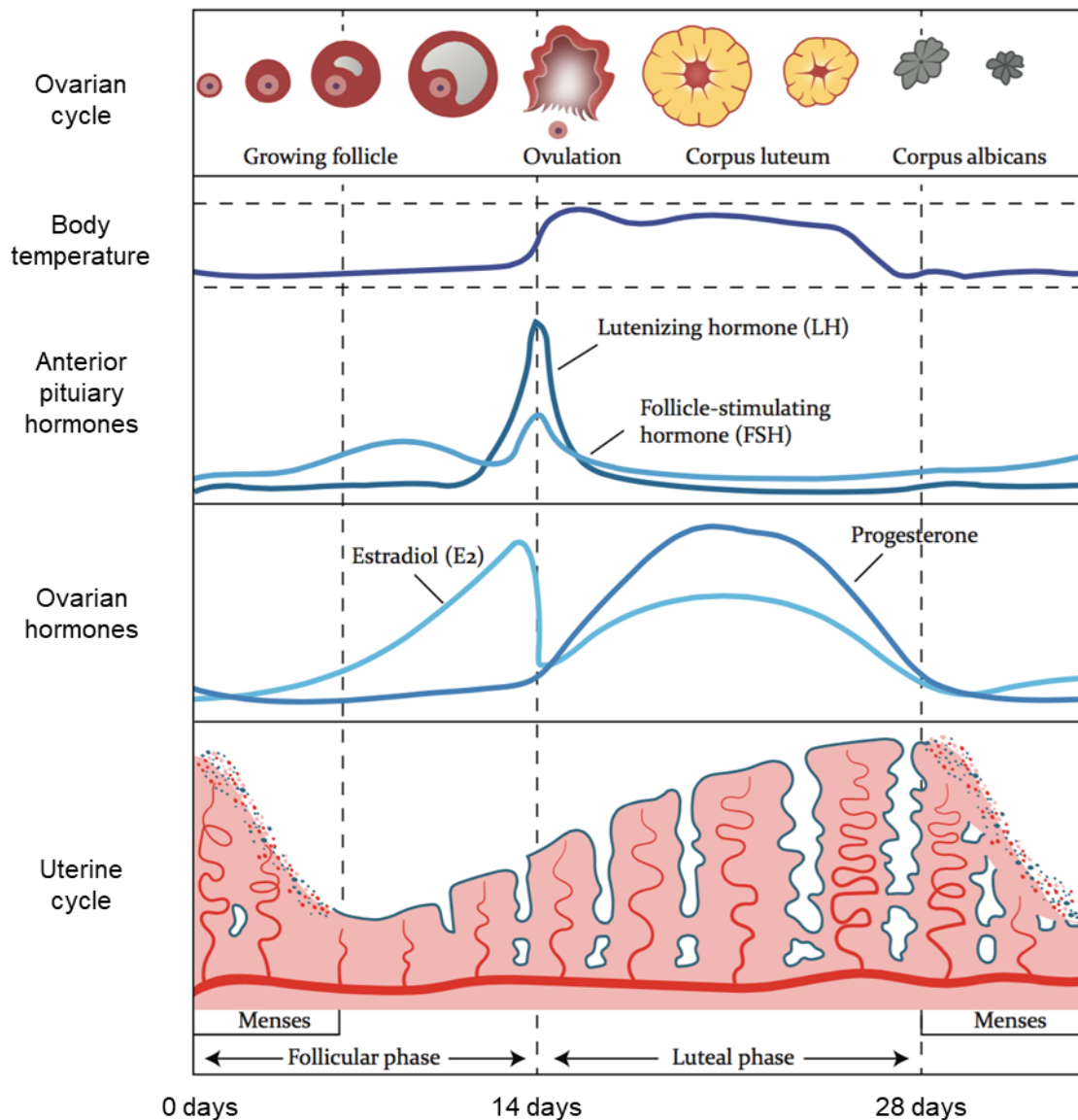
are characterised by a greater capacity to produce estradiol, a key estrogen steroid hormone, and by the appearance of luteinizing hormone (LH) receptors on the granulosa cells.

At each menstrual cycle, only one of the several primordial follicles will complete development and reach ovulation through the release of the oocyte into the fallopian tubes ready for fertilisation. The other follicles then degenerate through atresia, and granulosa cells undergo apoptosis (cell death) and are replaced by fibrous material (Gura and Freiman, 2018).

The process of transformation from primordial to preovulatory follicles is known as folliculogenesis, and is controlled by the cells within the follicle as well as the endocrine system (Hannon and Curry, 2018). It can take up to one year for follicle to develop from the primordial stage to ovulation (Williams and Erickson, 2000). Throughout this evolution, some primordial follicles will die before entering the growth stage and so will all selected follicles except preovulatory follicles. Folliculogenesis begins at puberty and will contribute to the ovulation of around 400 to 500 oocytes in total (i.e. 1 follicle in a 1000) between then and the end of the reproductive life (Findlay *et al.*, 2019).

Whilst the development of a follicle to reach the antral stage can take several months, the selection of dominant follicles within the pool of antral follicles takes place during the follicular phase (14 days on average) of the menstrual cycle (Figure 4). During this phase, the FSH produced by the pituitary gland (gland located at the base of the brain below the hypothalamus gland) increases the levels of aromatase in granulosa cells and theca cells, converting androgens into estradiol, and giving rise to a dominant antral follicle. Estradiol also negatively feeds back to the hypothalamic-pituitary axis causing a decrease in FSH levels. A decrease in FSH then leads to atresia of the subordinate follicles (Richards, 2018).

The dominant follicle is now producing threshold levels of estradiol that positively feedback to the hypothalamic-pituitary axis initiating the ovulatory LH surge. The rapid rise in LH induces a cascade of events in the follicle, including meiotic resumption in the oocyte, cumulus granulosa cell expansion, breakdown of the follicular wall, and differentiation of follicular cells to luteal cells. These events lead to ovulation and the transformation of the follicle into a shell called the corpus luteum.



**Figure 4. Ovarian, hormonal and uterine events during the menstrual cycle and pregnancy (from Richards, 2018)**

After ovulation, the remaining parts of the dominant follicle transform into the corpus luteum which produces estrogen and progesterone to signal the suppression of the production of the FSH (Devoto *et al.*, 2009). This will cause the corpus luteum to atrophy and disappear 9 to 11 days after ovulation, allowing the hypothalamic-pituitary axis to increase FSH levels again to start a new cycle.

### 1.2.2. Measuring the ovarian reserve

Because the ovarian reserve is a key determinant of female reproductive health, it is important to measure it as accurately as possible. Several methods have been developed and used in the last decades for that purpose.

The first test was introduced more than 30 years ago (Tal and Seifer, 2017) and was based on early (day 2-5) FSH levels. This is based on the principle that when the follicular pool declines, the early follicular FSH increases because there is an insufficient production of estradiol and inhibin B to fully inhibit FSH production. FSH measurements are therefore often combined to estradiol levels to enhance the sensitivity of the test because FSH can increase estradiol levels in women with a low ovarian reserve, which in turn lowers FSH levels to normal ranges (Ulrich and Marsh, 2019).

In the following years, the clomiphene citrate challenge test, gonadotropin releasing-hormone agonist, and inhibin B were suggested as measures of the ovarian reserve but were rapidly discredited for having poor predictive values of the ovarian reserve and/or being too costly (Tal and Seifer, 2017).

The antral follicle count (AFC), made possible via a transvaginal ultrasound, was then proposed. This test is a direct quantitative measurement in both ovaries of the antral follicles which measure between 2 and 10 mm in diameter (Broekmans *et al.*, 2010). It is recommended to perform this count between days 2 and 4 of a menstrual cycle, as the number of antral follicles fluctuates throughout the cycle.

The last method of ovarian assessment relies on the serum concentrations of AMH levels. This was suggested because AMH levels correlate highly with the number of growing follicles and decreases over time along with the ovarian reserve (Moolhuijsen and Visser, 2020).

The three tests (Table 1) still used today (FSH, AFC and AMH) are all indirect evaluations of the ovarian reserve, making the accuracy of these tests difficult to compare. However, AFC and AMH have better clinical value than FSH, especially since AMH declines before FSH rises (Ulrich and Marsh, 2019). AFC is a direct evaluation of the primordial pool while AMH is produced by preantral and small antral follicles. AFC and FSH have high intra-cycle variability, and AFC and AMH have better inter-cycle variability than FSH.

Moreover, AMH assessment suffers from comparability issues due to a diversity of standardised assays and AFC requires an invasive procedure and can be affected by inter-sonographer variability. AFC and AMH seem to have the best predictive values for poor response to ovarian stimulation using ART but FSH and AMH have slightly better predictive values for predicting the likelihood of conceiving using ART, though their sensitivity is pretty low overall (Broekmans *et al.*, 2006; La Marca *et al.*, 2010; Tal and Seifer, 2017). For these reasons and while admittedly not perfect, the ESHRE has established that the preferred measurement methods to study the ovarian reserve are the AFC and the measurements of AMH levels (Ferraretti *et al.*, 2011). This is further corroborated by a survey of 796 fertility centres across the world that indicated that 51% of centres considered AMH was the best test for measuring the ovarian reserve, 40% for AFC and FSH was trailing with 6% (Pastore *et al.*, 2018).

**Table 1. Comparison of ovarian reserve markers**

<b>Criteria</b>	<b>FSH</b>	<b>AFC</b>	<b>AMH</b>
<b>Timing in the menstrual cycle</b>	Day 2-5	Day 2-4	Anytime
<b>Methodology</b>	Blood sample	Endovaginal ultrasound	Blood sample
<b>What is measured</b>	Antral follicle pool	Small antral follicle pool	Preantral and small antral follicle pool
<b>Sensitivity for POR, %</b>	11-86	9-73	44-97
<b>Specificity for POR, %</b>	45-100	73-97	41-100
<b>AUC for POR</b>	0.68 (95% CI 0.61-0.74)	0.76 (95% CI 0.70-0.82)	0.78 (95% CI 0.72-0.84)
<b>Sensitivity for nonpregnancy, %</b>	3-65	7-34	19-66
<b>Specificity for nonpregnancy, %</b>	50-100	64-98	55-89
<b>Advantages</b>	-Widespread use	-Immediate results, -Good predictive value for stimulation ovarian response	-Reliable, -High POR sensitivity, -Good predictive value for stimulation ovarian response
<b>Limitations</b>	-Reliability, -Low sensitivity, -Dependent on functional HPO axis, -Less precision due to intercycle and intracycle variability -Less relevant at younger ages	-Invasive procedure, -Interobserver variability (sonographer-dependent), -Requires cost of ultrasound technician and availability of ultrasound machine	-Lack of international standardized assay, -Requires careful sample preparation and storage

Adapted from Tal & Seifer, 2017.

FSH, follicle-stimulating hormone; AFC, antral follicle count; AMH, anti-mullerian hormone; POR, poor ovarian response; AUC, area under the curve; CI, confidence interval; HPO, hypothalamus-pituitary-ovarian.

More than one ovarian reserve test is often performed in a clinical setting, sometimes resulting in discordant results up to 35% of the time (Tal and Seifer, 2017). In this case, it is possible to use intermediate values between the tests (e.g. when establishing an IVF dosing), however, several studies have found that including two tests was not needed to improve the accuracy of predictive models (Penzias *et al.*, 2020). Others also combine the results of both tests or even three tests (including FSH) (Tian *et al.*, 2023).

### 1.2.3. Diminished ovarian reserve

We can measure the ovarian reserve with relative accuracy but what does it mean for the patient? When is there not enough follicles to reproduce? To answer these questions, reproduction experts

have tried to establish a threshold of ovarian reserve called DOR under which the response to ovarian stimulation or fecundity is reduced (Practice Committee of the American Society for Reproductive Medicine, 2015). This is a normal state for women when they reach the mid-40s but is pathologic at younger ages.

DOR is a particularly crucial public health matter due to its impact on ART in the context of an increasing of age at first conception and due to the absence of a validated treatment to regenerate the ovarian reserve.

#### 1.2.3.1. DOR evaluation

Various cut-off values for DOR exist in the literature. A 2015 review (Cohen *et al.*, 2015) found 13 definitions of DOR among 121 articles with 107 of these rejected for not giving a clear definition of DOR. As a conclusion, the authors suggest using the 2012 ESHRE consensus values coined “the Bologna criteria” used for poor ovarian response (POR) as an a priori definition of DOR (AFC < 7 or AMH ≤ 1.1 ng/mL) but excluding the criteria of a previous POR and an age over 40. This would help standardised a definition and facilitate comparisons between studies. However, not necessarily all studies have the capacity to measure both the AFC and AMH levels, and some may wish to avoid measuring the AFC due to its invasive nature.

Even among clinicians (Pastore *et al.*, 2018), the term DOR is often confused with other ovarian conditions including primary ovarian insufficiency (POI) formerly called premature ovarian failure or premature menopause, and POR. POI is defined as the loss of ovarian activity before the age of 40 (European Society for Human Reproduction and Embryology (ESHRE) Guideline Group on POI *et al.*, 2016). It is similar to DOR because it results in a depleted ovarian reserve but differs from it in the fact that women with POI experience a cessation of menses for at least 4 months along with post-menopausal levels of FSH > 25 IU/l on two occasions more than four weeks apart (Park *et al.*, 2021). There is a potential overlap between the two conditions, since in theory DOR could precede POI, but no connection has been statistically documented yet (Christin-Maitre, 2023). Poor response to IVF stimulation (POR), defined as having at least two of the following three characteristics: maternal age ≥ 40; a previous POR such as a history of cycle cancelation or fewer than four oocytes retrieved after gonadotropin stimulation; and an abnormal ovarian reserve test (Ferraretti *et al.*, 2011). Consequently, POR patients can present with DOR but DOR patients don't have a POR yet, although ovarian reserve tests have been used to predict POR, as mentioned above.

### 1.2.3.2. Prevalence

Little is known about the prevalence of DOR in the general population. However, a couple of studies have analysed the prevalence of DOR in ART cycles from clinics based in the US. The first study found that the prevalence of a DOR diagnosis as clinically assigned (DOR definitions were not available) in 181,536 fresh ART cycles in 2004 was 19% and 26% in 2011 (Devine *et al.*, 2015). They also found a decrease incidence of POR between both periods, and determined that 69% of stimulated cycles assigned as DOR failed to meet the Bologna definition for poor response, suggesting either an over-diagnosis of DOR or a lack of correlation between DOR and POR. The second study (Butts *et al.*, 2013) analysed 182,779 fresh initial ART cycles between 2004–2007 in women up to age 40 and identified DOR as clinically assigned (DOR definitions were not available) was diagnosed in 13.9% in states with no ART insurance mandates and 10.3% in states with ART insurance mandates. They also observed an increase in DOR diagnosis throughout the years, which they attribute to a growing use of ovarian reserve tests. Unfortunately, both studies suffer from a measurement bias due to the absence of a homogenous DOR definition. DOR prevalence in the population is most likely lower than the prevalence in an ART population, although fertility clinics in the US often over-represent women with high income due to the nature of their heavily privatised medical insurance system. Women who want to conceive but never visit a fertility centre are also excluded from the calculations.

### 1.2.4. Ovarian reserve treatments

Treatments that either increase the number of follicles and/or improve the quality of oocytes are being researched, however, some techniques are showing more promising results than others.

#### 1.2.4.1. Mitochondrial transfer

Several methods for mitochondrial supplementation have been employed to improve the integrity, functionality, and quantity of mitochondria in oocytes with compromised quality (Jiang and Shen, 2022). Mitochondria play a crucial role in providing cellular energy through adenosine triphosphate (ATP) production and are involved in several cellular processes such as ion fluxes and management of reduction-oxidation status, which are essential for oocyte development. However, the development of this technology is hampered by biased study design, methodological issues, and a lack of information from animal models (Jiao and Bukulmez, 2021).

#### 1.2.4.2. Activation of primordial follicles

Another strategy to counter DOR relies on activating and growing residual dormant primordial follicles to generate mature oocytes. Clinical studies have shown that methods such as intraovarian autologous platelet-rich plasma infusion can enhance ovarian reserve parameters, leading to an increase in the number of mature oocytes, fertilisation rates, and the production of high-quality embryos (Jiao and Bukulmez, 2021). However, further high-quality randomised controlled trials are necessary to accurately assess the efficacy of this technique in terms of clinical pregnancy rates and live birth rates. Furthermore, bone marrow mesenchymal stem cell therapy has been proposed as a way to produce and secrete a variety of cytokines, chemokines, and growth factors, which may be involved in tissue repair and could activate dormant follicles. Because stem cells are cell types that are insufficiently differentiated and immature, and capable of self-renewal, they can proliferate and differentiate into various tissues and organs (Huang *et al.*, 2022). Clinical findings suggest that stem cell transplantation improves ovarian function, as demonstrated by the resumption of menstruation, regulation of hormone levels, and restored fertility in some patients. As shown by the high number of clinical trials registration in various countries, this therapy is rapidly gaining interest even though many details need to be improved and explored (Umer *et al.*, 2023).

#### 1.2.4.3. In vitro growth and maturation of oocytes

The idea of primordial follicle growth in vitro is to retrieve a primordial follicle and bring it to maturity in vitro before fertilisation and implantation (Labrune *et al.*, 2022). Nevertheless, isolating and culturing primordial follicles in humans poses significant challenges due to their small size, the limited interconnections between granulosa cells and oocytes, and the intricate extracellular matrix environment in which they develop. Many methodological challenges therefore need to be overcome before this method can be considered for clinical use.

#### 1.2.4.4. Generating new oocytes

Other approaches are looking at a way of creating new oocytes from various types of stem cells including embryonic stem cells, induced pluripotent stem cells or germline stem cells. In theory, these could be used to generate granulosa cells which could then be used to build new follicles that could subsequently be transplanted back into the ovary or combined with IVF protocols (Jiao and Bukulmez, 2021). Some studies have reported achieving the first step but these cells have not been converted into competent oocytes yet.



#### 1.2.4.5. Conclusion

As shown, many avenues of treatments are being explored, from the boosting of follicle mitochondria and of follicle maturation via bone marrow mesenchymal stem cell therapy, to in vitro folliculogenesis, to neo-oogenesis. Unfortunately, all are work in progress and will required several years at minimum to become true ovarian reserve treatments. An alternative consists in preventing a future need in healthy oocytes by freezing some ahead of infertile problems. In France, oocyte preservation is free without a medical justification between the ages of 29 and 37 (Assurance Maladie, 2023b). This method is specifically useful to patients about to receive cancer treatment but is not scalable to the entire population. The lack of effective treatments of the ovarian reserve highlights the importance of reducing exposure to all infertility risk factors including EDCs.

#### 1.2.5. Known ovarian reserve modifiable factors

Age is naturally an important risk factor of the ovarian reserve (Richardson *et al.*, 2014), but other factors, including genetic, medical and lifestyle factors, can also impact the ovarian reserve (Table 2).

**Table 2. Summary of ovarian reserve modifiable factors based on a review of the literature**

Factor	Effect on the ovarian reserve	Degree of impact	Confidence levels of the effect
Age	Decrease	High	Very high
Turner syndrome	Decrease	Very high	Very high
Fragile X syndrome	Decrease	High	Very high
Ethnicity	Lower in black and Hispanic women compared to Caucasian women	Low	Medium
Age at menarche	Inconsistent	Low	Low
Chemotherapy	Decrease	High	Very high
Radiotherapy	Decrease	Very high	Very high
Oral contraception	Inconsistent	Low	Low
Endometriosis	Decrease depending on type	High	High
Polycystic Ovary Syndrome	Increase	Very high	Very high
Body mass index	Lower in higher BMI, especially in obese patients	Medium	Medium
Tobacco	Decrease	High	Very high
Alcohol	Decrease for weekly binge drinking only	Low	Low
Air pollution	Decrease	Low	Medium
Temperature	Decrease	Low	Low

### *Genetic background*

Animal and human studies have identified genetic factors that play a role in folliculogenesis including one gene mutation (FMR1), seven genes differentially expressed (AMH, LHCGR, IGF1, IGF2, IGF1R, IGF2R and GREM1) and three polymorphisms (on genes GDF9, FSHR, and ESR1) (Greene *et al.*, 2014). In addition, several genetic syndromes are known to affect the ovarian reserve. The Turner syndrome, the most common sex chromosome abnormality in women, will often have their ovarian reserve depleted before reaching adulthood (Oktay *et al.*, 2016). Most of them will further develop POI and be diagnosed as infertile. The fragile X syndrome, on the other hand, is the most common cause of inherited intellectual disability in males, but can also develop in women and cause an early decay of follicles. Whilst the prevalence of DOR in women with fragile X is not known, up to 20% will develop POI (Man *et al.*, 2017).

### *Ethnic background*

Populations from different ethnic backgrounds have exhibited different levels of ovarian reserve, independently of common risk factors. Trends amongst 33 studies on the topic show that AMH levels tend to be lower in black and Hispanic women compared to Caucasian women (Seifer *et al.*, 2009). They also tend to be higher in Chinese women compared to Caucasians under the age of 25 but lower after 25 (Kotlyar and Seifer, 2021), though there were no difference found between South Asian and Caucasian women (Iglesias *et al.*, 2014). Nevertheless, more studies are needed to confirm whether these differences are indeed related to genetics or are an indirect consequence of cultural and/or environmental factors.

### *Age at menarche*

Furthermore, there are conflicting results (Bragg *et al.*, 2012; Schuh *et al.*, 2019) on the associations between early age at menarche and the ovarian reserve, independently of age. Early age at menarche could impact later levels of ovarian reserve because its decline tends to be steeper during late childhood/early adolescent years. An alternative hypothesis states that higher estrogen levels results in larger follicles pools and a lower age at menarche, resulting in a high ovarian reserve later in life.

### *Chemotherapy and radiotherapy*

The use of chemotherapy and radiotherapy in the treatment of cancer, especially at a young age, can also have effects on the ovarian reserve. Female cancer survivors are approximately 38% less likely to conceive after a cancer treatment. Follicles are extremely sensitive to radiation and 90% of patients with total abdominal irradiation are diagnosed with POI later on (Anderson and Wallace, 2013). A recent review on the effects of chemotherapy on the ovary (Spears *et al.*, 2019) found that the three

most commonly used chemotherapeutic drugs (cyclophosphamide, cisplatin and doxorubicin) cause a loss of follicles resulting in POI by inducing death and/or accelerated activation of primordial follicles and increased atresia of growing follicles. One study (Larsen *et al.*, 2003) also found a dose-dependent loss in number of antral follicles based on severity of treatments combining chemotherapy and radiotherapy in childhood cancer survivors.

#### *Oral contraception*

Due to the influence of oral contraceptives on FSH, several studies have questioned whether their use could result in a lower ovarian reserve. However, results have been inconsistent (Jung *et al.*, 2017), with some of the studies showing a significant decrease in AMH levels also reporting a reverse trend shortly after the discontinuation of oral contraceptives. This supports the notion that effects of oral contraceptives on AMH levels are reversible. Indeed, the decrease in FSH from oral contraceptives could be suspending follicular growth, which in turn decreases AMH levels while preserving the pool of follicles overall and allowing the AMH levels to go back to normal levels when FSH suppression is removed by the end of oral contraceptive intakes (Dólleman *et al.*, 2013).

#### *Female reproductive disorders*

Several female reproductive disorders are known to be associated with the ovarian reserve. Endometriosis has been associated with a decrease in ovarian reserve in various studies (Chiang *et al.*, 2015; Kitajima and Masuzaki, 2014). However, these associations could possibly be due to endometrioma surgery and not necessarily due to endometriosis itself (Streuli *et al.*, 2012). Ovarian and tubal surgeries (e.g. salpingectomy, cystectomy) can have an effect on ovarian reserve levels depending on localisation (unilateral or bilateral) and size (Henes *et al.*, 2018; Noventa *et al.*, 2016), possibly as a consequence of a reduction of the ovarian tissue. Nonetheless, the presence of endometrioma in ovaries has also been associated with a decreased AFC (Tian *et al.*, 2021) and AMH (Muzii *et al.*, 2018) independently of surgeries. Lastly, PCOS results in 2 to 4 times higher levels of AMH as a result of an overexpression of AMH by the granulosa cells (di Clemente *et al.*, 2021).

#### *Parity*

Some studies have investigated whether parity had an effect on the ovarian reserve and found conflicting results (La Marca *et al.*, 2012; Moini *et al.*, 2016). Whilst most of them indicate some form of selection bias or methodological misgivings, the hypothesis of causality between parity and the ovarian reserve raises questions. Multiparity demonstrates a capacity to reproduce some time ago, which indicates a healthy ovarian reserve. The issue of reverse causality therefore needs to be

addressed before we can ascertain that parity can contribute to increase or decrease the ovarian reserve (independently of age).

#### *Tobacco consumption*

Moreover, lifestyle factors can have a consequential role on the ovarian reserve. In a systematic review on the impact of cigarette smoking on the different stages of the reproductive function (Dechanet *et al.*, 2011), the authors found that cigarette smoke components target and impact each stage of the reproduction function including folliculogenesis, steroidogenesis, embryo transport, and endometrial receptivity, in a dose-dependent manner. Follicle loss has further been reported at all stages of folliculogenesis, especially on primordial follicles, in animal studies. Smoking is also associated with lower fecundity rates and a poor prognosis risk in IVF (Freour *et al.*, 2008). Nonetheless, some studies (Camlin *et al.*, 2014) suggest that cessation of smoking could potentially improve ovarian quality.

#### *Body mass index*

According to a meta-analysis of 45 studies on the associations between body mass index (BMI) and obesity on the ovarian reserve (Moslehi *et al.*, 2018), AMH and FSH are significantly lower in obese than in nonobese women. BMI is also negatively correlated with AMH and FSH but not in all studies (Jung *et al.*, 2017). However, in the aforementioned meta-analysis, BMI and AFC were not significantly associated, a result that could be due to a lack of statistical power. It is also possible that because the AFC does not differentiate between healthy and atretic follicles (Broekmans *et al.*, 2010), the number of follicles could be overestimated, thus reducing associations with BMI. The review further hypothesises that increased production of sex steroid hormones due to adiposity could send a negative feedback to the hypothalamic-pituitary-gonadal axis, reducing ovarian folliculogenesis, but an increase in the number of small antral follicles secreting AMH has also been identified in obese women experiencing anovulation (Jung *et al.*, 2017).

#### *Alcohol*

Alcohol consumption has been associated in dose-dependent way with a reduction in fecundability, however, these results have high heterogeneity due to inconsistencies in the types of outcomes or of beverages evaluated (Fan *et al.*, 2017). Very few studies have investigated the role of alcohol drinking on the ovarian reserve specifically (de Angelis *et al.*, 2020). One study of 1654 young African-American adults found no significant associations between almost all of the drinking behaviours assessed, including past and current drinkers, and AMH levels, except for those who binge drink twice a week who had a 21% lower anti-Müllerian hormone level compared with those who never drink (Hawkins

Bressler *et al.*, 2016), indicating a potential threshold effect although more studies are needed to fully comprehend this relationship.

### *Environmental factors*

The ovarian reserve is also influenced by environmental factors. Recent studies have shown associations between particulate matter (PM) of various diameters, sulfur dioxide (SO<sub>2</sub>), nitrous dioxide (NO<sub>2</sub>), and both AMH levels and AFC (Abareshi *et al.*, 2020; Feng *et al.*, 2021; Gaskins *et al.*, 2019) but not in all cases (La Marca *et al.*, 2020). Furthermore, exposure to higher temperatures has been associated with lower AFC (Gaskins *et al.*, 2021), which is of particular concern in a warming world and because increased temperature affect concentrations of PM (Sario *et al.*, 2013). The biological mechanisms for these associations are not fully explained but could involve inflammation of the ovarian tissue and oxidative stress resulting in follicular apoptosis.

Despite the high diagnosis rate of DOR in fertility centres, studies examining the determinants of DOR specifically are rare. However, since DOR represents a threshold value of the ovarian reserve, it seems pertinent to consider the same risk factors for DOR than for continuous indicators of the ovarian reserve.

While several risk factors of the ovarian reserve are known, the role of environmental pollutants, and in particular EDCs, on the ovarian reserve is much less understood (Jurewicz *et al.*, 2020; Mínguez-Alarcón and Gaskins, 2017; Souter *et al.*, 2013).

## **1.3. ENDOCRINE DISRUPTING CHEMICALS**

### **1.3.1. Definition and history**

EDCs are exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations as defined by the WHO (Damstra *et al.*, 2002). The term EDC was first coined at the Wingspread conference in 1991 in the US but its first indications trace back to the 1920s when pig farmers became concerned by lack of fertility in swines (Darbre, 2019). However, it is not until the 1960s-70s that awareness of the impacts of on environmental exposures on health started to spread, notably due to Rachel Carson's 1962 book 'Silent Spring' (Schug *et al.*, 2016). In her work (Carson, 1962), Rachel Carson brings attention to an increase in mortality and decrease in reproductive functions in birds observed in areas where dichlorodiphenyltrichloroethane (DDT) was being used as a pesticide. This period saw

the creation of many governmental agencies dedicated to investigate environmental effects on human health including the National Institute of Environmental Health Sciences (1966) and the Environmental Protection Agency (EPA) (1970), as well as environmental non-governmental organisations like the World Wildlife Fund for Nature (1961), Friends of the Earth (1969) and Greenpeace (1971). Scientists also began documenting abnormal events in alligators, fish, gulls, and minks including unexplained deaths and reproductive abnormalities (Darbre, 2019; Schug *et al.*, 2016), and worries that similar effects could happen in humans grew. Experiments regarding xenoestrogens showed that chemicals could bioaccumulate and biodegrade in living organisms, and could cause adverse effects. For instance, diethylstilbestrol (DES), a synthetic molecule prescribed to millions of women between the 1940s and 1980s in various parts of the world to prevent threatened miscarriage in the first trimester of pregnancy, was identified to cause a rare vaginal cancer in daughters born to women who had taken DES during pregnancy (Marty *et al.*, 2011). Several conditions such as hypospadias and cryptorchidism, reduced sperm counts, testicular cancer, as well as early puberty in humans were also linked with chemical exposures (Schug *et al.*, 2016). Events of mass poisoning via contamination of rice oil with polychlorinated biphenyls (PCBs) in 1968 in Japan and 1979 in Taiwan resulted a high rate of babies delivered by women pregnant during the incidents with low birth weight and delays in neurological development (Aoki, 2001).

As it became certain that “a large number of man-made chemicals that have been released into the environment, [...], have the potential to disrupt the endocrine system of animals, including humans” (The Wingspread Conference, 1991), more and more attention was given to EDCs in various fields including chemistry, toxicology, ecology, endocrinology, and epidemiology. Thanks to the development of in vitro technology, the list of EDCs increased rapidly from a few pesticides (such as DDT and chlordecone) and industrial chemicals (such as PCB congeners) to include new compounds found in plastic, and personal care products (Darbre, 2019). It also became possible to document their health effects even at extremely low doses and demonstrate their non-monotonic dose response. EDCs were associated with many types of cancers and reproductive, metabolic and other health issues. As the concept of Developmental Origins of Health and Disease (DOHAD) expanded, it also became apparent that human pathologies could be influenced by early development or in utero exposure (Gore *et al.*, 2015).

In 2009, a scientific statement released by the Endocrine Society summarised current knowledge on EDCs and their effects on human health concluding that “results from animal models, human clinical observations, and epidemiological studies converge to implicate EDCs as a significant concern to public health” (Diamanti-Kandarakis *et al.*, 2009). As scientific and public concern grew, institutions and agreements governing the use of EDCs by manufacturers became necessary. The registration, evaluation and authorisation of chemicals (REACH) program was created by the European Union (EU)

in 2007 in order to “improve the protection of human health and the environment from the risks that can be posed by chemicals while enhancing the competitiveness of the EU chemicals industry”. International agreements like the 1998 Rotterdam Convention and the 2004 Stockholm Convention were signed to reduce the use of pesticides and industrial chemicals, and persistent organic pollutants (POPs), of participating countries, respectively.

In 2016, the International Panel on Chemical Pollution listed 77 chemicals as identified EDCs or potential EDCs with high certainty (United Nations Environment Programme, 2016), with several hundreds of molecules being under investigation out of the 80 000 known chemicals present in our environment. REACH separates chemicals into different categories of EDCs under its new 2023 classification:

- ED HH 1: May cause endocrine disruption in humans.
- ED HH 2: Suspected of causing endocrine disruption in humans.
- ED ENV 1: May cause endocrine disruption in the environment.
- ED ENV 2: Suspected of causing endocrine disruption in the environment.

The previous REACH list of substances identified as endocrine disruptors at EU level included 108 molecules (The Danish Environmental Protection Agency, 2023). Furthermore, the database of endocrine disrupting chemicals and their toxicity profiles (DEDuCT) aiming to compile potential EDCs with supporting evidence for endocrine disruption in published literature specific to humans or rodents, has identified a total of 792 potential EDCs with supporting evidence for endocrine disruption from 2,218 research articles as of October 2020 (Karthikeyan *et al.*, 2021). Table 3 summarises the main families of EDCs or suspected EDCs and what products they were used in.

**Table 3. Examples of families of EDCs or suspected EDCs and their use in commercial products**

<b>Chemical family</b>	<b>Used in</b>
Organochlorine pesticides (OCPs)	Pesticides, herbicides, fungicides, mosquito nets
Polychlorinated biphenyls (PCBs)	Paint, sealants, transformer oils, carbonless copy paper, and plastics
Dioxins	Results from the manufacturing processes of smelting, chlorine bleaching, and some herbicides and pesticides
Furans	Results from the thermal degradation of natural food constituents
Polycyclic aromatic hydrocarbons (PAHs)	Results from the combustion of organic materials
Polychlorinated naphthalenes (PCNs)	Lubricants, electric cable insulation, wood, paper and fabric preservatives, cutting and grinding fluids, and plastics
Polybrominated diphenyl ethers (PBDEs)	Furnishings, bedding, foams, vehicle seats, computers, small appliances, wire insulation
Per-polyfluoroalkyl substances (PFAS)	Surfactants, non-stick coatings, and stain repellents in non-stick cookware, textiles, grease-resistant food packaging, carpets, leather products, textiles that enhance water, grease, and soil repellency
Organophosphate flame retardants (OPFRs)	Furnishings, textiles, foams, and PVC
Phenols (bisphenols, parabens, triclosan, ultraviolet filters)	Plastic items, PVC, epoxy resins, detergents, cosmetics, personal care products, medical devices, medicine, water pipes, thermal receipt paper, cigarette filters and dental products
Phthalates	Cosmetics, personal care products, plastic items, PVC, food packaging, medical devices, varnishes, waterproof clothing, detergents and coatings
Metals	Batteries, paint, coating, plating, cosmetics, hair dyes, jewelry, cigarettes, traditional or folk remedies, wood treatment, and food packaging

EDC, Endocrine Disrupting Chemicals; PVC, polyvinyl chloride.

Despite these efforts, EDCs are still widely used in commercial products with new molecules being regularly added to the market, and many research questions persist regarding their impacts on various windows of susceptibility, possible chemical interactions, and their dose-response relationships with health outcomes.

### 1.3.2. Sources of exposure to EDCs

EDCs include both natural and man-made molecules. Natural molecules include substances found in plants (phytoestrogens in legumes and soy, lignanes in whole grains, fruits and vegetables), fungi, and natural hormones such as steroids.



Synthetic molecules are released in the environment either intentionally (pesticides, fungicides, insecticides, etc), or unintentionally as part of the chemical's life cycle (by-products, leakage, disposal, or deterioration through use) or through industrial accidents. EDCs can then reach human exposure pathways through different means depending on the characteristics of the chemicals.

#### *Air*

Volatile and semi-volatile compounds such as persistent halogenated compounds, and aromatic hydrocarbons can bound to PM in the air which can then be inhaled by humans or be deposited on agricultural land, soil and water currents. Their travelling potential is so high that several have been measured in the Arctic (Hung *et al.*, 2005), and even at the bottom of the 8,000-meter deep Atacama Trench in the Pacific Ocean (Sobek *et al.*, 2023).

#### *Surface waters*

A number of pesticides and several other semipersistent and persistent organic chemicals have been detected in surface waters. These can then be ingested by fish which is consumed by humans or can end up in drinking water circuits that may or may not be treated to remove some of its content.

#### *Food*

Similarly, soil contains PCBs, polybrominated diphenyl ethers (PBDEs) and other dioxins, which can also make their way to human consumption via the food chain and is considered to be the major exposure route of EDCs for humans due to their bioaccumulation and biomagnification properties, especially for POPs (Djien Liem *et al.*, 2000).

#### *Consumer products*

EDCs are also present in a variety of consumer products, creating opportunities for exposure via inhalation, ingestion, and dermal contact. Few products of day-to-day life completely escape EDC or potential EDC use. Bisphenols and phthalates are used as plasticisers in detergents, lubricants, medical devices, pharmaceuticals, clothing, food packaging, toys, and personal care products (Heudorf *et al.*, 2007). Parabens (a subgroup of phenols) are commonly used as antimicrobial preservatives in toothpaste, soaps, shampoos, sunscreens, deodorants, cosmetic and pharmaceutical products, and detergents (Mínguez-Alarcón and Gaskins, 2017). Per-polyfluoroalkyl substances (PFAS) are also found in non-stick cookware (Teflon), food packaging, water-resistant coating for clothing, furniture and carpets (Ding, Harlow, Randolph Jr, *et al.*, 2020).

### *Occupational exposure*

A multitude of occupations are more exposed to certain categories of chemicals than the general population. In fact, EDC concentrations can be between 9 and 45 times higher in occupationally-exposed workers than in the general population (Prichystalova *et al.*, 2021). Agriculture workers use pesticides with possible endocrine disruption effects very regularly but so do gardeners, hygiene and pest control workers, wood workers, veterinarians, and even firefighters. Some industries (e.g. metallurgy) are much more exposed to heavy metals than the general population. Hairdressers and cosmetologists also use many products that contain phthalates and parabens. In fact, a job exposure matrix was developed specifically to estimate occupational exposure to potential endocrine disruptors in epidemiological research (Brouwers *et al.*, 2009).

#### 1.3.3. Toxicokinetic

Once the EDCs are inhaled, ingested, or in dermal contact, they are absorbed in the human body via the lungs, gastrointestinal tract and liver, and the skin, respectively, and transported by blood throughout the system to different tissues, where it can be metabolised, stored or excreted.

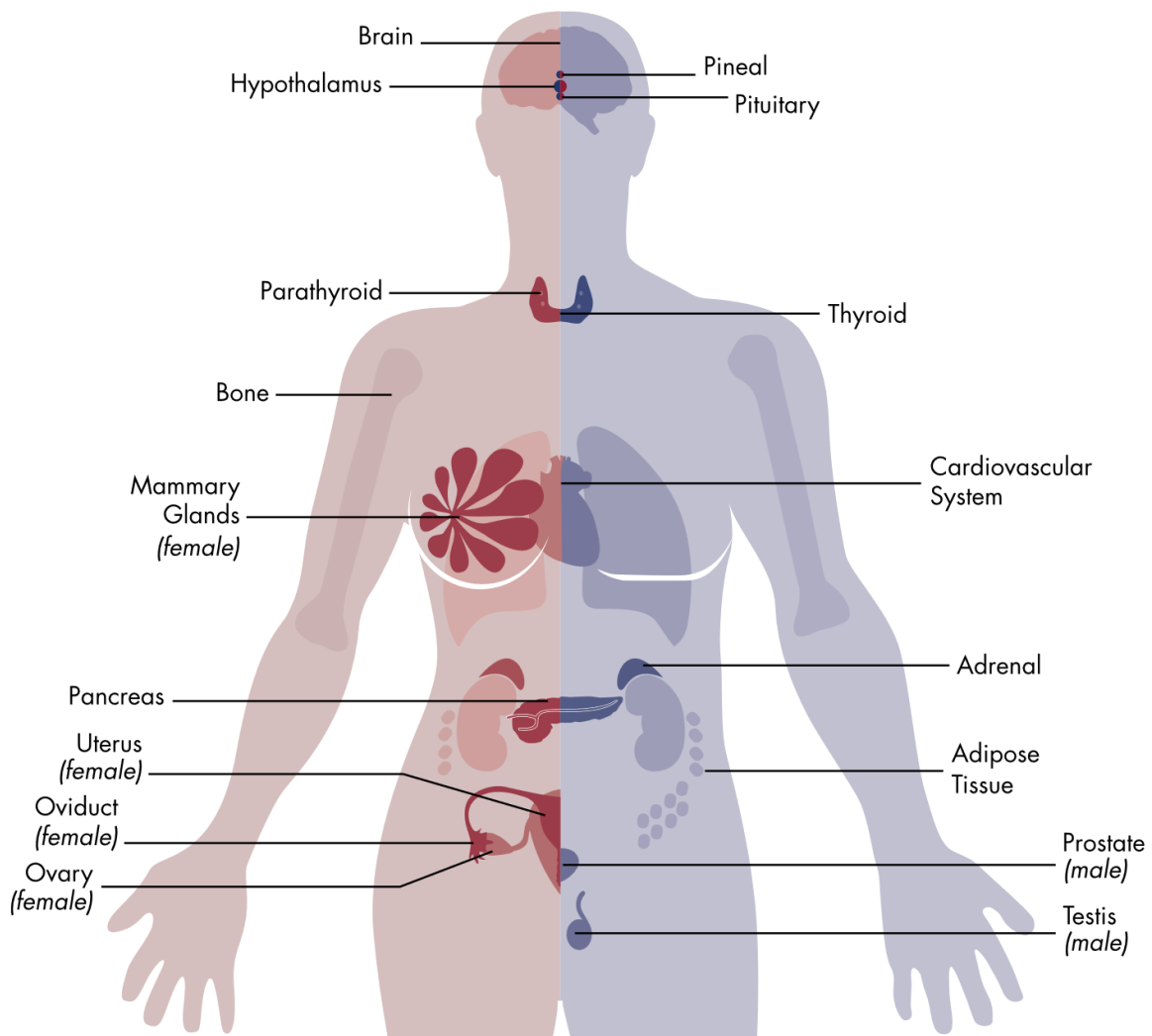
Some EDCs are hydrophilic (soluble in water) and others are lipophilic (soluble in lipids). Hydrophilic EDCs rapidly degrade and are only present in the human body for short periods of time (usually less than 24 hours) before they are excreted, though continuous exposure can help maintain EDC concentration levels more or less constant (Yilmaz *et al.*, 2020). Lipophilic EDCs are stored in fatty tissues (liver, brain, adipose), proteins (liver, muscle), and in the bones, and are often referred to as persistent chemicals. These storage units are then mobilised when a source of energy is required, which releases the chemicals in the blood circulation (World Health Organization, 2012).

Once the EDCs have been absorbed into the body, they can be metabolised by the liver. Metabolism is the sum of processes by which large molecules are broken down into smaller ones (metabolites) to make energy available to run cellular functions or to make parts for building cell parts. Unfortunately, this process can sometimes make EDCs more harmful by creating metabolites that have stronger endocrine disrupting potential than their parent compound or are more persistent compounds.

Hydrophilic EDCs are usually metabolised rapidly by the liver and excreted via urine or faeces. On the other hand, lipophilic EDCs are excreted much more slowly because they first accumulate in different parts of the body. Some chemicals can also exit the body of pregnant women or new mothers via the placenta or through breast milk, respectively.

### 1.3.4. Mechanisms of action of EDCs on the endocrine system

Once EDCs are released into the blood circulation, they are able to reach different parts of the endocrine system. This one is comprised of a series of glands in different parts of the body (Figure 5) that secrete hormones which travel to cells and tissues to regulate various bodily functions (World Health Organization, 2012). These hormones regulate many key features including reproductive processes, metabolism, sleep, blood pressure, growth, development, stress, and several gut, cardiovascular, and kidney functions (Damstra *et al.*, 2002). They can also produce different effects at different times throughout life (e.g. menopause when the follicle pool is depleted). Endocrine glands include the pituitary gland at the base of the brain, the thyroid gland in the neck, the uterus, the uterine tubes, and ovaries, which are key to the functioning of female reproduction, but also the mammary glands, the adrenal gland, and the pancreas to list a few.



**Figure 5. Representation of the human body's main endocrine glands in women (left) and men (right)**  
**(from Gore *et al.* 2015)**

EDCs can interfere with the endocrine system through several biological mechanisms. A 2020 consensus statement identified ten key characteristics of endocrine-disrupting chemicals as a basis for hazard identification (La Merrill *et al.*, 2020). However, these can be summarised into five main categories: (1) mimicking hormones by activating (agonist effect) or blocking (antagonist effect) hormone receptors, (2) disrupting the synthesis of hormones, and (3) their transportation, (4) altering DNA, and (5) causing cell degradation.

#### *Activating or blocking hormone receptors*

Hormones produce biological effects by acting on specialised proteins called receptors, which attract and bind to specific hormones. The number of hormone receptors is naturally limited and can either be localised to a specific part of the body (e.g. receptors for the thyroid stimulating hormones are mainly found in the thyroid gland) or throughout the body (e.g. insulin receptors). Different types of receptors have different effects. Nuclear receptors, located inside cells, bind to steroid and thyroid hormones to regulate gene expression. Membrane receptors, located on the plasma membrane of cells, bind to protein and amine hormones to produce effects inside the cell (e.g. eat glucose) (World Health Organization, 2012). EDCs can bind to these receptors, altering the careful balance organised by the endocrine system, either agonistically or antagonistically. For instance, in the context of the reproductive system, methoxychlor (an organochlorine pesticide) and bisphenol A (BPA) can cause estrogenic action by binding to the estrogen receptor ER $\alpha$ , which has in turn been associated with breast cancer (Clemons and Goss, 2001).

#### *Disrupting hormone synthesis*

EDC can also inhibit the synthesis of hormones, which is particularly important in steroidogenesis, a succession of enzymatic reactions converting cholesterol in steroid hormones (androgens, estrogens and progestogens). In fact, several classes of fungicides have been developed specifically to inhibit fungal membrane synthesis and growth by inhibiting specific CYP450 enzymes. Unfortunately these can also affect steroidogenesis in mammals since several CYP450 enzymes are involved in the steroid pathway (Damstra *et al.*, 2002). Phthalates can also inhibit steroidogenesis in ovaries by decreasing testosterone and estradiol levels (Gore *et al.*, 2015), and have been shown to inhibit testosterone synthesis in Leydig cells which are involved in spermatogenesis, the process in which sperm cells develop inside the testes (Yilmaz *et al.*, 2020).

#### *Disrupting hormone transportation*

Most of the hormones are transported in the bloodstream by a transport protein (Johnstone *et al.*, 2014) which can be a target of EDCs. By binding to this protein, these interrupt the delivery process of

the hormone to its target without even altering the serum concentrations of this hormone, making analyses more complicated in the process. For instance, some PCBs are known to bind the T4-binding globulin and transthyretin proteins which are used to transport T4, the main hormone produced by the thyroid hormone, thereby blocking the binding sites for T4 which can no longer reach the liver and kidneys to help regulate the metabolic rate or other digestive functions (Diamanti-Kandarakis *et al.*, 2009). This phenomenon can also affect the SHBG protein, used to transport 98% of testosterone and estradiol (Damstra *et al.*, 2002).

### *Altering DNA*

In addition, it is possible that EDCs can cause epigenetic changes. These are heritable changes in gene expression that are not due to changes in DNA sequence. They can occur via several mechanisms: DNA methylation, post-translational modification of histones, and altered micro ribonucleic acid (RNA) expression (Rissman and Adli, 2014). These epigenetic processes control tissue development by controlling gene expression. DNA methylation is the most widely studied epigenetic marker. It regulates gene expression by recruiting specific proteins and adding methyl groups to them as well as other molecular targets, including neurotransmitters, lipids, and DNA, or by inhibiting the binding of transcription factors to DNA (Moore *et al.*, 2013). It plays an important role in developmental and reproductive processes such as gametogenesis and embryogenesis. Histone proteins are involved in genomic DNA organisation. MicroRNAs are short non-coding RNA that suppress target gene expression through the inhibition of gene translation and the increase of the degradation of target mRNAs (Derghal *et al.*, 2016). EDCs bind to specific receptors, changing the expression of histones, DNA modifiers and microRNAs, thereby causing gene expression anomalies that can have transgenerational effects. DES has notably been shown to result in the hypermethylation of the Hoxa10 gene (a gene critical to uterine organogenesis) and have resulted in higher incidences of rare reproductive disorders in grandchildren of women exposed to DES (Christensen and Marsit, 2011).

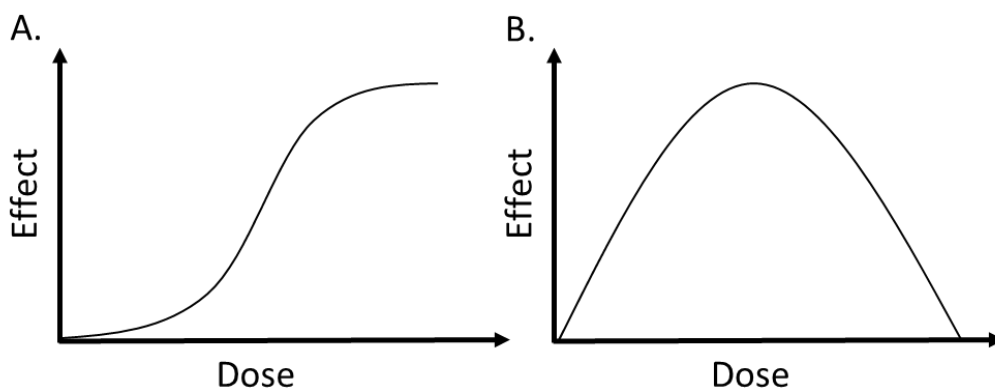
### *Causing cell degradation*

Lastly, apoptosis (cell death) can be triggered due to the absence of specific cell surface receptors or by molecules that can penetrate the cell directly, or can alternatively prevent their planned death. Heavy metals, including cadmium (Cd), have been shown to activate the Caspase-8 protein inducing the apoptosis of primary cortical neurons but also of testicular tissue (Rotimi and Singh, 2022).

Furthermore, EDCs can disrupt hormones via multiple mechanisms at the same time. This is often possible when they are broken down and transformed into metabolites that have differing properties. For instance, the estrogen agonist DDT can be metabolised into the androgen antagonist dichlorodiphenyldichloroethylene (DDE) (Diamanti-Kandarakis *et al.*, 2009).

### 1.3.5. Effects on hormones exhibit nonlinear dose-response characteristics

An additional challenge that EDCs pose lies in their often-unusual concentration-response patterns. Since the role of receptors is to mediate hormone effects, they tend to exhibit nonlinear dose-response characteristics, which will be replicated by EDCs due to their ability to attach to cell receptors (Zoeller *et al.*, 2012). The most classic hormone dose-response pattern is that of a sigmoidal curve (Figure 6.A). As the dose of a hormone increases, the response increases in a logarithmic manner until it reaches a point of saturation. This implies that a change in EDC concentration at lower doses, after a low threshold, can have a proportionally bigger response than at higher doses. Very low concentrations can initiate important biological effects simply because hormone receptors are high-affinity receptors. Moreover, once the concentrations reach a certain level, no further increase in response will be observed because receptors are saturated. It is also possible to observe a maximum response when receptor occupancy is far below 100%, even as little as 1% (Gore *et al.*, 2015), leaving “spare receptors” free at different points in time.



**Figure 6. Examples of non-linear dose-response relationships**

Sigmoidal curves are monotonic since the response does not decrease at any point as the hormone or chemical concentration increases, but this is not always the case. Dose-response curves can often be “U-shaped” (when maximal responses are observed at low and high doses) or “inverted U-shaped” (when maximal responses are observed at intermediate doses) (Figure 6.B) as a result of a variety of possible mechanisms. For instance, when hormone concentrations reach a certain threshold, a degradation of receptors occurs but the capacity of the cells to replace these receptors can be slower than its capacity to remove the bound receptors from the system (Bergman *et al.*, 2013). Thus, when concentrations are high, the response is decreased.

A non-monotonic response of a hormone can also be caused by the increased toxicity at higher doses of this hormone (cytotoxicity), resulting in the death of the cell it was bound to. This is the case for the

MCF7 breast cancer cells' response to estrogen, implying that estradiol does not have endocrine disrupting effect at high doses, even though it remains toxic generally (Gore *et al.*, 2015).

Another mechanism of non-monotonic dose-response is linked to the ability of a hormone to bind, though weakly, to other hormone receptors (Diamanti-Kandarakis *et al.*, 2009). For instance, low-dose estrogen can bind exclusively to estrogen receptors, but can also bind to androgen receptors and thyroid hormone receptors at higher dose, thereby decreasing the response of estrogen receptors.

Finally, non-monotonic responses can transpire when a cell line (a collection of cells originating from one cell) actually contains two populations of cells, with one that proliferates in response to a hormone while the other is inhibited by the same hormone. At low doses, the first cell type has minimal proliferation, and at high doses the second population has a low level of proliferation because it is being inhibited. This has been notably documented in the response of prostate cells to testosterone (Bergman *et al.*, 2013). If investigated independently each cell type generates a monotonic response, but when looking at the overall cell number, an inverted U-shaped curve can be observed.

This context of non-linear or non-monotonic dose-response has particular implications for health research and is particularly complicated to take into consideration in epidemiological research when it is not possible to control the exposure of participants, and therefore necessitates experimental research to complement it.

#### 1.3.6. Suspected impacts of endocrine disruptors on the female reproductive system

EDCs have been associated with common debilitating or life-threatening non-communicable diseases (NCDs) whose prevalence has increased in the last decades: cancers, obesity, cardiovascular diseases, chronic respiratory diseases, and diabetes (Gore *et al.*, 2015; Yilmaz *et al.*, 2020). They can also disrupt the thyroid function, and the prostate gland, and have effects on the brain's neurodevelopment, but also on pathologies associated with the reproductive system.

Specifically, there is a growing body of evidence showing that EDCs contribute to female reproductive disorders (altered cycle length, endometriosis, PCOS, fibroids, POI, earlier menopause). However, the WHO concluded in 2012 in its extensive summary of EDCs that a lot of gaps still existed in the research on the effect of EDCs on the female reproductive system (World Health Organization, 2012). A few years later the Endocrine Society also concluded that many potential EDCs have not been studied in regards to the female reproduction and that there was a real need for studies to focus on the effects and mechanisms by which EDCs affect it (Gore *et al.*, 2015). This call was further amplified by the International Federation of Gynaecology and Obstetrics (Di Renzo *et al.*, 2015).

Nevertheless, most of the literature on the subject of EDCs and fertility tend to focus on child development or male fertility, partially due to the fact that female fertility can be difficult to assess

(Karwacka *et al.*, 2019). Still, exposure assessment studies have confirmed that EDCs were present in follicular fluid (Richardson *et al.*, 2014), indicating the potential for harm on the reproductive system. Since these previous statements, several studies have been carried out and seems to overall suggest an association between EDCs and disorders in the female reproductive system (Sifakis *et al.*, 2017). Table 4 provides a non-exhaustive review of the literature reporting evidence of detrimental associations between EDC families and female reproductive disorders including longer time-to-pregnancy (TTP), altered cycle length, endometriosis, PCOS, uterine fibroids, POI, and early menopause. The heterogeneity in the results can be explained by methodological differences in study design, population, sample size, assessment of exposure, confounding and modeling strategy, lack of detection (e.g. PBDEs) and variability (e.g. non-persistent chemicals such as phthalates) of certain chemicals. The impact of EDCs on certain outcomes such as longer TTP and endometriosis seem to have been investigated more thoroughly than on others such as uterine fibroids or POI. Parabens and triclosan, followed by PBDEs, have the lowest amount of studies (6, 7, and 10 studies respectively), perhaps due to difficulties of detection. We can also observe that some associations have not been explored at all (e.g. PBDEs and early menopause).



**Table 4. Non-exhaustive review of the literature reporting evidence of detrimental associations between EDC families and female reproductive disorders**

Chemical family	Female reproductive disorder <sup>1</sup>						
	Longer time-to-pregnancy	Altered cycle length	Endometriosis	PCOS	Uterine fibroids	POI	Early menopause
OCPs	<b>Associations:</b> Axmon <i>et al.</i> (2006), Chevrier <i>et al.</i> (2013), Louis <i>et al.</i> (2013)	<b>Associations:</b> Chen <i>et al.</i> (2018), Ouyang <i>et al.</i> (2005), Toft <i>et al.</i> (2008), Windham <i>et al.</i> (2005)	<b>Associations:</b> Buck Louis <i>et al.</i> (2012), Matta <i>et al.</i> (2022), Ploteau <i>et al.</i> (2017), Pollack <i>et al.</i> (2021), Upson <i>et al.</i> (2013)	<b>Associations:</b> Yang <i>et al.</i> (2015)  <b>No associations:</b> Vagi <i>et al.</i> (2014)	<b>Associations:</b> Trabert <i>et al.</i> (2015)	<b>Associations:</b> Pan <i>et al.</i> (2019)	<b>Associations:</b> Akkina <i>et al.</i> (2004)
	<b>No associations:</b> Cole <i>et al.</i> (2006), Law <i>et al.</i> (2005)	<b>No associations:</b> Chen <i>et al.</i> (2005), Cooper <i>et al.</i> (2005)					
PCBs	<b>Associations:</b> Axmon <i>et al.</i> (2006), Chevrier <i>et al.</i> (2013), Louis <i>et al.</i> (2013), Yang <i>et al.</i> (2008)	<b>Associations:</b> Buck Louis <i>et al.</i> (2011), Cooper <i>et al.</i> (2005), Toft <i>et al.</i> (2008), Yang <i>et al.</i> (2011)	<b>Associations:</b> Buck Louis <i>et al.</i> (2012), Ploteau <i>et al.</i> (2016), Pollack <i>et al.</i> (2021), Roy <i>et al.</i> (2012), Vichi <i>et al.</i> (2012)	<b>Associations:</b> Vagi <i>et al.</i> (2014), Yang <i>et al.</i> (2015)	<b>Associations:</b> Trabert <i>et al.</i> (2015)	<b>Associations:</b> Pan <i>et al.</i> (2019)	<b>Associations:</b> Grindler <i>et al.</i> (2015)  <b>No associations:</b> Blanck <i>et al.</i> (2004), Cooper <i>et al.</i> (2002)
	<b>No associations:</b> Axmon <i>et al.</i> (2004), Law <i>et al.</i> (2005)	<b>No associations:</b> Windham <i>et al.</i> (2005)	<b>No associations:</b> Neblett <i>et al.</i> (2020)				
PBDEs	<b>Associations:</b> Gao <i>et al.</i> (2016), Harley <i>et al.</i> (2010)	<b>Associations:</b> Chao <i>et al.</i> 2010, Shi <i>et al.</i> (2022)  <b>No associations:</b> Harley <i>et al.</i> (2010)	<b>Associations:</b> Ploteau <i>et al.</i> (2017)  <b>No associations:</b> Buck Louis <i>et al.</i> (2012), Pollack <i>et al.</i> (2021)	<b>Associations:</b> Vagi <i>et al.</i> (2014)	No study	<b>No associations:</b> Pan <i>et al.</i> (2019)	No study
PFAS	<b>Associations:</b> Bach <i>et al.</i> (2015), Cohen <i>et al.</i> (2023), Fei <i>et al.</i> (2009), Jørgensen <i>et al.</i> , (2014), Louis <i>et al.</i> (2013), Luo <i>et al.</i> (2022), Vélez <i>et al.</i> (2015a)	<b>Associations:</b> Lum <i>et al.</i> (2017), Lyngsø <i>et al.</i> (2014), Zhou <i>et al.</i> (2017)	<b>Associations:</b> Buck Louis <i>et al.</i> (2012), Campbell <i>et al.</i> (2016), Wang <i>et al.</i> (2017)  <b>No associations:</b> Hammarstrand <i>et al.</i> (2021), Matta <i>et al.</i> (2022)	<b>Associations:</b> Vagi <i>et al.</i> (2014), Wang <i>et al.</i> (2019)	No study	<b>Associations:</b> Zhang <i>et al.</i> (2018)	<b>Associations:</b> Ding <i>et al.</i> (2020), Taylor <i>et al.</i> (2014)

Female reproductive disorder<sup>1</sup>

Chemical family	Longer time-to-pregnancy	Altered cycle length	Endometriosis	PCOS	Uterine fibroids	POI	Early menopause
Phthalates	<b>Associations:</b> Burdorf <i>et al.</i> (2011), Louis <i>et al.</i> (2014), Snijder <i>et al.</i> (2011), Thomsen <i>et al.</i> (2017)	<b>Associations:</b> Jukic <i>et al.</i> (2016)	<b>Associations:</b> Buck Louis <i>et al.</i> (2013), Cobellis <i>et al.</i> (2003)	<b>Associations:</b> Al-Saleh (2022), Jin <i>et al.</i> (2019), Luo <i>et al.</i> (2020), Vagi <i>et al.</i> (2014)	<b>Associations:</b> Lee <i>et al.</i> (2020), Zota <i>et al.</i> (2019, 2020)	<b>Associations:</b> Özel <i>et al.</i> (2019)	<b>Associations:</b> Grindler <i>et al.</i> (2015)
	<b>No associations:</b> Buck Louis <i>et al.</i> (2018), Jukic <i>et al.</i> (2016), Philips <i>et al.</i> (2018), Vélez <i>et al.</i> (2015b)			<b>No associations:</b> Akgül <i>et al.</i> (2019), Akın <i>et al.</i> (2020)			<b>No associations:</b> Ding <i>et al.</i> (2023)
Parabens	<b>Associations:</b> Ao <i>et al.</i> (2023), Smarr <i>et al.</i> (2017)	<b>Associations:</b> Nishihama <i>et al.</i> (2016)	<b>Associations:</b> Peinado <i>et al.</i> (2021)	<b>No associations:</b> Šimková <i>et al.</i> (2020)	<b>Associations:</b> Lee <i>et al.</i> (2020)	No study	No study
Bisphenol A	<b>Associations:</b> Philips <i>et al.</i> (2018), Wang <i>et al.</i> (2018)	<b>Associations:</b> Jukic <i>et al.</i> (2016)	<b>Associations:</b> Lee and Eata (2022), Peinado <i>et al.</i> (2020), Simonelli <i>et al.</i> (2017), Upson <i>et al.</i> (2014), Wen <i>et al.</i> (2020)	<b>Associations:</b> Akgül <i>et al.</i> (2019), Kandarakı <i>et al.</i> (2011), Konieczna <i>et al.</i> (2018), Rashidi <i>et al.</i> (2017)	<b>No associations:</b> Lee and Eata (2022)	<b>No associations:</b> Li <i>et al.</i> (2021), Özel <i>et al.</i> (2019)	No study
	<b>No associations:</b> Jukic <i>et al.</i> , (2016), Louis <i>et al.</i> (2014), Vélez <i>et al.</i> (2015b), Yeum <i>et al.</i> (2019)		<b>No associations:</b> Buck Louis <i>et al.</i> (2013), Itoh <i>et al.</i> (2007)	<b>No associations:</b> Gu <i>et al.</i> (2019), Vagi <i>et al.</i> (2014)			
Triclosan	<b>Associations:</b> Vélez <i>et al.</i> (2015b), Zhu <i>et al.</i> (2019)	<b>Associations:</b> Zhu <i>et al.</i> (2019)	<b>No associations:</b> Lee and Eata (2022)	<b>Associations:</b> Ye <i>et al.</i> (2018)	<b>No associations:</b> Lee and Eata (2022)	No study	No study
	<b>No associations:</b> Smarr <i>et al.</i> (2017)			<b>No associations:</b> Gu <i>et al.</i> (2019)			
Heavy metals	<b>Associations:</b> Buck Louis <i>et al.</i> (2012), Cole <i>et al.</i> (2006), Sallmén <i>et al.</i> (2000)	No study	<b>Associations:</b> Kim <i>et al.</i> , 2021, Pollack <i>et al.</i> (2013), Silva <i>et al.</i> (2013), Tian <i>et al.</i> (2021), Yilmaz <i>et al.</i> (2020), Zhang <i>et al.</i> (2021)	<b>Associations:</b> Kurdoglu <i>et al.</i> (2012), Sharma <i>et al.</i> (2022)	<b>Associations:</b> Ye <i>et al.</i> (2017)	<b>Associations:</b> Pan <i>et al.</i> (2021)	<b>Associations:</b> Eum <i>et al.</i> (2014) Mendola <i>et al.</i> (2013) Popovic <i>et al.</i> (2005), Wang <i>et al.</i> (2021)
	<b>No associations:</b> Bloom <i>et al.</i> (2011)		<b>No associations:</b> Lai <i>et al.</i> (2017)				<b>No associations:</b> White <i>et al.</i> (2020)

<sup>1</sup>Diminished ovarian reserve is not included.

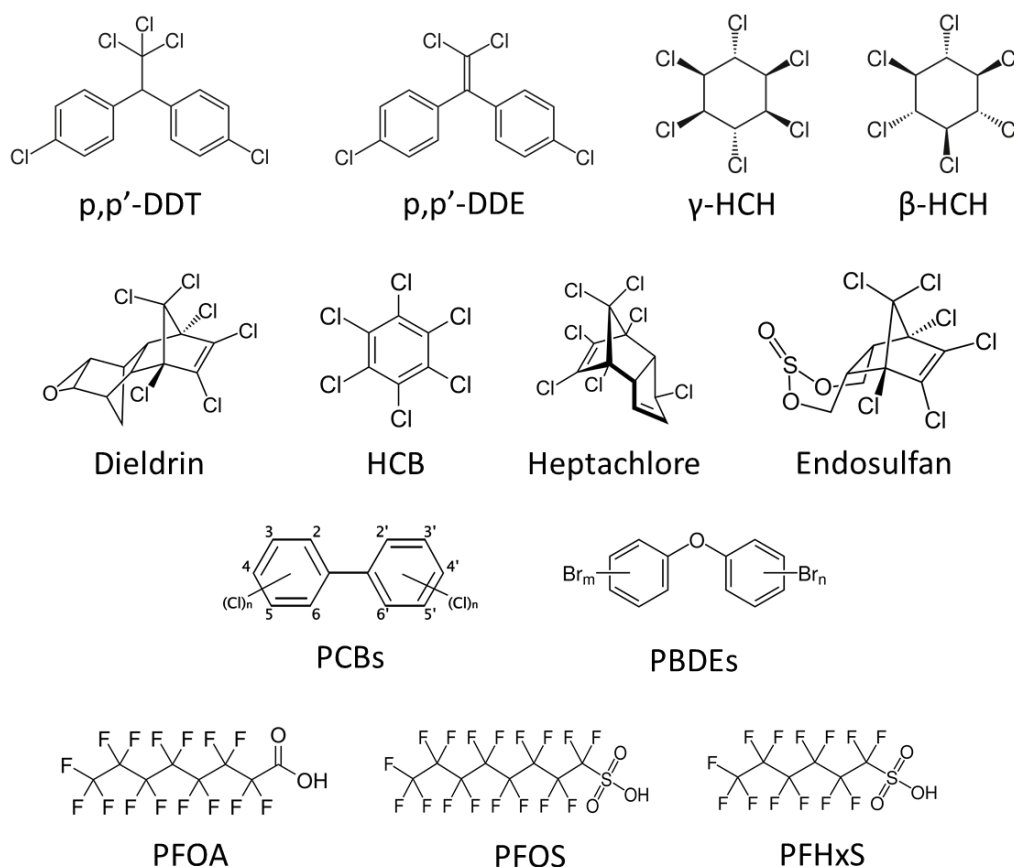
EDC, endocrine disrupting chemical; PCOS, polycystic ovary syndrome; POI, primary ovarian insufficiency; OCP, organochlorine pesticides; PCB, polychlorinated biphenyls; PBDE, polybrominated diphenyl ethers; PFAS, per-polyfluoroalkyl substances.

The biological adverse effects of EDCs on the reproductive system have been reported extensively in the animal research (Sifakis *et al.*, 2017), however, the ovarian reserve is more difficult to assess in women without the use of invasive procedures. In fact, evidence on the impact of EDCs on the ovarian reserve in women is rather limited, especially for (but not limited to) our families of interest: POPs, heavy metals and non-persistent chemicals.

### 1.3.7. Persistent Organic Pollutants

#### 1.3.7.1. Definition, sources and regulation

POPs are poly-halogenated chemical substances that either contain chlorine, bromine, or fluorine atoms (World Health Organization, 2010). The main categories of POPs include organochlorine pesticides (OCPs), PCBs, and PBDEs which are lipophilic and PFAS which are hydrophilic and hydrophobic at the same time.



**Figure 7. Molecule structures of selected POPs**

### *OCPs*

OCPs are molecules that have at least one ring structure and multiple chlorine atoms (Figure 7). They are divided into five classes: (1) DDT and its derivatives, (2) hexachlorocyclohexane (HCH) including lindane ( $\gamma$ -HCH), (3) cyclodienes including aldrin, dieldrin, endrin, heptachlor, chlordane, and endosulfan, (4) toxaphene, and (5) mirex and chlordecone (Sparling, 2016). OCPs affect pest by targeting their nervous system. Some, DDT and related compounds, act so that the transfer of information along the nerve is disrupted, while others, the cycloienes, act on the receptors of neurons to stop the transfer of information to the next neuron, creating convulsions, agitation, excitability, seizures, and other nervous disorders in exposed animals.

These chemicals were mostly used in agriculture but also in disease control. DDT was one of the first chemicals (1940) to be used on farms massively. It was also used to protect soldiers from insect-borne diseases such as malaria and typhus during World War II. However, thanks to Rachel Carson's 'Silent Spring' and other research work showing the effects of the molecule on eggshells of birds of prey including the America symbol, the bald eagle, the EPA cancelled the registration of DDT in 1972 (Jarman and Ballschmiter, 2012; Turusov *et al.*, 2002). However, DDT is still used to treat mosquito netting and house-wall spraying in South America, India, and Africa. Chlordecone is also infamous, especially in France, due to the health impacts it has created as a result of its massive use between 1972-1993 in the French West Indies to control banana root borers (Multigner *et al.*, 2016).

### *PCBs*

PCBs form a chemical family of 209 chlorinated aromatic compounds derived from biphenyl (there are 209 possible combinations for adding chlorine on the biphenyl molecule). These molecules were mainly used in the industry in electric transformers and capacitors as heat exchange fluids due to their excellent thermodynamic stability (stable at elevated temperature) (Sander *et al.*, 2013). They were also used as additives in paint, sealants, paints, carbonless copy paper, and plastics.

PCBs were mostly used between the 1930s and the 1980s before its production was progressively banned throughout the world. However, it was estimated in 2016 that 14 million tonnes of equipment and materials containing PCB (transformers account for the largest share of the total mass) and waste subsist (United Nation Environment Program, 2016). This represent 83% of its estimated total peak amount in 1990. In fact, transformers containing PCBs are still used in Cuba and Paraguay and PCB oils are used in several African countries. Some Nigerians even apply PCB transformer oils on their skin to soften and bleach it.

### *PBDEs*

Similarly to PCBs, there are 209 congeners of PBDEs (also called BDEs) which are classified according to the average number of bromine atoms on the molecule (Sander *et al.*, 2013). PBDEs were the first type of brominated flame retardants used commercially. They are usually not used as single molecules but in mixtures (three main ones) of several molecules in order to produce polymers to reduce flammability in many products. PentaBDE (BDE congeners 99/47/100/153/154) is found in household furnishings, bedding, foams and vehicle seats; octaBDE (BDE congeners 183/197/196/207) is used in plastics for personal computers and small appliances; and decaBDE (BDE congeners 209/206) is incorporated into plastics for television cabinets, vehicle interiors, consumer electronics, and wire insulation (Agency for Toxic Substances and Disease Registry, 2017). These mixtures were either removed from markets voluntarily or banned in the US and the EU in the 2000s-10s.

### *PFAS*

PFAS are fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom, though exceptions exist. Individual molecules differ mainly in fluorinated carbon chain length (Panieri *et al.*, 2022). PFAS are publicly known as “forever chemicals” because they could remain in the environment for anything between 20 years and over 1000 years (Agency for Toxic Substances and Disease Registry, 2021). The process of their degradation is not well understood but PFAS are resistant to biodegradation, photooxidation, direct photolysis, and hydrolysis due to the strength of their carbon-fluorine bonds.

These molecules have enhanced water-resistant properties due to their hydrophobic tail. As a result, they have been used since the 1950s as surfactants, non-stick coatings, and stain repellents in non-stick cookware, textiles, grease-resistant food packaging, carpets, leather products, textiles that enhance water, grease, and soil repellency. However, they were first created in response to a fatal U.S. Navy aircraft carrier fire, and are still used today in firefighting foams. Restrictions of PFAS exist but only concern a handful of molecules (mainly perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA)) among hundreds or possibly thousands.

### *Other POPs*

Polycyclic aromatic hydrocarbons (PAHs) are a large group of chemicals with at least two fused aromatic ring structures which are produced by all types of combustion of organic materials (e.g. fuels, garbage, plant material) (Kim *et al.*, 2013). Dioxins are by-products of organochloride manufacturing or incineration of chlorine-containing substances with its most infamous member (2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD) being a chemical involved in Agent Orange, an herbicide used by the US military during the Vietnam War (Wahlang, 2018). As opposed to other POPs, PAHs and

dioxins are an unintentional by-product of an anthropic activity rather than an intentionally produced chemical. Polychlorinated naphthalenes or PCNs were sold between the 1930s-80s as mixtures to be used either on its own or mixed with oils for use as lubricants, electric cable insulation, wood, paper and fabric preservatives, cutting and grinding fluids, and plasticisers (Fernandes *et al.*, 2017).

#### *International regulation*

Due to their impacts on the environment and severe health effects in humans and other species including cancers, birth defects, immune and reproductive dysfunctions, greater susceptibility to disease, and damages to the central and peripheral nervous systems, POPs have been the subject of many national regulations in all parts of the world. Nonetheless, because they are able to travel over great distances, a necessity to regulate their use on an international level rose to prominence in the 1990s. The Stockholm Convention on Persistent Organic Pollutants was born a few years later (2001) in Sweden and was entered into force in 2004 (United Nation Environment Program, 2004). The text initially applied to 12 chemicals called “the dirty dozen” by allocating them in three non-exclusive categories:

-Annex A: Prohibit and/or eliminate the production and use, as well as the import and export, of the intentionally produced POPs,

-Annex B: Restrict the production and use, as well as the import and export, of the intentionally produced POPs,

-Annex C: Reduce or eliminate releases from unintentionally produced POPs.

The text of the convention was subsequently amended in different conferences to modify annexes and the list of POPs to reach 28 chemicals. It has now been signed by 152 countries, although not every signatory (including the US) has ratified it in their own country (United Nation Environment Program, 2004). REACH has drawn up a very similar list with a few additions including PAHs, and dioxins.

A recent review (Li *et al.*, 2023) reported that as of 2020, a cumulative total of 31,306 kilotons of POPs had been synthesized and commercialized worldwide, with a peak annual global production occurring in the 1970s.  $\alpha$ -HCH had the largest historical global cumulative environmental releases among 25 POPs. The US and Europe were responsible for the biggest environmental releases in the 1960-70s, transitioning to China in the 2000s-10s.

#### 1.3.7.2. Characteristics

POPs are traditionally described as having four main characteristics: persistence, bioaccumulation, mobility, and toxicity.

### *Persistence*

POPs have a very high persistence in the environment and in biota due to their resistance to biotic and abiotic degradation. Their half-lives in the environment last a few days for the less persistent ones, and can depend on the type of landscape. For instance, the amount of POPs stored in tropical forests is higher than in boreal forests which is higher than in temperate forests (Gong *et al.*, 2021). The persistence in soil is also 30 times higher than that of foliage.

In humans, POP half-lives can reach up to 30 years (Bu *et al.*, 2015). However, estimations of half-lives for individual POPs are quite inconsistent. Ritter *et al.* (2011) estimated that the maximum intrinsic elimination half-life for PCBs is approximately 10–15 years. However, it is possible to find values ranging from 0.34 years to 16.7 years for PCB 180 (Masuda, 2001) or even 29.2 years (Aylward *et al.*, 2014), and from 7.6 years to 17 years for p,p'-DDE (Bu *et al.*, 2015). Overall, we can estimate that half-lives for OCPs and PCBs is 10-20 years, 5-10 years for PBDEs (Bu *et al.*, 2015; Ritter *et al.*, 2011) and 3-7 years for PFAS (Ding, Harlow, Randolph Jr, *et al.*, 2020; Lin *et al.*, 2021).

### *Bioaccumulation*

POPs can not only persist in the environment, but can also bioaccumulate i.e. accumulate in an organism at a faster rate at which it is eliminated. They can bind strongly to solids, and in particular to organic matter, and evade aqueous media including the cytoplasm of cells. This gives them the capacity to enter the lipids of organisms more easily and accumulate in adipose tissue, allowing them to persevere in animals where the metabolism rate is low (Ashraf, 2017). Consequently, POPs also climb the food chain within which they enter a process of biomagnification. Animals such as zooplankton assimilate these chemicals and get eaten by insects which are consumed by small fish that are then ingested by bigger fish, etc. At each of these steps, the concentrations of POPs increase and they can reach a point where they can be 10 million times those in water (Sparling, 2016). Because levels are low at the bottom of the food chain, no negative effects might be seen until they reach higher levels further up the food chain. Humans, being at the top, are therefore highly susceptible to be exposed to these higher levels.

### *Mobility*

In addition, POPs have long-range transport potential. POPs can move into a gas phase under different environmental temperatures, allowing them to volatilise from soils, vegetation or water sources (Ashraf, 2017). Once in the air, they can migrate over long distances before being re-deposited due to new meteorological conditions (usually in colder settings). This translocation of POPs through the atmosphere is referred to as the “grasshopper effect” and can be repeated several times (Jones, 2021). As a result, POPs are often transported into the Arctic, Antarctic, and mountainous areas (Hung *et al.*,

2005). Environmental variables such as temperature, wind speed, precipitation, and solar radiation can influence the transport of POPs, which could be of particular concern in a global warming context depending on the chemical (Nadal *et al.*, 2015).

### *Toxicity*

A defining characteristic of POPs is their toxicity on both the environment and animal health as shown by a wealth of evidence accumulated over the years. The health consequences associated with POP exposure can depend on the dose and duration of the exposure. Several accidental exposures have generated acute exposure in the past. Several mass PCB poisonings have occurred throughout the decades. The 1968 Yusho accident in Japan and the 1979 Yucheng accident in Taiwan mentioned previously exposed thousands of people to PCBs through rice oil contamination (Aoki, 2001). Vast amounts of Belgian poultry and Irish pork products were found to be contaminated with PCBs and dioxins in 1999 and 2008 respectively (Pratt *et al.*, 2012). Italy also saw a massive industrial accident – the Seveso disaster – in 1976 exposing residential populations to high levels of TCDD (Wahlang, 2018). The non-accidental contamination of the Vietnamese population by the aforementioned Agent Orange mixture should also be highlighted. Furthermore, water contamination to PFAS occurred when PFOA was released by a DuPont chemical facility in the early 2000s (Domingo and Nadal, 2019). These types of events have often been associated with short-term consequences including high rates of birth defects and cancers. Chronic exposure to POPs, on the other hand, are low dose exposures that occur over several years or decades that can often lead to medical implications in the long term. It is usually associated with metabolic and cardiovascular diseases, reproductive disorders, metabolic diseases such as diabetes, but also cancers, including breast (Mouly and Toms, 2016), ovarian, uterine, prostate, and testicular cancers (Alharbi *et al.*, 2018).

#### 1.3.7.3. Measuring exposure to POPs

Blood is often used to measure persistent chemicals that bind to blood fats. Thus, lipophilic POPs are usually measured in plasma or serum, whilst PFAs are usually measured specifically in serum because they are proteinophilic. Because POPs, especially OCPs and PCBs, are persistent chemicals with long half-lives that accumulate in fat tissues over time (Bu *et al.*, 2015), their concentrations in serum is generally considered as an accurate marker of long-term exposure.



#### 1.3.7.4. Factors affecting POP concentrations

Despite international efforts to curb the production and use of POPs, and accelerate their elimination, exposure in the population remain at a certain level for the various geopolitical, biological and chemical reasons seen above. POPs have been used in various industrial and domestic products, however, the main source of exposure for humans is less from direct contact with these products than from the contamination of soils, water and the air. These are the results of factory waste or combustion at the manufacturing level or from the residues of pesticide spraying.

##### *Diet*

Food is considered to be the main source of human exposure to lipophilic POPs, especially for PCBs which are estimated to account for up to 90% of human exposure (Djien Liem *et al.*, 2000; Weitekamp *et al.*, 2021). Nonetheless, dietary intake of POPs could proportionally decline in the coming years as POPs get slowly eliminated from the environment and as indoor inhalation exposure from consumer products increase (Weitekamp *et al.*, 2021).

Lipophilic POPs tend to have higher concentrations in fat rich products including fish, dairies, oils, meats and delicatessen (Gasull *et al.*, 2011). PFAS seems to be more present in fish and meats but also in products distributed in PFAS-coated wrapper. Therefore, differences in diets (e.g. Mediterranean versus vegan) could influence concentration levels (Henríquez-Hernández *et al.*, 2017).

##### *BMI*

Because of their relations to lipids, POP concentration levels are also affected by several related factors. Body fat content, which is usually approximated through the use of BMI in epidemiological studies, has been associated with POP concentrations but in an inconsistent manner. Higher BMI tends to be associated with higher levels of OCPs and PBDEs, lower levels of PCBs, and with varying effect on PFAS concentrations (Agudo *et al.*, 2009; Mehta *et al.*, 2020; Park *et al.*, 2019; Wolff *et al.*, 2005). The relationship with OCPs also often tends to be non-monotonic (inversed U-shape) (Achour *et al.*, 2017; Agudo *et al.*, 2009). Several explanations have been proposed for these varying directions of associations. Inverse associations could be the result of simple dilution during the uptake phase. In this case, people with high BMI who also have larger adipose tissue would have lower concentration than people with regular BMI and adipose tissue given equal food intake. BMI could alter the pharmacokinetics of POP causing longer half-lives in overweight individuals compared to lean ones, and thus higher concentrations in the former. If concentrations are measured after recent higher or lower intake of food with high levels of POPs, a measurement bias could also alter the results.

### *Female factors*

Similarly, the timing of POP analyses in pregnant or post-partum women can significantly alter the results since pregnancy mobilises body stores of fat. As a result women with higher parity usually have lower PCB and PFAS concentrations (Hardell *et al.*, 2010; Manzano-Salgado *et al.*, 2016) but results are less consistent for OCPs (Sousa *et al.*, 2022). Additionally, up to 20 percent of the maternal body burden of some POPs (especially PCBs) can be transferred to the child during months of lactation (Milbrath *et al.*, 2009). Women who have breastfed therefore have lower levels of POPs on average than women who have not. Even menstrual bleeding can discharge some amount of PFAS concentrations (Park *et al.*, 2019). For all of these reasons, POP concentrations in women can sometimes be lower than concentrations in men but the significance and direction of the associations tend to be very inconsistent (Fernández-Rodríguez *et al.*, 2015; Santé Publique France, 2021).

### *Age*

Due to the bioaccumulation property of POPs, their concentration levels will increase as people age. This association has been shown consistently across all lipophilic POPs (Ben Hassine *et al.*, 2014; Hardell *et al.*, 2010) and less consistently with PFAS (Tian *et al.*, 2018).

### *Socio-economic levels*

Generally, POP concentrations tend to increase with education levels and socio-economic status (Lin *et al.*, 2021; Montazeri *et al.*, 2019), however, it is not clear if these associations are mediated by dietary intake and by occupation. In the case of PFAS, some associations with smoking have also been attributed to unmeasured confounding associated with indicators of socio-economic levels (Park *et al.*, 2019). Because they affect the volatility of the compounds, temperature and by consequence seasons, have shown fluctuations of POP concentrations in the air (Estellano *et al.*, 2017; He *et al.*, 2019).

### *Time trends*

POPs that are included in the Stockholm convention have generally observed declines in environmental concentrations in their populations. Several time trend studies in humans have found significant decreased concentrations in blood samples (Schoeters *et al.*, 2017; Stubleski *et al.*, 2018). However, some analyses have also shown high correlations between POPs between time points (Bányiová *et al.*, 2017) as well as very stable relative contributions of individual POPs to their sum (Nøst *et al.*, 2019). PFAS are more recent and face fewer legal restrictions than other POPs, resulting in more stable concentration (Ding, Harlow, Randolph Jr, *et al.*, 2020), though certain PFAS (PFOA and PFOS) have slightly decreased in the last 15-20 years (Ding, Harlow, Batterman, *et al.*, 2020).

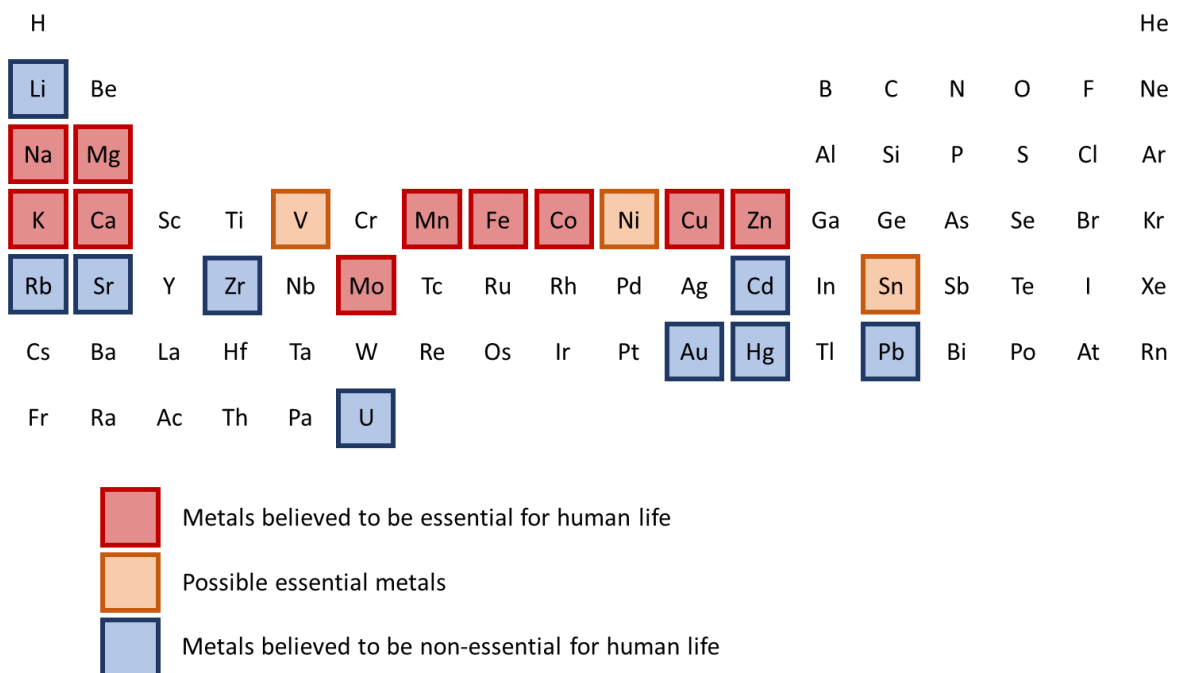
### 1.3.8. Heavy metals

#### 1.3.8.1. Definition and sources

Metals are natural constituents of the earth’s crust and are included in the periodic table of the chemical elements. Metals cannot be broken down and are nonbiodegradable (Briffa *et al.*, 2020). The human body requires low amounts of about 10 metals, called “essential metals”, for a variety of biochemical and physiological functions (Figure 8). In order to be categorised as essential a metal has to fulfil several conditions (Zoroddu *et al.*, 2019):

1. It must be present in human tissues.
2. Its total absence in our organism causes irreversible and severe damage to the vital functions.
3. The reduction in physiological function can be normalised by appropriate supplementation of the element.

Essential metals include potassium (K), magnesium (Mg), calcium (Ca), iron (Fe), manganese (Mn), cobalt (Co), copper (Cu), zinc (Zn), and molybdenum (Mo). Chromium (Cr) used to be considered as an essential metal but evidence of its toxicity effects (interference with iron absorption, blocking glucose use, and even cancer) have emerged. Other essential metals also have the potential to become toxic when reaching high concentrations or when they are introduced in different forms (Rzymiski *et al.*, 2015). Non-essential metals, such as lead (Pb), mercury (Hg) and cadmium (Cd), on the other hand, can be toxic even at very low concentrations (Briffa *et al.*, 2020).



**Figure 8. Periodic table of the elements including metal classification**

(adapted from Zoroddu *et al.*, 2019)

The term “heavy metals” can refer to different meanings depending on the field of study. In the medical literature, it has often been defined as metals with a density greater than 5 g/cm<sup>3</sup>, but it can also be defined according to atomic weight, atomic number or based on toxicity (Duffus, 2002). However, higher density or atomic number doesn’t necessarily imply significant biological impacts. Metalloids - elements that have the physical appearance and properties of metals but behave chemically like non-metals - such as Arsenic (As), are also sometimes categorised as heavy metals. By convention, we will use the term “heavy” for metals studied here (Lead (Pb), Mercury (Hg), Cadmium (Cd) and Cr), as they have been associated with human toxicity and also have a density greater than 5 g/cm<sup>3</sup>.

The details for each source of exposure for our metals of interest are available in Table 5, and a general description is provided below.

**Table 5. Description of forms, sources and use of metals investigated**

Metal	Forms	Source of anthropogenic emissions	Used in	Sources of exposure in the general population	Sources of occupational exposure
<b>Cadmium (Cd)</b>	Pure cadmium is a soft, silver-white metal. Cadmium chloride and cadmium sulfate are soluble in water.	Byproduct during the production of other metals such as zinc, lead, or copper.	Batteries, pigments, coatings, platings, stabilisers for plastics, nonferrous alloys, and photovoltaic devices.	Food and smoking.	Processes involving heating cadmium containing materials such as smelting and electroplating.
<b>Chromium (Cr)</b>	The three main forms of chromium (depending on its oxydation state) are chromium(0), chromium(III) and chromium(VI), which are all detectable in the human body. Small amounts of chromium(III) are needed for human health.	Released from industries involved in electroplating, leather tanning, textile production, and in the manufacture of chromium-based products. Chromium can also be released into the environment from the burning of natural gas, oil, or coal.	Wood treatment, leather tanning, and stainless steel cookware, and metal hip replacements.	Food and smoking.	Metallurgy and tanning industries.
<b>Mercury (Hg)</b>	The most common natural forms of mercury found in the environment are metallic mercury (pure), mercuric sulfide (cinnabar ore), mercuric chloride, and methylmercury (white crystalline solids).	Approximately 80% is released into the air, primarily from fossil fuel combustion, mining, and smelting, and from solid waste incineration. About 15% of the total is released to the soil from fertilizers, fungicides, and municipal solid waste (e.g. discarded batteries). An additional 5% is released from industrial wastewater to water into the environment.	Batteries, dental amalgams, jewelry, lamps, paint, and thermometers.	Food, especially fish products.	Manufacture of electrical equipment or automotive parts that contain mercury, chemical processing plants that use mercury, and metal processing. Mercury vapor can be released from amalgam fillings (dentists and assistants).

Metal	Forms	Source of anthropogenic emissions	Used in	Sources of exposure in the general population	Sources of occupational exposure
<b>Lead (Pb)</b>	Odorless, silver-bluish-white metal present in ore deposits that is insoluble in water, but can be divided into lead compounds (soluble and insoluble).	The major source has historically been anthropogenic emissions to the atmosphere from the combustion of leaded gasoline (banned in 1995). Other sources include deteriorating Pb-based paints, the combination of corrosive water and Pb pipes, use of Pb-containing products, the combustion of coal and oil, and waste incineration.	Storage batteries, solders, paint, pigments, electrical shielding, plumping, ammunition, pottery glazes, cosmetics, hair dyes, jewelry, imported children's toys, traditional or folk remedies, and food packaging.	Air, food, and drinking water.	Mining and smelting of ore, and the manufacture of Pb-containing products and pesticides.

#### 1.3.8.2. Characteristics

Although heavy metals are naturally occurring chemicals, they have increasingly become a source of pollution concern due to anthropogenic activity. They are emitted into the air through combustion, metal mining, smelting and foundries, landfills, waste dumps, livestock and manure, as well as roadworks, and onto surface waters and soils through pesticides and fertilisers (Agency for Toxic Substances and Disease Registry, 1999, 2020; Streets *et al.*, 2017). Pollution from natural causes also contribute marginally from soil erosion, and metal corrosion, and less marginally in the context of volcanic activity.

Because they are non-biodegradable, metals remain present in the soils until they are moved or react with other elements. They tend to bind covalently (pairing two atoms sharing one or more electrons) with organic compounds, rendering them toxic and difficult to get rid of in the process (Briffa *et al.*, 2020). This property allows them to bioaccumulate inside organisms. Depending on the metals, they might accumulate in different organs or tissues after entering the organism through the respiratory and digestive tracts. More than 90% of the total body burden of Pb is found in the bones where they exhibit a half-life of 20–30 years (Agency for Toxic Substances and Disease Registry, 2020), while Hg, Cd and Cr are mostly stored in the kidneys and then the liver with a biological half-time of around 10–30 years (Järup and Åkesson, 2009). After storage, small amounts of metals are progressively remobilised and detoxified by the body by hiding them within a protein or transforming them in insoluble forms to be slowly excreted from the organism. Pb mobilisation through the use of bone tissue can also be induced by a pregnancy when constituting the foetus's skeleton or when lactating (de Souza *et al.*, 2018).

#### 1.3.8.3. Measuring exposure to heavy metals

Hair is often used to measure long-term exposure to metals (White *et al.*, 2020), though metals can be measured efficiently in whole blood and urine as well. Whist matrix comparisons are rare, whole blood and urine concentrations seem to correlate well for metals, though this could depend on the process of absorption of each metal by the human body or on the metal form being absorbed (Järup and Åkesson, 2009).

#### 1.3.8.4. Factors affecting heavy metals exposure

Human exposure to heavy metals in the general population occurs via the air, dust, food and water (Qu *et al.*, 2021) or via direct contact and fumes in occupations of various industries. However, not

every metal has been studied in relation to every biological and lifestyle factor. For instance, the determinants of Cr have not been researched as thoroughly as those of Pb, Hg and Cd, presumably because it was considered as an essential metal in the past.

### *Age*

Since metals can accumulate in the body, it is possible that circulating concentrations increase with age depending on the target tissue. A progressive rise in Cd levels has indeed been documented until the age of 60 years, as it seems to be the case for other metals (Fløtre *et al.*, 2017), but after that Cd concentrations seem to plateau or even decrease (Chaumont *et al.*, 2013). This effect could be influenced by either less Cd intake due to reduced food intake and movement, or a reduced capacity of the kidneys to absorb Cd. Nonetheless, higher concentrations in older groups could also reflect the decrease in exposure over the past decades as bone Pb has been shown to maintain blood Pb levels long after exposure has ended (Agency for Toxic Substances and Disease Registry, 2020). Deteriorating bones in the elderly could also be a major contributor of circulating levels.

### *Sex*

Males seem to have higher blood concentrations of cadmium, mercury and lead (Almerud *et al.*, 2021), though these could depend on age classes (Davies *et al.*, 1997; Mannelje *et al.*, 2020) and are not consistent in all studies (Garner and Levallois, 2016). These associations can also disappear in adjusted analyses, which indicates the potential role of confounders such as smoking on this relationship (Martins *et al.*, 2020). As explained above, women can discharge some amount of Pb during pregnancy and lactation (de Souza *et al.*, 2018), which could also be responsible for some of these differences.

### *Diet*

Fish products are a well-known source of mercury (Birgisdottir *et al.*, 2013). In a similar process than for POPs, mercury is released into water as a consequence of anthropogenic activities and bioaccumulate from species to species (algae, zooplankton, krill, etc) and builds up in the muscles of fish (Agency for Toxic Substances and Disease Registry, 1999) which is then eaten by humans. Cd levels tend to decrease with higher meat intake because the latter is an important source of dietary iron which is inversely associated with intestinal Cd absorption (Almerud *et al.*, 2021). Pb in dairy products has a similar relationship with calcium, explaining why dairy products might be associated with decreased Pb levels. On the other hand, cereals, potatoes, root crops, and vegetables are important sources of heavy metals including Cd, Pb, and Hg, most likely as a consequence of fertiliser and pesticide use (Manwani *et al.*, 2022). Anecdotally, Pb bullets have been shown to significantly increase the Pb concentrations of game meat (Meltzer *et al.*, 2013).



### *Cosmetics*

Although the use of metals in cosmetics is prohibited or restricted in the EU, the lack of legislation in other countries and the possibility of accidental contamination of cosmetic products with these elements means that several metals including Pb, Cd, nickel (Ni), arsenic (As), and Hg, can still be detected in various types of commercially available cosmetics (Witkowska *et al.*, 2021). This can affect colour cosmetics, face and body care products, hair cosmetics, herbal cosmetics, etc. The use of cosmetic products has been associated with increased levels of Pb, Cd, Hg and Al concentrations in the blood, urine or internal organs (Borowska and Brzóška, 2015). Increased Pb concentrations have also been associated with the number of cosmetics used (Dix-Cooper and Kosatsky, 2018) and with the use of popular traditional cosmetics such as kohl, surma, or tiro (Etchevers *et al.*, 2015).

### *Socio-economic levels*

Socio-economic factors including education and ethnicity, have been identified for various metals but are also likely to be the product of dietary and cosmetic habits as indicated by mediation analyses (Etchevers *et al.*, 2015; Gump *et al.*, 2020). This can especially be true in the case of Hg, which is highly present in fish (Mínguez-Alarcón *et al.*, 2021). Differences could also be attributed to occupational exposure or poor housing structures. Furthermore, Pb has been associated with motor vehicle traffic density, which is itself associated with urbanity and socio-economic levels (García-Esquinas *et al.*, 2011).

### *Tobacco and alcohol consumption*

Although metal presence and concentrations in cigarettes can vary, most contain high levels of Pb and Cd, due to the *Nicotiana tabacum* plant's "preference" to absorb them from the soil (Rubio Armendáriz *et al.*, 2015). As a consequence, these metals are also present in e-cigarette liquids and aerosols and are considered to be a major source of exposure among smokers (Jain, 2019). However, smoking does not seem to have an impact on Hg exposure (Almerud *et al.*, 2021). In addition, studies have found contradictory results regarding the effect of alcohol consumption on metal exposure (Martins *et al.*, 2020), even when adjusting on tobacco usage which can be correlated to alcohol consumption.

### *Seasons*

Seasonality can influence metal exposure through a variety of means. Higher temperatures, humidity, solar radiation combined with more photochemical reactions and seasonal pollution which is at peak in the summer increase metal exposure either through the air, dust or via animal and plant increased intake (Levin *et al.*, 2020).

### *Time trends*

Mercury levels in humans seem to have globally decrease since the 1990s, most likely due to changes in dietary and occupational exposures, though these changes are less marked in Western countries (Sharma *et al.*, 2019). A sharp decline in Pb concentrations explained by the ban of lead in petrol was observed in the 1990s but levels have remained stable since, and so have Cd levels (Wennberg *et al.*, 2017).

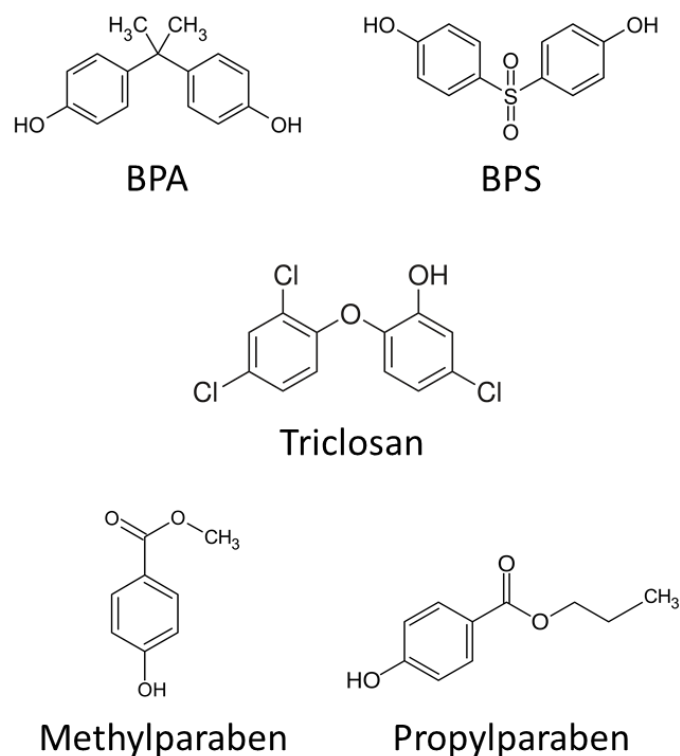
### 1.3.9. Non-persistent chemicals

#### 1.3.9.1. Definition, sources and regulation

Non-persistent EDCs are a class of chemicals that have relatively short biologic half-lives in biota (ranging from hours to days) due to their fast metabolism and elimination from the body following exposure, and that rapidly breakdown in the environment. Many of them were created to replace persistent chemicals following their bans. Here, we will focus more specifically on phenols and phthalates.

##### 1.3.9.1.1. Phenols

Phenols are compounds of one or more hydroxyl groups (-OH) bonded directly to an aromatic hydrocarbon group (Figure 9). They can be further divided include several categories of molecules including bisphenols, triclosan, and parabens, though others exist.



**Figure 9. Molecule structures of selected phenols**

### *Bisphenols*

Bisphenols are produced through a condensation reaction combining two phenols and an acetone molecule (Agency for Toxic Substances and Disease Registry, 2008). They are usually absorbed by the organism through oral ingestion (Roggeman *et al.*, 2022). The main route of metabolism of bisphenols in humans is through a process of glucuronidation (transfer of the glucuronic acid component) which occurs primarily in the liver. The bisphenol glucuronide then enters the blood stream to be delivered to kidneys which is then excreted with urine. The half-life of BPA, one of the most studied bisphenols, in the body is short (< 7–8 h), with almost complete excretion through urine within 24 hours of exposure (Völkel *et al.*, 2002).

Bisphenols are used in the manufacture of polycarbonate plastics present in many common consumer products (including plastic bottles and polyvinyl chloride (PVC)), but also of epoxy resins used for the interior lacquer coating of food and beverage cans, water pipes, thermal receipt paper, cigarette filters and some dental products (Souter *et al.*, 2013). Bisphenols can leach into food or beverages, especially when exposed to light and heat, is in contact with cleaning products, or simply when aging (Karwacka *et al.*, 2019). As a result of the scientific consensus on the health effects of BPA, this molecule has been heavily restricted in the EU and is being replaced in many countries by substitutes such as bisphenol F (BPF) and bisphenol S (BPS) (Bousoumah *et al.*, 2021). These molecules could possess similar

toxicological properties than BPA, but unfortunately significantly less information about their potential adverse health outcomes is available.

### *Triclosan*

Triclosan is another highly monitored phenolic compound. Its broad-spectrum antibacterial properties (Schildroth *et al.*, 2022) have made it a go-to molecule to add as an ingredient in many personal care products such as detergents, soaps, lotions, toothpaste and shampoos. Triclosan is also used as a plastic additive impregnated into toys, medical devices, household, veterinary, and industrial items (Karwacka *et al.*, 2019). As a result, it can penetrate the organism through dermal and mucosal contact with these products. Triclosan has a plasma half-life of approximately 19 hours, and the primary mode of elimination is through urine within the initial 24-hour period. The use of triclosan was only recently regulated in the USA due to its Food and Drug Administration (FDA) classification as “generally recognised as safe and effective” (Mínguez-Alarcón and Gaskins, 2017).

### *Parabens*

Parabens are a group of compounds formed by the esterification (replacement of at least one acidic hydroxyl group (–OH) by an organyl group (–R)) of the *p*-hydroxybenzoic acid (Wei *et al.*, 2021). Natural parabens are produced by plants and different microorganisms that serve to protect them against fungi and specific bacteria (Nowak *et al.*, 2018). Parabens are metabolised through two main processes: hydrolysis and conjugation. First, an enzyme (esterase) splits the molecule into an acid and alcohol (through hydrolysis) in the intestines. Then, the newly-formed metabolites are linked covalently to another molecule to increase their water solubility to be excreted (mostly) in urine (through conjugation). The main metabolite of parabens is para-hydroxybenzoic acid (Boberg *et al.*, 2010) and is usually conjugated as para-hydroxyhippuric acid. Parabens are considered to be completely excreted 72 hours after administration (Shin *et al.*, 2023).

Because of their anti-bacteria property, but also owing to their chemical stability when mixing with products, their low cost, and their low risk of inducing allergic reactions, industrial parabens have been synthesised to be used as antimicrobial preservatives in cosmetics, personal care products, and pharmaceuticals such as shampoos, conditioners, creams, soaps, deodorants, make-up products, wet wipes, menstrual pads, panty liners, tampons, and lotions (Mínguez-Alarcón and Gaskins, 2017). In fact, they are the most prevalent ingredients after water in personal care products (Wei *et al.*, 2021). They are also used as preservatives in the prevention of mould growth on paper products but also in processed food. For these reasons, parabens can enter our system through ingestion and dermal contact including the oral cavity. The most prevalent parabens are methyl paraben (MP), propyl paraben (PP), ethyl paraben (EP), and butyl paraben (BP) in order of detection (Nowak *et al.*, 2018). In

the EU, the proportion of paraben allowed in cosmetic products is restricted to 0.4% for each and 0.8% for total parabens (Błędzka *et al.*, 2014).

### 1.3.9.1.2. Phthalates

Phthalates constitute another class of chemicals widely used. They are esters of phthalic acids, which themselves come from derivatives of benzene, a natural constituent of petroleum. They are usually split into two groups based on the number of carbon atoms in their alkyl chains, the low molecular weight (1-4 carbons) and the high molecular weight ( $\geq 5$  carbons) (Schildroth *et al.*, 2022). Diester phthalates are hydrolysed into their primary metabolites, monoester phthalates. However, long-branched phthalates undergo an extra step of hydroxylation before being conjugated and excreted. For example, di(2-ethylhexyl)phthalate (DEHP) (Table 6) is first quickly converted into its primary metabolite (mono(2-ethylhexyl) phthalate (MEHP)) and then in several secondary metabolites including mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) (Frederiksen *et al.*, 2007). These metabolites are usually combined as a molar sum to measure DEHP exposure while other phthalates exposures are usually approximated using their main metabolite concentration in urine. The half-lives of the first elimination phase of di-*n*-butyl phthalate (DnBP), di-iso-butyl phthalate (DiBP), and DEHP metabolites are less than 4 hours and the half-lives of DEHP and di-iso-nonyl phthalate (DiNP) metabolites in the terminal phase of elimination are approximately 5 hours and 12–18 hours, respectively (Roggeman *et al.*, 2022).

**Table 6. List of diester phthalates and their most common metabolites**

Phthalate		Metabolites (primary and secondary)	
Dimethyl phthalate	DMP	Monomethyl phthalate	MMP
Diethyl phthalate	DEP	Monoethyl phthalate	MEP
Di- <i>n</i> -butyl phthalate	DnBP	Mono- <i>n</i> -butyl phthalate	MnBP
Di-iso-butyl phthalate	DiBP	Mono-iso-butyl phthalate	MiBP
Butylbenzyl phthalate	BBzP	Monobenzyl phthalate	MBzP
Di(2-ethylhexyl) phthalate	DEHP	Mono(2-ethylhexyl) phthalate	MEHP
		Mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP
		Mono(2-ethyl-5-oxohexyl) phthalate	MEOHP
		Mono(2-ethyl-5-carboxypentyl) phthalate	MECPP
		Mono(2-carboxy-hexyl) phthalate	MCMHP
Di-iso-nonyl phthalate	DiNP	Mono-iso-nonyl phthalate	MiNP
		Mono(hydroxy-iso-nonyl) phthalate	MHiNP
		Mono(oxo-iso-nonyl) phthalate	MOiNP
		Mono(carboxy-iso-octyl) phthalate	MCIOP

Low molecular weight phthalates include diethyl phthalate (DEP), DnBP, and DiBP, which are used in cosmetics and personal care products, varnishes, waterproof clothing, detergents and in coatings (including the ones used for orally administered medications) (Mínguez-Alarcón and Gaskins, 2017). High molecular weight phthalates include di(2-ethylhexyl) phthalate (DEHP), diisodecyl phthalate (DiDP) and DiNP, which are used as plasticisers to impart flexibility and durability in building materials (including flooring and wall coverings), PVC, food packaging, children's toys, and medical devices. Because phthalates do not chemically bound to products, they can leach, migrate or evaporate into indoor air and atmosphere, food, and other materials, and be easily be inhaled, or ingested (Heudorf *et al.*, 2007). Some phthalates (DEHP, DnBP and butylbenzyl phthalate (BBzP)) have been banned from the production of toys and personal care products within the EU.

#### 1.3.9.2. Characteristics

All non-persistent EDCs can be absorbed into the human body through ingestion, inhalation or dermally, however, humans are mostly exposed to bisphenols via ingestion, and parabens and phthalates through both oral and dermal administrations due to their use plastic food packaging and cosmetics.

The main challenge in studying non-persistent chemicals comes from their rapid metabolism in the human body, as it may contribute to measurement error and exposure misclassification. Consequently, it is possible for the estimated effects of the chemicals assessed in epidemiological studies to be attenuated or inflated, making it more difficult to interpret results (Lassen *et al.*, 2013). Another source of concern in understanding the health effects of non-persistent chemicals is the characterisation of the long-term exposure (Casas *et al.*, 2018).

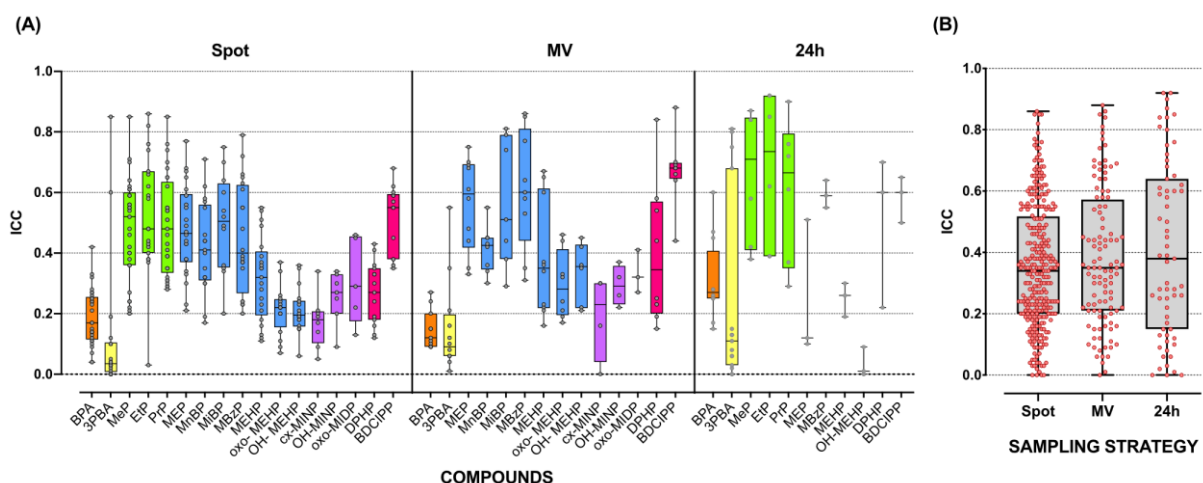
For these reasons, assessing the extent of a measurement bias when possible and more accurately measuring exposure have been a major source of focus in the literature on non-persistent chemicals.

#### 1.3.9.3. Measuring exposure to non-persistent chemicals

Due to their short half-lives, accurately measuring exposure to non-persistent chemicals in humans has remained a challenge. To remedy this, urine is considered to be the matrix of choice and several urine sampling methods have been employed to improve the accuracy of the measures as much as possible including spot (one sample), first morning void (first urine of the day), 24-hour samples (collecting of voids of urine for 24 hours), or pooled samples (average of several spot samples over a longer period of time). A 2022 review (Roggeman *et al.*, 2022) of the variability in urinary concentrations of non-persistent organic chemicals (including 21 articles on bisphenols, 17 articles on

parabens and 23 articles on phthalate metabolites) shared several observations. BPA showed the highest variability and parabens the lowest, but none of the chemicals showed very high intraclass correlation coefficients (ICC) (Figure 10). These differences in variability could be attributed to half-lives but also to usage. Parabens and low-molecular weight phthalates, present in personal care products and therefore used daily without frequent changes, had higher ICCs compared to BPA and high-molecular weight phthalates whose main source of exposure is the diet which can vary both daily and seasonally (Bastiaensen *et al.*, 2020; Mok *et al.*, 2022). However, chemicals used in sun protection products can vary depending on the season (Lassen *et al.*, 2013). Furthermore, absorption is usually slower through the skin than through the gastrointestinal tract.

When comparing between non-adjusted versus creatinine-adjusted versus specific gravity-adjusted concentrations, most of the chemicals with low ICCs (<0.4) still showed low reproducibility no matter the method. Among higher ICCs, both adjustment techniques showed similar ICCs except for two phthalate metabolites (monoethyl phthalate (MEP) and MEHP) which were higher for specific gravity and two parabens (EP and PP) which were slightly higher for creatinine. This evidence suggests that dilution adjustments of urine concentrations do not significantly affect the ICCs of most chemicals.



**Figure 10. Variation in the ICCs for short-lives chemicals or their metabolites in spot urine compared to morning void and 24 h urine (from Roggeman *et al.*, 2022)**

(A) ICCs of each class of compound; (B) Each class of compound was summarized according to sampling strategies. Boxplots show median and interquartile range, and upper and bottom bars represent max and minimum, respectively. Each dot represents an individual ICC from the studies included in this review. Studies of adults and pregnant women were included (not considering dilution adjustments).

Nonetheless, this review did not consider studies using pooled samples of urine across several timepoints. A study (Shin *et al.*, 2019) measuring 14 metabolites of eight phthalates in 577 urine

samples collected from 188 pregnancies over two trimesters observed higher ICCs in pooled samples than in first morning voids (except for MEP), and almost two times larger for metabolites with relatively variable exposure sources mainly through diet. In a later study (Shin *et al.*, 2021), the researchers also found that ICCs across two trimesters for BPA, MP, and PP, were higher in pools compared to first morning voids. Furthermore, ICCs were higher for MP and PP in women who provided three or more samples than those who provided only two samples. In addition, a simulation study (Vernet *et al.*, 2019) based on data from the French SEPAGES cohort on phenol concentrations showed that a sampling approach relying on repeated within-subject pooling of three daily spot samples can accurately estimate exposure averages except for triclosan. However, another study (Bastiaensen *et al.*, 2020) found that morning voids samples were consistent over 5 consecutive days and that phthalate ICCs increased significantly when values were corrected for urinary dilution and when only morning void samples were used.

Overall, these findings that the number of measurements required and adjustment method to accurately characterize long-term average exposure to non-persistent chemicals may vary by biomarker and by population socio-demographics.

#### 1.3.9.4. Factors affecting non-persistent EDC exposure

The variability highlighted above has a direct impact on the evaluations of non-persistent chemical exposure determinants as associations are generally less definite than for persistent chemicals.

##### *Diet*

Specifically, dietary studies have found that phenols were associated with meat, bread, beverages and butter and oil. BPA exposure may depend more on the food packaging than the food item itself, with canned foods being known to contain high levels of BPA (Larsson *et al.*, 2014). High-molecular weight phthalates have been associated with the consumption of ice cream, sweets and chocolate, most likely due to the migration of these phthalates from the packaging into the food (Husøy *et al.*, 2019). However, fish, meat, poultry, bread and dairy products seem to also be heavy contributors to the intake of DEHP.

##### *Personal care products*

The use of many PCPs has been associated with increased urinary concentrations of parabens and BPS (but not triclosan or BPA) in a French study (Nakiwala *et al.*, 2020) using a panel design with intense urine sample collection (8-80 urine samples per participant). These increases in urinary concentrations were at their peak within 0-6 h for EP and BP, and 12-24 h for BPS after using personal care products.



Higher triclosan levels have been observed in relation to sunscreen use (Schildroth *et al.*, 2022). Parabens are highly present in mouthwashes and in other oral hygiene products due to their antimicrobial properties (Ferguson *et al.*, 2017) and more generally in lotions, cleansers, creams, coloured cosmetics, toothpaste, hair care products, and soaps. BPA and BPS have been reported in lotions, shampoo, conditioner, facial cleansers, sunscreens, lipsticks and toothpaste (Nakiwala *et al.*, 2020). Urinary concentrations of the phthalate metabolite MEP have been associated with use of lotion, eye make-up and roll-on/stick deodorant and higher concentrations of MEP and mono-n-butyl phthalate (MBP) with several types of makeup (Berger *et al.*, 2019). Higher levels of DiNP metabolites were also correlated with frequent use of fragrance (Larsson *et al.*, 2014).

Personal care products and exposure to their chemicals can in turn affect ethnic groups differently. It has been reported that Afro-American women in the US purchase nine times more hair and beauty products than any other racial groups (Pagoni *et al.*, 2022). On the other hand, Causasian women tend to buy more hair straighteners, skin lighteners, and odour masking products. The use of personal care products is further affected by sex and age. For instance, greater urinary concentrations of phenols have been reported in women than in men (Ao *et al.*, 2022; Nowak *et al.*, 2018).

#### *Socio-economic levels*

Several studies have found inconsistent results regarding the relationship between education or household income and levels of non-persistent chemicals. Higher BPA concentrations were detected among participants with low household incomes but not everywhere (Bethea *et al.*, 2020), and higher bisphenol concentrations were sometimes observed in families with higher income (Ao *et al.*, 2022). Parabens, benzophenone-3 (BP-3) and triclosan were associated with a university education, but lower education has also been associated with higher BPA levels. In the HELIX study pooling six European mother-child cohorts, the concentrations of several parabens (MP, EP, BP, PP) in low educated pregnant women were lower than of those in high educated women but no associations were found for phthalate metabolites (Montazeri *et al.*, 2019). Moreover, unemployment did not change concentrations levels. These differences could reflect varying dietary patterns and to the use of plastics including PVC as suggested by a study reporting high levels of phthalates in people living in rural areas (Larsson *et al.*, 2014).

#### *BMI*

The impact of BMI on concentrations of non-persistent chemicals reflect similar trends to what is observed with POPs. Higher BMI has been associated with higher concentrations of bisphenols (Bethea *et al.*, 2020) but an inverse association was found between BMI and concentrations of triclosan, BP-3, parabens and phthalates (Ribeiro *et al.*, 2019; Vindenes *et al.*, 2021). Although they are not persistent,

these chemicals could still exert some lipophilic properties and accumulate in adipose tissues (Bethea *et al.*, 2020; Ribeiro *et al.*, 2019). This would lead individuals with a higher BMI to excrete lower levels of phthalates compounds in urine because there is more storage capacity. However, a high amount of chemical circulation could lead to the saturation of storage in adipose tissue that would only be observed for chemicals to which we are more exposed. There is also a possibility of reverse causation as chemicals such as bisphenols could act as obesogens and interfere with the body's natural weight control mechanisms (Bethea *et al.*, 2020; Ribeiro *et al.*, 2019).

#### *Time trends*

A decrease in BPA concentrations has been observed while BPF has remained stable and BPS has increased both in the US (Bommarito *et al.*, 2023) and in Japan (Gys *et al.*, 2020). Paraben levels in the general population seem to have increased until 2006 but has decreased since then in response to agency restrictions (Wei *et al.*, 2021). Several European studies have also observed a decrease in exposure by a factor of 2-8 for legally restricted phthalates but an increase for DiNP, di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) and di-2-ethylhexyl terephthalate (DEHTP), corresponding to a market shift towards shorter-chained phthalates (Göen *et al.*, 2011; Vogel *et al.*, 2023).

## **1.4. EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON THE OVARIAN RESERVE**

### **1.4.1. Persistent Organic Pollutants**

This section will focus specifically on OCPs, PCBs, PBDEs, and PFAS. Other classes of POPs are included in the Stockholm convention or in the REACH list, but are not included in this work due to a lack of biological or human studies on their effects on reproduction and/or on concentration levels in the general population and were not available for analysis.

#### **1.4.1.1. Experimental studies**

Animal studies have reported associations between exposure to OCPs (DDT,  $\gamma$ -HCH, and hexachlorobenzene (HCB)) and PFAS (PFOS, and PFOA), and impaired oocyte maturation (Lindenau *et al.*, 1994), and different steps of folliculogenesis (Alvarez *et al.*, 2000; Chen *et al.*, 2017; Du *et al.*, 2019). Higher doses of in utero exposure to selected POPs (PBDE 47 and 99, Aroclor 1016 (a commercial PCB mixture), hexabromocyclododecane (HBCD), and perfluorobutanesulfonic acid (PFBS) were also associated with a decreased number of follicles (Baldrige *et al.*, 2003; Ema *et al.*, 2008; Feng *et al.*, 2017; Lilienthal *et al.*, 2006; Talsness *et al.*, 2008). Moreover, in-vitro studies have shown impacts of

OCPs ( $\gamma$ -HCH, DDT and DDE) and PCB 126 on oocyte maturation (Alm *et al.*, 1998; Krogenæs *et al.*, 1998) and folliculogenesis (Holloway *et al.*, 2007).

#### 1.4.1.2. Epidemiological studies

##### *Post-natal exposure*

In humans, few studies have investigated the effects of POPs on indicators of the ovarian reserve, such as AMH and AFC treated as continuous variables, and none have looked at the effects of POPs on DOR (Table 7). In a population of 50 pregnant women, one study (Björvang *et al.*, 2021) found associations between higher POP levels (HCB, trans-nonachlor, PCB 74 and 99) and decreased non-growing follicle densities measured in ovarian cortical tissue, which disappeared for all POPs when they were adjusted for lipid levels, except for HCB. In the same study, no associations were observed with serum AMH in the first trimester of pregnancy. In a subsequent paper investigating 30 POPs (including OCPs, PCBs, PBDEs and PFAS) in a cohort of 185 women undergoing assisted reproductive technologies (Björvang *et al.*, 2022), the authors also found higher HCB concentrations (wet-weight) to be the only POP measured in serum to be associated with lower AMH levels, whereas none were associated with AFC. In a study of 420 null-parous women aged 20 to 30 years from a rural community in South Africa (Whitworth *et al.*, 2015), p,p'-DDT and p,p'-DDE were not associated with AMH levels. Bloom *et al.* (2017) identified associations between high levels of three PCBs (namely PCB 151, 170, and 180) amongst 45 POPs measured in follicular fluid and a lower number of antral follicles in a pilot study of 32 women (Bloom *et al.*, 2017). One POI case-control study (Zhang *et al.*, 2018) also investigated the role of five PFAS (PFOA, PFOS, perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), and PFBS) on day-3 FSH concentrations and found that increased exposure to PFOS and PFHxS in POI patients was associated in an increase in FSH levels, but not in the POI control population. However, Harlow *et al.* found in a cohort of 1371 women (45-56 year old) from the general population that higher levels of PFOA and PFOS were associated with increased day 2-5 FSH levels (Harlow *et al.*, 2021).

##### *Pre-natal exposure*

A limited number of epidemiological also explored associations between in utero exposure to POPs and the ovarian reserve at various ages. One Danish team found no effects of in utero PFOA or PFOS on AMH, AFC or FSH in daughters (mean age of 19) (Kristensen *et al.*, 2013), but found in a subsequent analysis that higher terciles of p,p'-DDE and HCB in utero (but not PCBs) resulted in a lower AFC but did not affect AMH in daughters (mean age of 20) (Kristensen *et al.*, 2016). Additionally, no significant associations were observed between in utero PFAS exposure (PFOS, PFOA, PFHxS, and PFNA) and AMH

levels in daughters (mean age of 15 years) in a cohort based in the United Kingdom (UK) (Donley *et al.*, 2019).

**Table 7. Summary of the associations between persistent organic pollutants and ovarian reserve outcomes**

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
<b>Post-natal</b>									
Whitworth <i>et al.</i> (2015)	South Africa	Cross-sectional	20–30 year old women living in rural villages	426	p,p'-DDT, p,p'-DDE	Plasma	AMH	Age, body mass index, parity, education and total lipids	No associations (p,p'-DDT: -1% (-3 to1); p,p'-DDE: 0.1% (-0.6 to 1)).
Bloom <i>et al.</i> (2017)	United States	Cross-sectional	Fertility centre	32	p,p'-DDT, p,p'-DDE, PCB congeners 28, 44, 49, 52, 66, 74, 77, 81,87, 99, 101, 105, 110, 114, 118, 123, 126, 128, 138, 146, 149,151, 153, 156, 157, 158, 167, 169, 170, 172, 177, 178, 180,183, 187, 189, 194, 195, 196–203, 199, 201, 206, 209	Follicular fluid	AFC	Body mass index, cigarette smoking, race and age	Mostly non-significant decreases in AFC. PCB 149 (-0.63, 95% CI (-0.98, -0.29)), PCB 170 (-0.39, 95% CI (-0.72, -0.07)) and PCB 180 (-0.38, 95% CI (-0.75, -0.02)) were associated with significant decreases in AFC.
Zhang <i>et al.</i> (2018)	China	Case-control	20-40 year old women consulting at a general hospital	120 cases and 120 controls	PFOS, PFOA, PFHxS, and PFNA	Plasma	Day 3 FSH	Age, body mass index, education, household income, sleep and parity	Increased PFOS ( $\beta$ 0.26; 95% CI (0.15, 0.38)), and PFHxS ( $\beta$ 0.16; 95% CI (0.04, 0.28)) in cases associated with higher FSH concentrations but not in controls.
Björvang <i>et al.</i> (2021)	Sweden	Cohort	Women undergoing caesarean section	145	PeCP, HCB, $\alpha$ -HCH, $\beta$ -HCH, $\gamma$ -HCH, oxychlorane, transnonachlor, p,p'-DDT, p,p'-DDE, PCB congeners 74, 99, 118, 138, 153, 156, 170, 180, 183, and 187, PBDE congeners 47, 99, and 153, PFHxS, PFHpA, PFOS, PFOA, PFNA, PFDA, PFUnDA, PFDoDA	Serum	AMH and follicle densities	Body mass index, age, and parity	No associations for AMH. Significant associations between higher HCB, trans-nonachlor, PCB 74 and 99 levels and decreased non-growing follicle densities, but not when also adjusted for total lipid levels, except for HCB.
Harlow <i>et al.</i> (2021)	United States	Cohort	45-56 year old women whom had a menstrual period in the prior 3 months	1371	PFOS, PFOA, PFHxS, and PFNA	Serum	Day 2-5 FSH	Age, site, race, smoking status, menopausal status, parity, and body mass index	Increased concentrations of PFOA (3.12%, 95% CI (0.37%, 5.95%)), and PFOS (2.88%, 95% CI (0.21%, 5.63%)) were associated with a doubling in FSH concentration. These associations disappeared when body mass index was not included in the covariates.

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
Björvang <i>et al.</i> (2022)	Sweden	Cohort	Fertility centre	185	PeCP, HCB, $\alpha$ -HCH, $\beta$ -HCH, $\gamma$ -HCH, oxychlorane, transnonachlor, p,p'-DDT, p,p'-DDE, PCB congeners 74, 99, 118, 138, 153, 156, 170, 180, 183, and 187, PBDE congeners 47, 99, and 153, PFHxS, PFHpA, PFOS, PFOA, PFNA, PFDA, PFUnDA, and PFDoDA	Serum and follicular fluid	AMH and AFC	Age, BMI, parity, fatty fish intake and cause of infertility	Higher HCB concentrations (wet-weight) significantly associated with lower AMH levels, whereas none were associated with AFC.
<b>Pre-natal</b>									
Kristensen <i>et al.</i> (2013)	Denmark	Cohort	General population, (mean age of daughters = 19 years old)	245	PFOA and PFOS	Serum	AMH, AFC (only one ovary, N = 147) and FSH	Maternal smoking, household income, daughter's body mass index and daughter's smoking	No associations.
Kristensen <i>et al.</i> (2016)	Denmark	Cohort	General population, (mean age of daughters = 20 years old)	341	p,p'-DDE, HCB, and sum of PCB congeners (118, 138, 153, 156, 170 and 180)	Serum	AMH, AFC (only one ovary, N = 43) and FSH (N = 74)	Maternal smoking, household income, total lipids, and daughter's body mass index	Higher terciles of p,p'-DDE and HCB resulted in a lower AFC but did not affect AMH or FSH levels.
Donley <i>et al.</i> (2019)	United Kingdom	Cohort	General population, (mean age of daughters = 15 years old)	446	PFOS, PFOA, PFHxS, and PFNA	Serum	AMH	Body mass index, maternal education, prenatal smoking, and maternal age at delivery	No associations.

AMH, anti-müllerian hormones; CI, confidence interval; AFC, antral follicle count; FSH, follicle-stimulating hormones.

#### 1.4.1.3. Conclusion

In summary, there is a low and inconsistent amount of results concerning the relationship between POPs and continuous indicators of the ovarian reserve. Furthermore, none of these studies have examined the relationship between POPs and DOR specifically.

#### 1.4.2. Heavy metals

##### 1.4.2.1. Experimental studies

In animal studies, deleterious effects on folliculogenesis have been reported for Cd (Nad *et al.*, 2007; Weng *et al.*, 2014), Hg (Merlo *et al.*, 2019), and Pb (Taupeau *et al.*, 2001). A decreased number of follicles of various stages was also found in rats exposed to Cr via lactation (Banu *et al.*, 2008). In humans, there is conflicting evidence that heavy metals can interfere with reproductive functions (Henriques *et al.*, 2019; Pollack *et al.*, 2014).

##### 1.4.2.2. Epidemiological studies

###### *Post-natal exposure*

Cd has been detected in human ovaries (Varga *et al.*, 1993) and Pb, Hg, Cd, and Cr, have previously been measured in follicular fluid (Butts *et al.*, 2021; Wu *et al.*, 2020). Only a few studies have assessed associations between heavy metals and AFC or AMH as continuous outcomes (Table 8). A first study has investigated the associations between exposure to whole blood Pb or Cd and AMH levels in two cohorts of pregnant women based in Ukraine and Greenland and found no significant associations for either metal (Christensen *et al.*, 2016). However, a selection bias cannot be excluded since the participation rate in Ukraine (26%) and the availability of whole blood (18% for Ukraine and 25% for Greenland) were both low. Another study (Lee *et al.*, 2018) found that higher whole blood Cd concentrations was associated with lower AMH levels in women aged 30 to 35, but not in older groups. Lastly, a study (Mínguez-Alarcón *et al.*, 2021) found that higher concentrations of hair Hg were associated with higher AFC in women attending a fertility centre. Nonetheless, this association was attenuated when the model was adjusted for total omega-3 polyunsaturated fatty acids, a beneficial nutrient absorbed when eating fish or shellfish which are food products known to contain high levels of Hg.

*Pre-natal exposure*

To the best of our knowledge, no studies have investigated the associations between in utero exposure to heavy metals and the ovarian reserve.



**Table 8. Summary of the associations between metals of interest and ovarian reserve outcomes**

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
<b>Post-natal</b>									
Christensen <i>et al.</i> (2016)	Ukraine & Greenland	Cross-sectional	Pregnant women in the general population	117	Pb, Cd	Whole blood	AMH	Gestational age, maternal age, research site, parity, fish intake, body mass index, smoking status, pelvic diseases and infections	No associations.
Lee <i>et al.</i> (2018)	South Korea	Cross-sectional	30-49 year old women from the general population	283	Cd	Whole blood	AMH	Body mass index, parity, smoking status, and fish intake	Higher Cd concentrations were significantly associated with a reduction in AMH levels ( $\beta$ -0.34 (95% CI (-0.64, 0.05)). After stratifying by age group, this association remained only in the 30-35 age group.
Mínguez-Alarcón <i>et al.</i> (2021)	United States	Cohort	Fertility centre	353	Hg	Hair	AFC	Age, body mass index, smoking status, infertility diagnosis, and alcohol intake	Higher terciles of Hg levels increased AFC (T2 v. T1: $\beta$ 14.0, 95% CI (13.4, 14.7); T3 v. T1: $\beta$ 14.1, 95% CI (13.4,14.8). Further adjustment for total n3PUFA and total calorie intake attenuated these associations.

AMH, anti-müllerian hormones; CI, confidence interval; AFC, antral follicle count; T, tercile.

#### 1.4.2.3. Conclusion

Whilst a few studies have looked at AFC or AMH as continuous indicators of the ovarian reserve, no study so far has investigated associations between heavy metals and DOR.

#### 1.4.3. Non-persistent chemicals

##### 1.4.3.1. Experimental studies

Evidence from experimental studies show that BPA (Li *et al.*, 2014; Signorile *et al.*, 2012) and DEHP metabolites (Hannon *et al.*, 2014; Zhang *et al.*, 2013) can significantly reduce the number of follicles at various stages of folliculogenesis including primordial, primary/preantral follicles, and antral follicles as well as corpora lutea. BPA (Peretz *et al.*, 2013), DEHP (Hannon *et al.*, 2015), dimethyl phthalate (DMP) (Mei *et al.*, 2019), BP (Ara *et al.*, 2021) and PP (Gal *et al.*, 2019) can also have effects on steroidogenesis, particularly on the production of estradiol. These effects are explained by the fact that chemicals such as BPA can specifically interact with oestrogen receptors alpha and beta, and can mimic or antagonise the effects of estrogen, but also exert antioestrogenic actions by inhibiting the aromatase activity – an enzyme involved in the synthesis of oestrogens (Karwacka *et al.*, 2019).

##### 1.4.3.2. Epidemiological studies

###### *Post-natal exposure*

Several studies among women enrolled in the Environment and Reproductive Health (EARTH) study, a prospective cohort established to assess environmental and dietary determinants of fertility, have observed associations between non-persistent chemicals and the ovarian reserve (Table 9). Specifically, these found that higher single spot urinary concentrations of BPA (Souter *et al.*, 2013), single spot PP (Smith *et al.*, 2013), and several (pooled) phthalates including DEHP metabolites (Messerlian *et al.*, 2016) were associated with a lower AFC. Since then, two other studies taking place in fertility centres showed significant associations between higher single spot BPA concentrations and a reduction in AMH and AFC (Czubacka *et al.*, 2021), and a higher risk of DOR (undefined) (Zhang *et al.*, 2023), whilst another study in 30-49 years old women in the general population found no relations between single spot BPA and AFC or AMH (Park *et al.*, 2020). Recently, Zhang *et al.* found that higher levels of single spot BPS were associated with lower AMH and a higher risk of DOR but not for BPF (Zhang *et al.*, 2023). In a DOR case-control study with 64 cases (diagnosed when 2 of 3 of the following criteria were present:  $AFC \leq 7$ ,  $AMH \leq 1.1$  ng/L, and day 2-3 FSH  $\geq 10$  IU/L) and 86 controls, Tian *et al.*

showed that increased mono-iso-butyl phthalate (MiBP) and MECPP measured in follicular fluid were significantly associated with higher odds of DOR but this was not the case for any other phthalates (MEP, MEHP, MEHHP, MEOHP) or any parabens (MP and PP) (Tian *et al.*, 2023). Finally, among three studies investigating various single spot samples of phthalate metabolites, higher quartiles of urinary MEHHP and MEOHP levels were associated with AFC but not with AMH and DOR in one study (Tian *et al.*, 2023), single spot MEHHP and MiBP concentrations were associated with AMH in another (Li *et al.*, 2022), and no associations were found in the general population with AMH or AFC in the last (Park *et al.*, 2020).

Although these studies have looked at various chemicals on their own, the available epidemiologic literature on mixtures of these EDCs in relation to the ovarian reserve and other fertility measures is still scarce and inconsistent. Bellavia *et al.* evaluated mixtures of phthalates, parabens and PFAS measured in follicular fluid in relation to women's fertility assessed as ovarian sensitive index (OSI) as well as clinical pregnancy and live birth probabilities among 333 women in Sweden and Estonia (Bellavia *et al.*, 2022). While they found negative associations between follicular fluid DEHP and methylparaben (MP) with OSI and suggestive with certain PFAS, no associations were found for pregnancy outcomes. In a different study and using principal component analysis, Pollack *et al.* evaluated mixtures of several single spot urinary phenol biomarkers including bisphenol A and parabens, in relation to reproductive hormones among 143 premenopausal women (Pollack *et al.*, 2018). The authors found that the ultraviolet protection factor (which was characterized by higher urinary concentrations of benzophenone-1 and benzophenone-3) was negatively associated with FSH levels. Finally, in the DOR case-control study mentioned above (Tian *et al.*, 2023), an increase of exposure to a mixture of paraben, phenol, phthalate, and PFAS from the median to the 75<sup>th</sup> percentile increased the odds of DOR. Nonetheless, this association seems to be heavily influenced by the associations between benzophenone-4 as a single exposure and DOR.

#### *Pre-natal exposure*

One study (Hart *et al.*, 2014) has tested the unadjusted associations between multiple phthalate and phenol biomarkers in pregnant women in 1989–1991 and AMH levels, and AFC, in 121 teenage girls (between 13 and 17 years old), and found that higher MEP concentrations, but no other metabolite, was significantly correlated with lower AMH levels and none were correlated with AFC. To the best of our knowledge, no other studies have attempted to replicate these results.

**Table 9. Summary of the associations between non-persistent chemicals of interest and ovarian reserve outcomes**

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
<b>Post-natal</b>									
Smith <i>et al.</i> (2013)	United States	Cohort	Fertility centre	192	MP, PP, BP	Urine	AFC and day 3 FSH	Age	No significant results but trend towards lower AFC and higher FSH levels for increases of each paraben.
Souter <i>et al.</i> (2013)	United States	Cohort	Fertility centre	209	BPA	Urine	AFC and day 3 FSH	Age	Higher quartiles of BPA concentrations compared to the 1st quartile were significantly associated with a decrease in AFC (Q2 v. Q1: -12% (95% CI (-23%, -0.6%)), Q3 v. Q1: -22% (95% CI, (-31%, -11%)), Q4 v. Q1: -17% (95% CI, (-27%, -6%)). No significant associations between BPA and FSH.
Messerlian <i>et al.</i> (2016)	United States	Cohort	Fertility centre	215	MEP, MBP, MiBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, DEHP	Urine	AFC and day 3 FSH	Age, body mass index, and smoking	Significant decrease in mean AFC across quartiles of $\Sigma$ DEHP (Q2 v. Q1: -24% (95% CI (-32%, -16%)), Q3 v. Q1: -19% (95% CI (-27%, -9%)), Q4 v. Q1: -14% (95% CI (-23%, -5%)). Similar trends were observed among the four individual DEHP metabolites (MEHP, MEHHP, MEOHP, MECPP).
Zhou <i>et al.</i> (2017)	China	Cross-sectional	PCOS patients	268	BPA	Urine	AFC, AMH and FSH	Age, body mass index, and income	An increase in BPA was significantly associated with a decrease in AFC ( $\beta$ - 0.34, 95% CI (-0.60, -0.08)). A non-significant decrease was observed for FSH and AMH.

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
Du <i>et al.</i> (2018)	China	Retrospective cohort	Fertility centre	415	MEP, MBP, MBzP, MEHP, MEHHP, MEOHP, MECPP, DEHP	Urine	AFC, AMH and DOR	Age, body mass index, and creatinine	No associations.
Park <i>et al.</i> (2021)	South Korea	Cross-sectional	30-49-year-old women from the general population	307	BPA, MBP, MEHHP, MEOHP	Urine	AFC and AMH	AMH: Body mass index AFC: age and body mass index	No associations.
Czubacka <i>et al.</i> (2021)	Poland	Cross-sectional	Fertility centre	511	BPA	Urine	AFC, AMH and FSH	Infertility diagnosis, body mass index, age, and smoking	Increases in BPA concentrations were significantly associated with a decrease in AMH ( $\beta$ -0.04, 95% CI (-0.13; -0.01)) and AFC ( $\beta$ -0.01, 95% CI (-0.11; -0.00)) but not with FSH.
Li <i>et al.</i> (2022)	China	Cross-sectional	Fertility centre	297	MEP, MBP, MiBP, MBzP, MEHP, MEHHP, MEOHP, MECPP	Urine	AFC, AMH and FSH	Age, body mass index, smoking status, drinking status, long-term residence, education level, and household income	Linear increases in MiBP concentrations was significantly associated with lower AMH levels ( $\beta$ 0.99, 95% CI (1.67, 0.30)), while the fourth quantile of mEHHP concentrations significantly increased them ( $\beta$ 0.27, 95% CI (0.03, 0.52)). The third quantile of MEP significantly increased FSH concentrations ( $\beta$ 0.15, 95% CI (0.02, 0.28)) but the third quantile of MCMHP decreased them ( $\beta$ 0.15, 95% CI (0.27, 0.04)). MEHHP was significantly associated with an AFC decrease (OR 1.43, 95% CI: 1.06, 1.92)).
Tian <i>et al.</i> (2023)	China	Case-control study	Fertility centre	64 cases and 86 controls	BPs, MP, PP, EP, MEP, MiBP, MEHP, MEHHP, MEOHP, MECPP	Urine	DOR	Age and infertility diagnosis	Increases in MiBP (OR 2.70, 95% CI (1.30, 5.76)), and MECPP (OR 1.92, 95% CI (1.02–4.09)) were significantly associated with higher likelihoods of DOR.

Author	Country	Design	Population	Size of sample	Exposures	Exposure matrix	Outcome	Covariates	Key findings
Zhang <i>et al.</i> (2023)	China	Cross-sectional	Fertility centre	111	BPA, BPS, BPF	Urine	AFC, AMH and DOR	AMH and FSH: Age, body mass index, smoking history, passive smoking and infertility diagnosis DOR: age, body mass index and household income for DOR	Higher BPS concentrations were associated with a decrease in AMH levels ( $\beta$ 0.287, 95% CI: (0.505, 0.070)). Higher BPA (OR of 7.11 (95%CI: 1.247, 40.588)) and BPS concentrations were associated with a higher risk of DOR.
<b>Pre-natal</b>									
Hart <i>et al.</i> (2014)	Australia	Cohort	General population	121	MEP, MnBP, MiBP, MBzP, MPP, MEHP, MECPP, MOP, MiNP, MCIOP, MiDP	Serum	AFC, AMH and FSH	No adjustment	MEP was significantly associated with serum AMH concentrations ( $r = 0.21$ , $p = 0.031$ ). MEHP, but no other metabolite, was significantly negatively associated with serum FSH ( $r = 0.22$ , $p = 0.024$ ).

AMH, anti-müllerian hormones; CI, confidence interval; AFC, antral follicle count; DOR, diminished ovarian reserve; FSH, follicle-stimulating hormones; PCOS, polycystic ovary syndrome.

#### 1.4.3.3. Conclusion

Additional studies on mixtures of pooled samples of non-persistent chemicals and the ovarian reserve and studies applying specific mixture models could help to increase our understanding of the reproductive health effects of these chemicals.

### 1.5. STATISTICAL CONSIDERATIONS FOR THE STUDY OF MIXTURES

Historically, epidemiological research investigating the impact of chemicals on human health has primarily focused on understanding the effects of individual pollutants on health outcomes in isolation, often overlooking the complex interactions that could occur between chemicals. The creation and development in the last decade of statistical methods that explore these interactions have allowed the field to expand and adopt a more comprehensive approach that includes the study of mixtures.

#### 1.5.1. Definition

The evaluation of the exposure to a group of chemicals or mixtures has grown in interest in the last several years because chemicals may interact with each other in various ways, including additively, synergistically, or antagonistically (Le Magueresse-Battistoni *et al.*, 2018). This approach enables a more accurate representation of human exposure to chemicals, as the population is typically exposed to multiple pollutants simultaneously rather than one at a time. This topic also represents an area of increasing concern to regulatory authorities in the US and Europe (Cedergreen, 2014).

Additivity of chemicals supposes that the sum of single-exposure effects constitutes the overall mixture effect. However, when chemicals in a mixture exacerbate the toxic effects of one another, i.e. they jointly exert a larger effect than predicted by a simple addition of effects, this is referred to as synergy (sometimes dubbed the “cocktail effect”). In contrast, antagonism describes the situation where chemicals negate the toxic effects of one another. Finally, interactions encompass both synergistic or antagonistic effects.

In a 2021 review of 385 experimental mixture studies (Martin *et al.*, 2021), authors found that 20.2% of mixtures showed synergistic effects (22.7% for endocrine disruptor mixtures) and 15.1% showed antagonistic effects (9.1% for endocrine disruptor mixtures). However, about 80% of included 2 or 3 mixture components, with 62% involving binary mixtures.

It is also possible for chemicals that show no significant associations when studied individually to have additive or multiplicative effects when treated as a mixture. In fact, previous animal studies have demonstrated that mixtures containing chemicals that share a similar mechanism of action can have

additive effects at concentrations that individually do not result in observable adverse effects. This was first observed with a mixture of eight weak estrogenic chemicals present in personal care products including BPA and was dubbed the “something from nothing phenomenon” (Silva *et al.*, 2002). A subsequent literature review has estimated there was good evidence demonstrating significant mixture effects with combinations of chemicals well below their individual no observable adverse effect levels, both with mixtures composed of similarly and dissimilarly acting agents (Kortenkamp *et al.*, 2007). Nonetheless, to the best of our knowledge, no such review exists for epidemiological studies yet.

### 1.5.2. Methods

Modelling mixtures represents a significant challenge in epidemiology. For once, high correlation between chemicals in the same model can lead to multicollinearity, and provide unstable effect estimates and inflate standard errors. Furthermore, detecting synergistically or antagonistically effects between chemicals is not possible in standard models, and becomes more difficult as the number of pollutants increases. However, even though multiple methods currently exist for studying environmental mixtures (61 methods according to a recent review (Joubert *et al.*, 2022)), no method can answer all mixture questions concurrently (Gibson *et al.*, 2019). It is therefore important to consider the question one wishes to investigate before selecting the most appropriate method.

Mixture research can be broadly divided into five main questions:

1. Are there specific patterns of exposure in the study population?
2. Which are the toxic agents in the mixture? Or, what are the independent effects of each mixture member on the health outcome of interest?
3. Which combination of mixture members best predict the outcome of interest?
4. Are there synergistic effects or interactions among mixture members?
5. What is the overall effect of the mixture on the outcome of interest?

For the purpose of this thesis, we will focus on the last two questions. In this context, two popular classes of models for mixtures analyses are favoured : exposure-index methods and response-surface methods (McGee *et al.*, 2023).

#### 1.5.2.1. Exposure-index methods

Exposure-index methods decompose coefficients from a linear model into an overall easily interpretable mixture effect and individual index weights, but require certain assumptions to be unbiased. Two prevalent models of this class are quantile g-computation and weighted quantile sum



regression (WQS). Quantile g-computation (an extension of the g-computation algorithm) estimates the effect of simultaneously increasing all exposure biomarkers within a mixture by one quantile (Keil *et al.*, 2020). WQS accomplished the same objective by assigning weights to each exposure that are chosen to maximize the likelihood of the regression model of the outcome variable against the exposures using bootstrap samples (Carrico *et al.*, 2015). Both methods are particularly useful when the relationship between the mixture and the outcome is non-linear but they do not assess possible interactions between mixture components. Additionally, WQS requires that all exposures act in the same direction on the outcome which is a difficult assumption to confirm and does not necessarily provide a full answer when the objective is to measure the overall effect of the mixture on the outcome.

#### 1.5.2.2. Response-surface methods

Response-surface methods estimate high-dimensional surfaces and are thus highly flexible but difficult to interpret and can be inefficient and underpowered to detect small associations. Bayesian Kernel Machine Regression (BKMR) is a popular response-surface method. It is a non-parametric Bayesian method which uses kernel functions to handle the high-dimensional parameter space of the exposure–response function in a flexible way that allows for nonlinear and non-additive effects. It can provide an overall mixture effect when increasing mixture exposure from one percentile to another (thereby reducing the number of dimensions), but it also reduces the number of variables by selecting exposure variables in each iteration (even though all exposures will be selected at least once and hence contribute to the overall mixture effect) (Bobb *et al.*, 2015). Moreover, it can assess the presence of interactions between mixture components and can incorporate hierarchical selection when it is pertinent to divide components into different groups which can be useful when two or more of the exposures are highly correlated with one another. Nevertheless, BKMR can require substantial computing power to run on large samples where it can take a few days or weeks to run.

#### 1.5.2.3. Conclusion

Because no single method can provide the information made available by the two types of methods described, Gibson *et al.* (2019) have recommended using multiple methods to address a given research question and assess the robustness of findings. For example, quantile g-computation can be used to provide a more interpretable mixture effect overall, whilst BKMR can provide relative effect differences (between two percentiles) which can help confirm the trends of the associations (or lack

thereof) observed in the quantile g-computation model but also assess the presence of interactions between mixture components.

## 1.6. OBJECTIVES

The overarching aim of this thesis was to study the impact of EDCs on the ovarian reserve, with the hypothesis that increased levels of exposure to EDCs decrease the ovarian reserve and increase the risk of DOR. Specifically, we looked at the effects of four families of chemicals, as single-exposure elements and as mixtures, on different indicators of the ovarian reserve including continuous markers (AMH, AFC, and FSH) and a clinical outcome (DOR), in women consulting in fertility centres. In addition, we explored whether in utero exposure to POPs were associated with AMH in teenage girls. To fulfil this aim, four epidemiological studies were conducted within three study populations:

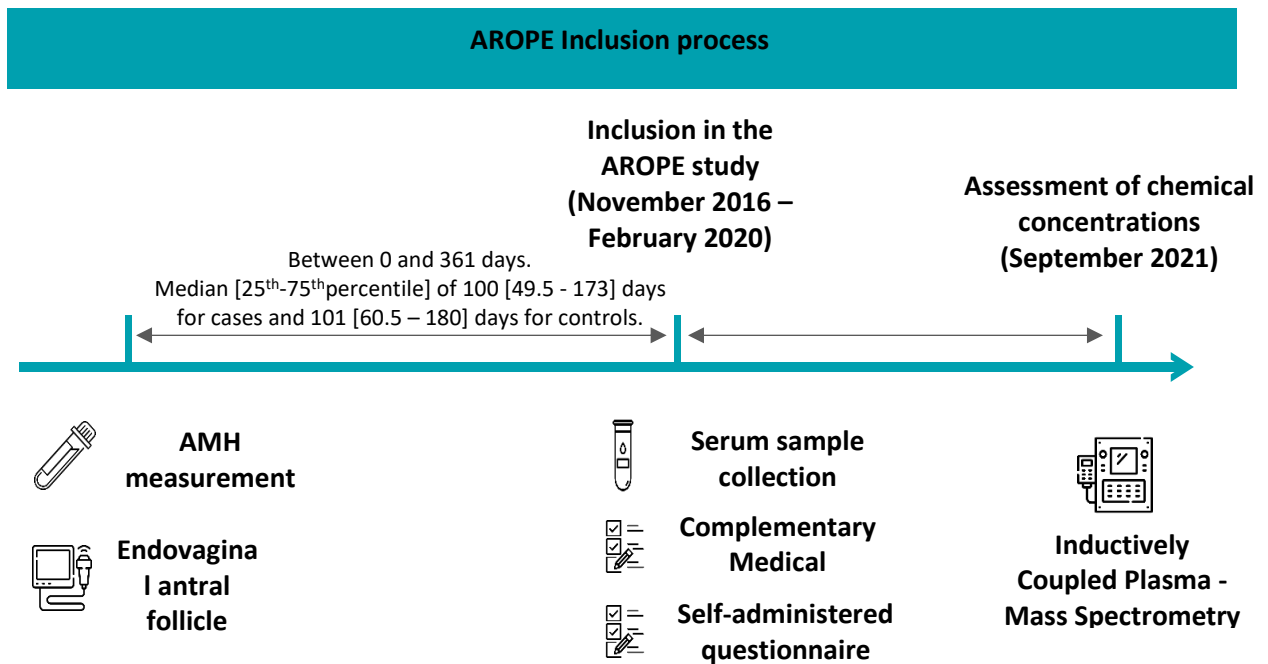
1. Associations between persistent organic pollutants and diminished ovarian reserve in the AROPE case-control study
2. Associations between heavy metals and diminished ovarian reserve in the AROPE case-control study
3. Associations between a mixture of phenols and phthalates, and the ovarian reserve in the EARTH cohort
4. Associations between in utero exposure to persistent organic pollutants and anti-müllerian hormone levels in 12-year old girls in the PELAGIE cohort

## CHAPTER 2: METHODS

### 2.1. POPULATIONS

#### 2.1.1. AROPE case-control study

The AROPE case-control study (“Altération de la réserve ovarienne : étude du rôle de l’exposition aux perturbateurs endocriniens persistants et aux solvants organiques”) included women between the ages of 18 and 40 amongst couples consulting for infertility in four fertility centres in the region of Bretagne – Pays de Loire in north-western France (Rennes University Hospital, Nantes University Hospital, Brest University Hospital, and La Sagesse, a semi-private clinic based in Rennes) between November 2016 and February 2020. All women consulting in these centres underwent a standardized endovaginal antral follicle count by a trained gynaecologist between days 2 and 4 of a spontaneous menstrual cycle (Broekmans *et al.*, 2010) as well as an AMH measurement performed at inclusion (36.2% of cases and 31.8% of controls) or within the year prior to the inclusion for women who had already started consulting in the clinics (63.8% of cases and 68.2% of controls) (Figure 11). AMH levels were measured using an automated Roche Elecsys immunoassay system in all AROPE centres, excepted for Rennes University Hospital, where the automated Beckman-Coulter Access Dxi assay was used (van Helden and Weiskirchen, 2015). Cases of DOR were defined using the threshold values for the AFC and AMH levels proposed in the ESHRE consensus Bologna criteria (Ferraretti *et al.*, 2011). Hence, cases were women presenting at least one of the following characteristics: a number of antral follicles lower than 7 and/or a blood level of AMH lower or equal to 1.1 ng/mL. Controls were selected by frequency matching per centre and per five-year age group among women whose fertility assessment was strictly normal (AMH levels between 1.1 and 5 ng/mL and AFC  $\geq 7$ , without genital malformation and with a menstrual cycle between 26 and 35 days). The non-inclusion criteria for cases and controls were endometriosis, polycystic ovary syndrome, a history of adnexal surgery, a history of cancer with chemotherapy or radiotherapy, a genetic or chromosomal syndrome (Turner or fragile X), morbid obesity (BMI  $\geq 35$  kg/m<sup>2</sup>), and women subject to legal protection or deprived of their liberty. At inclusion, a gynaecologist collected medical information and the participants completed a self-administered questionnaire that covered their social and demographic characteristics, personal habits, and lifestyle. Blood serum was also collected at inclusion to measure the chemicals of interest. In total, 149 cases and 161 controls were included in the AROPE study, with a participation rate of 65% for cases and 75% for controls. The sample sizes varied very slightly depending on the availability of blood samples used to measure chemical exposure.



**Figure 11. AROPE inclusion process**

The study was approved by the French ethical board for human participation in research study on health (authorisation n°2016-A00307-44) and the French national security agency for medicine and health products (ANSM). All participants provided informed consent for their participation in the study.

### 2.1.2. EARTH cohort

The Environment and Reproductive Health (EARTH) Study is a 2004-2019 prospective cohort established to assess environmental and dietary determinants of fertility at the Massachusetts General Hospital (MGH) Fertility Center (Messerlian *et al.*, 2018). Women between 18 and 45 years, and men between 18 and 55 years who have not had a vasectomy and who are not taking hormones at the time of enrolment, seeking fertility care at the centre were eligible to participate independently or as a couple and approximately 60% of those contacted by the research staff enrolled.

Women were followed from study entry throughout their fertility care, pregnancy, and labour and delivery. At enrolment, research staff administered sociodemographic, lifestyle, and medical history questionnaires to participants. The participant’s date of birth was collected at entry, and weight and height were measured by trained study staff. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Study participants also completed a comprehensive questionnaire on family, medical, reproductive and occupational history, perceived stress, consumer products use, smoking history, and physical activity. Primary infertility type was evaluated using the American Society of Assisted Reproductive Technology definitions, and classified as female factor (DOR,

endometriosis, ovulatory, tubal, uterine and other), male factor, or unexplained. Women provided one (23%) or two (77%) spot urine samples per IVF cycle (Mínguez-Alarcón *et al.*, 2019), with the first one collected between Day 3 and Day 9 of the gonadotrophin phase, and the second one collected on the day of oocyte retrieval (for fresh IVF cycles) or on day of embryo transfer (for cryo-thaw IVF cycles).

Ovarian reserve measures included lower AFC, circulating levels of day 3 FSH, and DOR. Lower AFC was defined using the Bologna criteria of AFC < 7 (Ferraretti *et al.*, 2011) and DOR was defined as AFC < 7, FSH > 10 UI/L or primary infertility diagnosis of DOR. All women participating in the study underwent an evaluation of ovarian AFC through transvaginal ultrasonography by one of the MGH reproductive endocrinology and infertility physicians in early follicular phase of an unstimulated cycle (Broekmans *et al.*, 2010). The levels of day-3 FSH were measured in serum samples with an automated electrochemiluminescence immunoassay using the Elecsys FSH reagent kit at the MGH Core Laboratory.

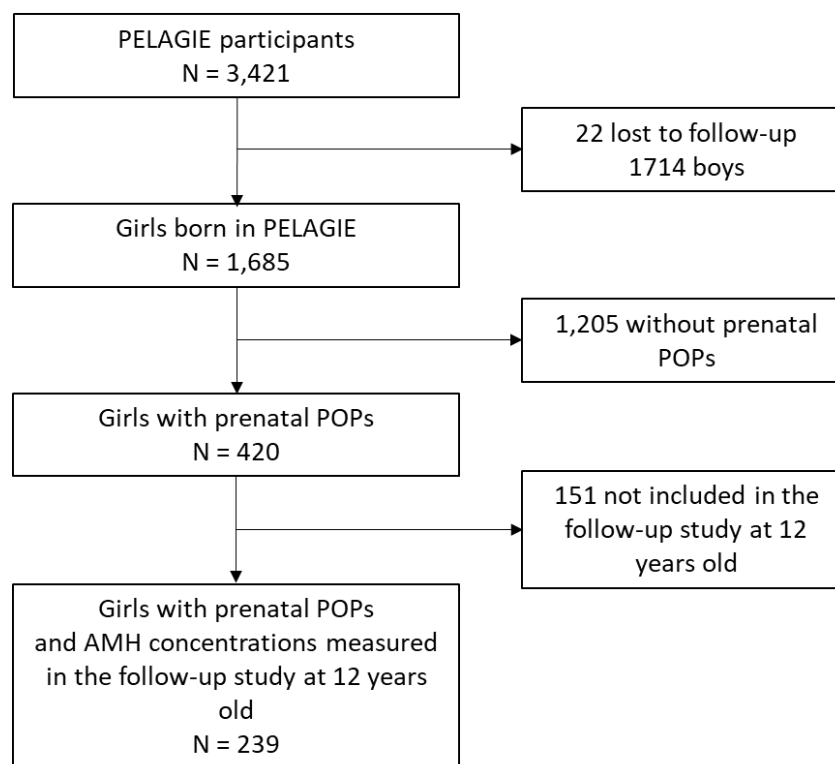
The study was approved by the Human Subjects Committees of the Harvard T.H. Chan School of Public Health, MGH, and the Centers for Disease Control and Prevention (CDC). Participants signed an informed consent after the study procedures were explained by trained research study staff and all the questions were answered.

### 2.1.3. PELAGIE cohort

PELAGIE (“Perturbateurs endocriniens: étude longitudinale sur les anomalies de la grossesse, l’infertilité et l’enfance”) is a mother–child cohort which enrolled 3,421 pregnant women in three districts of Brittany (northwestern France) from 2002 through 2006. Its aim was to study the role of environmental pollutants on pregnancy and on children’s health and development. Gynaecologists, obstetricians, and ultrasonographers recruited women during routine prenatal care visits before 19 weeks of gestation and followed until delivery. Women were given a questionnaire on family, social, and demographic characteristics, diet, and lifestyle, to complete at home. Midwives and paediatricians at the maternity units provided medical information about the pregnancy, delivery, and neonatal health. At the time of delivery, cord blood samples were collected from 2,138 women (62%).

When the child reached the age of 12, a questionnaire (“Your child at 12”) was sent to their parents to investigate the health and environment of their child. At 12, a clinical examination (including an assessment of the pubertal stage through Tanner and weight and height measures) was also proposed to children for whom a cord blood sample was collected at birth. If they agreed, a venous blood sample was also taken during the examination for an analysis of POP and sex hormones (AMH) measured using an automated Beckman-Coulter Access Dxi assay. Overall, 420 girls with POP concentrations were

included in the 12-year old follow-up. Among these, 239 girls had AMH concentrations and were included in the present analysis (Figure 12).



**Figure 12. Flowchart from inclusion in the PELAGIE cohort (N = 3,421) to our study population (N = 239)**

## 2.2. EXPOSURE EVALUATION

### 2.2.1. POPs

#### 2.2.1.1. POPs in the AROPE study

After clotting, blood samples were centrifuged and serum samples separated by centrifugation, transferred immediately to propylene vials with a Teflon stopper, and frozen at  $-20^{\circ}\text{C}$ . Samples were identified solely by a unique sample code and transferred, on dry ice, to the LERES (Laboratoire d'Etude et de Recherche en Environnement et Santé) laboratory in Rennes, France. All laboratory personnel were blind to the case/control status. A gas chromatograph (GC 7890B, Agilent Technologies, Santa Clara, California, US) coupled with a triple quadrupole mass spectrometer (7010B Triple Quadrupole GC/MS, Agilent Technologies) was used to determine the serum concentrations of 43 POPs: 15 organochlorines (aldrin, dieldrin, o,p'-DDE, o,p'-DDT, p,p'-DDE, p,p'-DDT,  $\alpha$ -endosulfan,  $\beta$ -endosulfan, heptachlor, cis-heptachlor epoxide, trans-heptachlor epoxide,  $\alpha$ -HCH,  $\beta$ -HCH,  $\gamma$ -HCH, HCB, 17 PCB

congeners (28, 31, 52, 74, 99, 101, 118, 138, 153, 163, 170, 180, 183, 187, 194, 196, and 203), 9 PBDEs congeners (28, 47, 66, 85, 99, 100, 153, 154, and 183), one polybromobiphenyl (PBB) congener (153), and HBCD (as a mixture of  $\alpha$ ,  $\beta$  and  $\gamma$  isomers). Concentrations are reported as wet weight (units of micrograms per litre). Total cholesterol (TC) and triglyceride (TG) levels were enzymatically measured in serum samples (in mmol/L). After conversion to g/L, total lipid levels were calculated as  $2.27*TC + TG + 0.623$  (Phillips et al., 1989).

#### 2.2.1.2. POPs in the PELAGIE cohort

POP determinations on cord blood samples were carried out at three different times by the Center of Toxicology of Quebec of the National Institute of Public Health, with gas chromatography-mass spectrometry. Plasma samples (2 ml) were enriched with  $^{13}C$ -labeled internal standards and denatured with formic acid. Organohalogenated compounds were automatically extracted with dichloromethane in a solid-phase extraction. Extracts were cleaned up on florisil columns and eluted by using a mixture of hexane/dichloromethane. The PCBs and OCPs (taken up in hexane) were then analyzed on a GC-MS agilent (agilent technologies; Mississauga, Ontario, Canada) with an agilent 60 m DB-XXL column (0.25 mm i.d., 0.25  $\mu$ m film thickness), whereas PBDEs were analysed on a GC-MS agilent with a 15 m DB-XXL column (0.25 mm i.d., 0.1  $\mu$ m film thickness). For both analytic instruments, the carrier gas was helium and all injections were in splitless mode. The MS used single ion monitoring and negative chemical ionization with methane as the reagent gas.

In the first analyses (2008), the levels of 14 OCPs ( $\alpha$ -HCH,  $\beta$ -HCH,  $\gamma$ -HCH, HCB, heptachlor, heptachlor epoxide, aldrin, dieldrin,  $\alpha$ -endosulfan,  $\beta$ -endosulfan, p,p'-DDE, p,p'-DDT, o,p'-DDE, and o,p'-DDT), 12 PCBs (congeners 74, 99, 101, 118, 138, 153, 170, 180, 183, 187, 194, and 203), 8 PBDEs (congeners 28, 47, 99, 100, 153, 154, 183, and 209), 1 PBB (congener 153), and HBCD, were determined from cord blood samples of a representative subcohort (N = 394). A second round of analyses on the same POPs occurred in 2013 when a second random subcohort was built for a follow up at 6 years old in order to complete the analyses for participants that were not previously included (N = 30). A final analysis that also included nine perfluorinated compounds (perfluorobutyric acid (PFBA), perfluorodecanoic acid (PFDA), perfluorohexanoic acid (PFHxA), PFNA, PFOA, perfluoroundecanoic acid (PFUDA), PFBS, PFHxS and PFOS) were measured in 2018 (N = 349).

Concentrations are reported as wet weight (units of mg/L). TC, free cholesterol (FC), TG, and phospholipid (PL) levels were also measured in these samples by an enzymatic method (in g/L) and allowed us to calculate the total lipid levels as  $1.677*(TC-FC)+FC+TG+PL$  (Phillips *et al.*, 1989).



### 2.2.2. Heavy metals in the AROPE study

Pb, Hg, Cd, and Cr were measured at inclusion in whole blood by inductively coupled plasma mass spectrometry, on an ICAP-TQ from Thermo Scientific® equipped with collision cell technology (Platform AEM2, University of Rennes 1 / Biochemistry Laboratory, Rennes University Hospital). The source of plasma was argon (Messer®) with high degree of purity (>99.999%). The collision/reaction cell used was pressurized with helium (Messer®). All samples were handled with care in order to avoid environmental contamination during their manipulation. All rinse, diluent, and standards were prepared with  $\geq 18$  M $\Omega$  cm ultrapure water using a Millipore® MilliQ Advantage A10 water station. Blood samples, stored at -80°C, were diluted 1:30 by addition of a diluent solution consisting of 0.5% (v/v) HNO<sub>3</sub> (Optima Grade Fischer Chemical®) and 0.01% (v/v) Triton X100 (Merck®). Calibration ranges preparation was carried out using a multi-element calibrator solution (SCP Science® Plasma Cal). Calibration and verification of instrument performance were realised using multi-element solutions (Thermo®). Quality control was Clincheck controls for trace elements (Recipe).

### 2.2.3. Phenols and phthalates in the EARTH study

Urine samples were collected in a sterile polypropylene specimen cup, divided into aliquots, frozen, and stored at -80 °C. Samples were shipped on dry ice overnight to the CDC for analysis. Following strict quality control/quality assurance practices, online solid-phase extraction coupled with isotope dilution-high-performance liquid chromatography-tandem mass spectrometry were used to quantify the urinary concentrations of four phenol biomarkers (bisphenol A, MP, propylparaben (PP), butylparaben (BP)) and eight phthalate metabolites: MEP; mono-n-butyl phthalate (MBP), MiBP, monobenzyl phthalate (MBzP), MEHP, MEHHP, MEOHP, and MECPP. Along with study samples, each analytical run included a set of calibrators, reagent blanks, and high- and low-concentration quality control (QC) materials. Concentrations of the QCs were evaluated using standard statistical probability rules (Caudill et al. 2008).

Specific gravity (SG) was measured within hours of collection at the Harvard T.H. Chan School of Public Health at room temperature using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) calibrated with deionized water before each measurement.

Overall, the number of urine samples available per woman (median, interquartile range (IQR)) was 1.0 (3.0) with a maximum of 14 samples.

## 2.3. STATISTICAL CONSIDERATIONS

### 2.3.1. Missing data under the limit of detection

Limited capacity of chemical assessment equipment to measure very low concentrations of chemicals in different matrices, leads to incomplete detection of chemicals in samples, resulting in missing data below the limit of detection (LOD). In turn, this reduces the statistical power of models and widens confidence intervals. However, we know the missing data will be to the left of the LOD value provided (“left-censored data”), a key piece of information not available for missing data for covariates which can influence the imputation model choice. Historically, simple methods such as an imputation of all missing values with  $LOD/2$  or  $\sqrt{LOD/2}$  have been employed, but these can provide model estimates that are biased when there is as little as 10% of missing data (Lubin *et al.*, 2004) and become increasingly biased as the percent of exposure below the LOD increases (Cole *et al.*, 2009). In the last fifteen years, an array of imputing methods has been developed in order to address this issue. Several simulation studies have compared different methods in the context of various amounts of missing data under the LOD, regression models used, sample sizes and the distribution of the variable(s) in question. Because the distribution of chemicals measured in human matrices often follows a log-normal distribution, maximum likelihood imputation can offer good performance, but not when the sample size is small (e.g.  $N < 50$ ) or other situations where the log-normal distribution assumption is not verified (Lynn, 2001; Nie *et al.*, 2010; Shoari *et al.*, 2015). In this case, regression on order statistics and its variations are suitable. Nevertheless, all techniques become biased under moderate and heavy censoring.

Due to various reasons to do with sampling, and resource constraints, exposure data are often analysed in multiple batches different years apart resulting in differing LOD values and proportions of censored observations across batches. Censored likelihood multiple imputation (an implementation of the multiple imputation that ensures that imputed values are below the LOD) has shown adequate performance in addressing this issue compared to complete-case analysis or standard multiple imputations using chained equations (Boss *et al.*, 2019). While this last method benefits from information from other variables, maximum likelihood imputation provides a better fit to a log-normal distribution but does not incorporate such information.

In consequence, in AROPE, POPs detected in fewer than 20% of samples were not included in the analyses. For POPs detected in between 20% and 50% of samples (PCB 28 only), exposure was dichotomised into two groups (detected versus not detected). For others (POPs detected in over 50% of samples), concentrations below the LOD were replaced using maximum likelihood estimations and were categorised in terciles. For these POPs, analyses were also performed on continuous exposure.

The same approach was used for the analysis of heavy metals in AROPE. In EARTH and in PELAGIE, where multiple LODs were present, concentrations below the LOD were imputed using censored likelihood multiple imputation using an R function developed specifically for the EARTH study. All imputations were performed by combining the results of five imputations, according to Rubin's rules (Rubin, 2004).

### 2.3.2. Lipid strategy

POPs stored in body fats are able to reach sensitive organs when mobilised by triglycerides, which means that a change in lipid content alters the content of POPs present in specific tissues (Phillips *et al.*, 1989; Rylander *et al.*, 2006). Different modelling strategies (standardisation, adjustment, or no correction) have been tested and compared in a simulation study (O'Brien *et al.*, 2016) which looks at different scenarios of the relation between POP exposure, lipids and outcomes. Considering the causal scenario of the AROPE study, i.e. when considering recent fat intake affects serum exposure concentrations and when other covariates affect both exposure concentrations and the outcome, standardisation (dividing POP concentrations by total lipids, also known as normalisation) seemed to perform best. This result was corroborated in a later comparative study (Cano-Sancho *et al.*, 2018) and was used in a study with a similar DAG on the associations between POPs and endometriosis (Ploteau *et al.*, 2017). In PELAGIE, however, the causal relationships are different because POPs are measured in utero while AMH concentrations at 12 years old. Additionally, lipids for POPs measured in 2008 showed concentrations beyond plausible range and were therefore deemed inaccurate. For these reasons, we decided not to adjust or correct POP concentrations for lipids.

### 2.3.3. Urine correction

The most widely used matrix to measure non-persistent chemicals is urine. However, a major disadvantage of this matrix is that the volume of urine varies from void to void depending on hydration status, which can dilute the concentrations of chemicals in a sample. Two main methods have been used to correct this issue.

The most widely used method is creatinine adjustment which involves dividing the analyte concentration by the creatinine concentration (Barr *et al.*, 2005). Creatinine is a waste product from skeletal muscle that is filtered and excreted by the kidneys and is constantly excreted into the urine. Because urinary creatinine is inversely proportional to urine volume (individuals with low urinary creatinine concentrations are well hydrated), it is a useful tool to correct chemical concentrations (O'Brien *et al.*, 2016). However, an increasing number of studies have shown that creatinine levels can

differ dramatically among different demographic groups (depending on sex, and ethnicity) (Barr *et al.*, 2005), but can also be confounded by other parameters such as diet, muscle mass, physical activity, co-existing medical conditions, and even the time of the day (Kuiper *et al.*, 2021). Because of a general decline in muscularity and glomerular filtration rate, urinary creatinine concentrations decrease with age (Barr *et al.*, 2005). Moreover, creatinine-correction might not be appropriate for some chemical metabolites, because these can be conjugated in the liver as glucuronides or sulfates and are actively excreted by the renal tubules, while creatinine is excreted mostly by glomerular filtration (Roggeman *et al.*, 2022).

Specific gravity, the second method, seems to offer an attractive alternative to creatinine. It is a measure of the relative density of a substance (here urine) compared to water, hence it can represent a closer proxy for hydration status than creatinine. Furthermore, its determination is simpler and cheaper than for creatinine (Sauvé *et al.*, 2015). Nonetheless, factors such as age, BMI, time of sample collection, ethnicity and sex, could possibly influence specific gravity but possibly less so than for creatinine (Kuiper *et al.*, 2021; Sauvé *et al.*, 2015).

Whilst certain formulas are used to correct chemical concentrations, some studies recommend to adjust regression models on either creatinine levels or specific gravity as a separate independent variable instead (Barr *et al.*, 2005). This allows the statistical significance of the model to be independent of the effects of creatinine levels of specific gravity and prevent a false discovery of a relation between the chemical of interest and the outcome simply because the outcome is related to creatinine levels of specific gravity (and not the chemical).

Nonetheless, further research is still required to investigate the most effective approach for correcting urine dilution.

In the work presented in this thesis, urine chemical concentrations were corrected by adjusting on specific gravity.

#### 2.3.4. Directed acyclic graphs

All models presented here used a directed acyclic graph (DAG) to select which covariates to include in the regression models as confounders. A DAG is a graphical representation of the relationships between the exposure, the outcome and other variables susceptible to influence or be influenced by the exposure or the outcome (Schisterman *et al.*, 2009). It is an a priori technique based on the existing literature and knowledge on the relations between factors. This process allows to determine the minimally sufficient adjustment sets of variables to estimate the total effects of the studied exposure on the outcome with minimal bias whilst reducing the variance caused by over-adjustment. DAGs can also help prevent adjusting for intermediate variables associated with both the exposure and the

outcome (Shrier and Platt, 2008). Alternatively, they can help avoid Berkson's bias which is created by adjusting on a variable that is dependent of both the exposure and the outcome (Hernán et al., 2004). Whilst these effects can be avoided in simple causal structures, there are more complicated to detect in more complex situations such as in the aetiology of the ovarian reserve.

#### 2.3.5. Single-exposure regression models

In the AROPE Study, associations between cases and control status and exposure variables were studied using unconditional multivariate logistic regressions as recommended for frequency matching (Pearce, 2016). In this matching method, controls are selected such that cases and controls have approximately similar, but not necessarily identical, distributions or proportions of the matching variables. In this context, the matched design does not require analysis at the pair level so long as the matching strata are retained (Kuo *et al.*, 2018). Frequency matching can be used if pair matching is not based on variables unique to very few individuals (e.g. sibling status, residence location, etc.), but rather over variables shared by multiple study subjects (e.g. sex, age categories, or centre of inclusion in our case) (Mansournia *et al.*, 2018). AROPE controls were matched through frequency matching by centre and age group only. The main objective in employing this design was to ensure the study did not only include older women as cases and younger women as controls, and maintained the best balance possible between groups.

Logistic regression results in AROPE are presented as odds ratios (OR) with their corresponding 95% CI. In PELAGIE, multivariate linear regressions were used to analyse the associations between POPs in cord blood and AMH at 12. The results are presented as Beta coefficients and their corresponding 95% CI.

In both AROPE and PELAGIE, when linearity was respected in single-exposure models, chemical biomarkers were modelled as continuous exposures in additions to models where they were divided in terciles. When modelled as continuous variables, log transformations were applied to chemical biomarkers to reduce skewness.

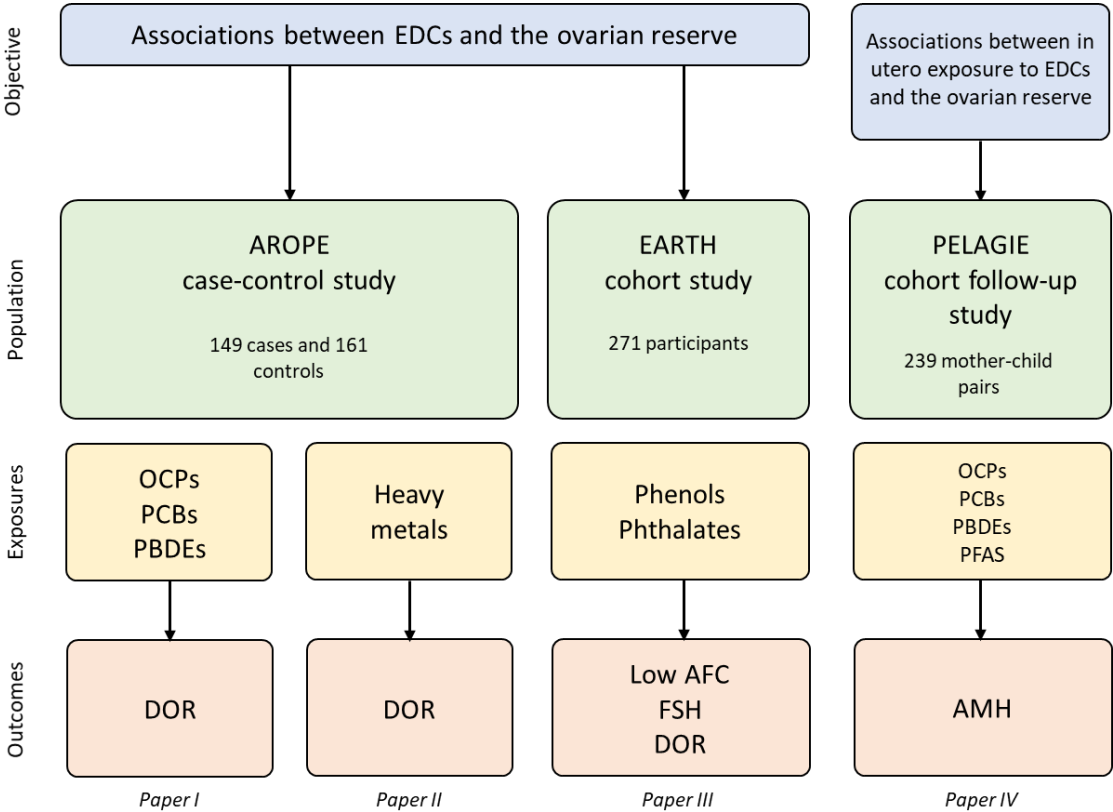
#### 2.3.6. Mixture approaches

Quantile g-computation results are presented as ORs with their corresponding 95% CI for binary outcomes and as adjusted mean differences and their corresponding CIs for continuous outcomes, when increasing all mixture components by one quartile. BKMR results are presented as difference in risk of experiencing the outcome for binary outcomes or mean differences for continuous outcomes for a change in mixture component concentrations between the 25th and 75th percentiles, along with

corresponding 95% credible intervals (CI). The posterior inclusion probabilities (PIPs) representing posterior probability of non-null association between an exposure and the outcome are also provided, along with several graphical outputs including the overall effect of the mixture comparing simultaneous increases in all mixture components by quantiles, exposure-response relationships for each biomarker while holding all other exposure biomarker at their median levels, and single-exposure associations when all the other exposure biomarker are fixed at the 25th, 50th, and 75th percentiles.

**2.4. OVERVIEW**

To summarise, our objectives, study populations, exposures of interest and outcomes per study are provided in Figure 13.



**Figure 13. Overview of research objectives, study populations, exposures and outcomes studied**

## CHAPTER 3: RESULTS

The results presented in this chapter are extracted from articles that are either published (article 1), accepted (article 2 and article 3), or in preparation (article 4). Sections of the articles containing information already shared in previous chapters have been removed to prevent redundancy. Supplementary data is available in the annexes.

### 3.1. ARTICLE 1: Associations between persistent organic pollutants and diminished ovarian reserve in the AROPE case-control study

**Status: Published in *Human Reproduction***

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## **Abstract**

### **Study question:**

Are persistent organic pollutants (POPs) associated with a diminished ovarian reserve (DOR) in women of reproductive age?

### **Summary answer:**

Amongst 17 POPs detected in over 20% of serum samples, only p,p'-DDE was significantly associated with an increased risk of DOR, and  $\beta$ -HCH was significantly associated with a decreased risk of DOR whilst mixture analyses yielded non-significant associations and did not detect any interactions between POPs.

### **What is known already:**

Animal studies have shown that several POPs can alter folliculogenesis and increase follicle depletion. However, only a few studies have been conducted in humans, with small sample sizes and inconsistent results.

### **Study design, size, duration:**

Our study included 138 cases and 151 controls from the AROPE case-control study. Study participants were women between 18 and 40 years of age recruited amongst couples consulting for infertility in four fertility centres in western France between 2016 and 2020.

### **Participants/materials, setting, methods:**

Cases of DOR were defined as women with AMH levels  $\leq 1.1$  ng/mL and/or AFC  $< 7$ , and controls were women with AMH levels between 1.1 and 5 ng/mL and AFC  $\geq 7$ , without genital malformations and with a menstrual cycle length between 26 and 35 days. Forty-three POPs (including 15 organochlorine pesticides, 17 polychlorinated biphenyls, and 9 polybromodiphenylethers) were measured in the serum at inclusion. We conducted logistic regression adjusted for potential confounders using a DAG to study the effect of each POP on DOR as single exposures and used Bayesian kernel machine regression (BKMR) to measure the mixture effect of POPs on DOR.

### **Main results and the role of chance:**

Seventeen POPs were detected in over 20% of the serum samples. In the single-exposure multivariate logistic regressions, p,p'-DDE (median 165.0 IQR 161.0 ng/L in controls) as a continuous exposure was significantly associated with an increased risk of DOR (OR 1.39, 95% CI 1.10 - 1.77) and non-significantly associated with an increased risk of DOR for the second and third terciles (OR 1.46, 95% CI 0.74 - 2.87, and OR 1.72, 95% CI 0.88 - 3.37, respectively).  $\beta$ -HCH (median 24.2 IQR 21.5 ng/L in controls) was significantly associated with a decreased risk of DOR when  $\beta$ -HCH was treated as a continuous exposure (OR 0.63, 95% CI 0.44 - 0.89) and for the third tercile of exposure (OR 0.43, 95% CI 0.21 - 0.84), and non-significantly associated with a decreased risk of DOR for the second tercile (OR 0.77,



95% CI 0.42 - 1.42). All sensitivity analyses confirmed our results. BKMR showed similar associations for single exposures but found no significant associations for the total mixture effect. In addition, the BKMR results did not suggest any interactions between POPs. The well-recognised anti-androgenic properties of p,p'-DDE and estrogenic properties of  $\beta$ -HCH could explain these associations of opposite direction.

**Limitations, reasons for caution:**

Controls were recruited amongst infertile couples and thus may not be representative of all women of reproductive age. However, their POP concentrations were in the same range as in the general French population.

**Wider implications of the findings:**

This study is the first to examine the associations between serum POPs and DOR. If these results are replicated elsewhere, this could have an impact on fertility prevention messages and help in understanding the impact of POPs on the female reproductive system.

**Study funding/competing interest(s):** This study was funded by the Fondation de France (grant number 2014-50537 and 00110196) and the French Biomedicine Agency (2016). None of the authors have any conflicts of interest to declare.

**Trial registration number:** N/A.

**Keywords:** Ovarian Reserve; Female Infertility; Persistent Organic Pollutants; Environmental Exposure; Pesticides; Polychlorinated Biphenyls.

**Résumé en Français**

**Question de l'étude :** Les polluants organiques persistants (POPs) sont-ils associés à une diminution de la réserve ovarienne (DOR) chez les femmes en âge de procréer ?

**Réponse résumée :** Parmi les 17 POPs détectés dans plus de 20 % des échantillons de sérum, seul le p,p'-DDE était significativement associé à un risque accru de DOR, et le  $\beta$ -HCH était significativement associé à un risque réduit de DOR, tandis que les analyses de mélange n'ont révélé aucune association significative et n'ont pas détecté d'interactions entre les POPs.

**Ce que l'on sait :** Des études sur des animaux ont montré que plusieurs POPs peuvent altérer la folliculogénèse et augmenter l'épuisement folliculaire. Cependant, peu d'études ont été réalisées chez la femme, avec des tailles d'échantillon réduites et des résultats contradictoires.

**Conception, taille, durée de l'étude :** Notre étude a inclus 138 cas et 151 témoins issus de l'étude de cas-témoins AROPE. Les participantes à l'étude étaient des femmes âgées de 18 à 40 ans recrutées

parmi les couples consultant pour infertilité dans quatre centres de fertilité de l'ouest de la France entre 2016 et 2020.

**Participants/matériel, cadre, méthodes :** Les cas de DOR étaient définis comme des femmes ayant des taux d'AMH  $\leq 1,1$  ng/mL et/ou AFC  $< 7$ , et les témoins étaient des femmes ayant des taux d'AMH entre 1,1 et 5 ng/mL et AFC  $\geq 7$ , sans malformations génitales et avec une longueur de cycle menstruel entre 26 et 35 jours. Quarante-trois POP (dont 15 pesticides organochlorés, 17 polychlorobiphényles et 9 polybromodiphényléthers) ont été mesurés dans le sérum à l'inclusion. Nous avons effectué des régressions logistiques ajustées sur des facteurs de confusion potentiels en utilisant un DAG pour étudier l'effet de chaque POP sur la DOR en tant qu'expositions individuelles, et nous avons utilisé la méthode Bayesian kernel machine regression (BKMR) pour mesurer l'effet de mélange des POPs sur la DOR.

**Principaux résultats et rôle du hasard :** Dix-sept POPs ont été détectés dans plus de 20 % des échantillons de sérum. Dans les régressions logistiques multivariées d'expositions individuelles, le p,p'-DDE (médiane 165.0, IQR 161.0 ng/L chez les témoins) en tant qu'exposition continue était significativement associé à un risque accru de DOR (OR 1.39, IC 95% 1.10 – 1.77) et associé de manière non significative à un risque accru de DOR pour le deuxième et le troisième terciles (OR 1.46, IC 95% 0.74 – 2.87, et OR 1.72, IC 95% 0.88 – 3.37, respectivement). Le  $\beta$ -HCH (médiane 24.2, IQR 21.5 ng/L chez les témoins) était significativement associé à un risque réduit de DOR lorsque le  $\beta$ -HCH était traité comme une exposition continue (OR 0.63, IC 95% 0.44 – 0.89) et pour le troisième tercile d'exposition (OR 0.43, IC 95% 0.21 – 0.84), et associé de manière non significative à un risque réduit de DOR pour le deuxième tercile (OR 0.77, IC 95% 0.42 – 1.42). Toutes les analyses de sensibilité ont confirmé nos résultats. Le modèle BKMR a montré des associations similaires pour les expositions individuelles, mais n'a trouvé aucune association significative pour l'effet total de mélange. De plus, les résultats du modèle BKMR n'ont suggéré aucune interaction entre les POP. Les propriétés anti-androgéniques reconnues du p,p'-DDE et les propriétés œstrogéniques du  $\beta$ -HCH pourraient expliquer ces associations de directions opposées.

**Limitations, raisons de prudence :** Les témoins ont été recrutés parmi les couples infertiles et pourraient donc ne pas être représentatifs de toutes les femmes en âge de procréer. Cependant, leurs concentrations de POPs étaient à des niveaux similaires à celles de la population française générale.

**Implications plus larges des résultats :** Cette étude est la première à examiner les associations entre les POPs sériques et la DOR. Si ces résultats sont reproduits ailleurs, cela pourrait avoir un impact sur les messages de prévention de la fertilité et aider à comprendre l'impact des POPs sur le système reproducteur féminin.

**Financement de l'étude/conflits d'intérêts :** Cette étude a été financée par la Fondation de France (numéro de bourse 2014-50537 et 00110196) et l'Agence de biomédecine française (2016). Aucun des auteurs n'a de conflits d'intérêts à déclarer.

**Numéro d'enregistrement de l'essai :** N/A.

**Mots-clés :** Réserve ovarienne ; Infertilité féminine ; Polluants organiques persistants ; Exposition environnementale ; Pesticides ; Polychlorobiphényles.

## Introduction

There are inconsistent results concerning the relationship between POPs and continuous indicators of the ovarian reserve. Here, we aimed to investigate the associations between serum concentrations of selected POPs and DOR defined using internationally recognised thresholds among women consulting for couple infertility in North-Western France.

## Methods

### *Population*

We used data from the AROPE case-control study (138 cases and 151 controls) as described in chapter 2.

### *Statistical analyses*

All POP concentrations in our study were standardised using total serum lipids, as recommended by O'Brien *et al.* (2016) (O'Brien *et al.*, 2016). POP concentrations were then log-2 transformed when treated as a continuous variable. POPs detected in fewer than 20% of samples were not included in the analyses. For POPs detected in between 20% and 50% of samples, exposure was dichotomised into two groups (detected versus not detected). For others (POPs detected in over 50% of samples), concentrations below the limit of detection (LOD) were replaced by combining the results of five imputations using maximum likelihood estimations (Cole *et al.*, 2009). We reviewed the relevant literature to develop a directed acyclic graph (DAG) (Shrier and Platt, 2008) (Supplementary Figure S1), which was used to determine the minimally sufficient adjustment sets of variables for regression to estimate the total effects of the studied exposure on the outcome. Based on this DAG, our models were systematically adjusted for age (continuous), education level (high school or lower, high school + 2 years of university, high school + 3 years of university and above), BMI (continuous), and season of inclusion (fall, winter, spring, summer). This set of adjustments was supplemented by an a priori adjustment on centre of inclusion. Covariates with missing data were imputed using multiple imputation by chained equations (five sets) (Kristman *et al.*, 2005). Additionally, we conducted sensitivity analyses using different definitions for cases: one based on the AFC outcome only (AFC < 7), one based on the AMH outcome only (AMH levels  $\leq 1.1$  ng/mL), and one based on the combination of both the AFC and AMH outcomes (AFC < 7 and AMH levels  $\leq 1.1$  ng/mL). In addition, as suggested by Ferraretti & Gianaroli (2014), we performed a sensitivity analysis in which cases were defined as AFC < 5 or AMH levels  $\leq 0.7$  ng/mL (Ferraretti and Gianaroli, 2014). All sensitivity analyses were performed using the same original group of controls (N=151). Finally, we carried out two sensitivity analyses, one

restricted to women who had not undergone any prior ovarian stimulation to remove the potential impact of previous ovarian stimulation on our models, and another restricted to women whose outcome were measured at inclusion. Furthermore, we used Bayesian kernel machine regression (BKMR) (Bobb *et al.*, 2015) for POPs with a detection rate above 50% to evaluate the mixture effect of POPs on DOR. Hierarchical variable selection was performed to estimate the posterior inclusion probabilities of the groups and individual POPs. The model was fit with 10,000 iterations using a Markov chain Monte Carlo (MCMC) algorithm. One individual was considered to exert an overly large leverage on the mixture effect and was therefore removed from the BKMR analysis. All analyses were performed using R (version 4.0.4). The threshold for significance for all analyses was 5%.

## Results

### *Population description*

The participant characteristics can be found in Table I. The mean age was 33.8 ( $\pm$  4.0) years for cases and 32.4 ( $\pm$  4.1) years for controls. Most study participants (66.6% for cases and 64.3% for controls) had an education level equivalent to high school +2 years university or above. Their ethnic origin was mostly European (87.4% of cases and 90.8% of controls). Almost half of the women were never smokers (48.4% in cases and 48.6% in controls) and close to two thirds of the participants were women with primary infertility (60.9% in cases and 58.3% in controls).

### *Serum concentrations of POPs*

Table II shows the detection rates and the serum concentrations of POPs in the control and case groups. Out of 43 persistent organic pollutants, 19 were detected in over 20% of samples and 15 were detected in over 90% of samples. The POP with the highest median serum concentration was p,p'-DDE (28.9 ng/g lipid base), followed by PCB 153 (21.1 ng/g lipid base), PCB 180 (15.2 ng/g lipid base), and HCB (7.4 ng/g lipid base). Correlation coefficients (Supplementary Figure S2) between PCBs were between 0.4 and 1, except for PCB 28, which were lower. OCPs were correlated together (between  $\rho = 0.3$  and  $\rho = 0.6$ ), except for p,p'-DDT, which only correlated with p,p'-DDE ( $\rho = 0.6$ ). p,p'-DDE, HCB, and  $\beta$ -HCH correlated moderately with PCBs ( $0.3 < \rho < 0.6$ ), with the exception of PCB 28.

### *Associations between POP exposures and DOR*

Associations between tercile and continuous POP exposures and DOR are presented in Table III. In multivariate logistic regressions, p,p'-DDE as a continuous exposure was significantly associated with an increased risk of DOR (OR 1.39, 95% CI 1.10 - 1.77) and non-significantly associated with an increased risk of DOR for the second and third terciles (OR 1.46, 95% CI 0.74 - 2.87, and OR 1.72, 95%

CI 0.88 - 3.37, respectively). By contrast,  $\beta$ -HCH was significantly associated with a decreased risk of DOR when  $\beta$ -HCH was treated as a continuous exposure (OR 0.63, 95% CI 0.44 - 0.89) and for the third tertile of exposure (OR 0.43, 95% CI 0.21 - 0.84), and non-significantly associated with a decreased risk of DOR for the second tertile (OR 0.77, 95% CI 0.42 - 1.42). There were no significant associations for any other POPs.

#### *Sensitivity analyses*

The associations found in the main analysis for p,p'-DDE and  $\beta$ -HCH were confirmed in all sensitivity analyses (Supplementary Tables SIII-SVIII). In addition, PCB 118 was significantly associated with an increased risk of DOR in multivariate regressions when cases were defined as AFC < 7 for the second tertile of exposure (OR 2.98, 95% CI 1.09 - 8.11).

#### *Associations between the POP mixture and DOR - BKMR analysis*

POPs were organised into two groups: the OCP group and the PCB group. The combined effect of POPs on DOR in BKMR analysis is shown in Figure 1. The group posterior inclusion probabilities (PIP) (Table IV) show the model favours the inclusion of the OCP group more (96.9%) than the PCB group (31.8%). The OCP PIP was mainly driven by  $\beta$ -HCH (91.4%) and then p,p'-DDE (6.8%). The strongest PIPs in the PCB group were PCB 99 (17.6%) and PCB 183 (11.5%). As we can observe in Figure 2, all plots, except those for p,p'-DDE and  $\beta$ -HCH, showed no impact of the exposure on the probability of having DOR. The plot for p,p'-DDE showed a quasi-linear relationship with DOR, whereas  $\beta$ -HCH showed a non-linear relationship. Nonetheless, the relationship with  $\beta$ -HCH indicates stabilisation of the effect on DOR in the higher quantiles of exposure. Furthermore, the overall mixture effect was Beta = -0.14, 95% CI -0.36 - 0.06, when all exposures were fixed at the 75th percentile compared to when they were all fixed at the 50th percentile. We did not detect any interaction between different POPs when investigating the individual effect of each POP comparing the 75th to the 25th percentile of exposure when all other POPs were fixed at the 25th, 50th, and 75th percentiles (Figure 3).

## **Discussion**

Amongst 17 POPs detected in more than 20% of our serum samples, only two – p,p'-DDE and  $\beta$ -HCH – were associated with DOR. p,p'-DDE was significantly associated with an increased risk of DOR, whereas  $\beta$ -HCH was significantly associated with a decreased risk of DOR, both in a dose-dependent manner. These results were stable in all sensitivity analyses including when DOR was only defined using AFC or AMH only. The BKMR analysis showed no significant mixture effect and an absence of interactions between POPs.

To the best of our knowledge, our study is the first to analyse the relationship between POPs and DOR (defined in this study as AFC < 7 and/or AMH  $\leq$  1.1 ng/L using ESHRE thresholds). However, four previous studies (Björvang et al., 2021, 2022; Bloom et al., 2017; Whitworth et al., 2015) investigated the relationship between p,p'-DDE and AMH levels and/or AFC as continuous variables and did not report any significant associations. Nevertheless, the populations in two of these studies were quite different compared to ours, namely, young rural South African women (Whitworth et al., 2015) and pregnant women (Björvang et al., 2021). Two studies also had limited sample sizes (50 women for Björvang et al. (2021) and 32 women for Bloom et al. (2017)). Furthermore, studies that previously attempted to look at the role of  $\beta$ -HCH on AMH levels or AFC were unable to detect the molecule in enough samples to carry out the analyses (Björvang et al., 2021, 2022; Cole et al., 2006).

Our results regarding p,p'-DDE are in accordance with previous in-vitro and in-vivo experimental data. In primary cultures of human granulosa cells, p,p'-DDE was reported to increase FSH stimulation of aromatase activity, resulting in the overproduction of estradiol, leading to an increase in follicular recruitment and the acceleration of follicular depletion (Younglai et al., 2004). In addition, p,p'-DDE was shown to reduce the synthesis of vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 of granulosa cells in primary human cultures and rat ovaries (Holloway et al., 2007), which may in turn alter ovarian follicular development. Moreover, Chedrese and Feyles (2001), reported that p,p'-DDE, but not estradiol, can decrease progesterone synthesis in hamster ovary cells. These results highlight that the effect of p,p'-DDE on ovarian cells can be mediated by non-estrogenic activities, which may include their well-known anti-androgenic properties (You et al., 1998, 1999). On the other hand,  $\beta$ -HCH has been shown to exert estrogen-like effects in MCF-7 human breast cells (Hatakeyama et al., 2002) and uterotrophic responses in ovariectomized mice (Bigsby et al., 1997). Furthermore, adult rats exposed to high exposure levels of  $\beta$ -HCH (> 50 mg/kg) showed atrophy of the ovaries, impaired oogenesis, and focal hyperplasia and metaplastic changes of the endometrial epithelium, which could be due to an estrogenic action of  $\beta$ -HCH (Van Velsen et al., 1986). We believe that the opposite direction of the associations between p,p'-DDE and  $\beta$ -HCH and DOR observed in our study may be explained, at least partially, by the different mechanisms and hormonal actions of these chemicals, i.e., the anti-androgenic properties of p,p'-DDE and the oestrogenic properties of  $\beta$ -HCH. The opposite direction of the association between POPs with anti-androgenic and estrogenic properties has been previously reported in other epidemiology studies, notably regarding time to pregnancy (Cohn et al., 2003) and prostate cancer (Emeville et al., 2015). Other studies are needed to replicate these observations on DOR as well as mechanistic studies to better understand these intricate phenomena.

This study has several limitations. POPs were either measured at the time of inclusion or less than one year after the ovarian reserve evaluations. There is therefore a possibility for reverse causation of the

associations. However, POPs, especially OCPs and PCBs, are persistent chemicals with long half-lives that accumulate in fat tissues over time (Bu et al., 2015) and their concentrations in human serum is hence considered as a marker of long-term exposure. Additionally, human exposure to POPs has gradually decreased over time (Bányiová et al., 2017; Schoeters et al., 2017). A study (Stubleski et al., 2018) also found that after a 5-year follow-up, POP concentrations in an elderly population decreased significantly with most reductions being in the 30–40% range but with lower declines for women compared to men. Nevertheless, correlations between POPs between time points remain high (Bányiová et al., 2017; Nøst et al., 2019), indicating that the overall effect (change in risk of DOR for one unit increase in POP concentrations) should be stable if POP concentrations were lower at the time of sampling.

In addition, if the infertility status of the control partners was related to POP levels as shown in studies on POPs and semen parameters (Vested et al., 2014), and exposure to POPs was shared between the members of the couple, it could imply that the controls included in the AROPE study were more exposed to POPs than the general population. If this was indeed the case, this phenomenon could have led to reduced associations between POPs and DOR in our study. Nevertheless, Góralczyk et al. (2015) reported low correlations of POP concentrations between partners living together. Moreover, the POP concentration levels of the AROPE controls are comparable to the levels of the general population. PCB levels were slightly lower than those reported in a French mother-child cohort (ELFE, 2011) (Dereumeaux et al., 2016) and OCP concentrations were comparable to concentrations found in a French national cross-sectional study (ESTEBAN, 2014-16) (Santé Publique France, 2021).

We chose a priori to study the relation between POPs and DOR rather than indicators of ovarian reserve treated as continuous variables such as in previous published studies in order to use a more clinically pertinent outcome. Additionally, doing so allowed us to ensure we could recruit more women with lower ovarian reserves, but with the cost of losing the added statistical power of modeling on continuous variables. Our definition of DOR uses threshold levels for AFC and AMH levels that were proposed by the Bologna ESHRE consensus for the purpose of predicting a poor ovarian response (POR). However, many fertility centres also use this definition to diagnose DOR (Pastore et al., 2018). We performed several sensitivity analyses using various definitions of the outcome to limit the risk of DOR misclassification: AFC < 7, AMH levels ≤ 1.1 ng/mL, and AFC < 7 & AMH levels ≤ 1.1 ng/mL. Furthermore, Ferraretti & Gianaroli (2014) suggested modifying the Bologna criteria to adjust the cut-off values. We therefore implemented an additional sensitivity analysis in which DOR was defined as AFC < 5 and/or AMH levels ≤ 0.7 ng/mL. The results of all sensitivity analyses were consistent with our main findings. Nonetheless, there is still a possibility that we missed a critical threshold which could be responsible for a lack of significant results in our findings. Finally, the analytical system for the determination of AMH levels was different in one centre (Rennes University Hospital), which used the



Beckman-Coulter Access assay, whereas the other centres used AMH Roche Elecsys Assays. Nevertheless, these assay systems have been shown to provide highly correlated results in comparative studies ( $r = 0.903$  (Li et al., 2016) and  $r = 0.991$  (van Helden and Weiskirchen, 2015)). Any residual difference should have been accounted for through our adjustment on centres of inclusion. In addition, the AMH concentrations used in this study were measured within the year before inclusion. This could result in a non-differential measurement bias, as AMH levels decrease over time. Despite this imprecision, the results of our sensitivity analysis restricted to women with no prior ovarian stimulation (Supplementary Table SVII) or women whose outcomes were measured at inclusion (Supplementary Table SVIII) did not deviate from the main results of our study.

Our study also has several strengths. The AROPE study recruited cases and controls from four of the most prevalent fertility centres in north-western France. The French national health insurance funds infertility consultations and the first four in vitro fertilisation attempts until the age of 43 (National Health Insurance, 2021), which may limit a potential selection bias when recruiting patients in fertility centres. However, it is still possible that the study participants could have higher levels of education than that of the general population, as shown in a British study on the prevalence of infertility and help seeking, in which the authors reported that women with lower levels of education are less likely to seek help (Datta et al., 2016). In addition, although the AROPE study's participation rate was 65% for cases and 75% for controls, there were no differences between patients included or not in our study in terms of age, BMI, or parity (data not shown). Another strength of our study, as mentioned above, is that we performed multiple sensitivity analyses varying our case definition using pertinent thresholds to avoid issues of measurement bias.

Furthermore, we used an a priori adjustment strategy using DAGs to limit over-adjustments (Shrier and Platt, 2008). Moreover, all our analyses were based on lipid-normalised POP levels (standardisation). We chose this strategy rather than that of lipid-adjusted models in light of results from a simulation study by O'Brien et al (2016), which investigated various scenarios for the relationship between POP exposure, lipid levels, and outcomes and standardisation and showed that standardisation performed best under the causal scenario of our study, i.e., when considering recent fat intake affects serum exposure concentrations and when other covariates affect both exposure concentrations and the outcome.

Lastly, we were able to analyse the effects of individual POPs on DOR as well as the effect of POPs as a mixture using BKMR. This method allowed us to include non-linear associations and check for interactions between pollutants. Evaluating the mixture effect is of particular importance because chemicals could either show no significant associations when studied individually and yet have an effect when treated as a mixture. Alternatively, they could have individual effects and either have an

even larger impact as a mixture or mitigate each other's impacts (Le Magueresse-Battistoni et al., 2018).

Although we found some evidence of associations with POP levels in adulthood, other windows of exposure, especially prenatal exposure, may be critical to understanding the impact of POPs on the ovarian reserve. Because the development of the ovarian reserve occurs in utero and is not resupplied afterwards (Richardson et al., 2014), the effects of exposure to POPs in utero on the pool of primordial follicles could have long-lasting impacts. Incidentally, animal data have reported evidence for an association between in-utero exposure to several POPs and a decreased number of follicles (Baldrige et al., 2003; Ema et al., 2008; Lilienthal et al., 2006; Talsness et al., 2008). Data is scarce for humans, but one study (Kristensen et al., 2016) found an association between prenatal p,p'-DDE levels and a reduced number of follicles at 19 years of age.

In conclusion, we found limited but suggestive evidence of associations between serum POPs and DOR in infertile couples, especially for p,p'-DDE and  $\beta$ -HCH, which showed opposite associations. These results may be explained by possible differences in the endocrine properties of these compounds and need to be replicated in further studies. In addition, future research should also focus on exploring different windows of susceptibility, including preconceptional, prenatal, and peripubertal exposure.

**Table I. Study population characteristics (N = 138 cases and 151 controls)**

<b>Characteristic - N (%)</b>	<b>Cases (N = 138)</b>	<b>Controls (N = 151)</b>
<b>Centre</b>		
Rennes University Hospital	34 (24.7)	42 (27.8)
Brest University Hospital	8 (5.8)	4 (2.6)
Nantes University Hospital	58 (42.0)	78 (51.7)
La Sagesse Clinic	38 (27.5)	27 (17.9)
<b>Season of inclusion</b>		
Fall	26 (18.8)	43 (28.5)
Spring	49 (35.5)	41 (27.2)
Summer	29 (21.0)	23 (15.2)
Winter	34 (24.7)	44 (29.1)
<b>Age</b>		
Mean ± SD	33.8 ± 4.0	32.4 ± 4.1
[18,25[	6 (4.3)	10 (6.6)
[25,30[	14 (10.2)	29 (19.3)
[30,35[	52 (37.7)	65 (43.0)
[35,40]	66 (47.8)	47 (31.1)
<b>Ethnic background</b>		
Europe	111 (87.4)	128 (90.8)
Outside Europe	16 (12.6)	13 (9.2)
Missing data	11	10
<b>Education</b>		
High school or lower	42 (33.4)	49 (35.8)
High school +2 years of university	25 (19.8)	22 (16.1)
High school +3 years or higher of university	59 (46.8)	66 (48.2)
Missing data	12	14
<b>Body-Mass Index (kg/m<sup>2</sup>)</b>		
Mean ± SD	22.9 ± 3.4	23.6 ± 3.8
< 20	24 (17.4)	27 (18.0)
[20,25[	83 (60.2)	75 (50.0)
[25,30[	25 (18.1)	36 (24.0)
[30,35[	6 (4.3)	12 (8.0)
Missing data	0	1
<b>Infertility status</b>		
Primary	84 (60.9)	88 (58.3)
Secondary	54 (39.1)	63 (41.7)
<b>Parity</b>		
No children	84 (60.9)	88 (58.3)
1 child	33 (23.9)	33 (21.9)
≥ 2 children	21 (15.2)	30 (19.9)
<b>Anti-müllerian hormone levels (ng/mL)</b>		
Mean ± SD	0.61 ± 0.34	2.94 ± 1.00
<b>Antral follicle count</b>		
Mean ± SD	8 ± 5	21 ± 9
<b>Follicle-stimulating hormone (IU/L)</b>		

Mean ± SD	12.3 ± 12.4	6.7 ± 2.1
Missing data	4	3
<b>Luteinising hormone (IU/L)</b>		
Mean ± SD	7.7 ± 8.3	5.8 ± 2.4
Missing data	9	4
<b>Estradiol (pg/mL)</b>		
Mean ± SD	44.3 ± 20.8	37.0 ± 16.1
Missing data	19	8
<b>Age at menarche</b>		
Mean ± SD	12.9 ± 1.6	13.0 ± 1.7
Missing data	17	19
<b>Smoking status</b>		
Never smoker	61 (48.4)	68 (48.6)
Past smoker	46 (36.5)	49 (35.0)
Current smoker	19 (15.1)	23 (16.4)
Missing data	12	11
<b>Cigarette pack-years</b>		
Non smoker	61 (52.1)	67 (51.1)
]0,3[	26 (22.2)	18 (13.7)
[3,6[	14 (12.0)	16 (12.2)
≥ 6	16 (13.7)	30 (22.9)
Missing data	21	20
<b>Alcohol (number of drinks per week)</b>		
Non drinker	36 (29.5)	22 (16.2)
]0,7]	78 (63.9)	101 (74.3)
> 7	8 (6.6)	13 (9.6)
Missing data	16	15

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Abbreviations: N, number of subjects; SD, standard deviation.

**Table II. Serum persistent organic pollutant (POP) concentrations in ng/L (ng/g lipid base) in the AROPE study (N = 138 cases and 151 controls)**

POPs <sup>1</sup>	Groupe	LOD (ng/L)	Detection N (%)	Minimum	Percentiles			Maximum
					25	50	75	
<i>Organochlorine pesticides</i>								
Dieldrine	Controls	25	26 (17.2)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	121 (21.3)
	Cases	25	18 (13)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	82 (14.2)
o,p'-DDE	Controls	2.5	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	2.7 (0.5)
	Cases	2.5	0	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)
p,p'-DDE	Controls	10	151 (100)	25 (4.4)	111.5 (19.9)	165.0 (28.9)	272.5 (48.8)	1839 (324.2)
	Cases	10	138 (100)	41 (7)	132.5 (24.0)	202.0 (35.5)	355.2 (62.1)	7532 (1229.1)
p,p'-DDT	Controls	5	51 (33.8)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	6.2 (1.1)	186 (33.4)
	Cases	5	52 (37.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	7.4 (1.3)	170.3 (29)
Heptachlore	Controls	2.5	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	3.6 (0.6)
	Cases	2.5	0	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)
cis-Heptachlor epoxide	Controls	5	143 (94.7)	<LOD (<LOD)	8.2 (1.5)	11.1 (2)	15.1 (2.6)	34.4 (5.3)
	Cases	5	133 (96.4)	<LOD (<LOD)	8.1 (1.6)	10.3 (1.9)	14 (2.4)	26.6 (5.5)
HCB	Controls	2.5	151 (100)	11.3 (2)	34 (6.1)	41.5 (7.4)	50.2 (9)	149 (25.7)
	Cases	2.5	138 (100)	15.6 (2.6)	35.7 (6.4)	41.7 (7.5)	47.7 (9)	108.3 (15.1)
α-HCH	Controls	2.5	6 (4)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	7.9 (1.4)

	Cases	2.5	7 (5.1)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	23.4 (3.7)
β-HCH	Controls	2.5	151 (100)	6.4 (1.3)	16.7 (3.3)	24.2 (4.5)	38.2 (6.3)	262.6 (47.2)
	Cases	2.5	138 (100)	2.8 (0.6)	17.1 (3.1)	22.5 (4.1)	34.5 (5.9)	587.3 (110.7)
γ-HCH	Controls	2.5	12 (7.9)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	13 (2.4)
	Cases	2.5	13 (9.4)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	48.5 (7.7)
<i>Polychlorinated biphenyls</i>								
PCB 28	Controls	2.5	75 (49.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	4.2 (0.8)	32.8 (6.6)
	Cases	2.5	68 (49.3)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	4.1 (0.7)	22.1 (3.8)
PCB 52	Controls	2.5	9 (6)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	20.4 (2.5)
	Cases	2.5	8 (5.8)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	11 (1.9)
PCB 74	Controls	2.5	147 (97.4)	<LOD (<LOD)	8.2 (1.6)	11.8 (2.1)	17.2 (2.9)	106.4 (21.2)
	Cases	2.5	137 (99.3)	<LOD (<LOD)	8.6 (1.6)	12.1 (2.2)	17.3 (3)	88.5 (15.1)
PCB 99	Controls	2.5	150 (99.3)	<LOD (<LOD)	8.9 (1.6)	12.2 (2.2)	16.8 (3.1)	65.6 (13.1)
	Cases	2.5	137 (99.3)	<LOD (<LOD)	10.1 (1.8)	13.3 (2.5)	18.1 (3.2)	43.5 (7.4)
PCB 101	Controls	2.5	19 (12.6)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	9.8 (1.9)
	Cases	2.5	20 (14.5)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	16.2 (2.7)
PCB 118	Controls	2.5	151 (100)	5.3 (0.9)	17.8 (3.1)	24.3 (4.3)	35.5 (6.2)	107.9 (21.5)
	Cases	2.5	138 (100)	5.2 (1)	19.9 (3.5)	26.8 (4.9)	34.3 (6.3)	90.2 (17.3)
PCB 138+163 <sup>2</sup>	Controls	2.5	151 (100)	14.5 (2.6)	56.6 (10.4)	76 (13.5)	105.2 (19.2)	276.1 (55)
	Cases	2.5	138 (100)	16.6 (2.8)	64.5 (11.1)	87.1 (16.7)	114.6 (19.4)	285.7 (47.8)
PCB 153	Controls	2.5	151 (100)	21.3 (3.8)	80 (15.3)	120.6 (21.1)	162.9 (29.1)	401.5 (80)
	Cases	2.5	138 (100)	24.4 (4.1)	97.9 (16.3)	130.1 (24.3)	170.4 (30.5)	452.4 (76.2)

PCB 170	Controls	2.5	151 (100)	4.5 (0.8)	17.6 (3.3)	27.2 (5.2)	43 (7.6)	137 (24.6)
	Cases	2.5	138 (100)	3.7 (0.6)	20.8 (3.9)	32.8 (6.1)	46.1 (7.8)	164.8 (30.4)
PCB 180	Controls	2.5	151 (100)	12.3 (2.2)	50 (9.3)	78.8 (15.2)	121.9 (21.6)	362.9 (65.2)
	Cases	2.5	138 (100)	10 (1.7)	63.7 (11.6)	98.7 (18.1)	127.5 (23)	456.6 (84.2)
PCB 183	Controls	2.5	144 (95.4)	<LOD (<LOD)	4.7 (0.8)	6.6 (1.2)	9.4 (1.7)	28 (5.6)
	Cases	2.5	133 (96.4)	<LOD (<LOD)	5.5 (1)	7.9 (1.4)	10.6 (1.9)	27.1 (5.6)
PCB 187	Controls	2.5	149 (98.7)	<LOD (<LOD)	9.7 (1.7)	15.3 (2.7)	25.2 (4.5)	117.9 (21.2)
	Cases	2.5	138 (100)	3.1 (0.5)	11.5 (2.1)	18.3 (3.3)	25 (4.5)	86.1 (12.7)
PCB 194	Controls	2.5	141 (93.4)	<LOD (<LOD)	5.4 (1.1)	8.8 (1.6)	15.1 (2.6)	52.8 (9.5)
	Cases	2.5	133 (96.4)	<LOD (<LOD)	7.1 (1.4)	11.1 (2.1)	16.7 (2.9)	68.7 (12.7)
PCB 196+203 <sup>2</sup>	Controls	2.5	141 (93.4)	<LOD (<LOD)	5.1 (0.9)	8 (1.4)	12.4 (2.1)	35.7 (7.1)
	Cases	2.5	133 (96.4)	<LOD (<LOD)	6.6 (1.2)	9.6 (1.8)	13.1 (2.3)	45.8 (8.4)
<i>Polybromodiphenylethers</i>								
BDE 28	Controls	2.5	8 (5.3)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	54.4 (8)
	Cases	2.5	3 (2.2)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	3.6 (0.6)
BDE 47	Controls	5	4 (2.6)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	131.1 (19.2)
	Cases	5	7 (5.1)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	176.2 (29.9)
BDE 66	Controls	5	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	5.3 (0.9)
	Cases	5	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	11.7 (1.9)
BDE 99	Controls	5	2 (1.3)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	161.6 (23.7)
	Cases	5	5 (3.6)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	32.7 (5.6)

BDE 100	Controls	5	2 (1.3)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	146.3 (21.5)
	Cases	5	4 (2.9)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	29.6 (5)
BDE 153	Controls	130	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	150 (22)
	Cases	130	0	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)
BDE 154	Controls	25	3 (2)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	125 (18.3)
	Cases	25	1 (0.7)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	<LOD (<LOD)	72 (11.8)

<sup>1</sup>Aldrin, o,p'-DDT,  $\alpha$ -endosulfan,  $\beta$ -endosulfan, trans-heptachlor epoxide, PCB 31, BDE congener 85 & 183, polybromobiphenyl 153, and  $\alpha\beta\gamma$ -hexabromocyclododecane concentrations were below the LOD in all cases and controls.

<sup>2</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: N, number of subjects; LOD, limit of detection.



**Table III. Associations between serum persistent organic pollutants and diminished ovarian reserve (defined as AFC < 7 and/or AMH ≤ 1.1 ng/ml) in the AROPE study (N = 138 cases and 151 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 138)	Number of controls (N = 151)	Diminished ovarian reserve			
				Univariate logistic regression		Multivariate <sup>1</sup> logistic regression	
				OR	CI	OR	CI
<b>Organochlorine pesticides</b>							
p,p'-DDE	≤ 22.39	29	51	Ref.	Ref.	Ref.	Ref.
	(22.39-40.74]	51	50	1.76	0.96 - 3.21	1.46	0.74 - 2.87
	> 40.74	58	50	<b>1.96</b>	<b>1.08 - 3.55</b>	1.72	0.88 - 3.37
	Continuous	138	151	<b>1.40</b>	<b>1.13 - 1.74</b>	<b>1.39</b>	<b>1.10 - 1.77</b>
p,p'-DDT	< LOD	52	51	Ref.	Ref.	Ref.	Ref.
	≥ LOD	86	100	1.19	0.73 - 1.92	1.13	0.66 - 1.94
cis-Heptachlor epoxide	≤ 1.71	53	50	Ref.	Ref.	Ref.	Ref.
	(1.71-2.42]	50	51	0.94	0.54 - 1.64	0.84	0.47 - 1.52
	> 2.42	35	50	0.65	0.36 - 1.15	0.64	0.35 - 1.18
	Continuous	138	151	0.89	0.58 - 1.36	0.86	0.55 - 1.35
HCB	≤ 6.43	38	51	Ref.	Ref.	Ref.	Ref.
	(6.43-8.42]	54	50	1.42	0.80 - 2.51	1.07	0.57 - 2.01
	> 8.42	46	50	1.19	0.66 - 2.21	0.89	0.45 - 1.77
	Continuous	138	151	1.08	0.66 - 1.79	0.85	0.47 - 1.53
β-HCH	≤ 3.62	49	51	Ref.	Ref.	Ref.	Ref.
	(3.62-5.77]	53	50	1.08	0.62 - 1.88	0.77	0.42 - 1.42

	> 5.77	36	50	0.72	0.40 - 1.29	<b>0.43</b>	<b>0.21 - 0.84</b>
	Continuous	138	151	0.81	0.62 - 1.07	<b>0.63</b>	<b>0.44 - 0.89</b>
<b>Polychlorinated biphenyls</b>							
PCB 28	≤ 0.41	41	49	Ref.	Ref.	Ref.	Ref.
	(0.41-0.60]	45	51	0.97	0.46 - 2.04	0.97	0.44 - 2.12
	> 0.60	52	51	1.25	0.68 - 2.30	1.24	0.64 - 2.41
	Continuous	138	151	1.05	0.77 - 1.43	1.00	0.71- 1.39
PCB 74	≤ 1.65	39	51	Ref.	Ref.	Ref.	Ref.
	(1.65-2.52]	48	49	1.21	0.68 - 2.15	1.00	0.53 - 1.89
	> 2.52	51	51	1.31	0.74 - 2.31	0.91	0.47 - 1.76
	Continuous	138	151	1.02	0.78 - 1.32	0.84	0.61 - 1.15
PCB 99	≤ 1.82	35	51	Ref.	Ref.	Ref.	Ref.
	(1.82-2.69]	43	50	1.29	0.71 - 2.35	1.24	0.65 - 2.39
	> 2.69	60	50	1.73	0.97 - 3.07	1.52	0.80 - 2.90
	Continuous	138	151	1.20	0.87 - 1.65	1.04	0.72 - 1.50
PCB 118	≤ 3.45	33	51	Ref.	Ref.	Ref.	Ref.
	(3.45-5.50]	55	50	1.67	0.93 - 2.99	1.43	0.76 - 2.70
	> 5.50	50	50	1.49	0.83 - 2.67	1.17	0.60 - 2.29
	Continuous	138	151	1.10	0.81 - 1.51	0.94	0.65 - 1.37
PCB 138+163 <sup>3</sup>	≤ 3.71	33	51	Ref.	Ref.	Ref.	Ref.
	(3.71-6.44]	42	50	1.27	0.70 - 2.32	1.01	0.50 - 2.04
	> 6.44	63	50	<b>1.87</b>	<b>1.05 - 3.32</b>	1.27	0.57 - 2.81
	Continuous	138	151	1.26	0.97 - 1.63	0.97	0.65 - 1.43
PCB 153	≤ 16.36	35	51	Ref.	Ref.	Ref.	Ref.
	(16.36-26.21]	50	50	1.43	0.80 - 2.56	1.09	0.56 - 2.16
	> 26.21	53	50	1.48	0.83 - 2.65	1.01	0.48 - 2.12
	Continuous	138	151	1.29	0.94 - 1.77	1.00	0.65 - 1.55
PCB 170	≤ 11.34	35	51	Ref.	Ref.	Ref.	Ref.
	(11.34-16.43]	33	50	0.94	0.51 - 1.75	0.75	0.38 - 1.49
	> 16.43	70	50	<b>1.96</b>	<b>1.12 - 3.44</b>	1.56	0.78 - 3.12
	Continuous	138	151	1.27	0.92 - 1.75	1.00	0.66 - 1.51

PCB 180	≤ 10.85	32	51	Ref.	Ref.	Ref.	Ref.
	(10.85-18.91]	45	50	1.41	0.77 - 2.56	1.10	0.54 - 2.21
	> 18.91	61	50	<b>1.87</b>	<b>1.05 - 3.33</b>	1.20	0.54 - 2.67
	Continuous	138	151	1.30	0.99 - 1.69	1.01	0.68 - 1.51
PCB 183	≤ 0.94	30	51	Ref.	Ref.	Ref.	Ref.
	(0.94-1.52]	51	51	1.70	0.94 - 3.09	1.43	0.73 - 2.81
	> 1.52	57	49	<b>1.86</b>	<b>1.03 - 3.36</b>	1.43	0.69 - 2.96
	Continuous	138	151	<b>1.42</b>	<b>1.04 - 1.94</b>	1.23	0.81 - 1.85
PCB 187	≤ 2.06	40	52	Ref.	Ref.	Ref.	Ref.
	(2.06-3.54]	41	52	1.17	0.64 - 2.13	0.99	0.49 - 1.97
	> 3.54	57	47	1.74	0.98 - 3.07	1.38	0.68 - 2.77
	Continuous	138	151	1.23	0.96 - 1.57	1.07	0.78 - 1.47
PCB 194	≤ 1.23	30	52	Ref.	Ref.	Ref.	Ref.
	(1.23-2.38]	59	49	<b>1.90</b>	<b>1.05 - 3.43</b>	1.40	0.69 - 2.85
	> 2.38	49	50	1.67	0.92 - 3.03	0.87	0.37 - 2.05
	Continuous	138	151	<b>1.34</b>	<b>1.05 - 1.71</b>	1.09	0.74 - 1.60
PCB 196+203 <sup>3</sup>	≤ 1.12	29	51	Ref.	Ref.	Ref.	Ref.
	(1.12-1.89]	53	51	<b>1.83</b>	<b>1.00 - 3.33</b>	1.50	0.73 - 3.09
	> 1.89	56	49	<b>1.89</b>	<b>1.04 - 3.43</b>	1.42	0.63 - 3.22
	Continuous	138	151	<b>1.37</b>	<b>1.05 - 1.80</b>	1.16	0.78 - 1.72

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>All POPs were log2-transformed when treated as continuous variables.

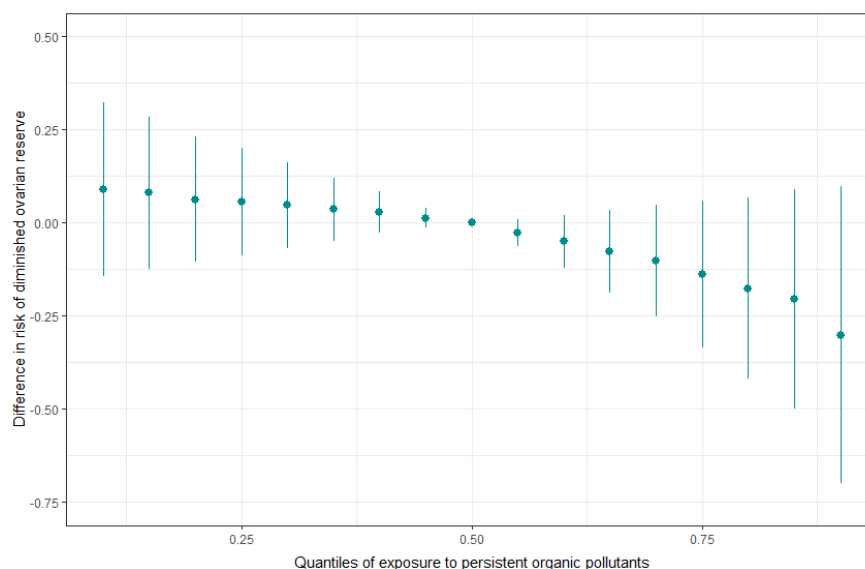
<sup>3</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

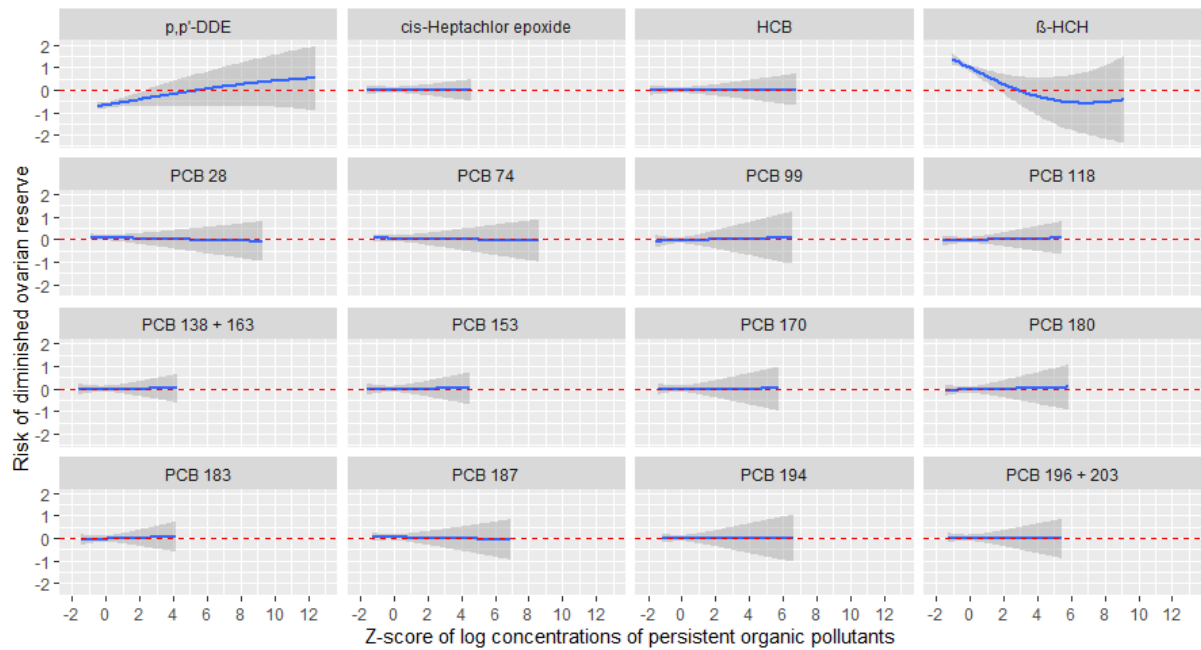
**Table IV. Posterior inclusion probabilities for group inclusion and conditional inclusion in the Bayesian Kernel Machine Regression model (N = 137 cases and 151 controls)**

Persistent organic pollutant	Group	Diminished ovarian reserve	
		Group PIP (%)	Conditional PIP (%)
p,p'-DDE	1	96.9	6.8
cis-Heptachlor epoxide	1	96.9	1.0
HCB	1	96.9	1.0
$\beta$ -HCH	1	96.9	91.4
PCB 28	2	31.8	6.9
PCB 74	2	31.8	3.0
PCB 99	2	31.8	17.6
PCB 118	2	31.8	6.0
PCB 138+163	2	31.8	6.0
PCB 153	2	31.8	9.2
PCB 170	2	31.8	7.1
PCB 180	2	31.8	9.3
PCB 183	2	31.8	11.5
PCB 187	2	31.8	7.2
PCB 194	2	31.8	7.0
PCB 196+203	2	31.8	9.1

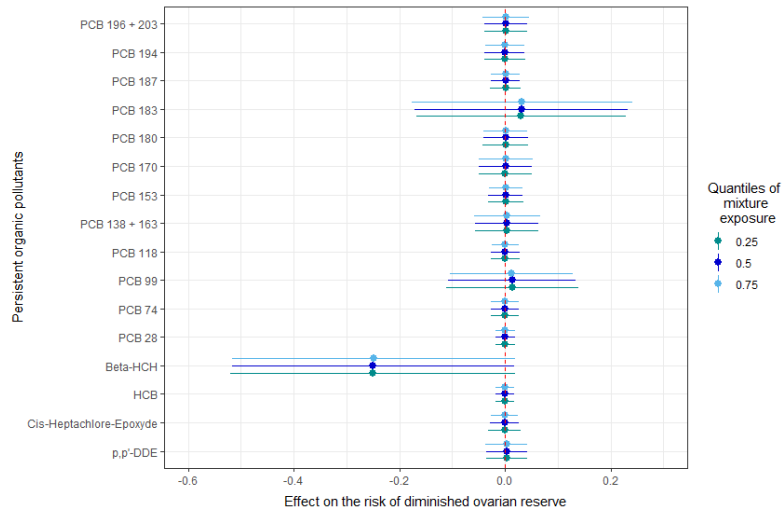
Abbreviations: PIP, posterior inclusion probability.



**Figure 1.** Difference in risk (and their 95% credible intervals) of diminished ovarian reserve due to exposure to the persistent organic pollutant mixture when all pollutant concentrations are fixed at different percentiles compared to when they are fixed at their median levels. The model was adjusted for age, centre, education level, body-mass index, and season of inclusion.



**Figure 2.** p,p'-DDE, p,p'-dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; β-HCH, β-hexachlorocyclohexane; and PCB, polychlorinated biphenyl. Difference in risks (blue lines) and their 95% credible intervals (shaded areas) of diminished ovarian reserve at a given exposure level of each persistent organic pollutant compared to the median exposure level of that pollutant while holding all other chemical exposure at their median levels. The model was adjusted for age, centre, education level, body-mass index, and season of inclusion.



**Figure 3.** p,p'-DDE, p,p'-dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; β-HCH, β-hexachlorocyclohexane; and PCB, polychlorinated biphenyl. Effects of individual persistent organic pollutants (and their 95% credible intervals) on the risk of diminished ovarian reserve with all other pollutant exposures fixed at different quantiles (0.25, 0.50, 0.75). The model was adjusted for age, centre, education level, body-mass index, and season of inclusion.

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### 3.2. ARTICLE 2: Associations between heavy metals and diminished ovarian reserve in the AROPE case-control study

**Status: Published in *Reproductive Biomedicine Online***

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Heavy metals and diminished ovarian reserve: single-exposure and mixture analyses amongst women consulting in French fertility centres. *Reprod Biomed Online*. 2 June 2023. Available at:

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## Abstract

**Research question:** Do heavy metals affect the risk of diminished ovarian reserve (DOR) in women of reproductive age?

**Design:** We included 139 cases and 153 controls between 2016-2020. The participants were aged 18-40 and consulted for couple infertility in one of four fertility centres in western France. Cases of DOR were defined as women with an antral follicle count  $< 7$  and/or anti-müllerian hormone levels  $\leq 1.1$  ng/ml. Controls were frequency matched on age groups and centres and were women with normal ovarian reserve evaluations, no malformations, and menstrual cycles between 26-35 days. Heavy metals (lead, mercury, cadmium and chromium) were measured in whole blood at inclusion. Single-exposure associations were examined with multivariable logistic regressions adjusted on potential confounders. Mixture effects were investigated with quantile g-computation and Bayesian kernel machine regression (BKMR).

**Results:** We observed non-significant increased risks of DOR for cadmium (OR 1.89, 95% CI 0.98 - 3.67, for the second tercile of exposure and OR 1.18, 95% CI 0.55 - 2.52, for the third tercile) and chromium (OR 1.24, 95% CI 0.65 - 2.39, for the second tercile of exposure and OR 1.77, 95% CI 0.84 - 3.70, for the third tercile). Quantile g-computation and BKMR both yielded a non-significant increased risk of DOR for the mixture of metals, with no evidence of interaction.

**Conclusions:** We detected weak signals that some heavy metals could be associated with DOR. These findings should be replicated in other studies.

**Keywords:** Reproductive outcomes, Female fertility, Heavy metals, Chemical exposures, Mixtures, Environmental epidemiology.

## Résumé en Français

**Question de recherche :** Les métaux lourds affectent-ils le risque de diminution de la réserve ovarienne (DOR) chez les femmes en âge de procréer ?

**Méthodes :** Nous avons inclus 139 cas et 153 témoins entre 2016 et 2020. Les participantes étaient âgées de 18 à 40 ans et consultaient pour infertilité de couple dans l'un des quatre centres de fertilité de l'ouest de la France. Les cas de DOR étaient définis comme des femmes ayant un compte folliculaire antral  $< 7$  et/ou des taux d'hormone anti-müllérienne  $\leq 1.1$  ng/ml. Les témoins étaient appariés en fréquence selon les groupes d'âge et les centres, et étaient des femmes avec des évaluations normales de la réserve ovarienne, sans malformations et avec des cycles menstruels de 26 à 35 jours. Les métaux lourds (plomb, mercure, cadmium et chrome) ont été mesurés dans le sang total à l'inclusion. Les associations individuelles ont été examinées avec des régressions logistiques multivariées ajustées sur

les facteurs de confusion potentiels. Les effets de mélange ont été étudiés par quantile g-computation et Bayesian kernel machine regression (BKMR).

**Résultats :** Nous avons observé des risques accrus non significatifs de DOR pour le cadmium (OR 1.89, IC 95% 0.98 - 3.67, pour le deuxième tercile d'exposition, et OR 1.18, IC 95% 0.55 - 2.52, pour le troisième tercile) et le chrome (OR 1.24, IC 95% 0.65 - 2.39, pour le deuxième tercile d'exposition, et OR 1.77, IC 95% 0.84 - 3.70, pour le troisième tercile). Les méthodes quantile g-computation et BKMR ont toutes deux abouti à un risque accru non significatif de DOR pour le mélange de métaux, sans montrer d'indication d'interaction.

**Conclusions :** Nous avons détecté des signaux faibles indiquant que certains métaux lourds pourraient être associés à la DOR. Ces résultats doivent être reproduits dans d'autres études.

**Mots-clés :** Reproduction, Fertilité féminine, Métaux lourds, Expositions chimiques, Mélanges, Épidémiologie environnementale.

## **Introduction**

Whilst these studies have looked at AFC or AMH as continuous indicators of the ovarian reserve, no study so far has investigated associations between heavy metals and DOR as a clinical outcome. In this study our aim was to examine the associations between whole blood Pb, Hg, Cd and Cr, and DOR in four French fertility centres.

## **Materials and Methods**

### *Population*

We used data from the AROPE case-control study (139 cases and 153 controls) as described in chapter 2.

### *Statistical analyses*

Heavy metals were analysed both as tertiles based on control concentrations in order to better represent exposure in the general population, and as continuous variables (log-10 transformed).

We used a directed acyclic graph (DAG) (Supplementary Material S2) to a priori determine the minimally sufficient set of confounders to adjust our models on. The variables selected were age (continuous), centre (Brest and Rennes University Hospitals; La Sagesse Clinic; Nantes University Hospital), education level (high school or lower, high school + 2 years of university, high school + 3 years of university and above), ethnic background (European origin and non-European origin), smoking status (past, current and never users), and season of inclusion (fall, winter, spring, summer). We used multiple imputation by chained equations (five sets) (Kristman *et al.*, 2005) to impute covariates with missing data (9.2% for education level, 7.9% for ethnic background, and 7.5% for smoking status).

Mixture analyses was carried out using two different multi-pollutant models, quantile g-computation and Bayesian kernel machine regression (BKMR), in order to obtain complementary information on the mixture of the heavy metals on DOR.

We performed three sensitivity analyses where we modified the definition of DOR in order to account for potential misclassification of the outcome. A first sensitivity was defined using only the AFC outcome with the same threshold of  $AFC < 7$  (N = 55 cases & 153 controls) as in the main outcome. Similarly, a second sensitivity analysis was defined using only the AMH outcome as  $AMH \leq 1.1$  (N = 135 cases & 153 controls). Finally, the outcome in the third analysis was defined as  $AFC < 7$  &  $AMH \leq 1.1$  ng/mL rather than and/or (N = 90 cases & 153 controls). Additionally, it has been suggested that the ESHRE Bologna Criteria should be revisited in light of more recent studies. We therefore conducted an extra sensitivity analysis (N = 90 cases & 153 controls) using the new proposed values ( $AFC < 5$  and/or  $AMH \leq 0.7$  ng/mL) (Ferraretti and Gianaroli, 2014). The control group (N=153) remained the same between all sensitivity analyses and the main analysis. Finally, we performed a sensitivity analysis

restricted to women with no prior ovarian stimulation (N = 107 cases & 110 controls) in order to assess whether previous ovarian stimulations could modify the effects of metals on DOR.

All analyses were performed using R (version 4.0.5). The threshold for significant results for all our analyses was 5%.

## **Results**

### *Population description*

The mean age in the case group was 33.8 years ( $\pm$  4.0) whilst the mean age in the control group was 32.4 years ( $\pm$  4.1) (Table 1). Close to half of the population had an education level equivalent to high school + 3 years of university or above (47.2% for cases and 47.8% for controls). Cases and controls had similar smoking habits (37% of cases and 34.8% of controls were current smokers, and 48.0% of cases and 48.9% of controls had never smoked). The majority of cases (90.8%) and controls (87.5%) had a European ethnic background and more than half of both groups were women with primary infertility (61.2% for cases and 58.8% for controls).

### *Whole blood concentrations of heavy metals*

Detection rates and the concentrations of heavy metals in whole blood in the control group are available in Table 2. The detection rates of Pb, Cd, Cr and Hg were 100%, 98.7%, 88.9%, and 93.5%, respectively. The metal with the highest median concentration was Pb (9.1  $\mu\text{g/L}$ ), followed by Hg (1.5  $\mu\text{g/L}$ ), Cr (1.1  $\mu\text{g/L}$ ) and Cd (0.1  $\mu\text{g/L}$ ). The highest correlation among metals (Supplementary Material S3) was between Pb and Cd ( $\rho = 0.15$ ).

### *Effects of heavy metal exposures on DOR*

Associations between tercile and continuous heavy metals and DOR are presented in Table 3. We observed a close-to-statistically significant association for the second tercile of Cd exposure (OR 1.89, 95% CI 0.98 - 3.67) but the association was not significant for the third tercile (OR 1.18, 95% CI 0.55 - 2.52) or when Cd was treated as a continuous exposure (OR 0.94, 95% CI 0.45 - 1.99). Cr (continuous) also showed a non-statistically significant increased risk of DOR (OR 2.75, 95% CI 0.88 - 8.60). This effect was also observed when Cr was treated as terciles (OR 1.24, 95% CI 0.65 - 2.39, for the second tercile, and OR 1.77, 95% CI 0.84 - 3.70, for the third tercile).

### *Mixture analyses*

Quantile g-computation weights can be found in Figure 1. Increasing all metal exposures by one quartile was non-statistically associated with an increased risk of DOR (OR 1.40, 95% CI 0.85 - 2.32). Cr

and Cd had positive weights (0.80 and 0.20 respectively), and Pb and Hg had negative weights (0.73 and 0.27 respectively).

The mixture effect of heavy metals on DOR in the BKMR analysis is shown in Figure 2. According to the posterior inclusion probabilities (PIP) (Table 4), the model favoured the inclusion of Cr more (59.0%) than Cd (29.2%), Pb (28.2%) and Hg (25.4%). The overall mixture effect had a non-monotonic relationship with DOR (Figure 2). Increasing the mixture exposure from the 25<sup>th</sup> percentile to the 75<sup>th</sup> percentile was associated with a statistically non-significant increase in the risk of DOR (Beta 0.11, 95% CI -0.22; 0.48). Plots of the exposure-response relationships between individual metals and DOR while holding all other chemical exposures at their median levels (Figure 3) showed no statistically significant impacts on DOR, though Cr exhibited a linear increased risk of DOR and Pb a linear decreased risk of DOR. Graphical inspection (Figure 4) of the effect of individual metals on the risk of DOR when all other metal exposures are fixed at different quantiles (.25, .50, .75) did not detect strong evidence of interactions between metals.

#### *Sensitivity analyses*

The sensitivity analyses where we modified the case definition are available in Supplementary Material S4-S7. When DOR was defined as AMH  $\leq$  1.1 ng/ml only, the second tercile of Cd exposure became statistically significant (OR 1.98, 95% CI 1.01 - 3.87). Other sensitivity analyses did not report any statistically significant increased risk when DOR was defined using AFC only or in combination with AMH, and we did not observe any increased risks of DOR for the rest of the heavy metals. The sensitivity analysis restricted to women with no prior ovarian stimulation did not change our results (Supplementary Material S8).

#### **Discussion**

In this case-control study in four fertility centres in Western France, we investigated the associations between four heavy metals (Pb, Hg, Cd, and Cr) and DOR. We observed non-significant increased risks of DOR for Cd and Cr with a close to significant association for the second tercile of Cd. Sensitivity analyses with alternative DOR definitions showed reinforced associations when DOR was defined by AMH only but not when DOR was defined using AFC only or in combination with AMH. Other metals (Pb and Hg) were not significantly associated with an increased risk of DOR. Quantile g-computation and BKMR both showed a non-significant increased risk between the metal mixture and DOR, with no evidence of interaction between metals.

To the best of our knowledge, this is the first study to examine the associations between heavy metals and DOR as a clinical outcome. Our results are in accordance with animal studies that reported possible impacts of Cd and Cr on different indicators of the ovarian reserve. Indeed, these have reported effects



of Cd on every stage of folliculogenesis such as the decrease in volume of growing follicles, the decrease in follicle number, the increase in the number of atretic follicles, and degenerative changes of the corpus luteum (Massányi et al., 2020), and on the concentrations of steroid hormones including estradiol, progesterone, follicle stimulating hormone and luteinizing hormone (Nasiadek et al., 2019; Nna et al., 2017). Regarding Cr, one study showed changes in the number of atretic follicles, the number of follicles at different stages of maturation and the number of oocytes collected (Murthy et al., 1996). Some authors have suggested that Cd and Cr may impact female fertility through cellular apoptosis induced by oxidative stress or through gene expression modifications, which could lead to follicular death or disrupted steroidogenesis (Dutta et al., 2021). However, the complete underlying biological mechanisms remain poorly understood (Dutta et al., 2021; Henriques et al., 2019; Pollack et al., 2014). In humans, previous studies have reported inconsistent associations between heavy metals and continuous indicators of the ovarian reserve. One study (Christensen et al., 2016) found non-significant associations between increased whole blood Cd and higher AMH levels during pregnancy. However, the comparison with our study population is limited because AMH levels in pregnant women can decrease with advancing gestational age (McCredie et al., 2017), but also because bone mobilisation during this period can affect blood metal concentrations. A study on women between 30 and 49 years old found decreased AMH levels due to increased whole blood Cd in the 30-35 age group specifically (Lee et al., 2018). This was corroborated by another case-control study investigating primary ovarian insufficiency (Pan et al., 2021) whom also found a non-significant relationship between increased urinary Cd and decreased AMH levels.

Compared to studies that investigated the relationship between heavy metals and markers of ovarian reserve, the Cd concentrations in the AROPE study were lower than in the studies reporting significant associations with the ovarian reserve (minimum median of 0.3 µg/L compared to 0.1 µg/L in controls in AROPE) (Lee et al., 2018; Pan et al., 2021). In addition, Pb concentrations were lower than in the study from Christensen (2016) which reported no significant associations between Pb and AMH (Christensen et al., 2016) (median of 14.6 µg/L vs 9.1 µg/L in AROPE). It is possible that heavy metals only show a significant impact on DOR after reaching a certain threshold concentration level, as it can be the case for many endocrine disrupting chemicals, which could explain why we did not detect associations in our study whilst others did. Compared to biomonitoring studies that included males and females at all ages, AROPE Cd levels were also lower than the concentrations found in biomonitoring studies from France (Nisse et al., 2017), Belgium (Hoet et al., 2021) and the United States (Shim et al., 2017) (minimum median of 0.21 µg/L), but Pb levels were half of those found in a French national cross-sectional study (Esteban, 2014-16) taking place in the general adult population (Santé Publique France, 2021). In addition, Cr concentrations in AROPE were almost twice as high as the concentrations found in the French IMEPOGE study (Nisse et al., 2017); but not Pb, Cd and Hg

which had similar levels. AROPE metal concentrations therefore seem in line with these different studies considering their population characteristics (especially age and sex) except perhaps for Cr which is rarely measured.

Our study has several limitations. Our exposure assessment relies on a single heavy metal measurement instead of measures repeated over several years. However, the blood half-life of all the metals studied here is longer than one month and up to four months for some (Agency for Toxic Substances and Disease Registry, 2020, 2012b; Díez et al., 2009; Järup and Åkesson, 2009). In the cases of Pb, Cd and Cr, organs and tissues such as bones, kidneys and the liver can sustain blood levels when these are excreted (de Souza et al., 2018; Godt et al., 2006). Thus, whole blood metal concentrations can easily be maintained over long periods of time, and this despite the seasonality variability of some metals (Levin et al., 2020) which we took into account in our adjustment strategy.

Other studies exploring the impact of heavy metals on in vitro fertilisation outcomes have measured metals in follicular fluid instead of blood. Follicular fluid is a more direct measure of metals in the ovaries than whole blood, but it is restrained to women that underwent IVF which is not the case for women with a very low ovarian reserve due to low IVF success rates, and could lead to a potential selection bias. To the best of our knowledge only one study (Bloom et al., 2012) has compared metal concentrations in blood and follicular fluid and found high correlations for all studied metals excepted for Pb. The impact of using whole blood in our study on our results should therefore be moderate and non-differential.

AMH concentrations used in this study were measured either at inclusion or within the year prior to the inclusion. The delay between the whole blood sampling used for metal concentrations and the AMH measurement could therefore result in a non-differential measurement bias since AMH levels decrease over time. However, our sensitivity analysis restricted to women with no prior ovarian stimulation showed similar results than in the whole population. Furthermore, AMH concentrations were measured using two different assays (the Beckman-Coulter Access assay in Rennes University Hospital and the AMH Roche Elecsys assay for the three other centres). Nonetheless, comparative studies (Li et al., 2016; van Helden and Weiskirchen, 2015) where assays ran in parallel on serum samples frozen at  $-20^{\circ}\text{C}$  have yielded highly correlated results. Our multivariate analyses also included centres as an adjustment variable partly to account for any residual differences due to the use of different assays.

Our main results were confirmed in sensitivity analyses only when DOR was defined by AMH only. For other metals, the direction of their effects on DOR in sensitivity analyses seem to fluctuate depending on whether DOR is defined by AMH only or AFC only, with only one of those associations (second tercile of Cadmium when DOR is defined by  $\text{AMH} \leq 1.1 \text{ ng/ml}$ ) being statistically significant. These differences can be attributed to different factors. Previous studies have reported that AMH had at least the same

level of accuracy and clinical value for the prediction of poor response and nonpregnancy as AFC (Broer et al., 2009). However, one article (Zhang et al., 2019) recently showed measurement discordances between low AFC (< 7) and low AMH (< 1.1 ng/ml) in 1121 infertile women undergoing in vitro fertilisation. Some studies have also observed that AMH levels in younger women did not correlate with the number of growing follicles, while in women of late reproductive age a significant correlation was observed with the primordial follicle density (Moolhuijsen and Visser, 2020); indicating that AMH and AFC could reflect different aspects of the ovarian reserve at older ages (the ovarian function and the recruitable follicles respectively). Accordingly, a hypothesis that could explain the inconsistencies in our sensitivity analyses based on the different definitions of DOR is the fact that metals may impact primordial follicles and not antral follicles.

Lastly, although we recruited patients over a wide area of France and for a period of four years, study power for this analysis was still limited by the sample size.

Our study also has several strengths. The centres included in the AROPE study are four of the most prevalent fertility centres in North-western France where access to treatments is facilitated by the French national health insurance which funds infertility consultations and the first four in vitro fertilisation attempts until the age of 43 (National Health Insurance, 2021). Patients with prevalent female infertility factors (endometriosis, polycystic ovary syndrome, adnexal surgery, cancer with chemotherapy or radiotherapy, and a genetic or chromosomal syndrome), were excluded of the study during the recruitment process, thus preventing other infertility biology mechanisms to interfere in the assessment of the causal relationship between our exposures and outcome. In addition, the AROPE study's participation rates were 65% for cases and 75% for controls and there were no differences between patients included and not included in our study regarding age, BMI and parity (data not shown).

We made the decision a priori to study DOR rather than AMH or AFC as continuous variables in order to use a more clinically pertinent outcome. To that end, we used threshold levels for AFC and AMH that were put forward by the Bologna ESHRE consensus for the purpose of predicting a poor ovarian response (POR). Indeed, many fertility centres also use this definition to diagnose DOR (Pastore et al., 2018). However, we performed several sensitivity analyses where we varied the threshold levels used to limit the risk of DOR misclassification.

Another important strength of this study was the evaluation of the joint impact of exposure to multiple metals using two mixture methods: quantile g-computation and BKMR. Apart from being able to measure mixture effects of the metals on DOR, we were interested in checking the consistency of the results between both models. Whilst some slight differences can be observed due to non-linearity and difference in weights, the overall results were very consistent.

In conclusion, the study detected weak signals that some heavy metals, especially Cd and Cr, could be associated with DOR. These findings should be replicated in other studies. In particular, future research should focus on exploring different windows of susceptibility, especially prenatal exposure, and on understanding the biological mechanisms that could lead heavy metals to impact female fertility.

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**Table 1. Study population characteristics (N = 139 cases and 153 controls)**

<b>Characteristic - N (%)</b>	<b>Cases (N = 139)</b>	<b>Controls (N = 153)</b>
<b>Centre</b>		
Rennes University Hospital	34 (24.5)	42 (27.5)
Brest University Hospital	8 (5.8)	4 (2.6)
Nantes University Hospital	59 (42.4)	80 (52.3)
La Sagesse clinic	38 (27.3)	27 (17.6)
<b>Season</b>		
Fall	35 (25.1)	45 (29.4)
Spring	26 (18.7)	45 (29.4)
Summer	49 (35.3)	40 (26.1)
Winter	29 (20.9)	23 (15.1)
<b>Age</b>		
Mean $\pm$ SD	33.8 $\pm$ 4.0	32.4 $\pm$ 4.1
18 - 24	6 (4.3)	10 (6.5)
25 - 29	15 (10.8)	29 (19.0)
30 - 34	52 (37.4)	67 (43.8)
35 - 40	66 (47.5)	47 (30.7)
<b>Ethnic background</b>		
Europe	112 (87.5)	128 (90.8)
Outside Europe	16 (12.5)	13 (9.2)
Missing data	11	12
<b>Education</b>		
High school or lower	42 (33.1)	49 (35.5)
High school + 2 years of university	25 (19.7)	23 (16.7)
High school + 3 years of university and above	60 (47.2)	66 (47.8)
Missing data	12	15
<b>Body-Mass Index (kg/m<sup>2</sup>)</b>		
Mean $\pm$ SD	22.9 $\pm$ 3.4	23.6 $\pm$ 3.8
<20	24 (17.3)	27 (17.9)
20 - 24	84 (60.4)	75 (49.7)
25 - 29	25 (18.0)	37 (24.5)
30 - 34	6 (4.3)	12 (7.9)
Missing data	0	2
<b>Infertility status</b>		
Primary	85 (61.2)	90 (58.8)
Secondary	54 (38.8)	63 (41.2)
<b>Parity</b>		
No children	85 (61.2)	90 (58.8)
1 child	33 (23.7)	33 (21.6)
$\geq$ 2 children	21 (15.1)	30 (19.6)
<b>Anti-müllerian hormone levels (ng/L)</b>		
Mean $\pm$ SD	0.61 $\pm$ 0.34	2.93 $\pm$ 1.00
<b>Antral follicle count</b>		
Mean $\pm$ SD	8 $\pm$ 5	21 $\pm$ 9
<b>Age at menarche</b>		

Mean ± SD	12.91 ± 1.57	12.96 ± 1.61
Missing data	17	20
<b>Smoking status</b>		
Never smoker	61 (48.0)	69 (48.9)
Past smoker	19 (15.0)	23 (16.3)
Current smoker	47 (37.0)	49 (34.8)
Missing data	12	12
<b>Cigarette pack-years</b>		
Non smoker	61 (51.6)	68 (51.5)
< 3	27 (22.9)	19 (14.4)
3 - 5	14 (11.9)	16 (12.1)
≥ 6	16 (13.6)	29 (22.0)
Missing data	21	21
<b>Alcohol (number of drinks per week)</b>		
Non drinker	36 (29.3)	23 (16.9)
≤ 7	79 (64.2)	100 (73.5)
> 7	8 (6.5)	13 (9.6)
Missing data	16	17

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Note: N, number of subjects; SD, standard deviation.

**Table 2. Whole blood heavy metal concentrations in µg/L by group (N = 139 cases and 153 controls)**

Metal	LOD (ug/L)	Group	Detection N (%)	Minimum	Percentiles			Maximum
					25th	50th	75th	
Pb	0.5	Control	153 (100)	2.8	6.5	9.1	12.5	70.1
		Case	139 (100)	3.6	6.6	8.4	11.3	110.8
Hg	0.5	Control	151 (98.7)	<LOD	0.9	1.5	2.4	9.2
		Case	137 (98.6)	<LOD	1.0	1.6	2.7	6.7
Cd	0.05	Control	136 (88.9)	<LOD	0.1	0.1	0.3	4.8
		Case	127 (91.4)	<LOD	0.1	0.2	0.3	1.7
Cr	0.5	Control	143 (93.5)	<LOD	0.6	1.1	1.9	6.0
		Case	128 (92.1)	<LOD	0.8	1.6	2.1	11.7

Abbreviations: N, number of subjects; LOD, limit of detection.

**Table 3. Associations between heavy metals and diminished ovarian reserve in the AROPE study (N = 139 cases and 153 controls)**

<b>Diminished Ovarian Reserve 139 cases and 153 controls</b>							
				Univariate logistic regression		Multivariate <sup>1</sup> logistic regression	
Metal	Concentration (µg/L) <sup>2</sup>	Number of cases	Number of controls	OR (95% CI)	p-value	OR (95% CI)	p-value
Pb	≤ 7.40	48	51	Ref.		Ref.	
	(7.40-11.47]	57	51	1.19 (0.69, 2.05)	0.538	0.99 (0.55, 1.79)	0.981
	> 11.47	34	51	0.71 (0.39, 1.27)	0.250	0.63 (0.33, 1.19)	0.153
	Continuous	139	153	0.75 (0.26, 2.18)	0.596	0.53 (0.16, 1.71)	0.288
Hg	≤ 1.09	41	51	Ref.		Ref.	
	(1.09-1.96]	41	51	1.04 (0.59, 1.84)	0.885	0.92 (0.51, 1.67)	0.782
	> 1.96	57	51	1.42 (0.81, 2.49)	0.223	1.06 (0.56, 2.01)	0.852
	Continuous	139	153	1.36 (0.72, 2.55)	0.318	0.92 (0.46, 1.84)	0.814
Cd	≤ 0.10	37	51	Ref.		Ref.	
	(0.10-0.24]	62	51	1.87 (1.06, 3.30)	0.032	1.89 (0.98, 3.67)	0.058
	> 0.24	40	51	1.32 (0.75, 2.32)	0.344	1.18 (0.55, 2.52)	0.664
	Continuous	139	153	1.04 (0.66, 1.62)	0.877	2.38 (0.85, 6.72)	0.097
Cr	≤ 0.72	24	51	Ref.		Ref.	
	(0.72-1.71]	50	51	1.34 (0.76, 2.38)	0.310	1.24 (0.65, 2.39)	0.512
	> 1.71	64	51	2.03 (1.15, 3.60)	0.015	1.77 (0.84, 3.70)	0.132
	Continuous	138	153	2.07 (1.04, 4.13)	0.039	1.54 (0.61, 3.88)	0.355

<sup>1</sup> Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

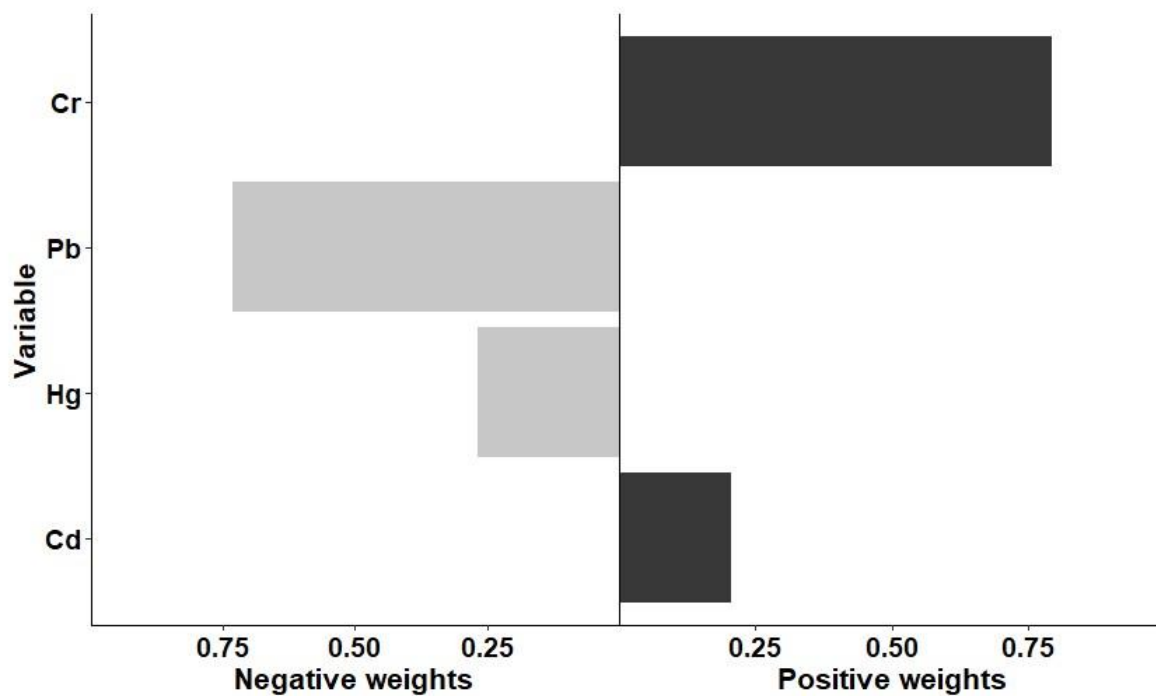
<sup>2</sup> All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Note: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

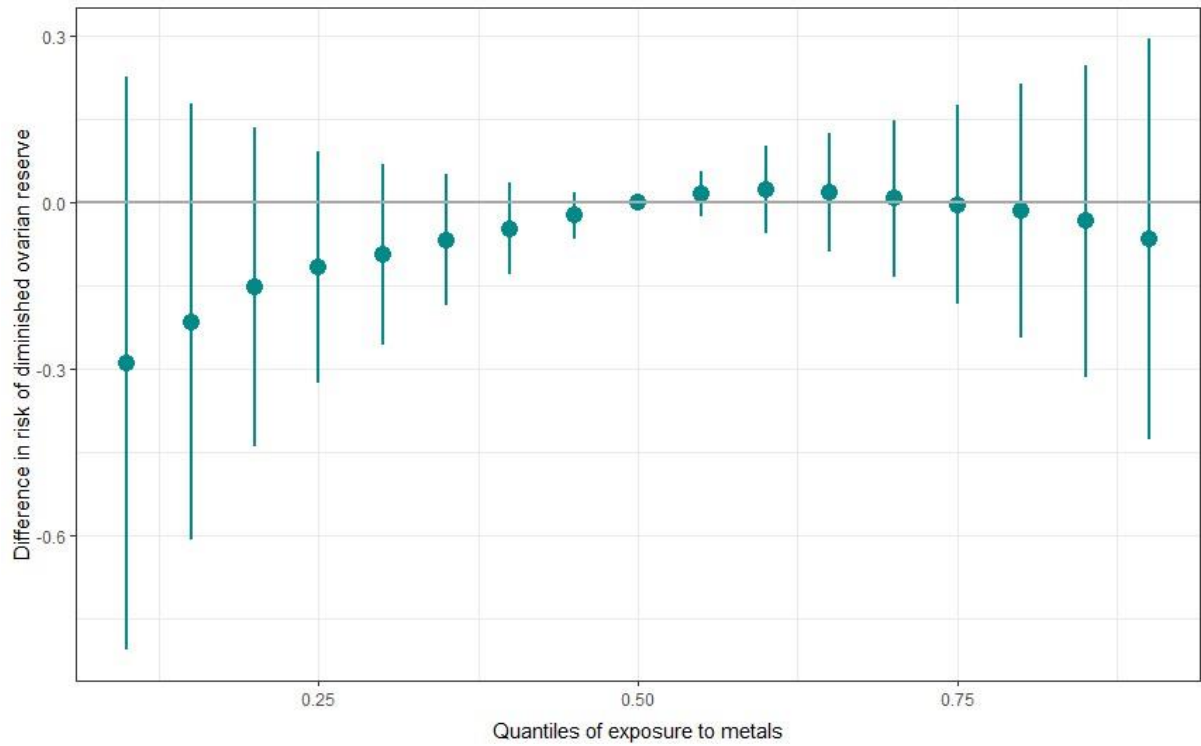


**Table 4. Posterior inclusion probabilities for conditional inclusion in the BKMR model (AROPE study: N = 138 cases and 153 controls)**

Heavy metal	Posterior inclusion probability (%)
Pb	0,2822
Hg	0,254
Cd	0,292
Cr	0,590

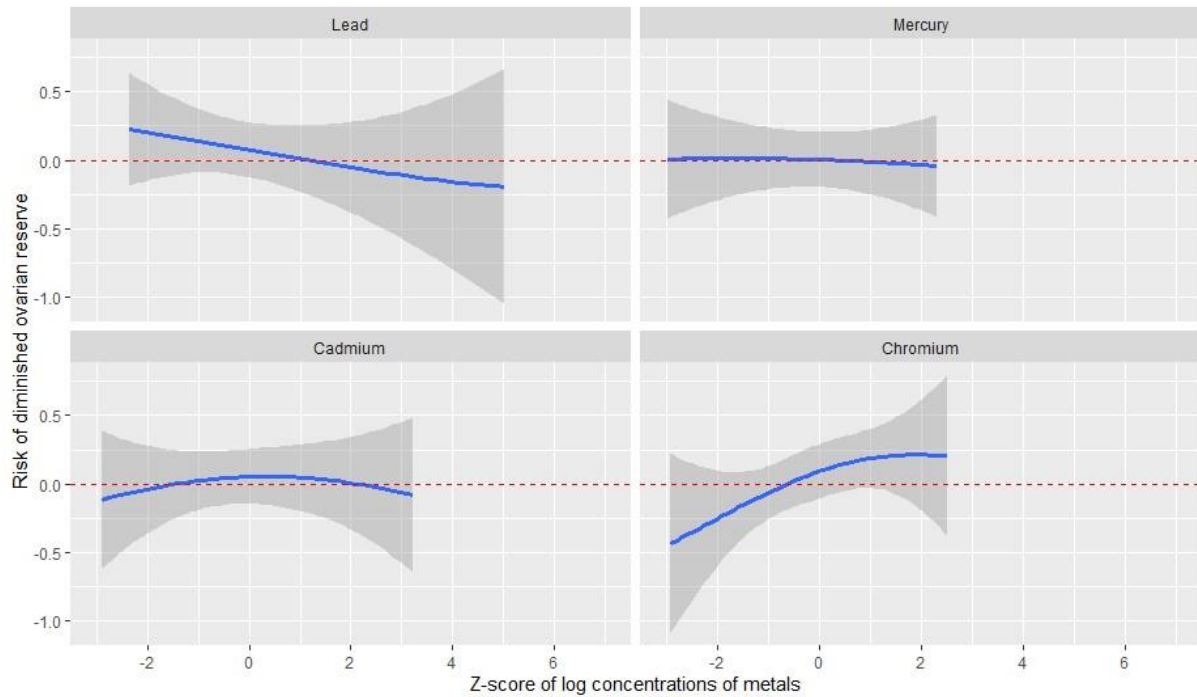


**Figure 1.** Title: Quantile g-computation weights of heavy metals on the metal mixture effect on diminished ovarian reserve (AROPE study: N = 139 cases and 153 controls)



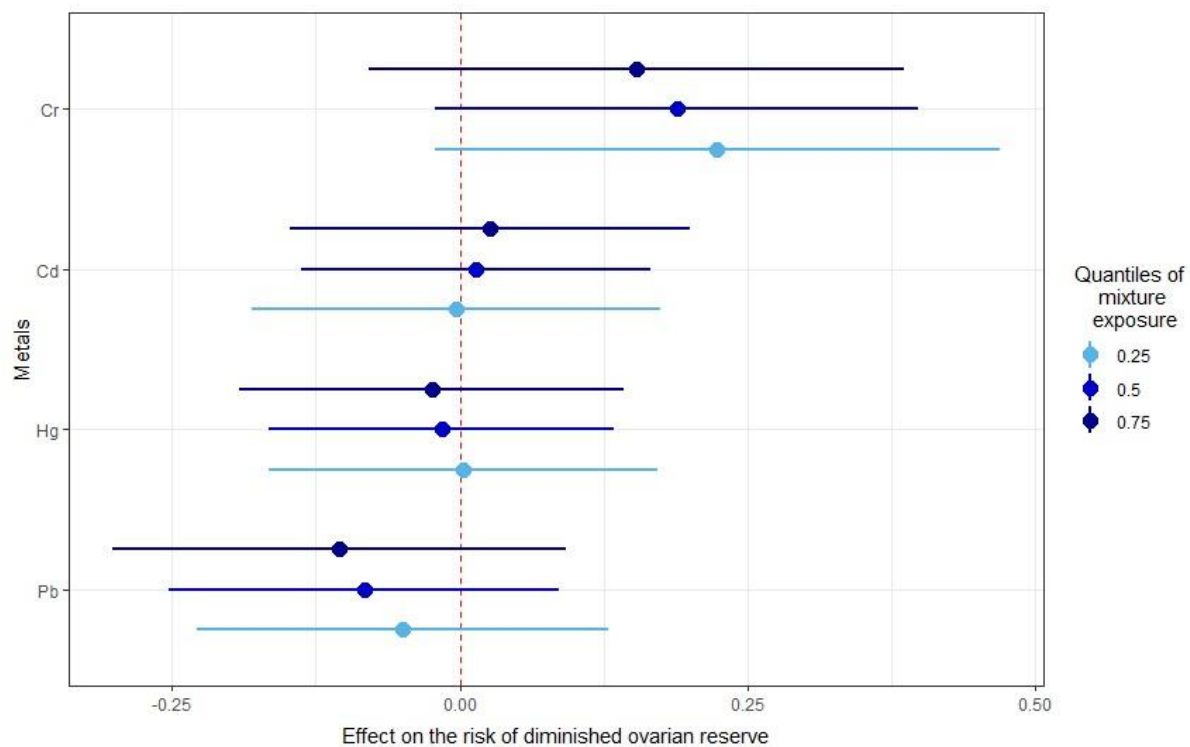
**Figure 2.** Title: Mixture effect of heavy metals on diminished ovarian reserve at different percentiles of exposure compared to the 50th percentile

Caption: Difference in risk and its 95% credible interval of diminished ovarian reserve due to exposure to heavy metals when all metals are at different percentiles compared to when they are at their median levels (AROPE study: N = 139 cases and 153 controls). The model was adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.



**Figure 3.** Title: Mixture analysis of the exposure-response relationships between individual heavy metals and diminished ovarian reserve

Caption: Difference in risk (blue lines) and its 95% credible interval (shaded areas) of diminished ovarian reserve at a given exposure level of each heavy metal compared to the median exposure level of that metal while holding all other metals at their median levels. The model was adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion. AROPE study: N = 139 cases and 153 controls.



**Figure 4.** Title: Effects of heavy metals on the risk of diminished ovarian reserve when the mixture exposure is fixed at different quantiles

Caption: The model was adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion. All other metal exposures are fixed at different quantiles (0.25, 0.50, 0.75). AROPE study: N = 139 cases and 153 controls.

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### 3.3. ARTICLE 3: Associations between a mixture of phenols and phthalates, and the ovarian reserve in the EARTH cohort

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## Abstract

Although prior studies have found associations of the ovarian reserve with urinary concentrations of some individual phenols and phthalate metabolites, little is known about the potential associations of these chemicals as a mixture with the ovarian reserve. We investigated whether mixtures of four urinary phenols (bisphenol A, butylparaben, methylparaben, propylparaben) and eight metabolites of five phthalate diesters including di(2-ethylhexyl) phthalate were associated with markers of the ovarian reserve among 271 women attending a fertility center who enrolled in the Environment and Reproductive Health study (2004-2017). The analysis was restricted to one outcome per study participant using the closest outcome after the last exposure assessment. Ovarian reserve markers included lower antral follicle count (AFC) defined as  $AFC < 7$ , circulating serum levels of day 3 follicle stimulating hormone (FSH) assessed by immunoassays, and diminished ovarian reserve (DOR) defined as either  $AFC < 7$ ,  $FSH > 10$  UI/L or primary infertility diagnosis of DOR. We applied Bayesian Kernel Machine Regression (BKMR) and quantile g-computation to estimate the joint effects and assess the interactions between chemical exposure biomarkers on the markers of the ovarian reserve while adjusting for confounders. Among all 271 women, 738 urine samples were collected. In quantile g-computation models, a quartile increase in the exposure biomarkers mixture was not significantly associated with lower AFC (OR = 1.10, 95% CI = 0.52, 2.30), day 3 FSH levels (Beta = 0.30, 95% CI = -0.32, 0.93) or DOR (OR = 1.02, 95% CI = 0.52, 2.05). Similarly, BKMR did not show any evidence of associations between the mixture and any of the studied outcomes, or interactions between chemicals. Despite the lack of associations, these results need to be explored among women in other study cohorts.

**Keywords:** Mixtures; Ovarian reserve; Phenols; Phthalates.

## Résumé en français

Bien que des études antérieures aient trouvé des associations entre la réserve ovarienne et les concentrations urinaires de certaines molécules de phénols et métabolites de phtalates individuels, on sait peu de choses sur les associations potentielles de ces produits chimiques en tant que mélange avec la réserve ovarienne. Nous avons étudié si les mélanges de quatre phénols urinaires (bisphénol A, butylparabène, méthylparabène, propylparabène) et huit métabolites de cinq phtalates, y compris le phtalate de di(2-éthylhexyle), étaient associés à des marqueurs de la réserve ovarienne chez 271 femmes consultant un centre de fertilité et inscrites à l'étude sur l'environnement et la santé reproductive (2004-2017). L'analyse a été limitée à un seul résultat par participant à l'étude en utilisant le résultat le plus proche après la dernière évaluation de l'exposition. Les marqueurs de la réserve ovarienne comprenaient un compte folliculaire antral (AFC) inférieur, défini comme  $AFC < 7$ , des taux sériques circulants de l'hormone folliculo-stimulante (FSH) du jour 3 mesurés par des dosages immuno-

enzymatiques, et une diminution de la réserve ovarienne (DOR) définie comme AFC < 7, FSH > 10 UI/L ou diagnostic d'infertilité primaire de DOR. Nous avons appliqué les méthodes de Bayesian Kernel Machine Regression (BKMR) et quantile g-computation pour estimer les effets conjoints et évaluer les interactions entre les biomarqueurs d'exposition chimique sur les marqueurs de la réserve ovarienne tout en ajustant pour les facteurs de confusion. Parmi les 271 femmes incluses dans cette analyse, 738 échantillons d'urine ont été collectés. Dans les modèles de quantile g-computation, une augmentation d'un quartile dans le mélange de biomarqueurs d'exposition n'était pas significativement associée à un AFC inférieur (OR = 1.10, IC 95% = 0.52, 2.30), à des niveaux de FSH du jour 3 (Beta = 0.30, IC 95% = -0.32, 0.93) ou à une DOR (OR = 1.02, IC 95% = 0.52, 2.05). De même, la BKMR n'a montré aucune associations avec le mélange, ni d'interactions entre les produits chimiques. Malgré l'absence d'associations, ces résultats doivent être explorés chez les femmes dans d'autres études de cohortes.

**Mots-clés :** Mélanges ; Réserve ovarienne ; Phénols ; Phtalates.

## Introduction

Endocrine disrupting chemicals (EDCs) measured as a mixture are associated with the ovarian reserve has been less well studied (Bellavia et al. 2023; Pollack et al. 2018; Zhan et al. 2022). To fill this knowledge gap, we evaluated the joint effects of urinary phenol and phthalate metabolite concentrations, as a mixture, in relation to the ovarian reserve among women consulting in a fertility clinic.

## Material and methods

### Study population

We evaluated women enrolled in the EARTH Study (N = 271) as described in chapter 2.

### Statistical analysis

The clinical and demographic characteristics of the study population were examined using means  $\pm$  standard deviations (SDs) for continuous variables, medians and inter-quartile ranges for discrete variables, and numbers and percentages for categorical variables. We used Spearman correlation coefficients to summarize the correlations between urinary biomarker concentrations. Concentrations below the LOD were imputed using multiple imputation through chained equations for multiple LODs (five sets) using a truncated normal imputation (Lapidus et al. 2014). Missing data for covariates (1%) were imputed within this process. Covariates were selected a priori through the use of a directed acyclic graph (Schisterman et al. 2009) (Supplemental Material 1). Chemical metabolites concentrations were averaged for women with more than one urine sample, and subsequently log-transformed. To avoid modeling issues due to multicollinearity, a summary measure of di(2-ethylhexyl)phthalate (DEHP) metabolites (i.e. MEHP, MEHHP, MEOHP, and MECPP) was calculated by dividing each metabolite concentration by their molecular weight and summing them. Mixture analyses were performed using quantile g-computation (Keil et al. 2020) and Bayesian Kernel Machine Regression (BKMR). We selected a set of covariates a priori using substantive knowledge and included age (continuous), BMI (categorized as  $\leq 20$ , between 20 and 25 and between 25 and 55 kg/m<sup>2</sup>), education level (lower than college graduate, college graduate and graduate degree), and specific gravity (as previously explained) as adjustment variables in all our models. A sensitivity analysis was performed by further adjusting for the number of urine samples per woman (women provided urine samples both at study enrolment and per infertility treatment) since women with more collected urines may be more infertile because they participated longer in the study before achieving a pregnancy. All analyses were performed using R (version 4.2.2). The threshold for significant results for all our analyses was 5%.

## Results

Women had an average age at enrollment of 35.6 ( $\pm$  4.6) years (Table 1). Most women were Caucasian (80%), whilst 3% were Black, 9% were Asian and 8% identified with another racial group. The majority of women (61%) had a graduate degree. The main cause of infertility at enrollment was female factor (41%) followed by unexplained (32%) and male factor (24%). The participants provided a median (range) 1 (1-14) urine samples and the average (inter-quartile range) number of days between the collection of urinary samples and the assessment of AFC and day 3 FSH concentrations was 151 (241) days. Among all 271 women, 738 urine samples were collected with biomarker detection frequencies ranging from 72% (butylparaben) to 100% (methylparaben, MEP, and MEOHP). Median urinary biomarker concentrations were similar to those found among all female participants in the National Health and Nutrition Examination Survey (NHANES) (CDC 2022). Urinary DEHP metabolite concentrations were highly correlated ( $r > 0.95$ ) as was urinary methylparaben and propylparaben ( $r = 0.87$ ) (Supplementary Material 2). Other urinary biomarkers were moderately correlated with each other ( $r$  ranging from 0.17 – 0.80). A total of 17% women had lower AFC, the mean ( $\pm$  standard deviation) day 3 FSH levels was 7.5 ( $\pm$  2.8) UI/L and 21% of women were diagnosed with DOR.

Quantile g-computation results showed no significant associations between a quartile increase in mixture exposure biomarkers and lower AFC (OR = 1.10, 95% CI = 0.52, 2.30), day 3 FSH levels (Beta = 0.30, 95% CI = -0.32, 0.93) and DOR (OR = 1.02, 95% CI = 0.52, 2.05) (Supplementary Material 3). The underlying regression results of the five-quantile g-computation models were all consistent with the pooled model (data not shown).

These results were confirmed in the BKMR analyses (Supplementary Material 4). The mean difference in outcome when comparing mixture exposure biomarker concentrations at the 75th to the 25th percentile was not associated with lower AFC (Beta = -0.01, 95% CI = -0.17, 0.14), or changes in serum day 3 FSH (Beta = 0.05, 95% CI = -0.24, 0.35) or DOR (Beta = -0.05, 95% CI = -0.60, 0.50). Moreover, we observed no associations between a simultaneous change in mixture component concentration quantiles and the outcomes (Figures 1A, 2A and 3A). Plots of the relationships between individual mixture components and the outcomes while holding all other components at their median levels (Figures 1B, 2B and 3B) did not suggest significant associations of any of the chemicals on the examined outcomes. Similarly, the associations between the individual biomarkers and the outcomes when all other chemicals exposure biomarkers were fixed at different quantiles (.25, .50, .75) did not indicate signs of interactions between chemicals (Figures 1C, 2C and 3C). PIPs (Table 3) were similar for all chemicals for each outcome (7-21% for lower AFC, 23-33% for serum day 3 FSH, and 34-38% for DOR) and were either low (for lower AFC and serum day 3 FSH) or moderate (for DOR). In the sensitivity analysis further adjusting for the number of urine samples per woman, findings remained similar and

no associations between mixtures of these biomarkers in relation to the ovarian reserve were observed (data not shown).

## **Discussion**

We investigated the associations between a mixture of four phenol and eight phthalate biomarker concentrations measured in multiple urine samples per participant (median of 3) with ovarian reserve markers including lower AFC and day 3 FSH levels, and with DOR among women participating in the EARTH Study. We applied quantile g-computation and BKMR approaches to evaluate the mixture, and overall, we found no evidence of associations between the concentrations of the chemical mixtures and the ovarian reserve. We also observed no evidence of any interaction among EDCs. Given the high prevalence of infertility worldwide (Mascarenhas et al. 2012), the ubiquitous exposure to EDCs among women of reproductive age (CDC 2022) and the scarce literature on mixtures of phenols and phthalates in relation to the ovarian reserve in women, these results need to be verified and validated among women in other study cohorts such as among women from general population rather than fertility clinics.

The results of the current analysis are not all consistent with our previously published EARTH study results for individual biomarkers of phenols and phthalate metabolites in relation to AFC and day 3 FSH levels. Specifically, we previously reported an association of lower mean AFC with higher urinary concentrations of bisphenol A (N = 154, years = 2004-2010, median urinary concentrations = 1.2 µg/L, no association with serum day 3 FSH) (Souter et al. 2013), propylparaben (N = 190, years = 2004-2010, median urinary concentrations = 49.6 µg/L, positively associated with serum day 3 FSH) (Smith et al. 2013), and DEHP metabolites (N = 215, years = 2004-2012, median urinary concentrations = 0.21 µg/L, did not investigate day 3 FSH) (Messerlian et al. 2016). One potential explanation for the different results between previous and the current EARTH analysis may be the higher urinary biomarkers concentrations observed in earlier years of the study compared to those observed in this analysis. As a result, the threshold value necessary for the biomarkers to have an impact on the outcome may not have been reached. In fact, these downward trends in EDCs over time have also been demonstrated among 171 pregnant United States women participating in the Environmental influences on Child Health Outcomes (ECHO) Program, in which authors noted that concentrations of certain chemicals decreased with calendar year, whereas concentrations of their replacements increased (Buckley et al. 2022). Similar trends were reported among women participating in other study cohorts (Ashrap et al. 2018; Kim et al. 2021). Another major difference with our previous publications is the assessment of continuous AFC as an outcome and the assessment as a binary outcome, DOR, in this analysis (BKMR package is not developed for Poisson distribution variables such as AFC). Nonetheless, the associations previously found in EARTH were not consistent across categories of exposure. Additionally, and similar

to current results, we previously found no associations of butylparaben and methylparaben (Smith et al. 2013), as well as non-DEHP metabolites (Messerlian et al. 2016) with AFC assessed continuously. Also, urinary bisphenol A concentrations were not related to day 3 FSH levels in earlier years of the study (Souter et al. 2013).

The available epidemiologic literature on mixtures of these EDCs in relation to the ovarian reserve and other fertility measures is still scarce and inconsistent (Bellavia et al. 2023; Pollack et al. 2018; Zhan et al. 2022). Applying BKMR, Bellavia and colleagues evaluated mixtures of phthalates, parabens and PFAS measured in follicular fluid in relation to women's fertility assessed as ovarian sensitive index (OSI) as well as clinical pregnancy and live birth probabilities among 333 women in Sweden and Estonia (Bellavia et al. 2023). While they found negative associations between follicular fluid DEHP and methylparaben with OSI and suggestive with certain PFAS, no associations were found for pregnancy outcomes. In a different study and using principal component analysis, Pollack and colleagues evaluated mixtures of several urinary phenol biomarkers including bisphenol A and parabens among others, in relation to reproductive hormones among 143 premenopausal women (Pollack et al. 2018). The authors found that the ultraviolet protection factor (which was characterized by higher urinary concentrations of benzophenone-1 and benzophenone-3) was negatively associated with FSH levels. In a different investigation applying principal component analysis and BKMR, Zhan et al. explored mixtures of urinary phenols and phthalate metabolites in relation to self-reported infertility among 857 reproductive-aged women participating in 2013-2016 NHANES (Zhan et al. 2022). Authors observed that urinary bisphenol A and certain DEHP metabolites were associated with higher infertility when adjusting for the other examined biomarkers. Of note, none of these mixture studies used quantile-g computation. Clearly, additional studies on mixtures of EDCs and the ovarian reserve as well as fertility endpoints applying sophisticated approaches will help to increase our understanding of the reproductive health effects of chemical mixtures.

An important strength of this study is the evaluation of the mixture effect of several chemicals on our outcomes of interest using two complementary methods: quantile g-computation and BKMR. Quantile g-computation estimates the ovarian reserve association for a simultaneous increase in all exposure biomarkers by one quantile. This is highly interpretable and efficient, but assumes the association is linear on the quantile scale — i.e. the association comparing the first to second quartile is the same as comparing the third to fourth quartile — and moreover assumes no exposures interact non-additively. BKMR, on the other hand, is not as interpretable or efficient, but it allows each exposure to have its own potentially non-linear exposure response relationship, and further allows exposures to interact synergistically or otherwise. In tandem, BKMR can support the results of the quantile g-computation analysis or indicate that some of its assumptions are not appropriate — e.g. evidence of interactions or of different functional relationships between exposures and outcomes.



The current study has several limitations. First, it may not be possible to generalize our findings to couples from the general population attempting conception. Nevertheless, approximately 15% to 25% of couples trying to achieve pregnancy are diagnosed with infertility (Slama et al. 2012; Thoma et al. 2013), which makes our study findings applicable to a significant proportion of couples seeking treatment. Chemical biomarker concentrations were also similar to U.S. women of the general population for parabens and some of the phthalate metabolites (MiBP, MEHP, MEOHP) although slightly lower for others (MEP, MBzP, MEHHP, MECPP) (CDC 2019). However, not all infertile couples seek treatment, especially in case of secondary infertility implying that our study population is not representative of all couples with fertility issues (Tielemans et al. 2002). Women with secondary infertility who had initially conceived using IVF might also have modified their behaviors regarding personal care products following professional advice given during the first procedure. Furthermore, if exposure to phenols and phthalates is high enough to have contributed to the infertility of male partners with fertile female partners, it is possible that these female partners are more exposed to these chemicals than the general population. Nevertheless, exposure to this type of chemicals does not appear to be correlated within couples (Buck Louis et al. 2014), presumably because partners do not use the same personal care products. We also considered how the selection process could artificially create non-causal associations between exposures and outcomes using our DAG (Arah 2019) but did not identify clear evidence of such a risk using our set of covariates. Ethnicity has been shown to impact access to any form of infertility treatment in the USA (Dongarwar et al. 2022), suggesting the potential for some form selection bias. However, this relationship is likely confounded by sociodemographic factors such as education, which is included in our models. Second, exposure misclassification is possible given the short biological half-lives (< 24 hours) of these non-persistent chemicals (Koch et al. 2004; Shin et al. 2023; Völkel et al. 2002) and their episodic exposure (Braun et al. 2012). However, this misclassification is likely non-differential. Moreover, we have previously demonstrated in the EARTH cohort that a single urine sample may reasonably represent an individual's exposure to short half-lived chemicals such as BPA and phthalates over several months (Braun et al. 2012; Smith et al. 2012). Nevertheless, we have also employed several methods that have been shown to partially reduce variability, and thus exposure misclassification, including the use of morning voids, the collection of several urine samples to calculate an average concentration (for 89% of the sample), and the correction of concentrations by adjusting for specific gravity (Bastiaensen et al. 2020; Hoppin et al. 2002; Roggeman et al. 2022). Third, residual confounding is still possible because other EDCs, which may be correlated with those included in the current analysis were not measured or excluded a priori from this analysis because of relatively low sample size or detection frequencies (e.g. triclosan, benzophenone-3, bisphenol and phthalate replacements). Fourth, only outcomes measured after the assessment of biomarker exposures were considered in order to avoid issues of reverse causality.

Indeed, several studies have looked at the intra-individual variations of AFC between menstrual cycles and have found moderate inter-cycle variations (van Disseldorp et al. 2009). This has nonetheless resulted in the exclusion of individuals from the analyses and thus limited statistical power. In addition, due to the nature of our design we cannot interpret our findings as causal. For this reason, we have interpreted estimates as associations rather than effects. Finally, we only have a few women with data on serum AMH so this ovarian reserve biomarker was excluded from this analysis. The biggest strength of this study is the use of sophisticated methods to analyze mixtures of exposure biomarkers in relation to the ovarian reserve. Other strengths include the observational design, which minimizes the risk of reverse causation, comprehensive adjustment for other reproductive and lifestyle factors that could result in residual confounding and the collection of multiple urine samples to reduce exposure misclassification.

### **Conclusions**

In conclusion, we evaluated mixtures of several endocrine disrupting chemicals in relation to ovarian reserve biomarkers and applied modern approaches to analyze chemical mixtures such as quantile g-computation and BKMR. Overall, we found no associations between mixtures of urinary biomarker concentrations of bisphenol A, parabens and phthalates with lower AFC, day 3 FSH levels and DOR among women attending a fertility center. We also observed no evidence of any interaction between the examined EDCs. Given the high prevalence of infertility worldwide, the ubiquitous exposure to EDCs among women of reproductive age and the scarce literature on mixtures of phenols and phthalates in relation to the ovarian reserve in women, these results need to be explored among women in other study cohorts including among the general population rather than a fertility clinic cohort.

**Table 1. Baseline demographic and reproductive characteristics among 271 women enrolled in the EARTH Study between 2004 and 2017.**

<b>Characteristic<sup>1</sup></b>	<b>Total cohort</b>
Age (years), mean ( $\pm$ SD)	35.6 ( $\pm$ 4.6)
Race	
White/Caucasian	218 (80)
Black	9 (3)
Asian	23 (9)
Other	21 (8)
Education level	
< College graduate	18 (7)
College graduate	86 (32)
Graduate degree	167 (61)
Body mass index (kg/m <sup>2</sup> )	
<20	29 (11)
20-25	144 (53)
>25-55	96 (36)
Smoking status	
Never smoker	203 (75)
Past smoker	58 (21)
Current smoker	10 (4)
Infertility diagnosis	
Female Factor	
Diminished ovarian reserve	42 (16)
Endometriosis	26 (10)
Other	8 (3)
Ovulatory	23 (8)
Tubal	14 (5)
Uterine	5 (2)
Male Factor	65 (24)
Unexplained	86 (32)
Previous pregnancy	110 (41)
Previous infertility examination	195 (74)
Previous infertility treatment	108 (49)
Low AFC (<7)	47 (17)
Day 3 FSH Levels (IU/L), mean ( $\pm$ SD)	7.5 ( $\pm$ 2.8)
Total physical activity (hours per week), mean ( $\pm$ SD)	6.5 (7.5)
Menstrual cycle length (days), mean ( $\pm$ SD)	29.9 ( $\pm$ 7.2)
Regular menstrual cycle	247 (93)
Urine dilution (specific gravity), mean ( $\pm$ SD)	1.015 ( $\pm$ 0.007)
Number of urine samples, median (IQR)	1.0 (3.0)

Abbreviations: N, number; IQR, interquartile range; SD, standard deviation.

<sup>1</sup>Values are presented as N (%) unless otherwise noted.

Missing data for the following characteristics: BMI (N=2), infertility diagnosis (N=2), previous infertility examination (N=6), previous infertility treatment (N=1), physical activity (N=1), menstrual cycle length (N=3), regular menstrual cycle (N=4).

**Table 2. Unadjusted urinary phenol and phthalate biomarker concentrations ( $\mu\text{g/L}$ ) among 271 women contributing 738 urine samples (mean of 3) and enrolled in the EARTH Study between 2004 and 2017.**

Biomarker	LOD range	Detection			Geometric mean	SD	75th	95th
		Frequency %	25th	Median				
Bisphenol A	0.1 - 0.4	89	0.5	1.0	1.0	2.7	1.9	5.0
Methylparaben	0.4 - 0.6	100	45.9	125.0	113.8	786.7	294.2	1137.0
Propylparaben	0.1 - 0.2	99	9.0	30.9	22.9	245.8	70.8	280.8
Butylparaben	0.1 - 0.2	72	0.1	0.5	0.7	15.7	3.6	28.0
MBP	0.4 - 0.6	99	4.4	9.5	9.5	59.4	22.1	64.4
MiBP	0.2 - 0.8	99	2.6	5.6	5.5	17.1	11.8	42.1
MEP	0.4 - 0.4	100	17.2	39.5	43.1	539.8	120.5	478.2
MBzP	0.2 - 0.7	99	1.2	2.8	2.6	15.0	6.0	23.2
MEHP	0.5 - 1.2	81	0.9	2.0	2.3	17.0	5.4	22.1
MEHHP	0.2 - 0.7	99	4.1	10.3	11.3	117.8	27.0	125.9
MEOHP	1.2 - 1.2	100	2.7	6.6	7.2	73.4	17.2	76.2
MECPP	0.2 - 0.3	94	7.1	17.6	18.9	144.1	41.9	223.5
$\Sigma\text{DEHP}^1$	-	-	0.1	0.1	0.1	1.2	0.3	1.5

N: number of participants; LOD: limit of detection; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; DEHP: di(2-ethylhexyl) phthalate.

<sup>1</sup> $\Sigma\text{DEHP}$ : Molar sum of DEHP metabolites (MEHP, MEHHP, MEOHP and MECPP) expressed in mmol/l.

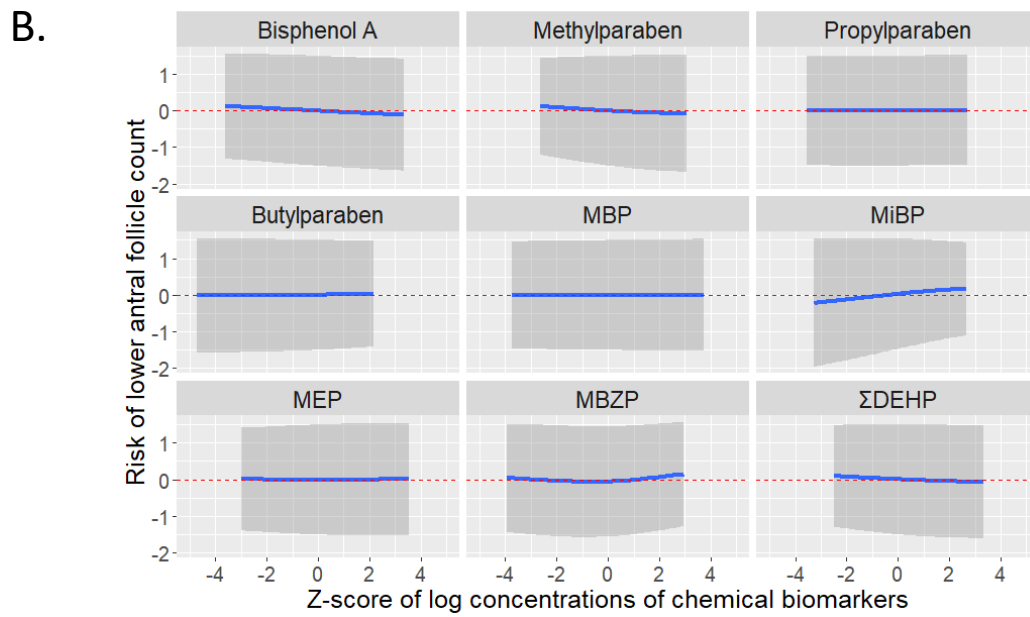
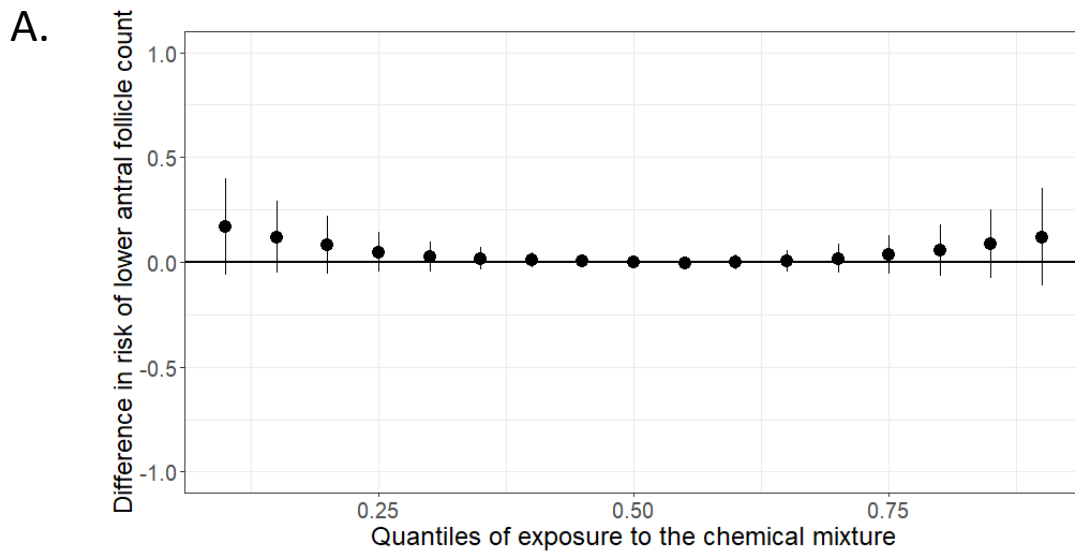
**Table 3. Bayesian Kernel Machine Regression posterior inclusion probabilities (%) for the lower antral follicle count (N = 262), day-3 FSH (N = 261) and DOR (N = 271) outcomes**

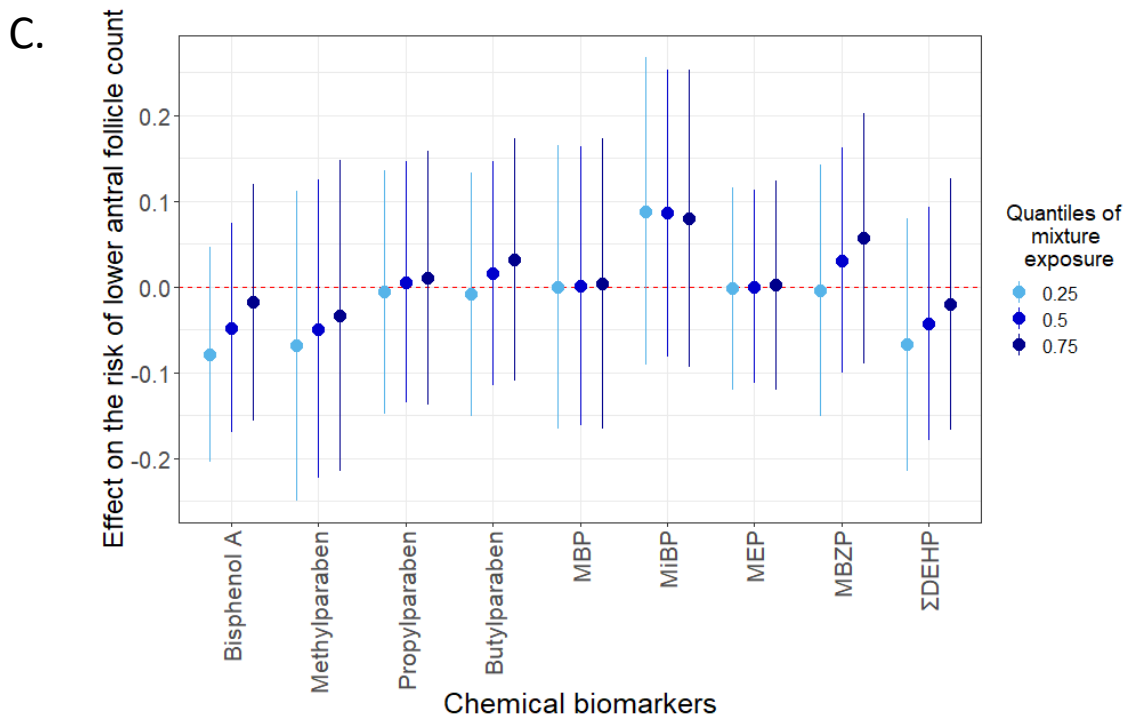
Biomarker	Outcome		
	Lower Antral Follicle Count	Day 3 Follicle Stimulating Hormone levels	Diminished Ovarian Reserve
Bisphenol A	16.2	27.9	36.4
Methylparaben	19.8	24.5	33.7
Propylparaben	9.6	25.2	35.2
Butylparaben	8.3	25.2	36.2
MBP	7.3	26.3	38.2
MiBP	19.2	27.5	34.5
MEP	8.0	32.6	34.0
MBZP	20.5	30.9	37.1
$\Sigma$ DEHP <sup>1</sup>	10.7	22.9	34.7

MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate.

<sup>1</sup> $\Sigma$ DEHP: Molar sum of DEHP metabolites (mono(2-ethylhexyl) phthalate, mono(2-ethyl-5-hydroxyhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, and mono(2-ethyl-5-carboxypentyl) phthalatePP).

Figure 1. BKMR mixture effects of phenol and phthalate biomarkers on lower antral follicle count (N = 262)

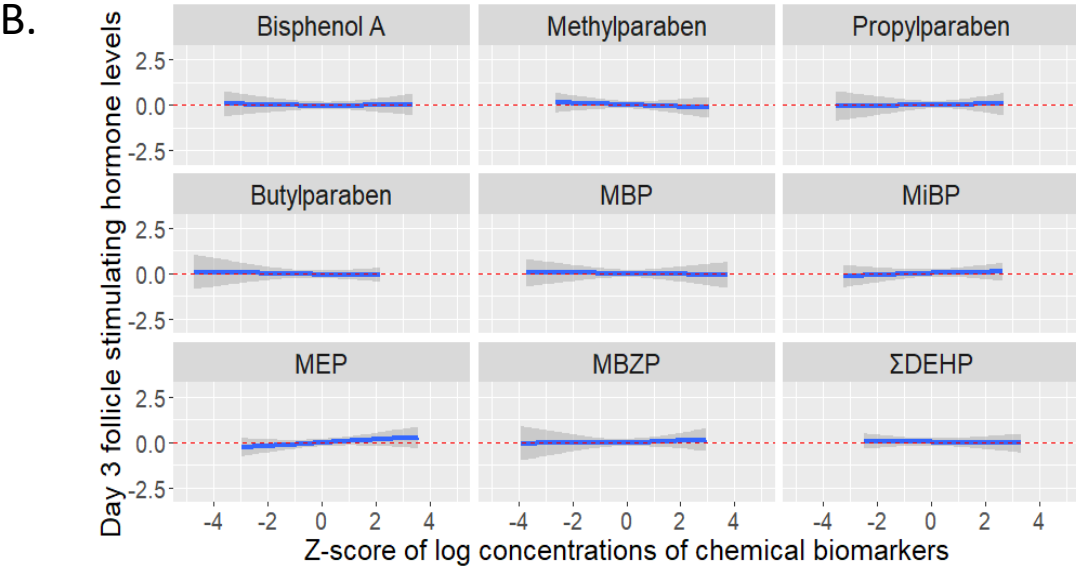
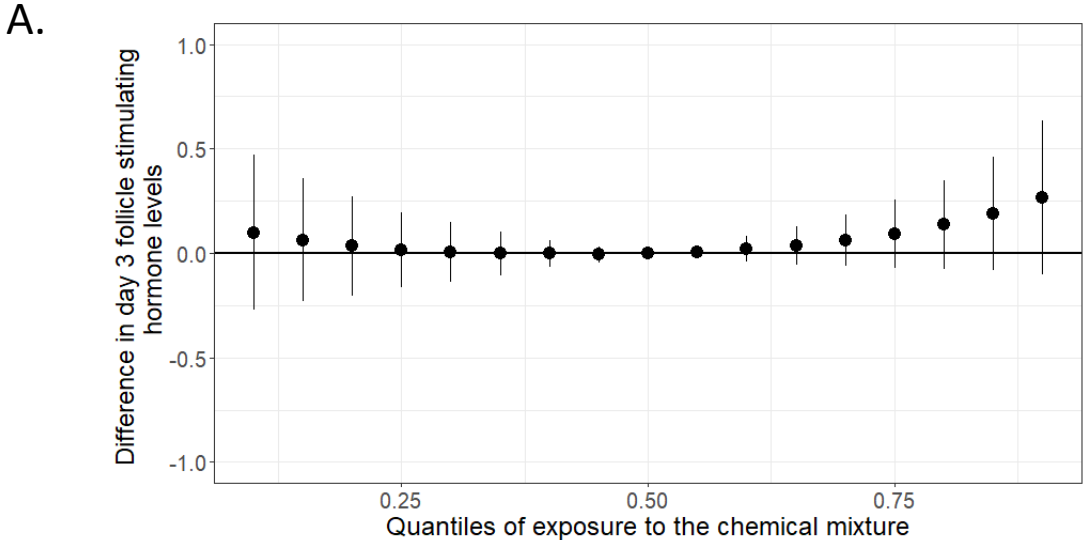




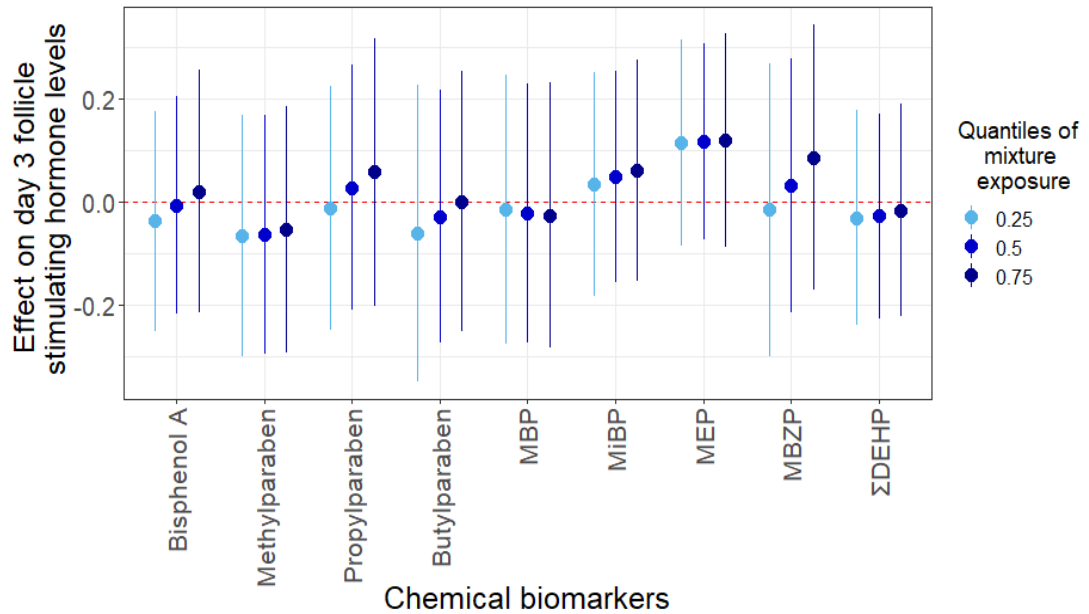
Adjusted for age, body mass index, education level and specific gravity. A. Overall effect of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75<sup>th</sup> to 25<sup>th</sup> percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate;  $\Sigma$ DEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.



**Figure 2. BKMR mixture effects of phenol and phthalate biomarkers on day-3 follicle stimulating hormone levels (N = 261)**

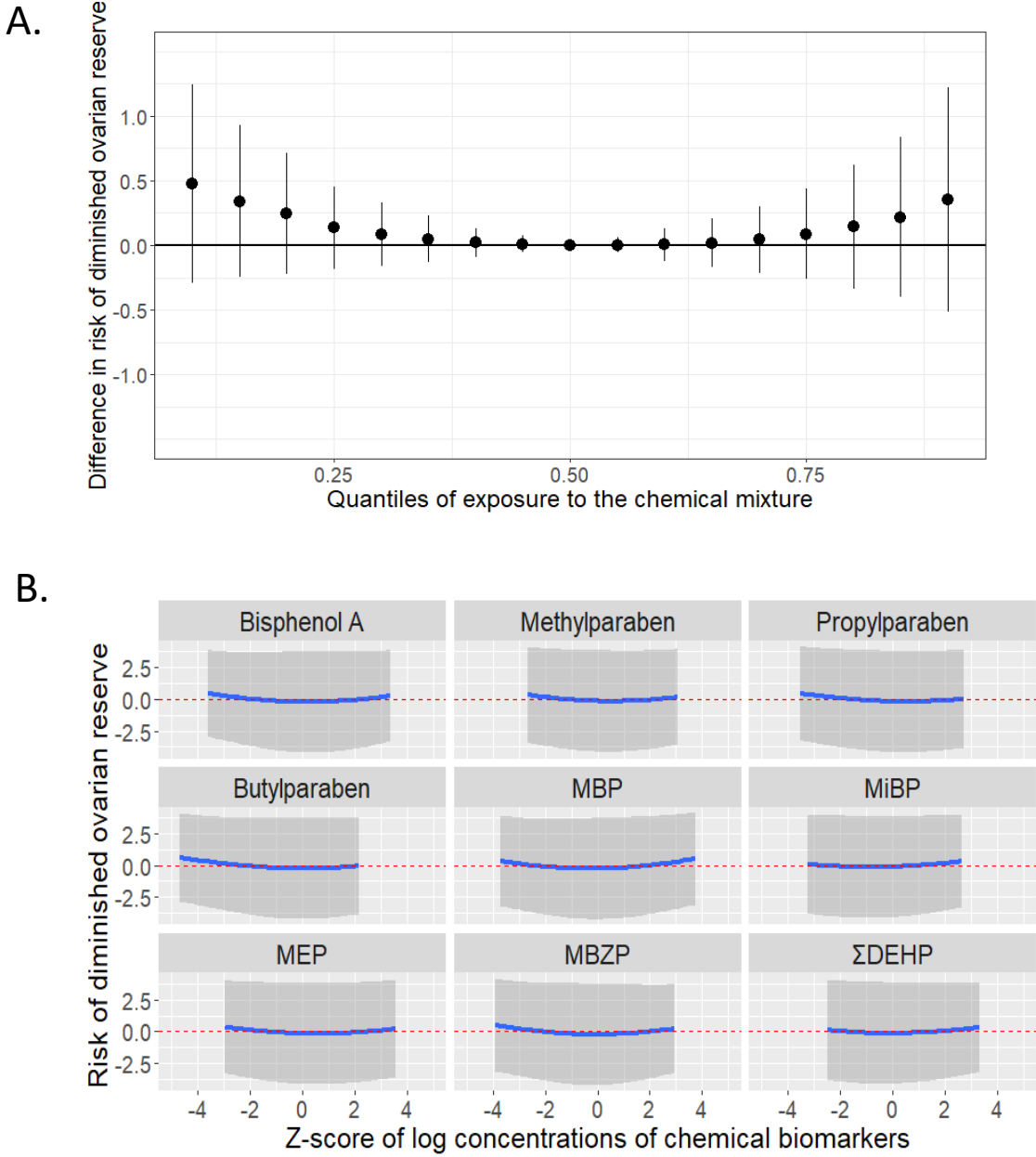


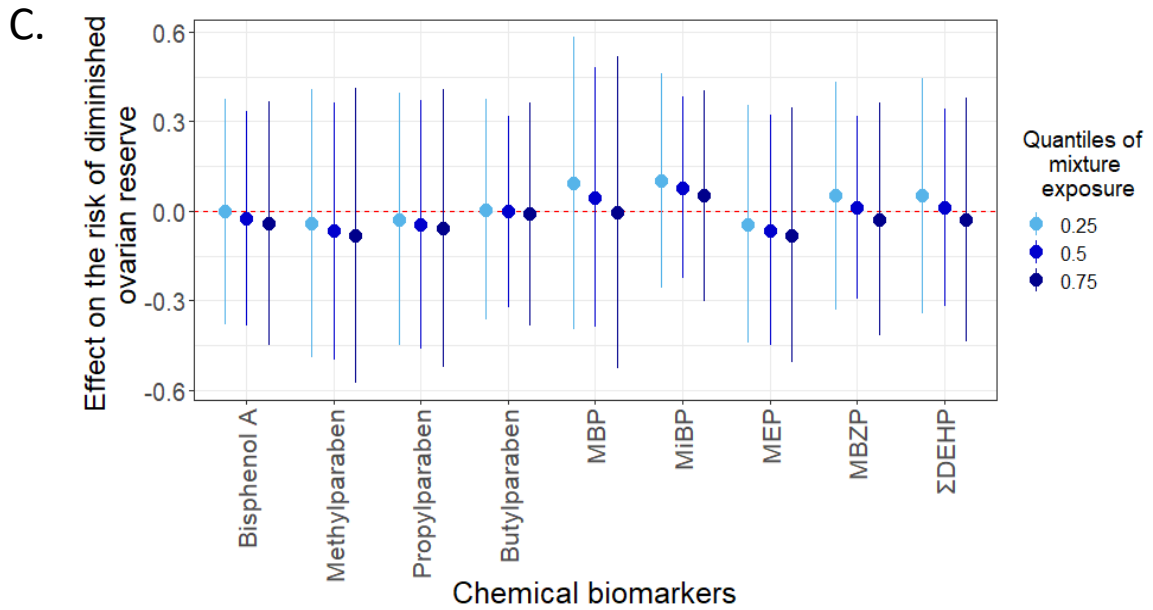
C.



Adjusted for age, body mass index, education level and specific gravity. A. Overall effect of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75<sup>th</sup> to 25<sup>th</sup> percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; ΣDEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

**Figure 3. BKMR mixture effects of phenol and phthalate biomarkers on diminished ovarian reserve (N = 271)**





Adjusted for age, body mass index, education level and specific gravity. A. Overall effect of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. B. Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. C. Mean difference comparing the 75th to 25th percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25th, 50th, and 75th percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate;  $\Sigma$ DEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

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**3.4. ARTICLE 4: Associations between in utero exposure to persistent organic pollutants and anti-müllerian hormone levels in 12-year old girls in the PELAGIE cohort**

**Status: In preparation**

**Prenatal exposure to persistent organic pollutants and its impact on the ovarian reserve in the PELAGIE mother-child cohort**

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## Abstract

Although the ovarian reserve is constituted in utero, the literature on the effects of persistent organic pollutants (POPs) during this period on the ovarian reserve evaluated later in life is limited. We investigated whether cord blood concentrations of POPs were associated with anti-Müllerian hormone (AMH) levels in girls at the age of 12.

We included 239 girls from the French mother-child PELAGIE cohort. POP concentrations of 14 organochlorine pesticides (OCPs), 17 polychlorinated biphenyls (PCBs), 5 polybrominated diphenyl ethers (PBDEs), and 9 per-polyfluoroalkyl substances (PFAS) were measured on cord blood sampled at birth. During a follow-up study at 12 years old blood samples were collected to measure AMH levels using an automated Beckman-Coulter Access assay. Single-exposure associations were examined with multivariable linear regressions adjusted a priori on potential confounders. Mixture effects were investigated using quantile g-computation and Bayesian kernel machine regression (BKMR).

Overall, 18 POPs were measured in at least 30% of samples. No significant associations were found in multivariate linear regressions, except for the third tercile of exposure to PCB 180 which was significantly associated with an increase in AMH levels at 12 ( $\beta = 0.51$ , 95% CI = 0.02; 0.99). Quantile g-computation results showed no significant associations between a quartile increase in mixture exposures and AMH levels ( $\beta = 0.04$ , 95% CI = -0.33; 0.41). In BKMR, the mean difference in AMH levels did not change significantly when comparing mixture exposure concentrations at the 75th to the 25th percentile ( $\beta = 0.04$ , 95% CI = -0.37; 0.46). The BKMR analysis did not indicate any signs of interactions between POPs.

Despite the lack of associations in this study, we recommend exploring these associations in larger mother-child cohorts and at older ages.

**Keywords:** Ovarian Reserve; Female Infertility; Persistent Organic Pollutants; Mixtures.

## Résumé en Français

Bien que la réserve ovarienne se constitue in utero, la littérature sur les effets des polluants organiques persistants (POPs) pendant cette période sur la réserve ovarienne évaluée plus tard dans la vie est limitée. Nous avons étudié si les concentrations de POP dans le sang de cordon étaient associées aux niveaux d'hormone anti-Müllérienne (AMH) chez les filles à l'âge de 12 ans.

Nous avons inclus 239 filles de la cohorte française mère-enfant PELAGIE. Les concentrations de POPs de 14 pesticides organochlorés (OCP), 17 polychlorobiphényles (PCB), 5 polybromodiphényléthers (PBDE) et 9 substances per-polyfluoroalkyl (PFAS) ont été mesurées dans le sang de cordon prélevé à la

naissance. Lors d'une étude de suivi à 12 ans, des échantillons de sang ont été prélevés pour mesurer les niveaux d'AMH à l'aide d'un dosage automatisé Beckman-Coulter Access. Les associations d'expositions individuelles ont été examinées avec des régressions linéaires multivariées ajustées a priori sur les facteurs de confusion potentiels. Les effets de mélange ont été étudiés à l'aide des méthodes de quantile g-computation et de Bayesian kernel machine regression (BKMR).

Au total, 18 POPs ont été détectés dans au moins 30% des échantillons. Aucune association significative n'a été trouvée dans les régressions linéaires multivariées, sauf pour le troisième tercile d'exposition au PCB 180, qui était significativement associé à une augmentation des niveaux d'AMH à 12 ans (Beta = 0.51, IC 95% = 0.02 ; 0.99). Les résultats de quantile g-computation n'ont montré aucune association significative entre une augmentation d'un quartile des expositions en mélange et les niveaux d'AMH (Beta = 0.04, IC 95% = -0.33 ; 0.41). Dans le modèle BKMR, la différence moyenne des niveaux d'AMH n'a pas changé de manière significative en comparant les concentrations d'exposition en mélange au 75e percentile par rapport au 25e percentile (Beta = 0.04, IC 95% = -0.37 ; 0.46). Cette analyse BKMR n'a pas montré également d'interactions entre les POPs.

Malgré l'absence d'associations dans cette étude, nous recommandons d'explorer ces associations dans de plus grandes cohortes mère-enfant et à des âges plus avancés.

**Mots-clés :** Réserve ovarienne ; Infertilité féminine ; Polluants organiques persistants ; Mélanges.

## **Introduction**

There are inconsistent results concerning the relationship between POPs measured in utero and continuous indicators of the ovarian reserve in teenage years. Our aim was to investigate the associations between cord blood concentrations of selected POPs and AMH levels at 12 in a French mother-child cohort.

## **Methods**

### *Population*

We used data from the PELAGIE cohort and its follow-up at age 12 (N = 239) as described in chapter 2.

### *Statistical analyses*

For POPs detected in at least 70% of samples, values under the LOD were imputed with chained equations. These exposures were then treated as continuous exposures and categorised into terciles. POPs detected between 50% and 70% of samples were categorised in three groups: one group missing data under the LOD, and two groups divided at the median of measured concentrations. For POPs detected in between 30% and 50% of samples, exposure was dichotomised into two groups (detected versus not detected). Lastly, POPs detected in fewer than 30% of samples were not included in the analyses. POP concentrations were log-2 transformed when treated as continuous variables. We used Spearman correlation coefficients to summarise the correlations between POPs using POPs detected in at least 70% of samples only. The set of confounders was determined using a directed acyclic graph (DAG) (Shrier and Platt, 2008) (Supplementary Material S1) and included maternal age at delivery (continuous), maternal education level at inclusion (no high school diploma, high school graduate, and higher education), and maternal pre-pregnancy BMI ( $\leq 25$  versus  $> 25$  kg/m<sup>2</sup>). We used Bayesian kernel machine regression (BKMR) (Bobb *et al.*, 2015) and quantile g-computation (Keil *et al.* 2020) to evaluate the mixture effect of POPs on AMH levels for POPs with a detection rate above 70%. Hierarchical variable selection was performed to estimate the posterior inclusion probabilities of the groups (OCPs, PCBs, and PFAS) and individual POPs. The model was fit with 20,000 iterations. The threshold for significance for all analyses was 5%.

## **Results**

### *Population description*

Both maternal and girl characteristics can be found in table 1. The mean age of mothers at delivery was 31.4 ( $\pm 3.8$ ) years old. The majority (72%) of mothers had a higher education and 20% were high school graduates. Only 3 women (1%) were not European. Most mothers (57%) had never smoked but

13% smoked in early pregnancy only and 7% were still smoking at inclusion. Girls had a mean age of 12.8 ( $\pm$  0.1) at inclusion in the follow-up study. The vast majority of girls (94%) were not exposed to passive smoking at inclusion. Among the girls, 44% had begun menstruating.

#### *Cord blood concentrations of POPs*

POP concentrations are available in table 2 and correlation coefficients in the Supplementary Material (S2). Out of 47 POPs, 18 were measured in at least 30% of samples and 15 in at least 70% of samples (Table 2). None of the PBDEs were detected in at least 30% of samples but all 9 PFAS molecules were detected in at least one sample. PCBs were highly correlated with one another (rho ranging from 0.63 – 0.98). Among OCPs, beta-HCH was highly correlated with hexachlorobenzene (rho = 0.75), and moderately with p,p'-DDE (rho = 0.55). Beta-HCH and hexachlorobenzene were also moderately correlated with PCBs (rho ranging from 0.45 – 0.75). Correlation coefficients between PFAS varied widely (rho ranging from 0.28 – 0.78).

#### *Associations between POP exposures and AMH*

Associations between tertiles and continuous POP exposures and AMH are presented in Table 3. No significant associations were found in multivariate linear regressions, except for the third tertile of exposure to PCB 180 which was significantly associated with an increase in AMH levels at 12 (Beta = 0.51, CI = 0.02; 0.99).

#### *Associations between the mixture of POPs and AMH*

Quantile g-computation results showed no significant associations between a quartile increase in mixture exposures and AMH levels (Beta = 0.04, 95% CI = -0.33, 0.41). The underlying regression results of the five quantile g-computation models were all consistent with the pooled model (data not shown). In BKMR, the mean difference in AMH levels did not change significantly when comparing mixture exposure concentrations at the 75th to the 25th percentile (Beta = 0.04, 95% CI = -0.37, 0.46). Moreover, we observed no associations between a simultaneous change in mixture component concentration quantiles and AMH levels (Figure 1). Plots of the relationships between individual POPs and AMH levels while holding all other POPs at their median levels (Figure 2) did not suggest any significant associations. Furthermore, the associations between each POP and AMH levels when all other POPs were fixed at different quantiles (.25, .50, .75) did not indicate any signs of interactions between POPs (Figure 3). Group PIPs were 30% for OCPs, 44% for PCBs and 31% for PFAS. In the OCP group, non-null associations were detected approximately 30-35% of the time for all pollutants. For PCBs, non-null associations were detected between 5% (PCB 138) and 33% (PCB 187) of the time. Finally, posterior probabilities for PFAS were between 10% and 26%.

## Discussion

In this study, we investigated the associations between cord blood POP concentrations and AMH levels at 12 years old in the PELAGIE mother-child cohort. Among 47 pollutants within 4 classes of POPs measured, 18 were found in more than 30% of samples, and one (PCB 180) was significantly associated with an increase in AMH levels. We used two mixture models, quantile g-computation and BKMR, which returned null associations in line with single-exposure results, and showed an absence of interactions between chemicals.

Our results are consistent with the epidemiological studies that have investigated the potential links between prenatal exposure to POPs and the ovarian reserve. One Danish team (Kristensen *et al.*, 2013) examined the effect of prenatal PFOA and PFOS levels on the ovarian reserve of 267 women stratified by hormonal contraceptive use (mean age of 19) and found no effects on either AMH levels, or AFC. However, in a subsequent analysis on 74 non-users of hormonal contraceptives, they found that higher prenatal levels of p,p'-DDE and HCB (but not for the sum of PCBs) led to a decreased AFC but did not impact AMH (Kristensen *et al.*, 2016). Another mother-child cohort study based in the UK (Donley *et al.*, 2019) did not find any significant associations between prenatal exposure to PFOS, PFOA, PFHxS, or PFNA, and AMH levels in 446 daughters (mean age of 15). Nevertheless, both of these cohorts used maternal serum samples during the 30th week of pregnancy, and at different weeks of pregnancy, respectively, instead of cord blood. Although we think that the impact of gestational age at collection may have a low impact on the comparison due to the long half-life of POPs, the effect of the matrix is known to be more important. Indeed, Vizcaino *et al.* (2014) reported that organochlorine pesticides, PCBs and PBDEs were generally threefold lower in the cord blood compared to maternal serum, even if their levels were highly correlated between cord blood and maternal serum ( $\rho > 0.9$ ), whereas Govarts *et al.* (2012) suggested a factor of 5 between cord blood and maternal serum. Nevertheless, POP concentrations were higher in the studies on the ovarian reserve that investigated prenatal POPs measured in maternal serum, than those in PELAGIE by a factor of 10 for p,p'-DDE, HCB and PCBs. Both of these cohorts measured concentrations in serum sampled in the early 1990s whilst cord blood in PELAGIE was sampled 15 years later, which may explain the differences in exposure levels.

The results regarding PCB 180 are contrary to our hypothesis that increased POP concentrations in utero could lead to decreased AMH levels in teenagers, and differ with the study from Kristensen *et al.* (2016) which found null results for PCBs were measured as a sum. However, a chance finding due to the number of analyses performed cannot be excluded. Thus, these results need to be replicated before making definite interpretations.

Our study has several limitations, including the measurement of POPs in three different batches due to the selection process. However, we believe that these different batches will have moderate



influence on the determination of POPs since they have been measured in the same laboratory with very good internal reference. Moreover, we decided not to adjust on batch since they could not be considered as confounder since POP measurements were determined independently of AMH measures. Another limitation of this study may be the evaluation of AMH at 12 years of age, when all girls had not reach puberty yet. Nevertheless, a recent longitudinal survey has investigated AMH levels from infancy (median 0.3 years) to adolescence (median age 15.9 years) including data during childhood (median age 7.3 years) and puberty (11.3 years) and on ovary ultrasound and magnetic resonance imaging (Hagen *et al.*, 2022). This study found that AMH was remarkably stable over the period and that the evaluation of AMH during puberty period is a good marker of the number of small antral follicles and a good predictor of later ovarian activity. However, since Kristensen *et al.* (2016) reported interactions between POPs and ovarian reserve depending on oral contraception use, it could be interesting to conduct additional analysis stratified on pubertal status when levels of hormones may also be different.

Our study has also several strengths including the prospective design with measurements of a large number of POPs at birth which are a good proxy of prenatal exposures considering the long half-life of these chemicals. Our study population was also very homogenous in terms of socio-economic characteristics with a high consumption of fish and selfish consumption which is considered to be the main contributor of PCB exposure (Chevrier *et al.* 2013). Nevertheless, we could not exclude a selection bias since mothers that had cord blood collection were women with higher educational levels and lower BMI than mothers without cord blood collection. Secondly, this study allows us to adjust on the main potential confounders based on a priori knowledge using DAG. Lastly, we used single-exposure and modern mixture methods to study the impact of pollutants on the ovarian reserve.

In this study, we evaluated the associations between prenatal POPs and AMH levels at 12 in the PELAGIE mother-child cohort. We also used quantile g-computation and BKMR to model POPs as a mixture. Overall, we found null associations between POPs and AMH. We also observed no evidence of any interaction between the POPs examined in this study. Given the ubiquitous nature of POPs, these associations should be explored in other mother-child cohorts with follow-ups at different periods including perinatality and in adulthood.

**Table 1. Study population characteristics (N = 239)**

<b>Characteristic</b>	<b>N (%)</b>	<b>Mean ± SD</b>
Maternal age at delivery		31.4 ± 3.8
Missing data		0
Maternal education level at inclusion		
< High school graduate	20 (8)	
High school graduate	48 (20)	
Higher education	171 (72)	
Missing data	0	
Ethnicity		
European	236 (99)	
Non-European	3 (1)	
Missing data	0	
Maternal pre-pregnancy Body Mass Index (kg/m <sup>2</sup> )		
[16,25]	205 (86)	
> 25	33 (14)	
Missing data	1	
Maternal inclusion year		
2002	31 (13)	
2003	101 (42)	
2004	78 (33)	
2005-2006	29 (12)	
Missing data	0	
Maternal smoking status at inclusion		
Never smoker	135 (57)	
Past smoker	55 (23)	
Smoker in early pregnancy only	31 (13)	
Smoker at inclusion	17 (7)	
Missing data	1	
Parity at inclusion		
No previous children	94 (39)	
At least one child	145 (61)	
Missing data	0	
Girl age		12.8 ± 0.1
Missing data		1
Girl Body Mass Index (kg/m <sup>2</sup> )		18.3 ± 2.7
Missing data		28
Girl passive smoking		
Not exposed	230 (96)	
Exposed	9 (3.8)	
Missing data	0	
Girl ménarche (yes v. no)	104 (44)	
Missing data	0	
Anti-Müllerian Hormone levels (ng/mL)		2.24 ± 1.34
Missing data		0

Abbreviations: N, number of subjects; SD, standard deviation.

**Table 2. Cord blood persistent organic pollutant concentrations in ug/L in the PELAGIE cohort (N = 239)**

Persistent Organic Pollutant	N tested	Detection N (%)	Min	q25	Median	q75	q95	Max
Aldrin	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
p,p'-DDT	239	2 (0.8)	<LOD	<LOD	<LOD	<LOD	<LOD	0.066
o,p'-DDT	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
p,p'-DDE	239	227 (95.0)	<LOD	0.120	0.190	0.330	0.672	3.300
o,p'-DDE	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dieldrin	238	143 (60.1)	<LOD	<LOD	0.010	0.022	0.041	0.150
Alpha-Endosulfan	238	8 (3.4)	<LOD	<LOD	<LOD	<LOD	<LOD	0.022
Beta-Endosulfane	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Heptachlor	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Heptachlorepoxyde	231	57 (24.7)	<LOD	<LOD	<LOD	<LOD	0.03	0.086
Hexachlorobenzene	239	219 (91.6)	<LOD	0.034	0.044	0.057	0.079	0.140
Alpha-HCH	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Beta-HCH	239	238 (99.6)	<LOD	0.036	0.051	0.076	0.131	0.220
Gamma-HCH	239	15 (6.3)	<LOD	<LOD	<LOD	<LOD	0.01	0.035
PCB 28	167	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 52	167	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 74	239	46 (19.2)	<LOD	<LOD	<LOD	<LOD	0.041	0.088
PCB 99	239	39 (16.3)	<LOD	<LOD	<LOD	<LOD	0.033	0.062
PCB 101	239	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PCB 118	239	237 (99.2)	<LOD	0.021	0.029	0.040	0.065	0.140
PCB 138	234	234 (100)	0.015	0.051	0.069	0.094	0.153	0.290
PCB 153	239	239 (100)	0.028	0.091	0.120	0.170	0.290	0.480
PCB 170	239	234 (97.9)	<LOD	0.022	0.031	0.043	0.071	0.110
PCB 180	239	239 (100)	0.014	0.057	0.080	0.120	0.201	0.280
PCB 183	239	71 (29.7)	<LOD	<LOD	<LOD	0.010	0.019	0.033
PCB 187	239	182 (76.2)	<LOD	0.010	0.014	0.020	0.038	0.08
PCB 194	239	104 (43.5)	<LOD	<LOD	<LOD	0.014	0.026	0.045
PCB 203	239	36 (15.1)	<LOD	<LOD	<LOD	<LOD	0.015	0.041
PBDE 28	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PBDE 47	239	49 (20.5)	<LOD	<LOD	<LOD	<LOD	0.023	0.098
PBDE 99	239	27 (11.3)	<LOD	<LOD	<LOD	<LOD	0.020	0.084
PBDE 100	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PBDE 153	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PBDE 154	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PBDE 183	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
PBDE 209	235	27 (11.5)	<LOD	<LOD	<LOD	<LOD	0.100	0.860
PBB 153	70	0 (0)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
HBCD	70	1 (1.4)	<LOD	<LOD	<LOD	<LOD	<LOD	0.100
PFBA	200	40 (20.0)	<LOD	<LOD	<LOD	<LOD	0.028	0.077
PFBS	200	12 (6.0)	<LOD	<LOD	<LOD	<LOD	0.008	0.251
PFDA	181	181 (100)	0.015	0.076	0.101	0.127	0.176	0.505
PFHxA	200	102 (51.0)	<LOD	<LOD	0.002	0.027	0.078	0.351
PFHxS	200	200 (100)	0.064	0.424	0.544	0.69	1.105	1.415

PFNA	196	196 (100)	0.068	0.247	0.356	0.44	0.637	1.541
PFOA	200	200 (100)	0.538	1.643	2.184	2.815	4.184	7.362
PFOS	200	200 (100)	0.538	3.431	4.37	5.612	8.105	15.556
PFUDA	190	176 (92.6)	<LOD	0.049	0.070	0.089	0.144	0.302

Abbreviations: N, number of subjects; LOD, limit of detection.

**Table 3. Associations between cord blood persistent organic pollutants and AMH levels at 12 in the mother-child PELAGIE cohort (N = 239)**

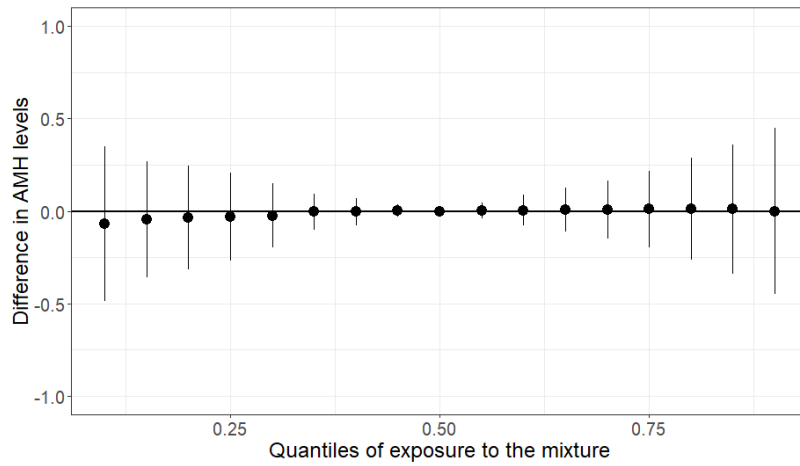
Persistent organic pollutant <sup>1</sup>		Beta <sup>2</sup>	CI	p-value
<b>Organochlorine pesticides</b>				
Beta-HCH (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.11	-0.31 ; 0.53	0.607
	Tercile 3	-0.06	-0.51 ; 0.39	0.784
	Continuous	-0.03	-0.24 ; 0.19	0.799
Dieldrin (N = 238)	< LOD	Ref.	Ref.	
	≥LOD - < median	-0.32	-0.76 ; 0.12	0.157
	≥ median	-0.23	-0.63 ; 0.17	0.262
Hexachlorobenzene (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.18	-0.25 ; 0.61	0.420
	Tercile 3	0.00	-0.43 ; 0.43	0.990
	Continuous	-0.03	-0.36 ; 0.30	0.863
p,p'-DDE (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	-0.37	-0.79 ; 0.05	0.089
	Tercile 3	0.00	-0.43 ; 0.44	0.990
	Continuous	0.04	-0.14 ; 0.22	0.642
<b>Polychlorinated biphenyls</b>				
PCB 118 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.29	-0.12 ; 0.70	0.171
	Tercile 3	0.02	-0.40 ; 0.44	0.936
	Continuous	0.01	-0.25 ; 0.26	0.950
PCB 138 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.15	-0.27 ; 0.57	0.493
	Tercile 3	-0.14	-0.59 ; 0.31	0.544
	Continuous	0.02	-0.24 ; 0.27	0.890
PCB 153 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.09	-0.33 ; 0.52	0.673
	Tercile 3	0.19	-0.27 ; 0.66	0.421
	Continuous	0.07	-0.20 ; 0.34	0.601
PCB 170 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.17	-0.26 ; 0.60	0.437
	Tercile 3	0.42	-0.07 ; 0.90	0.092
	Continuous	0.13	-0.14 ; 0.40	0.361

PCB 180 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.13	-0.29 ; 0.56	0.536
	<b>Tercile 3</b>	<b>0.51</b>	<b>0.02 ; 0.99</b>	<b>0.042</b>
	Continuous	0.14	-0.13 ; 0.41	0.311
PCB 187 (N = 239)	Tercile 1	Ref.	Ref.	
	Tercile 2	0.12	-0.31 ; 0.55	0.590
	Tercile 3	0.23	-0.23 ; 0.69	0.335
	Continuous	0.18	-0.12 ; 0.48	0.237
PCB 194 (N = 239)	< LOD	Ref.	Ref.	
	≥ LOD	0.36	-0.03 ; 0.75	0.074
<b>Per-polyfluoroalkyl substances</b>				
PFHxA (N = 200)	< LOD	Ref.	Ref.	
	≥LOD - < median	0.10	-0.42 ; 0.63	0.698
	≥ median	0.04	-0.39 ; 0.46	0.869
	Tercile 1	Ref.	Ref.	
PFOA (N = 200)	Tercile 2	-0.30	-0.77 ; 0.17	0.215
	Tercile 3	-0.31	-0.79 ; 0.16	0.201
	Continuous	-0.14	-0.47 ; 0.18	0.388
	Tercile 1	Ref.	Ref.	
PFNA (N = 196)	Tercile 2	-0.21	-0.69 ; 0.26	0.378
	Tercile 3	-0.26	-0.74 ; 0.22	0.285
	Continuous	-0.10	-0.40 ; 0.19	0.489
	Tercile 1	Ref.	Ref.	
PFDA (N = 181)	Tercile 2	0.15	-0.35 ; 0.66	0.550
	Tercile 3	0.01	-0.50 ; 0.51	0.980
	Continuous	-0.09	-0.40 ; 0.22	0.574
	Tercile 1	Ref.	Ref.	
PFUDA (N = 190)	Tercile 2	-0.02	-0.52 ; 0.48	0.932
	Tercile 3	0.20	-0.33 ; 0.72	0.463
	Continuous	0.06	-0.27 ; 0.39	0.739
	Tercile 1	Ref.	Ref.	
PFHxS (N = 200)	Tercile 2	-0.42	-0.89 ; 0.05	0.084
	Tercile 3	-0.06	-0.53 ; 0.41	0.798
	Continuous	-0.16	-0.46 ; 0.13	0.282
	Tercile 1	Ref.	Ref.	
PFOS (N = 200)	Tercile 2	-0.29	-0.76 ; 0.18	0.234
	Tercile 3	-0.24	-0.71 ; 0.23	0.319
	Continuous	-0.15	-0.47 ; 0.17	0.350

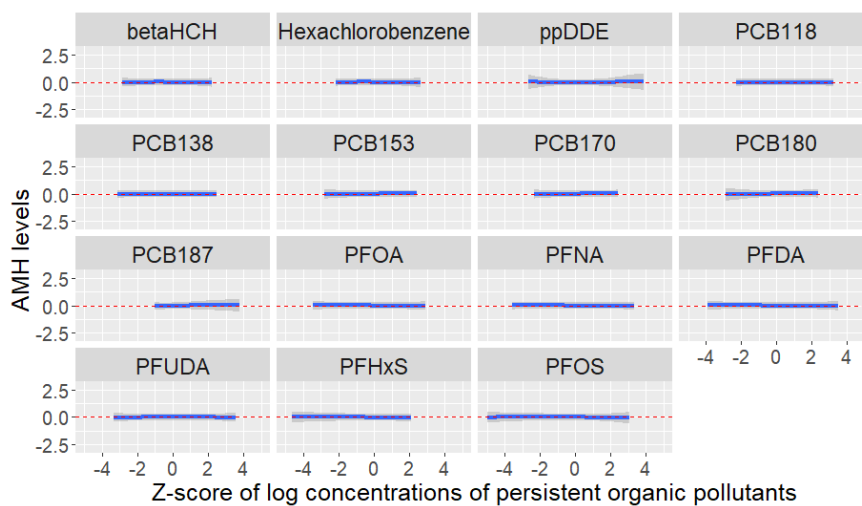
<sup>1</sup> All POPs were log2-transformed when treated as continuous variables.

<sup>2</sup> Adjusted for maternal age at delivery, maternal body-mass index, and maternal education level.

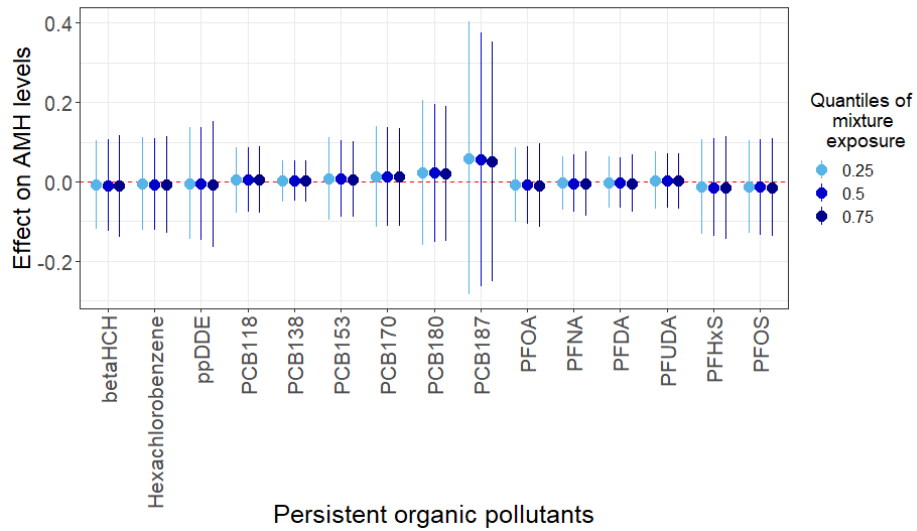
Abbreviations: CI, confidence interval; Ref, reference.



**Figure 1.** Overall effect of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. Adjusted for maternal age at delivery, maternal body-mass index, and maternal education level.



**Figure 2.** Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. Adjusted for maternal age at delivery, maternal body-mass index, and maternal education level.



**Figure 3.** Mean difference comparing the 75th to 25th percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25th, 50th, and 75th percentiles. Adjusted for maternal age at delivery, maternal body-mass index, and maternal education level.

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## CHAPTER 4: DISCUSSION

### 4.1. SYNTHESIS OF RESULTS

In this thesis, we have investigated the associations between EDCs and the ovarian reserve. We focused on assessing the effects of four families of EDCs (POPs, heavy metals, phenols, and phthalates), both individually and in mixtures, on various indicators of the ovarian reserve, as well as on DOR. We conducted our research among two populations of women seeking reproductive assistance at fertility centres in two countries (France and the USA). Additionally, we examined whether prenatal exposure to POPs was associated with AMH levels in adolescent girls in a French mother-child cohort.

In total, we measured the concentrations of 67 EDCs (53 POPs, 4 heavy metals, and 12 non-persistent chemicals) and assessed the impact on the ovarian reserve of the 41 EDCs we could detect in sufficient quantities. Three different markers (AFC, AMH, day-3 FSH) were used to measure the ovarian reserve. This work includes the first studies on the associations between POPs and heavy metals, and DOR.

Overall, we found limited evidence that EDCs were associated with markers of the ovarian reserve or with DOR. In the AROPE case-control study, p,p'-DDE was significantly associated with an increased risk of DOR, whereas  $\beta$ -HCH was significantly associated with a decreased risk of DOR. Furthermore, we observed non-significant increased risks of DOR for Cd and Cr with a close to significant association for the second tercile of Cd. We also observed that increased PCB 180 measured in cord blood significantly increased AMH levels in teenage girls in the PELAGIE cohort.

We also evaluated the mixture effects of various combinations of EDCs within each family of chemicals using BKMR and quantile g-computation. In the EARTH cohort, phenols and phthalates were only studied as a mixture and were not associated with various ovarian reserve markers (AFC, FSH or DOR). In AROPE, the heavy metal mixture showed a non-significant increase in the probability of DOR whilst the POP mixture showed a non-significant decrease in the probability of DOR, carried mainly by beta-HCH.

Nonetheless, it is important to interpret these findings in light of several methodological considerations.

## 4.2. METHODOLOGICAL CONSIDERATIONS

### 4.2.1. Population selection

Both the AROPE and EARTH studies recruited patients from fertility centres, while the PELAGIE cohort was implemented in the general population in order to study the impact of prenatal exposure on the ovarian reserve. In the PELAGIE follow-up study at 12 years old, the participation rate was 60% in children eligible for the clinical examination segment of the study that included the samples for AMH measurement. The same rate was found among those contacted by the research staff in EARTH. In AROPE, the participation rate was slightly higher for cases (65%) and controls (75%) according to the medical professionals in charge of inclusion. Whilst these numbers seem to be in line with the average participation rate for these types of study designs (Galea and Tracy, 2007; Silva Junior *et al.*, 2015; Sritharan *et al.*, 2020), they could still suffer from non-response bias, especially since the mothers of daughters selected in the PELAGIE follow-up at 12 years old were women with higher educational levels and lower BMI than mothers of daughters not included. However, there were no differences between patients included or not in AROPE in terms of age, BMI, or parity.

Furthermore, two of our study populations (ARPE and EARTH) were based in fertility centres. In France, the national health insurance funds infertility consultations and the first four IVF attempts until the age of 43 (National Health Insurance, 2021), which may limit bias when recruiting patients in fertility centres. However, in Spain, where IVF cover is similar (up to 3 ART cycles for women up to the age of 45), a study found women of lower socioeconomic characteristics had a lower likelihood of accessing ART services in general (Alon and Pinilla, 2021). However, Spanish patients usually spend around one year on waiting lists for access to public clinics thereby reducing ART success and pushing people towards using private clinics which represent 75% of all cycles. In the US, healthcare including infertility care depends on private insurances and is therefore not accessible to everyone. In fact, the cost of a single IVF cycle has been estimated at more than 50% of the average annual income (Dongarwar *et al.*, 2022), and the main concern of the vast majority of Americans regarding IVF treatments is financial (Iaconelli *et al.*, 2013). A retrospective cohort study using the US birth data files between 2011–2019 found that non-hispanic-Black and Hispanic women were approximately 70% less likely to access any form of infertility treatment (Dongarwar *et al.*, 2022). However, this association between ethnicity and infertility care in the US is likely confounded by several sociodemographic factors such as education, income level, institutionalised racism, and cultural stigmas. This situation could improve in the future as 20 states have recently (2022) passed legislation requiring private insurers to cover or offer coverage for infertility evaluation and treatment (Merkison *et al.*, 2023). We have attempted to explore the impact of selection bias in our EARTH study using DAGs as

recommended (Arah, 2019), but did not identify clear evidence of such a risk using our set of covariates, unless ethnicity was not completely mediated by socio-economic factors. Although we did not use DAGs for the exploration of selection biases in the AROPE study, a similar conclusion could be made considering the similarities between causal structures and the choice of regression covariates. Additionally, a selection bias would likely be reduced in France, compared to the US, considering accessibility to publicly-funded coverage as explained above.

#### 4.2.2. Exposure assessment

##### 4.2.2.1. Exposure matrix

Some studies exploring the impact of EDCs on in vitro fertilisation outcomes have measured exposures in follicular fluid instead of blood or urine. Follicular fluid is a more direct measure of exposure concentrations in the ovaries, but it is only sampled in women that have undergone IVF, which is not the case for women with a very low ovarian reserve due to their low probability of IVF success. Measuring exposure in follicular fluid would require selecting a specific population but could nonetheless be interesting to consider. Six studies summarised in a review on the associations between POPs and IVF outcomes (Lefebvre et al., 2021) have compared POP concentrations in serum and follicular fluid, and one study (Bloom et al., 2012) has compared metal concentrations in blood and follicular fluid. These all found moderate to high correlations between matrices for all studied exposures excepted for Pb (Bloom et al., 2012). The impact of using serum for POPs and whole blood for metals in our study should therefore be moderate and non-differential. By contrast, urinary concentrations of phenols and phthalates do not correlate well with follicular fluid concentrations (Yao *et al.*, 2020). Differences in concentrations could be attributed to differences in speed of clearance of these chemicals within the two matrices (Du *et al.*, 2016), but more research is needed on this topic to fully elucidate the issue and determine whether follicular fluid is a less biased exposure matrix.

##### 4.2.2.2. Variability of non-persistent EDCs

The variability of EDCs poses significant challenges when attempting to measure them accurately and evaluate their impact on human health in epidemiological studies. These challenges often depend on the persistence levels of exhibited by the chemicals. While lipophilic POP concentrations measured in serum are generally considered as an accurate marker of long-term exposure (Bu *et al.*, 2015), it is more difficult to have the same confidence for heavy metals for which the half-lives are between one

and four months (Järup and Åkesson, 2009). However, in the cases of Pb, Cd and Cr, organs and tissues can sustain their blood concentrations in a sufficiently stable manner (de Souza *et al.*, 2018).

Non-persistent chemicals are considerably more difficult to assess precisely due to their very short half-lives, and using specific study designs to compensate this issue is advised. Pooling urine sample concentrations have been shown to accurately estimate exposure averages (Vernet *et al.*, 2019). This method was employed in our study in the EARTH cohort but was not possible for all individuals included since some only had one sample collected. However, it has previously been shown in this specific cohort that a single urine sample may reasonably represent an individual's exposure to short half-lived chemicals such as BPA and phthalates over several months (Braun *et al.*, 2012).

Nonetheless, all concentrations of EDCs measured in the different studies included in this thesis were comparable to national biomonitoring studies or similar epidemiological studies except for metal concentrations, which at times were lower (Cd and Pb), and at other times, higher (Cr). When compared to older studies, these usually displayed lower concentrations, which is consistent with the decrease in usage of source products over time.

#### 4.2.3. Evaluation of the ovarian reserve

##### 4.2.3.1. DOR as a fertility indicator

In AROPE, we chose a priori to study the relation between POPs and DOR rather than continuous indicators of the ovarian reserve as in previous published studies in order to use a more clinically pertinent outcome. Doing so also allowed us to ensure we could recruit more women with lower ovarian reserves, but with the cost of losing the added statistical power of using models with continuous outcomes. DOR was also used as a diagnosis in the EARTH cohort and was used alongside other indicators to evaluate the associations between non-persistent chemicals and the ovarian reserve. In PELAGIE, we used AMH as a continuous outcome because recruitment was carried out in the general population, and because DOR thresholds are not appropriate in teenage girls. However, a follow-up study in adulthood is being considered with an evaluation of AMH levels and an AFC.

In AROPE and EARTH, we used threshold levels for AFC and AMH that were established by the Bologna ESHRE consensus for the purpose of predicting a POR. Indeed, most fertility centres use this definition to diagnose DOR (Pastore *et al.*, 2018). However, in AROPE, we performed several sensitivity analyses where we varied the threshold levels used to limit the risk of DOR misclassification. In EARTH, we used a composite definition of DOR based on AFC but also on FSH levels and infertility diagnosis.

However, several studies comparing AMH levels and AFC between fertile and infertile women (both groups with fertile male partners) have shown that these markers were similar in both groups

(Hvidman *et al.*, 2016; Khan *et al.*, 2019) and that the probability of not conceiving was not lower in women with low AMH (Steiner *et al.*, 2017). Nonetheless, in one of these studies (Hvidman *et al.*, 2016), the proportion of non-growing follicles was significantly lower in infertile patients whereas the proportion of large follicles was significantly higher (by approximately 5% in each case). Overall, these results may indicate that the thresholds used for DOR are not suited for the prediction of conception. Instead, it is possible that the quality of follicles has a larger impact on conception than the quantity of follicles, but research on this subject is lacking.

Regardless, DOR remains one of the most frequent pathologies diagnosed in women consulting in fertility centres (Pastore *et al.*, 2018) and remains a crucial public health matter due to its impact on IVF prognosis.

#### 4.2.3.2. Comparing ovarian reserve markers

##### *AMH versus AFC*

In AROPE, our main results regarding heavy metals were confirmed in sensitivity analyses when DOR was defined by AMH only. Indeed, the direction of the effects of metals on DOR in sensitivity analyses seem to fluctuate depending on whether DOR is defined by AMH only or AFC only, with only one of those associations (second tercile of Cd when DOR is defined by AMH  $\leq 1.1$  ng/ml) being statistically significant. These differences could be attributed to different factors. Previous studies have reported that AMH had at least the same level of accuracy and clinical value for the prediction of poor response as AFC (Broer *et al.*, 2009). However, one team (Zhang *et al.*, 2019) measured AFC and AMH levels in 1,121 women undergoing IVF and showed that one in five women had either low AFC but high AMH or low AMH but high AFC according to the Bologna criteria. Consistent with this study, this proportion reached 26% in AROPE. Some studies have also observed that AMH levels in younger women did not correlate with the number of growing follicles, while in women of late reproductive age a significant correlation was observed with the primordial follicle density (Moolhuijsen and Visser, 2020); indicating that AMH and AFC could reflect different aspects of the ovarian reserve at older ages (the ovarian function and the recruitable follicles respectively).

Accordingly, one hypothesis that could account for the inconsistencies observed in our sensitivity analyses based on varying definitions of DOR in our study on heavy metals, is that metals might affect primordial follicles rather than antral follicles. By contrast, our analyses regarding p,p'-DDE and the ovarian reserve were consistent across all definitions of DOR.

### *FSH versus AFC and AMH*

Whilst the results in the EARTH study all pointed towards null associations independently of the ovarian reserve marker, the discrepancy between lower AFC (AFC < 7) and day 3 FSH (> 10 UI/L) in our population was 18% and the correlation between FSH and AFC was moderate ( $\rho = -0.40$ ). These differences seem to be consistent with the literature. In fertility studies, 25 to 40% of the patients exhibit discordant values when comparing AMH and FSH thresholds depending on the definition of low AMH (< 0.8 of < 1.1 ng/ml, FSH > 10 IU/L). A study based in the general population has also reported a moderate correlation between FSH and AFC ( $\rho = -0.55$ ) and a high correlation between AFC and AMH ( $\rho = 0.88$ ).

As previously explained, AFC and FSH both have high intra-cycle variability but AFC and AMH are considered to have better clinical value than FSH (Ulrich and Marsh, 2019). A lack of consistency between FSH and ovarian markers could be accentuated by the fact that circulating FSH concentrations usually rise sharply in women older than 35 years, yet these represent a significant proportion of women in fertility centres. Differences between FSH concentrations and AFC are therefore to be expected.

### *Conclusion*

The general scientific consensus seems to point towards AFC and AMH as the more accurate indicators of the ovarian reserve (Penzias *et al.*, 2020). To address issues of intra-cycle variability and ensure comparability between studies, some authors (Melado *et al.*, 2018) have further suggested to measure AMH levels at the same time as the AFC is performed i.e. between day 2 and 4 of a spontaneous menstrual bleed. However, in the clinical setting, it is usually when this concentration is measured since FSH and LH also need to be measured within this time window.

#### 4.2.4. Study power

In AROPE, although we recruited patients over a wide area of France and for a period of four years, study power for this analysis was still limited by the sample size.

Furthermore, because of the previous analyses performed on the EARTH cohort on single-exposure associations, we originally believed that we had adequate power for this study on mixtures. For example, AFC has previously been used in a sample of 154 women from the EARTH study (Souter *et al.*, 2013) as a binary variable where an increase in BPA (quartiles) was significantly associated with an AFC  $\geq 15$ . In a different EARTH analysis (Messerlian *et al.*, 2016), significant associations were shown between phthalates (divided into quartiles) and AFC (continuous) in a sample of 215 women.



Lastly, the sample size for our analysis on PELAGIE was limited by the availability of cord blood samples and the number of girls whom accepted to return for a follow-up analysis.

#### 4.2.5. Confounders

In all our studies, we used an a priori adjustment strategy using DAGs to incorporate a comprehensive adjustment of confounding variables in our regression models whilst limiting a loss in precision due to over-adjustment (Shrier and Platt, 2008). Nonetheless, it is possible that our DAGs did not include potential confounders we may have missed in our literature review or which have not been identified yet.

#### 4.2.6. Multiple testing

Considering the number of pollutants analysed in our different studies, it is possible that our findings might have been influenced by the presence of both false positive and false negative results. However, the objective was to provide signals to be confirmed in further studies rather than to reach definite conclusions. All analyses were also planned a priori and consequently, we decided not to correct our results for false discoveries (Bender and Lange, 2001; Goeman and Solari, 2011).

#### 4.2.7. Mixture methods

An important strength of all the studies included in this thesis is the evaluation of the mixture effect of several chemicals on our outcomes of interest using two complementary methods: quantile g-computation and BKMR. Quantile g-computation estimates the ovarian reserve association for a simultaneous increase in all exposure biomarkers by one quantile. This is highly interpretable and efficient, but assumes the association is linear on the quantile scale — i.e. the association comparing the first to second quartile is the same as comparing the third to fourth quartile — and moreover assumes no exposures interact non-additively. BKMR, on the other hand, is not as interpretable or efficient, but it allows each exposure to have its own potentially non-linear exposure response relationship, and further allows exposures to interact synergistically or otherwise. In tandem, BKMR can support the results of the quantile g-computation analysis or indicate that some of its assumptions are not appropriate — e.g. evidence of interactions or of different functional relationships between exposures and outcomes.

### 4.3. IMPLICATIONS IN PUBLIC HEALTH

#### 4.3.1. Societal Impacts of EDCs

Although chemicals, including EDCs such as those present in plasticisers, pesticides, and food preservatives, can be seen as having provided valuable benefits to the economy and global trade, it is crucial to consider and balance these advantages with their associated costs.

Multiple economic evaluations were conducted to estimate the impact and financial costs of EDCs on various health outcomes, such as neurobehavioral deficits, female and male reproductive disorders, obesity, and diabetes. This economic burden amounted to €163 billion in the EU and \$340 billion in the USA annually (Kassotis *et al.*, 2020). However, these estimates are likely underestimations, as they focused on both a limited number of EDCs and a limited number of health outcomes. A study looking at the specific costs of EDCs on female reproductive disorders found that the impact of DDE and phthalates on fibroids and endometriosis could be associated with costs of approximately €1.4 billion annually in the EU alone (Hunt *et al.*, 2016). Moreover, the latency of health effects can delay the impacts of EDCs as POPs, which have been banned several decades ago, are still measured in humans and still have health consequences today. In addition, epigenetic processes caused by acetylation and methylation of deoxyribonucleic acid (DNA) as a result of EDC exposure could also have long-lasting transgenerational effects (La Merrill *et al.*, 2020) that have inadequately been investigated in cost evaluation.

Moreover, EDCs come with an inherent social cost. Exposure to EDCs is dependent on several factors that can vary according to socio-economic groups including profession, housing, and diet (Yilmaz *et al.*, 2020). Professional workers using EDCs such as pesticides, fungicides, and heavy metals are also at higher risk of exposure to EDCs. People living in poor housing may be more exposed to plastic material, deteriorating metal structures, and motor vehicle traffic (García-Esquinas *et al.*, 2011). Additionally, individuals from higher education backgrounds generally consume more fish and vegetables which contain higher concentrations of POPs and metals (Montazeri *et al.*, 2019), while individuals from lower education backgrounds consume a higher proportion fried foods, and processed foods, which contain higher levels of non-persistent chemicals (Galobardes *et al.*, 2001).

According to the Institute of Radioprotection and Nuclear Safety, in 2021, 53% of the French population considered that the risks posed by EDCs were high, a 16% increase from 37% in 2014 (IRSN, 2022). EDCs therefore represent a significant perceived risk by the French population and needs to be addressed, especially in conjunction with social scientists. From a societal perspective, it is necessary to weigh these social and economic costs against the costs of preventing EDC exposure, and/or developing safer alternatives to EDCs.

#### 4.3.2. Reducing EDC exposure

##### 4.3.2.1. Clearing EDCs from the environment

Several existing and emerging treatment technologies for the removal of phenolic-based EDCs have been developed, including advanced oxidation processes, adsorption processes, membrane-based filtration, bioremediation, phytoremediation and other integrated approaches (Werkneh *et al.*, 2022). EDCs removal can be performed through the use of combined processes but despite these efforts, the complete removal of EDCs remains challenging because of their persistency and complexity in water, wastewater and sediment. Furthermore, this process only affects the dispersion of phenolic EDCs in the environment at the production level and not in personal care products or food packaging, other consumer products, or occupational exposure to EDCs in general.

As we have described previously, eliminating POP waste has also been a significant international challenge (Li *et al.*, 2023). The United Nations Environment Program (UNEP) estimates that more than 80% of the total amount of PCBs in the world remains to be eliminated and countries are not currently on track to achieve the 2028 reduction goals (UNEP, 2017).

##### 4.3.2.2. Is EDC prevention a viable option?

In the absence of strong regulation and capacity to efficiently eliminate EDC waste, several strategies for the prevention of exposures to EDCs have been developed and tested. A 2022 review (Martin *et al.*, 2022) evaluated the impact of 21 lifestyle interventions on reducing endocrine-disrupting phthalate and phenol exposures among reproductive age men and women using studies that reported chemical concentrations. Overall, dietary and personal care products interventions showed a significant reduction but no intervention was successful in eliminating concentrations completely. The concentrations of high-molecular weight phthalate were more easily decreased than for low-molecular weight phthalates. A subsequent review (Yang *et al.*, 2023) of 26 interventions on the exposure to phenols and phthalates in children and adults from dietary intake and personal care products, concluded that interventions were generally successful but also highlighted the influence of participant motivation, compliance, ease of adherence, and the difficulties of identifying viable replacement products. Moreover, the reviewed suggested that policy which reduces or removes EDC in manufacturing across multiple sectors may be more impactful than focusing on individual behaviour change.

Other studies have shown that chronic physical activity and diets rich in fibres can increase the metabolism and elimination of EDCs by increasing biotransformation enzyme activity in the liver and/or increasing biliary clearance (Lee and Jacobs Jr, 2019).

In France, prevention messages focus particularly on the first 1,000 days of life mainly through a website advising couples on how to modify their life habits to reduce chemical exposure (Ministère de la Santé et de la Prévention, 2023) as part of a broader national program on risk reduction and health promotion in early life. However, a 2018 survey among perinatal health professionals (Marguillier *et al.*, 2020) showed that most of the responding professionals reported not to give any information on EDCs during pregnancy, partly due to the lack of medical information, training or tools to communicate effectively. It also showed that 74% of perinatal health professionals considered that information about health risks of endocrine disruptors was important and 92% wished they were better informed. The Clinical Practice Guidelines from the French National College of Midwives has highlighted the need to reduce domestic chemical exposures during the perinatal period, but also points toward the lack of time available for consultations and the multiplicity of prevention messages to transmit (Barasinski *et al.*, 2022). Nonetheless, various endeavours to support medical professionals in this EDC prevention effort have been implemented. Several centres attached to public hospitals, such as the PREVENIR network that includes the PRE<sup>2</sup>B centre (Prévention des Risques Environnementaux et REproduction en Bretagne) in Rennes and the ARTEMIS centre (Aquitaine Reproduction Enfance Maternité et Impact en Santé environnement) in Bordeaux, accompany infertile couples to evaluate their exposure to reprotoxic chemicals and provide them with individualised advice to reduce it. Several online training programs on the topic of exposure to EDCs have been developed including a course by the Ecole des Hautes Etudes en Santé Publique (EHESP, 2023) for medical professionals on EDCs and chemical risks surrounding perinatality and childhood.

A number of smartphone applications have also been developed by independent parties to better inform the consumers on the chemicals present in a given product and identify those that may be toxic, usually by providing a rating on their health impact. However, these sources should not be relied upon as the sole basis of information, and caution should be exercised when using them since they may lack toxicological expertise (Marie *et al.*, 2022).

In general, approaches to avoid exposure sources can have positive effects on chemical concentrations but cannot entirely reduce EDC exposure or effectively protect from the possible harms of low-dose of EDCs (Lee and Jacobs Jr, 2019).

#### 4.3.3. Infertility prevention

Most studies conducted in Western countries regarding fertility awareness indicate that a significant proportion of adults of reproductive age lacks adequate knowledge about age-related fertility decline and the risk factors associated with infertility. A 2018 systematic review of 71 studies (mostly Western) on fertility awareness and its associated factors concluded that fertility awareness levels were low to

moderate among people of reproductive age (Pedro *et al.*, 2018). In general, individuals exhibited awareness regarding the lower probability of achieving pregnancy at older ages but tended to significantly overestimate the likelihood of conceiving, both spontaneously and through ART. This overconfidence in ART was also observed in a French observational study conducted among female students of seven midwifery schools (Chelli *et al.*, 2015). Nonetheless, the majority of participants to these different studies was shown to possess high awareness levels of infertility risk factors related to lifestyle (e.g. smoking, weight, and drugs).

Despite this context, no government-led prevention program exists in France, a situation that has led the 2022 national report on the causes of infertility to call for a national infertility prevention strategy (Hamamah and Berlioux, 2022). Unfortunately, education programs in general have yielded mixed results in the past regarding their effectiveness on increasing fertility awareness with side effects such as increases in anxiety (Pedro *et al.*, 2018), and with certain campaigns sometimes being criticised as insensitive. Nonetheless, a number of initiatives have emerged in recent years across the world. Australia was the first country to fund a program that included an interactive website generating over five million visits per year, and learning modules for healthcare providers (Harper *et al.*, 2021). Denmark opened in 2011 the world's first Fertility Assessment and Counselling Clinic to provide individual fertility assessment and guidance to people with no known reproductive issues. The UK Department for Education introduced fertility education into the UK school curriculum in 2019. Moreover, a Portuguese study (Xavier *et al.*, 2023) has suggested using AMH levels as a screening method for predicting future low ovarian reserve, and has estimated that offering an AMH test at a family planning appointment to women aged 25-30 years who have not previously been pregnant, nor attempted to become pregnant, could save the health service €9.4 million and reduce the use of ART by 24%.

Considering the general lack of awareness on fertility capacity, a comprehensive national strategy involving transdisciplinary actors could help reduce infertility-related challenges as well as the reliance on IVF.

#### **4.4. FUTURE PERSPECTIVES**

##### **4.4.1. Studying other windows of vulnerability and the transgenerational effect**

Although we did not find any associations between POPs measured in cord blood and AMH levels at 12 years old, consistently with the literature, other periods of exposure and windows of vulnerability need to be examined. Repeated measures of exposure and markers of the ovarian reserve throughout the pregnancy, and surrounding perinatal, infancy, puberty and adolescence could help us

understand the relationship between EDCs and the ovarian reserve. A follow-up at 18 in the PELAGIE cohort is currently ongoing and the feasibility of doing a specific follow-up study on female reproductive health including the ovarian reserve but also reproductive disorders (e.g. endometriosis, PCOS) is being assessed, which could help us expand our study to adulthood. As described previously, the literature on the associations between prenatal exposure to heavy metals and non-persistent chemicals is also lacking and should be considered in further studies.

Pooling separate cohorts could also help gaining statistical power, although consistency in the timing of exposure and outcome measurements can be difficult to achieve. The ATHLETE consortium which comprises sixteen longitudinal population-based birth cohort studies in 11 countries (Vrijheid *et al.*, 2021), including PELAGIE, could be an interesting prospect, provided that ovarian reserve evaluations are carried out.

Furthermore, experimental studies have shown that EDC exposure to phthalates, BPA, and OCPs, can have transgenerational effects mediated via epigenetic mechanisms on multiple reproductive outcomes (including litter size, early puberty, estrous cyclicity disruption, follicle numbers alteration, early reproductive aging, decrease fertility, increase cysts in ovaries, sex steroid hormone level alterations) (Brehm and Flaws, 2019). Developing general population cohorts that span over several generations is necessary to understand if these effects are also present in humans. These could draw inspiration from ongoing research investigating the health effects observed in the grandchildren of women who were exposed to DES during their pregnancy in the 1960s (Zamora-León, 2021). Pursuing mother-child cohort follow-ups with the inclusion of pregnancies from the children of said cohorts and measuring the development of the subsequent generation may be especially relevant.

#### 4.4.2. Exploring less studied chemical exposures

Among tens of thousands of chemicals, only a minority has been investigated in epidemiological studies. Some have been prioritised due to mass accidents as a result of acute exposure, or due to their effects on animal species or on the environment. Others have been examined due to their high prevalence in biomonitoring studies, or in the context of water surveillance. Additionally, chemicals can be chosen because they are used in a significant number of products or over a wide geographical area. Nonetheless, some molecules might have escaped this net.

##### 4.4.2.1. The Missing EDCs

Although a wide range of EDCs have been studied in relations to female reproductive outcomes, some have escape scrutiny. This is notably the case for chlordecone, an EDC and organochlorine pesticide

with well-defined estrogenic and progestogenic properties in vivo and in vitro. Mammalian studies in females have consistently reported that chlordecone exposure impairs ovulation and can lead to a reduced ovarian reserve (increase in atretic antral follicles and decrease in the number of normal or median sized follicles) (Legoff *et al.*, 2019; Swartz and Mall, 1989). Despite these results to date, no study has been published concerning the link between chlordecone exposure and female fertility. This is the objective of the KARU-FERTIL project, a study modelled on AROPE, which will take place in Guadeloupe, within women from couples consulting for infertility.

Other chemical families including organophosphorus flame retardants (OPFRs), and ultraviolet (UV) filters, also deserve some attention as the literature on their impact on the ovarian reserve is limited. Regarding OPFRs, one study (Liu *et al.*, 2023) has shown that triethyl phosphate was associated with decreased LH, and tris(1,3-dichloro-2-propyl) phosphate with decreased AFC. A mixture analysis (Pollack *et al.*, 2018) including parabens, phenols, and UV filters also gave some evidence that UV filters (benzophenones 1 and 3) could decrease levels of estradiol, LH and FSH. However, toxicological studies on the topic seem to be lacking.

#### 4.4.2.2. Newer EDC replacements

Amongst replacements of chemicals that have now been qualified as EDCs, several are rapidly gaining interest due to their potential toxicity. More than 200 structural variants of bisphenols are present in consumer goods, however, only a few have been considered in epidemiological studies (Lucarini *et al.*, 2020). For instance, bisphenol AF, B, and tetrabromobisphenol A (TBBPA), are chemicals that may exert similar adverse effects on the reproductive system than bisphenol A, F or S, but that still have insufficient toxicological and epidemiological data to evaluate their effects on reproduction (Siracusa *et al.*, 2018).

In a similar fashion, di(isononyl) cyclohexane-1,2-dicarboxylic acid, diisononyl ester (DINCH) and DEHTP have been introduced to replace DEHP, and their exposure is rapidly increasing in the general population. However, there is evidence to suggest that these replacements also exhibit endocrine disrupting properties and may therefore pose risks to hormonally-regulated processes, including reproductive hormones (Bommarito *et al.*, 2023).

In addition, the production and use of alternative fluorinated compounds such as GenX, ADONA and F53B, have emerged as short-chained versions of PFAS, following the ban of certain PFAS molecules (Brandsma *et al.*, 2019). Evidence from animal and in vitro studies indicates that these chemicals similarly affect reproductive endpoints; however, epidemiological studies are scarce and inconsistent (Chambers *et al.*, 2021).

#### 4.4.2.3. Emerging chemicals

While chemicals replacing known EDCs or other toxic chemicals that have gained interest from short-term mass-scaled health events are simple to identify, this is not the case for new chemicals with potential endocrine disrupting properties or with other or unknown mechanisms of action that can impair the ovarian reserve. The term "emerging chemicals", or "contaminants of emerging concern" in the field of water surveillance, is often used to qualify chemicals that were not previously identified or have a new environmental health or toxicological understanding (Lazofsky and Buckley, 2022).

The use of new non-targeted approaches using high-resolution mass spectrometry could help us identify these chemicals. These tools can now enable the simultaneous detection, within the examined biological matrices, of tens of thousands of chemical signals (David *et al.*, 2021). The implementation of these non-targeted methods relies on the "omics" methodologies used in the meet-in-the-middle approach. In the context of identifying new chemicals, these cannot provide concrete concentrations but can provide frequencies of detection and can discern if one individual is more exposed than another. Nonetheless, a secondary targeted analysis on a new sample can subsequently quantify the concentrations.

#### 4.4.2.4. Other chemicals of interest: solvents

Solvents are liquids that have the property of dissolving, diluting, or extracting other substances without causing a chemical change in those substances and without undergoing any change themselves. These chemicals are present in a wide range of products, such as adhesives, paints, dyes, inks, cleaning products, and cosmetics (Enderle *et al.*, 2023) and can be classified into three families: oxygenated solvents (e.g. alcohols, esters, ethers), petroleum solvents (e.g. benzene, aromatic solvents), and chlorinated solvents (e.g. perchloroethylene, trichloroethylene). While very few solvents have been identified as EDCs, some glycol ethers have exhibit reprotoxic effects (Kelsey, 2022). Animal studies have reported that several glycol ethers may affect the ovarian function (INSERM, 2006) and metabolites of glycol ethers have been associated with a prolonged TTP and with prolonged menstrual cycles (Garlantézec *et al.*, 2013). However, the literature on the ovarian reserve or other reproductive disorders is lacking. Occupational exposure to solvents has been evaluated in AROPE and will be an interesting topic to explore in relation to DOR.



#### 4.4.2.5. Microplastics

Plastics are present everywhere in our daily life and in the environment, yet little attention has been devoted to the impact of microplastics on human health. Microplastics are the result of the degradation of large plastic objects, or come from particles directly synthesised and used in synthetic textiles (microfibers), cosmetics and health products (microbeads), and many other commercial products (Priya *et al.*, 2022). They are defined as plastic particles < 5 mm in diameter (< 1 µm for nanoplastics) and include chemicals identified as polyester, polyethylene, copolymers of polypropylene, and polyurethane (Jung *et al.*, 2022). Microplastics have been found in food, drinking water, dust, and in all kinds of human biological samples, including semen, placenta and breast milk (Kutralam-Muniasamy *et al.*, 2023). In experimental studies, microplastics have been shown to exert endocrine-disrupting-like properties such as decreasing the levels of steroid hormones (notably estradiol and testosterone) by disrupting the hypothalamic pituitary gonadal axis (Afreen *et al.*, 2023). They have also been associated with apoptosis of granulosa cells in rats likely due to oxidative stress, a decline in the number of antral follicle cells, affected oocyte maturation quality and development (Liu *et al.*, 2022) and a reduction in ovary size in mice (Wei *et al.*, 2022). Nonetheless, the development of methods of quantifying microplastics in human matrices are still required, particularly in order to avoid potential contamination of the samples (especially due to the widespread use of plastic materials in medical equipment). Given the prevalence of plastics around the globe which is predicted to triple by 2060 (OECD, 2022), the study of the effects of microplastics on the reproductive function seems urgent. As a matter of fact, the European AURORA project has the objective of developing new methods to characterise micro- and nanoplastics in placentas and assess their health effects on the foetus.

#### 4.4.3. Considering other factors

Other factors than chemicals that may impact the endocrine system and the ovarian reserve have not been explored to the same extent.

##### *Perceived stress*

The literature on perceived stress and markers of the ovarian reserve is still scarce and inconsistent, even though the central stress response system is primarily mediated by the hypothalamic-pituitary-adrenal axis (Mínguez-Alarcón *et al.*, 2023), which can affect the secretion of oxidative stress products. This relationship could be further modulated by the effects of working day and night shifts (Özyürek *et al.*, 2021).

### *Depression*

In patients with a major depressive disorder (MDD), stress can inhibit the hypothalamic-pituitary-adrenal axis and stress-induced glucocorticoids can inhibit gonadal activity (Gök *et al.*, 2023). MDD may also accelerate biological aging by interacting with cellular aging markers (Önel *et al.*, 2023). In fact, one study found an association between a lifetime history of depression and an earlier transition to perimenopause (Gök *et al.*, 2023). Furthermore, infertile women with depression are less likely to initiate treatment for infertility, though depression could still be twice as high in infertile patients compared to fertile patients (Crawford *et al.*, 2017). This can create a detrimental cycle in which depression contributes to early infertility, which, in turn, can amplify feelings of depression and stress. These factors can be further intensified by the use of IVF procedures. Because depression and psychological stress are strongly interrelated, one study has investigated their interactions and has found that stress was related to AFC in women with low positive affect but not in women with high positive affect (Bleil *et al.*, 2012).

### *Physical activity*

The amount of physical activity can affect hormonal profiles which can result in delayed menarche, primary or secondary amenorrhea, or oligomenorrhea (Miller *et al.*, 2022). However, exercise could also benefit folliculogenesis and steroidogenesis due to the positive impact the hypothalamus could have as a central regulator of energy homeostasis on leptins. It could also help in reducing oxidative stress (Kiranmayee *et al.*, 2017). This could be further mediated by the effect of physical activity on perceived stress.

#### 4.4.4. Using more advanced mixture models

The lack of a single model to assess all relevant aspects of the overall effect of a mixture on an outcome is limiting. Nonetheless, a new model called Bayesian multiple index model (BMIM) aims at combining response-surface methods (such as BKMR) and exposure-index methods (such as quantile g-computation) (McGee *et al.*, 2023). This model allows the modelling of non-linear and non-additive relationships between exposure indices and a health outcome, while reducing the dimensionality of the exposure vector and estimating index weights with variable selection. In practice, it provides the mean difference of the outcome for a simultaneous decile increase of all mixture components, and at the same time examines the interactions between exposures but also between families of exposures. This method could be implemented in future mixture studies when a coding package is made available.

#### 4.4.5. Assessing the exposome in relation to the ovarian reserve

While we have investigated the impact of mixtures of different classes of EDCs, it is important to acknowledge that these mixtures only represent a fraction of the real-world combinations of EDCs, other chemicals and all possible adverse environmental exposures. To comprehensively analyse such a combination of exposures would require to jointly model their effects on the reproductive outcome in question. This is essentially the objective of studying the exposome, which represents the totality of exposures from a variety of external and internal sources including chemical, biological, or physical agents, from conception onward, over a complete lifetime (Vineis *et al.*, 2020). Evaluating multiple families of environmental pollutants simultaneously could help unravel confounding factors caused by co-exposures and shed light on the mediating effects of specific groups of exposures that could lie along the causal pathway. Nonetheless, the correlation structure of the over 100 environmental exposures measured in HELIX revealed that the correlation coefficients were stronger within than between families of exposures, which may suggest that concentrating on a single family of exposures at a time may not be confounded to a great extent by unmeasured exposures from other families of exposures (Tamayo-Uria *et al.*, 2019). Additionally, as the number of exposures tested increases, the need for statistical power in order to correct for multiple testing increases too. The exposome is often divided into smaller parts like the chemical exposome or the urban exposome for reasons we will highlight below.

##### 4.4.5.1. The chemical exposome

The chemical exposome aims to comprehensively assess the complex and cumulative effects of chemical exposures on human health. An important notion to especially consider in mixtures or chemical exposome studies is the potential for interactions between chemicals. However, this can be difficult to assess in exposome statistical methods, and evidence regarding the interactions between EDCs in humans is limited (Dutta *et al.*, 2023). To shed light on this subject, an interesting prospect would be to perform a literature review of epidemiological studies using mixtures of chemicals and evaluate the amount reporting synergism and antagonism between chemicals, modelled on the toxicological review described in chapter 1. Studying the chemical exposome was not possible in the AROPE study because only a limited number of chemicals have been measured so far, but could in principle be explored in the EARTH study in a subfertile population or in the ATHLETE consortium in the general population, though ovarian reserve parameters have only been measured in a limited number of cohorts.

#### 4.4.5.2. The urban exposome

Relative to other parts of the exposome, exposures within the urban exposome can be highly correlated to one another. The urban exposome includes built environment characteristics that individuals are exposed to in the outdoor urban environment and can be assessed through common geospatial methods. These include variables like air pollution, proximity to green and blue spaces, climate and meteorological factors (including mean temperature, heat stress, atmospheric pressure, and extreme climate events), noise, population density, proximity to traffic and public transport. These factors have not been explored in much detail in regards to the ovarian reserve or other reproductive disorders. Whilst a few studies have investigated the associations between air pollution and AFC (Gaskins *et al.*, 2019), limited data is available for exposures such as green space (Abareshi *et al.*, 2020; Triebner *et al.*, 2019), and ambient temperature (Gaskins *et al.*, 2021); and no attention has been given to the other variables highlighted. Further studies could consider exploring these relationships, especially in an urban exposome context.

#### 4.4.5.3. Integrating omics data

The internal exposome, defined as the internal endogenous processes including epigenetic, gene expression, inflammation, and metabolism, can be assessed through molecular omics methodologies such as genome-wide DNA methylation, transcriptomics, proteomics and metabolomics, thus forming omics layers (Santos *et al.*, 2020). Omics signatures of these different layers can reflect both physiological responses to external exposures and internal signatures of health outcome.

One of the ways to integrate omics data in epidemiological studies is the meet-in-the-middle approach. This method involves measuring the internal exposome to study its association with exposures of interest, as well as with the health outcomes of interest (Vineis *et al.*, 2020). For instance, one study used this approach to use untargeted metabolomics to identify metabolites and pathways in the follicular fluid associated with periconceptional exposure to air pollutants, and found six metabolomic pathways associated with NO<sub>2</sub> and mature oocytes including vitamin D3 (cholecalciferol) metabolism (Hwang *et al.*, 2022). This method could be particularly useful in helping to decipher the biological mechanisms that could lead EDCs to interfere with the ovarian reserve. However, this method is exploratory, and any findings obtained through it necessitate confirmation through additional studies.

#### 4.4.6. From quantity to quality: developing new ovarian reserve measures

The progressive reduction in follicle numbers throughout reproductive life is usually accompanied by a decline in oocyte quality (Richardson *et al.*, 2014), however, recent studies have shown that this is

not necessarily the case (Khan *et al.*, 2019; Morin *et al.*, 2018). Indeed, this relationship may be confounded by the association between oocyte quality and age which is likely mostly due to increased oxidative stress from mitochondria (Moghadam *et al.*, 2022). Thus, oocyte quality might represent a better indicator of fertility or POR. Oocyte quality refers to the ability of the oocyte to complete meiotic maturation (including the proper segregation of chromosomes) as well as supporting fertilisation and embryonic development ultimately leading to a successful live birth (Homer, 2020). Unfortunately, no definite test exists to measure oocyte quality. In the context of IVF, the quality of oocytes often refers to post-fertilisation criteria including the blastulation rate, aneuploidy rate, or even the live birth rate (Morin *et al.*, 2018) but these represent indirect measures. Other criteria could involve each of the oocyte's cellular compartments and surroundings (e.g. zona pellucida, and cytoplasm), but studies on their impact on fertilisation often offer contradictory results (Lemseffer *et al.*, 2022). Moreover, because quality is not uniform among oocytes, no one single indicator should be used to unilaterally inform overall oocyte quality (Homer, 2020). Some authors have suggested a correlation between oocyte quality and the expression of low molecular weight proteins belonging to the insulin growth factor family in follicular fluid. Some studies also show that the degree of apoptosis of cumulus cells surrounding the oocyte is negatively correlated with oocyte quality (Lemseffer *et al.*, 2022). In addition, biochemical markers related to mitochondria could be useful in understanding the state of oocyte maturation. Nonetheless, research remains very limited.

Due to their invasive nature, these indicators can only be available in the context of oocyte retrieval during an IVF procedure. In consequence, etiological studies based on these indicators could only be retrospective which can represent a significant hurdle to study the impact of highly-variable exposures such as non-persistent chemicals. This research represents nonetheless an important field to explore, especially since a certain proportion of idiopathic infertility cases could perhaps be comprised of women with a normal quantity but low-quality oocytes.

#### 4.4.7. The need for a pre-conceptional cohort

The majority of fertility-related epidemiological studies are typically conducted within fertility centres, where at least one member of the couple is experiencing infertility. Although fertile partners can be included as the study focus, this approach carries the risk of including participants with idiopathic infertility and may overlook confounding factors attributed to the source of infertility in the affected partner. This can also raise concerns of population selection, especially in countries where access to fertility centres is limited by financial means.

In addition, estimating infertility prevalence is difficult in the absence of studies amongst the general population. In France, only one 2007-2008 cross-sectional study (Slama *et al.*, 2012), called the

Observatory of Fecundity, made such estimations but by using a current-duration design (using the time elapsed between the start of the period of unprotected intercourse and the phone interview, and estimating the probability of pregnancy by inferring the underlying distribution). Using a pre-conceptional cohort design, i.e. following couples when they first start trying to conceive, would allow us to measure TTP more accurately and monitor the evolution of lifestyle factors across trimesters. Furthermore, it would become possible to sample blood, urine and other matrices to measure exposures at inclusion and at repeated times after inclusion. Lastly, this design could also provide an opportunity to research the environmental risk factors of miscarriages.

The costs associated with establishing such a cohort may be significant, but they need to be weighted against the potential benefits it could offer in terms of research opportunities for the population and its potential to guide public health policies. Alternatively, this model could be implemented exclusively online, similar to the American PRESTO cohort (Wise *et al.*, 2015) and Danish SNART-GRAVID (Mikkelsen *et al.*, 2009) e-preconception cohorts. Nonetheless, measuring the chemical exposome would become impossible in this case.

Moreover, recruitment could represent a significant hurdle to overcome but inspiration can be taken from the aforementioned cohorts. For instance, couples could be recruited by using the health appointment phone application following medical appointments, and partnerships with midwives and gynaecologists could help cast a wide net for recruitment. Building a pre-conceptional cohort specifically amongst existing children of mother-child cohorts such as the PELAGIE children whom are now reaching the age of the majority could also be an interesting prospect considering the amount of prenatal exposure information available, and may ease recruitment difficulties.

#### 4.4.8. Monitoring ovarian reserve time trends

In the study of the Observatory of Fecundity in France (Slama *et al.*, 2012), the authors recommended repeating this exercise regularly to monitor fertility time trends. They further suggested monitoring semen quality in parallel. While several studies have explored the time trends of semen parameters, none seem to have explored the time trends of the ovarian reserve. Nevertheless, starting such an endeavour in the general population could take at least a decade. A temporary approach could be to focus on ART centres that have decades worth of patient records including information on their ovarian reserve, but could pose problems of heterogeneity of ovarian reserve measurement tools.

## CONCLUSION

The objective of this thesis was to study the impact of EDCs on the ovarian reserve. To achieve this, we conducted studies in fertility centres and within the general population, evaluated EDCs individually and as mixture, included prenatal and postnatal exposures, and measured the ovarian reserve as continuous indicators as well as a clinical outcome.

Overall, we found limited evidence that EDCs could diminish the ovarian reserve. However, further studies are necessary to validate our results and explore the influence of EDCs during various susceptibility windows on the ovarian reserve at different life stages. These studies should aim to include larger populations to enhance study power, which may have been insufficient in our analyses. Beyond the topics of windows of vulnerability, latency from exposure and the role of mixtures, there are numerous unanswered questions surrounding EDCs. The biological mechanisms of EDCs observed on folliculogenesis in animal studies need to be elucidated. Efforts should be directed towards evaluating the impact of low doses, exploring non-traditional dose-responses, in particular for chemicals with short half-lives, and investigating under-studied chemicals. Moreover, the possible epigenetic effects of EDCs on the subsequent generations of exposed individuals should be investigated. Finally, new tools such as non-targeted methods and the meet-in-the-middle approach offer promising avenues of research.

While these questions are still lingering, coupled with the constant introduction of new chemicals with potential EDC effects into domestic or occupational products, particular attention should be brought to preventing exposure to EDCs. Training medical professionals, running population awareness campaigns, proposing product replacements, and treating waste containing EDCs in the environment, represent some of the tools available to accomplish this objective. Given the ubiquitous nature of EDCs and their detrimental effects on various health issues, possibly including the ovarian reserve, implementing measures to minimise exposure is essential for protecting human health and the environment.

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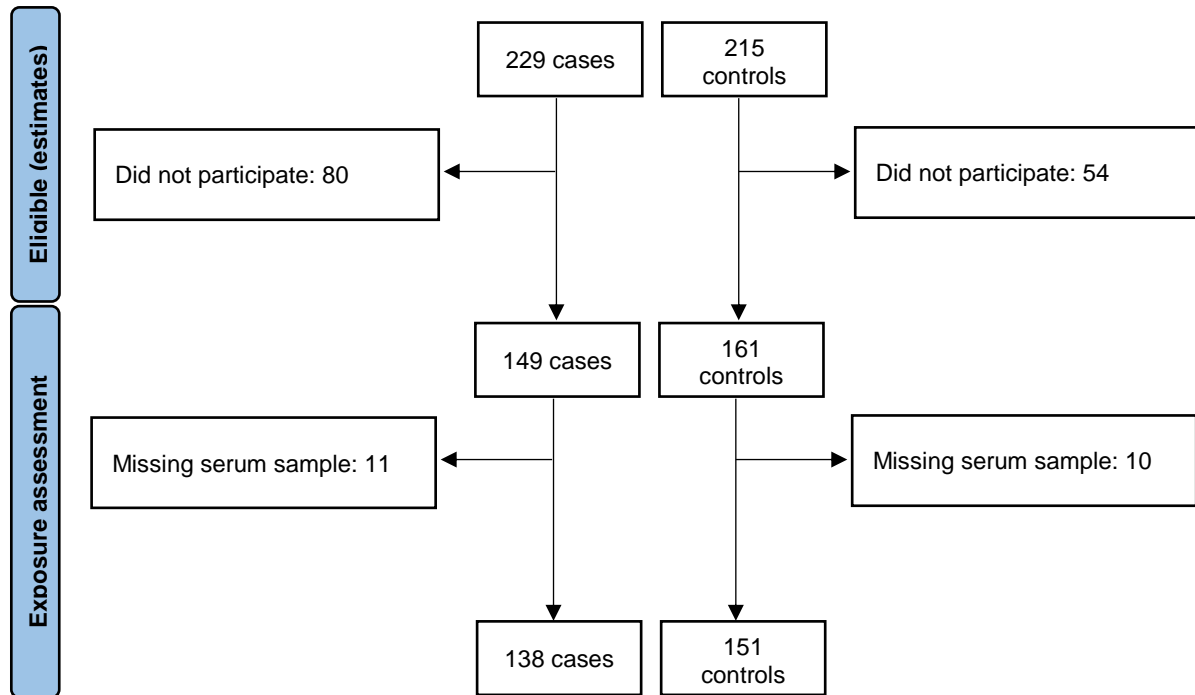
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## APPENDICES

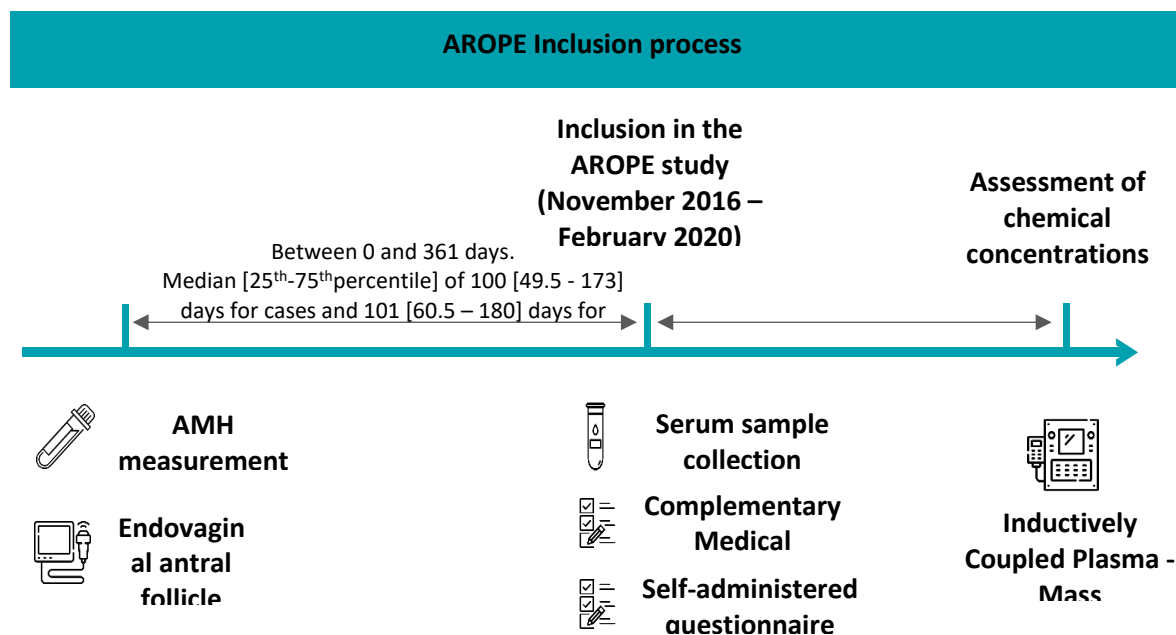
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Appendix 1. Supplementary material for article 1 “Serum persistent organic pollutants and diminished ovarian reserve: a single-exposure and mixture exposure approach from a French case-control study”.

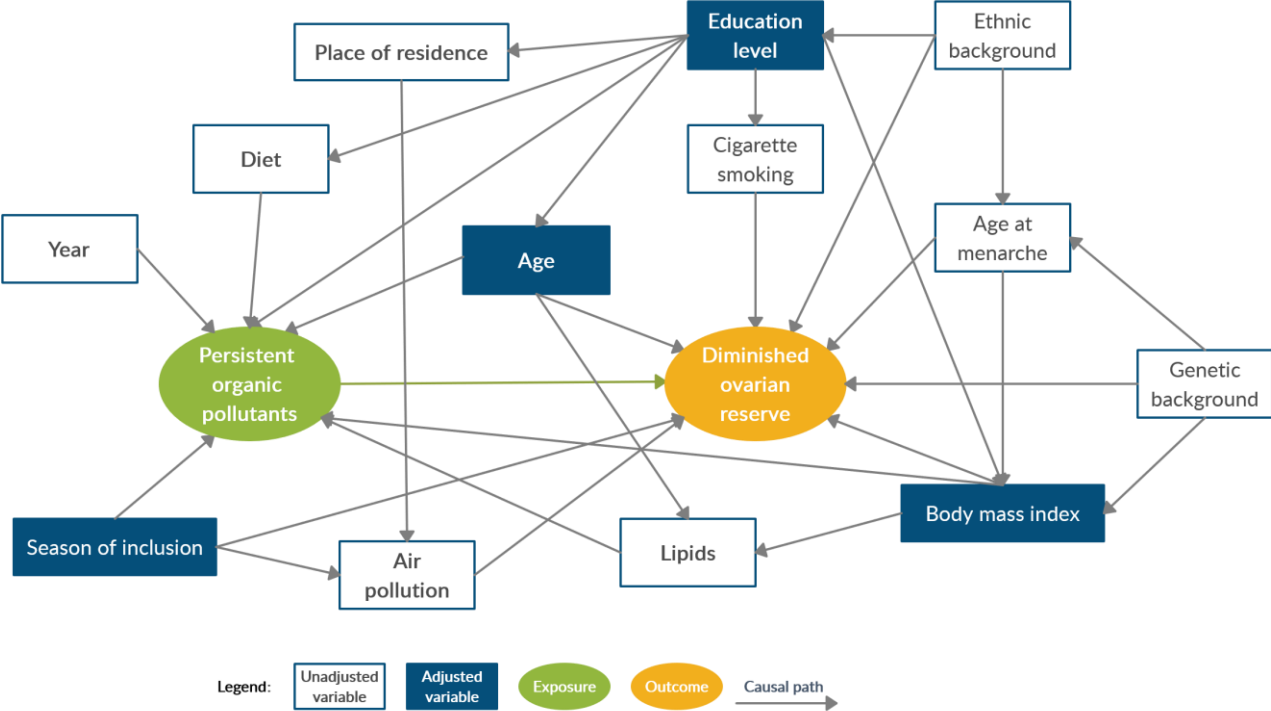
Supplementary Figure S1. AROPE study inclusion flowchart



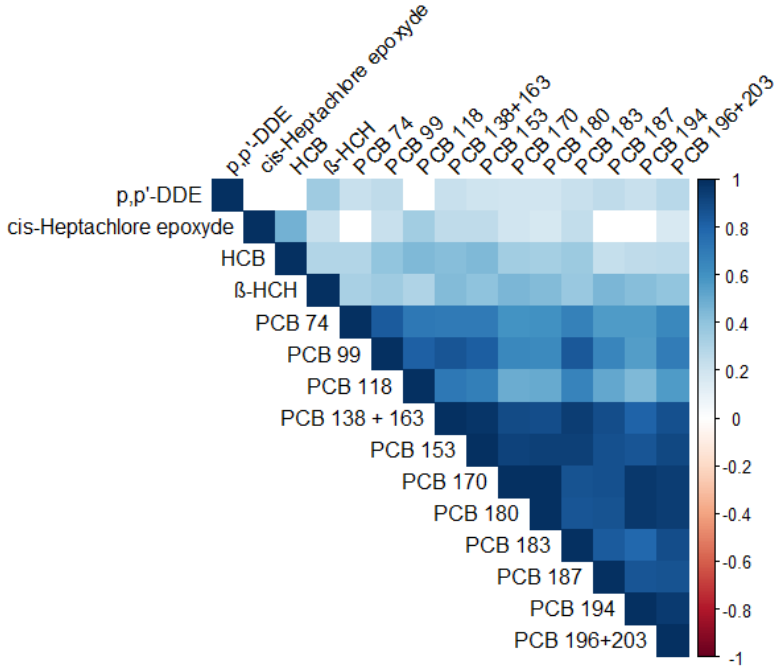
Supplementary Figure S2. AROPE study inclusion process



**Supplementary Figure S3. Directed acyclic graph between persistent organic pollutants and diminished ovarian reserve**



**Supplementary Figure S4. Spearman correlation plot for persistent organic pollutant with a detection frequency above 20% in the control population (N = 151)**



**Supplementary Table SI. Analytical characteristics of measured compounds**

Compound <sup>a</sup>	CAS number	Chemical family	ISTD	Time segment	t <sub>R</sub> (min)	Quantifier MRM transition Precursor > Product (CE (V))	Qualifier MRM transition Precursor > Product (CE (V))	LOD <sup>b</sup> (µg/L)	LOQ <sup>b</sup> (µg/L)	
<b>Target compounds</b>										
1	α-HCH	319-84-6	OCs	β-HCH <sup>13</sup> C <sub>6</sub>	2	8.8	216.9 > 181.0 (5)	180.9 > 145.0 (15)	0.0025	0.005
2	HCB	118-74-1	OCs	HCB <sup>13</sup> C <sub>6</sub>	2	8.8	283.8 > 213.9 (30)	281.8 > 211.9 (30)	0.0025	0.005
3	γ-HCH	58-89-9	OCs	γ-HCH <sup>13</sup> C <sub>6</sub>	2	9.1	216.9 > 181.0 (5)	181.0 > 145.0 (15)	0.0025	0.005
4	β-HCH	319-85-7	OCs	β-HCH <sup>13</sup> C <sub>6</sub>	3	9.4	216.9 > 181.1 (5)	181.0 > 145.0 (15)	0.0025	0.005
5	PCB 31	16606-02-3	PCBs	PCB 28 <sup>13</sup> C <sub>12</sub>	3	9.6	256.0 > 186.0 (25)	258.0 > 186.0 (25)	0.0025	0.005
6	PCB 28	7012-37-5	PCBs	PCB 28 <sup>13</sup> C <sub>12</sub>	3	9.6	256.0 > 186.0 (25)	258.0 > 186.0 (25)	0.0025	0.005
7	Heptachlor	76-44-8	OCs	cis-Heptachlor epoxide <sup>13</sup> C <sub>10</sub>	3	9.7	271.7 > 236.9 (15)	273.7 > 238.9 (15)	0.0025	0.005
8	PCB 52	35693-99-3	PCBs	PCB 52 <sup>13</sup> C <sub>12</sub>	3	9.8	289.9 > 219.9 (25)	291.9 > 221.9 (25)	0.0025	0.005
9	Aldrin	309-00-2	OCs	Aldrin <sup>13</sup> C <sub>12</sub>	3	10.0	262.9 > 192.9 (35)	254.9 > 220.0 (20)	0.025	0.05
10	cis-Heptachlor epoxide	1024-57-3	OCs	cis-Heptachlor epoxide <sup>13</sup> C <sub>10</sub>	4	10.5	352.8 > 262.9 (15)	354.8 > 264.9 (15)	0.005	0.01
11	PCB 74	32690-93-0	PCBs	PCB 52 <sup>13</sup> C <sub>12</sub>	4	10.5	289.9 > 219.9 (25)	291.9 > 221.9 (25)	0.0025	0.005
12	trans-Heptachlor epoxide	28044-83-9	OCs	cis-Heptachlor epoxide <sup>13</sup> C <sub>10</sub>	4	10.5	135.0 > 099.0 (15)	216.9 > 182.0 (20)	0.025	0.05
13	o,p'-DDE	3424-82-6	OCs	p,p'-DDE D <sub>8</sub>	4	10.6	246.0 > 176.2 (30)	248.0 > 176.2 (30)	0.0025	0.005
14	PCB 101	37680-73-2	PCBs	PCB 101 <sup>13</sup> C <sub>12</sub>	4	10.7	253.9 > 184.0 (35)	325.9 > 255.9 (30)	0.0025	0.005
15	PCB 99	38380-01-7	PCBs	PCB 101 <sup>13</sup> C <sub>12</sub>	4	10.7	253.9 > 184.0 (40)	325.9 > 255.9 (25)	0.0025	0.005
16	α-Endosulfan	959-98-8	OCs	α-Endosulfan D <sub>4</sub>	5	10.9	194.9 > 159.0 (5)	194.9 > 125.0 (20)	0.025	0.05
17	p,p'-DDE	72-55-9	OCs	p,p'-DDE D <sub>8</sub>	5	10.9	246.1 > 176.2 (30)	315.8 > 246.0 (15)	0.01	0.02
18	Dieldrin	60-57-1	OCs	Dieldrin <sup>13</sup> C <sub>12</sub>	6	11.2	277.0 > 241.0 (5)	262.9 > 193.0 (35)	0.025	0.05
19	o,p'-DDT	789-02-6	OCs	o,p'-DDT <sup>13</sup> C <sub>12</sub>	7	11.3	235.0 > 165.2 (20)	237.0 > 165.2 (20)	0.025	0.05
20	PCB 118	31508-00-6	PCBs	PCB 118 <sup>13</sup> C <sub>12</sub>	7	11.4	253.9 > 184.0 (35)	325.9 > 255.9 (30)	0.0025	0.005
21	BDE 28	41318-75-6	PBDEs	BDE 28 <sup>13</sup> C <sub>12</sub>	7	11.5	246.0 > 139.0 (30)	406.0 > 246.0 (20)	0.0025	0.005
22	PCB 153	35065-27-1	PCBs	PCB 153 <sup>13</sup> C <sub>12</sub>	7	11.5	359.9 > 289.9 (25)	287.9 > 217.9 (40)	0.0025	0.005
23	β-Endosulfan	33213-65-9	OCs	β-Endosulfan <sup>13</sup> C <sub>9</sub>	8	11.7	206.9 > 172.0 (15)	194.9 > 158.9 (10)	0.025	0.05
24	p,p'-DDT	50-29-3	OCs	p,p'-DDT <sup>13</sup> C <sub>12</sub>	8	11.8	235.0 > 165.2 (20)	235.0 > 199.2 (15)	0.005	0.01
25	PCB 138 + PCB 163	35065-28-2 + 74472-44-9	PCBs	PCB 138 <sup>13</sup> C <sub>12</sub>	8	11.8	359.9 > 289.9 (30)	287.9 > 217.9 (40)	0.0025	0.005
26	PCB 187	52663-68-0	PCBs	PCB 138 <sup>13</sup> C <sub>12</sub>	8	11.9	393.8 > 323.8 (30)	395.8 > 360.8 (15)	0.0025	0.005
27	PCB 183	52663-69-1	PCBs	PCB 180 <sup>13</sup> C <sub>12</sub>	8	11.9	393.8 > 323.8 (30)	323.8 > 253.8 (40)	0.0025	0.005
28	PCB 180	35065-29-3	PCBs	PCB 180 <sup>13</sup> C <sub>12</sub>	9	12.5	393.8 > 323.8 (30)	395.8 > 325.8 (30)	0.0025	0.005
29	BDE 47	5436-43-1	PBDEs	BDE 47 <sup>13</sup> C <sub>12</sub>	10	12.8	325.8 > 216.8 (30)	485.7 > 325.7 (20)	0.005	0.01
30	α + β + γ-HBCD	134237-50-6 + 134237-51-7 + 134237-52-8	HBCDs	BDE 47 <sup>13</sup> C <sub>12</sub>	10	12.9	238.9 > 159.1 (5)	320.9 > 159.1 (8)	2.5	5
31	PCB 170	35065-30-6	PCBs	PCB 180 <sup>13</sup> C <sub>12</sub>	10	13.0	393.8 > 323.8 (30)	395.8 > 360.8 (15)	0.0025	0.005
32	PCB 196 + PCB 203	42740-50-1 + 52663-76-0	PCBs	PCB 194 <sup>13</sup> C <sub>12</sub>	10	13.0	427.8 > 357.8 (30)	429.8 > 359.8 (30)	0.0025	0.005
33	BDE 66	189084-61-5	PBDEs	BDE 47 <sup>13</sup> C <sub>12</sub>	10	13.1	326.0 > 217.0 (30)	486.0 > 326.0 (20)	0.005	0.01
34	PCB 194	35694-08-7	PCBs	PCB 194 <sup>13</sup> C <sub>12</sub>	11	13.9	427.8 > 357.8 (30)	429.8 > 394.8 (15)	0.0025	0.005
35	BDE 100	189084-64-8	PBDEs	BDE 100 <sup>13</sup> C <sub>12</sub>	12	14.1	405.8 > 296.7 (35)	565.5 > 405.6 (20)	0.005	0.01
36	BDE 99	60348-60-9	PBDEs	BDE 99 <sup>13</sup> C <sub>12</sub>	13	14.6	565.6 > 405.6 (20)	405.7 > 296.7 (35)	0.005	0.01
37	BDE 85	182346-21-0	PBDEs	BDE 99 <sup>13</sup> C <sub>12</sub>	14	16.0	406.0 > 297.0 (35)	564.0 > 404.0 (25)	0.125	0.25
38	BDE 154	207122-15-4	PBDEs	BDE 154 <sup>13</sup> C <sub>12</sub>	14	16.1	643.6 > 483.6 (25)	483.6 > 376.8 (35)	0.025	0.05
39	PBB 153	59080-40-9	PBBs	BDE 154 <sup>13</sup> C <sub>12</sub>	14	16.4	467.6 > 307.6 (35)	307.7 > 147.8 (30)	0.025	0.05

	Compound <sup>a</sup>	CAS number	Chemical family	ISTD	Time segment	t <sub>R</sub> (min)	Quantifier MRM transition Precursor > Product (CE (V))	Qualifier MRM transition Precursor > Product (CE (V))	LOD <sup>b</sup> (µg/L)	LOQ <sup>b</sup> (µg/L)
40	BDE 153	68631-49-2	PBDEs	BDE 153 <sup>13</sup> C <sub>12</sub>	15	17.3	483.7 > 323.6 (40)	643.6 > 483.6 (20)	0.125	0.25
41	BDE 183	207122-16-5	PBDEs	BDE 183 <sup>13</sup> C <sub>12</sub>	16	21.7	721.8 > 561.7 (20)	561.7 > 454.6 (35)	2.5	5
<b>Labeled ISTDs</b>										
a	HCB <sup>13</sup> C <sub>6</sub>	93952-14-8	OCs		2	8.8	289.8 > 219.8 (35)	-		
b	γ-HCH <sup>13</sup> C <sub>6</sub>	58-89-9	OCs		2	9.1	186.8 > 151.0 (15)	-		
c	β-HCH <sup>13</sup> C <sub>6</sub>	222966-68-9	OCs		3	9.4	224.8 > 189.0 (10)	-		
d	PCB 28 <sup>13</sup> C <sub>12</sub>	208263-76-7	PCBs		3	9.6	267.9 > 198.1 (30)	-		
e	PCB 52 <sup>13</sup> C <sub>12</sub>	208263-80-3	PCBs		3	9.8	301.9 > 232.1 (30)	-		
f	Aldrin <sup>13</sup> C <sub>12</sub>	n/a	OCs		3	10.0	268.0 > 198.0 (35)	-		
g	cis-Heptachlor epoxide <sup>13</sup> C <sub>10</sub>	1024-57-3	OCs		4	10.5	363.0 > 269.9 (15)	-		
h	PCB 101 <sup>13</sup> C <sub>12</sub>	104130-39-4	PCBs		4	10.7	265.9 > 196.0 (35)	-		
i	α-Endosulfan D <sub>4</sub>	203645-57-2	OCs		5	10.9	163.9 > 129.0 (15)	-		
j	p,p'-DDE D <sub>8</sub>	93952-19-3	OCs		5	10.9	254.1 > 184.1 (35)	-		
k	Dieldrin <sup>13</sup> C <sub>12</sub>	475274-96-5	OCs		6	11.2	284.8 > 213.9 (30)	-		
l	o,p'-DDT <sup>13</sup> C <sub>12</sub>	1396995-26-8	OCs		7	11.3	249.0 > 177.1 (28)	-		
m	PCB 118 <sup>13</sup> C <sub>12</sub>	104130-40-7	PCBs		7	11.4	335.9 > 266.0 (35)	-		
n	BDE 28 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		7	11.5	257.9 > 150.1 (35)	-		
o	PCB 153 <sup>13</sup> C <sub>12</sub>	185376-58-3	PCBs		7	11.5	371.8 > 302.0 (32)	-		
p	β-Endosulfan <sup>13</sup> C <sub>9</sub>	n/a	OCs		8	11.7	202.0 > 166.0 (10)	-		
q	p,p'-DDT <sup>13</sup> C <sub>12</sub>	104215-84-1	OCs		8	11.8	249.0 > 177.1 (25)	-		
r	PCB 138 <sup>13</sup> C <sub>12</sub>	208263-66-5	PCBs		8	11.8	301.9 > 232.0 (40)	-		
s	PCB 180 <sup>13</sup> C <sub>12</sub>	160901-82-6	PCBs		9	12.5	405.8 > 336.0 (35)	-		
t	BDE 47 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		10	12.8	337.9 > 230.0 (32)	-		
u	PCB 194 <sup>13</sup> C <sub>12</sub>	208263-74-5	PCBs		11	13.9	441.8 > 371.9 (35)	-		
v	BDE 100 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		12	14.1	575.7 > 415.9 (25)	-		
w	BDE 99 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		13	14.6	415.8 > 307.9 (38)	-		
x	BDE 154 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		14	16.1	655.7 > 495.8 (25)	-		
y	BDE 153 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		15	17.3	655.7 > 495.8 (25)	-		
z	BDE 183 <sup>13</sup> C <sub>12</sub>	n/a	PBDEs		16	21.7	573.9 > 465.8 (40)	-		

<sup>a</sup> Compounds listed in order of retention times; <sup>b</sup> for a 2-mL sample of serum

**Supplementary Table SII. POP concentrations (ng/L) in the standard reference materials (SRMs) 1957 and 1958 (N = 7)**

Compound <sup>a</sup>	LOD (ng/L)	LOQ (ng/L)	POP concentrations (ng/L) in SRM 1957 (n = 7)			POP concentrations (ng/L) in SRM 1958 (n = 7)		
			Measured (RSD %)	Reported	Measured/ reported (%)	Measured (RSD %)	Reported	Measured/ reported (%)
α-HCH	2.5	5.0	< 5.0	n.r.	-	224 (10)	260 <sup>c</sup>	86
HCB	2.5	5.0	31.4 (15)	29.7 <sup>b</sup>	106	393 (15)	442 <sup>b</sup>	89
γ-HCH	2.5	5.0	< 5.0	n.r.	-	231 (9)	315 <sup>c</sup>	73
β-HCH	2.5	5.0	26.6 (8)	31.3 <sup>c</sup>	85	243 (4)	278 <sup>c</sup>	87
PCB 28	2.5	5.0	7.44 (20)	8.6 <sup>c</sup>	87	403 (3)	402 <sup>b</sup>	100
PCB 52	2.5	5.0	< 5.0	n.r.	-	416 (4)	401 <sup>b</sup>	104
Aldrin	25	50	< 50	n.r.	-	373 (5)	n.r.	-
cis-Heptachlor epoxide	5.0	10	14.8 (6)	n.r.	-	392 (5)	n.r.	-
PCB 74	2.5	5.0	11.8 (5)	13.8 <sup>c</sup>	86	394 (3)	414 <sup>b</sup>	95
o,p'-DDE	2.5	5.0	< 5.0	n.r.	-	460 (6)	450 <sup>b</sup>	102
PCB 101	2.5	5.0	< 5.0	n.r.	-	385 (4)	409 <sup>b</sup>	94
PCB 99	2.5	5.0	10.1 (11)	11.6 <sup>c</sup>	87	373 (4)	385 <sup>b</sup>	97
p,p'-DDE	10	20	928 (3)	921 <sup>b</sup>	101	1280 (4)	1250 <sup>b</sup>	102
Dieldrin	25	50	27.6 (19)	n.r.	-	483 (7)	n.r.	-
o,p'-DDT	25	50	< 50	n.r.	-	253 (3)	313 <sup>b</sup>	81
PCB 118	2.5	5.0	15.8 (7)	18.9 <sup>b</sup>	84	407 (5)	412 <sup>b</sup>	99
BDE 28	2.5	5.0	17.6 (21)	20.0 <sup>c</sup>	88	442 (15)	462 <sup>b</sup>	96
PCB 153	2.5	5.0	59.5 (4)	58.2 <sup>b</sup>	102	444 (5)	457 <sup>b</sup>	97
p,p'-DDT	5.0	10	< 10	n.r.	-	145 (4)	293 <sup>b</sup>	49
PCB 138 + PCB 163	2.5	5.0	43.4 (3)	36.9 <sup>b</sup>	118	451 (8)	473 <sup>b</sup>	95
PCB 187	2.5	5.0	12.6 (8)	15.5 <sup>b</sup>	82	357 (8)	411 <sup>b</sup>	87
PCB 183	2.5	5.0	4.49 (8)	5.77 <sup>c</sup>	78	355 (7)	407 <sup>b</sup>	87
PCB 180	2.5	5.0	49.1 (3)	54.5 <sup>b</sup>	90	431 (4)	459 <sup>b</sup>	94
BDE 47	5.0	10	237 (8)	268 <sup>b</sup>	88	577 (6)	651 <sup>b</sup>	89
PCB 170	2.5	5.0	14.3 (11)	16.2 <sup>b</sup>	88	395 (12)	422 <sup>b</sup>	94
PCB 196 + PCB 203	2.5	5.0	10.1 (6)	10.9 <sup>c</sup>	93	726 (9)	795 <sup>b</sup>	91
BDE 66	5.0	10	< 10	6.7 <sup>c</sup>	-	388 (7)	440 <sup>b</sup>	88
PCB 194	2.5	5.0	9.10 (6)	11.9 <sup>b</sup>	76	349 (8)	387 <sup>b</sup>	90
BDE 100	5.0	10	43.9 (26)	49.7 <sup>b</sup>	88	375 (7)	475 <sup>b</sup>	79
BDE 99	5.0	10	69.0 (28)	76.0 <sup>b</sup>	91	404 (7)	492 <sup>b</sup>	82
BDE 85	125	250	< 250	8.2 <sup>c</sup>	-	493 (14)	475 <sup>b</sup>	104
BDE 154	25	50	< 50	7.0 <sup>c</sup>	-	270 (6)	441 <sup>b</sup>	61
PBB 153	25	50	< 50	15.5 <sup>b</sup>	-	421 (10)	421 <sup>b</sup>	100
BDE 153	125	250	< 250	61.0 <sup>b</sup>	-	387 (6)	455 <sup>b</sup>	85
BDE 183	2500	5000	< 5000	n.r.	-	< 5000	453 <sup>b</sup>	-

n.r., not reported; <sup>a</sup> Compounds listed in order of retention times; <sup>b</sup> Certified concentration from the certificate of analysis of the Standard Reference Material; <sup>c</sup> Reference concentration from the certificate of analysis of the Standard Reference Material.

**Supplementary Material SIII. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – Cases = AFC < 7 & AMH ≤ 1.1 ng/ml (N = 51 cases & 151 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 51)	Number of controls (N = 151)	Diminished Ovarian Reserve Multivariate <sup>1</sup> logistic regression	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 22.39	10	51	Ref.	Ref.
	]22.39-40.74]	21	50	1.45	0.56 - 3.73
	> 40.74	20	50	1.47	0.55 - 3.92
	Continuous	51	151	1.44	1.05 - 1.98
p,p'-DDT	< LOD	21	51	Ref.	Ref.
	≥ LOD	30	100	0.94	0.43 - 2.04
cis-Heptachlore epoxyde	≤ 1.71	17	51	Ref.	Ref.
	]1.71-2.42]	17	50	0.72	0.30 - 1.74
	> 2.42	11	51	0.82	0.34 - 1.97
	Continuous	51	151	0.97	0.50 - 1.88
HCB	≤ 6.43	11	51	Ref.	Ref.
	]6.43-8.42]	16	50	1.04	0.39 - 2.78
	> 8.42	24	50	1.54	0.58 - 4.15
β-HCH	Continuous	51	151	1.60	0.69 - 3.70
	≤ 3.62	15	51	Ref.	Ref.
	]3.62-5.77]	22	50	1.11	0.47 - 2.61
	> 5.77	14	50	0.48	0.18 - 1.31
Continuous		51	151	0.58	0.33 - 1.00
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.41	15	49	Ref.	Ref.
	]0.41-0.60]	14	51	0.75	0.21 - 2.76
	> 0.60	22	51	1.45	0.57 - 3.70
	Continuous	51	151	0.94	0.58 - 1.52
PCB 74	≤ 1.65	11	51	Ref.	Ref.
	]1.65-2.52]	18	49	1.16	0.45 - 3.01
	> 2.52	22	51	1.32	0.50 - 3.44
PCB 99	Continuous	51	151	1.04	0.67 - 1.61
	≤ 1.82	12	51	Ref.	Ref.
	]1.82-2.69]	16	50	1.26	0.49 - 3.23
	> 2.69	23	50	1.80	0.72 - 4.47
PCB 118	Continuous	51	151	1.35	0.80 - 2.26
	≤ 3.45	8	51	Ref.	Ref.
	]3.45-5.50]	21	50	2.73	0.97 - 7.68
	> 5.50	22	50	2.60	0.91 - 7.39



PCB 138+163 <sup>3</sup>	Continuous	51	151	1.21	0.72 - 2.03
	≤ 3.71	12	51	Ref.	Ref.
	]3.71-6.44]	14	50	1.07	0.40 - 2.86
	> 6.44	25	50	1.52	0.50 - 4.59
PCB 153	Continuous	51	151	1.19	0.69 - 2.07
	≤ 16.36	10	51	Ref.	Ref.
	]16.36-26.21]	18	50	1.64	0.60 - 4.49
	> 26.21	23	50	1.87	0.64 - 5.44
PCB 170	Continuous	51	151	1.33	0.73 - 2.42
	≤ 11.34	11	51	Ref.	Ref.
	]11.34-16.43]	11	50	0.84	0.30 - 2.34
	> 16.43	29	50	2.29	0.85 - 6.17
PCB 180	Continuous	51	151	1.22	0.69 - 2.15
	≤ 10.85	8	51	Ref.	Ref.
	]10.85-18.91]	19	50	2.13	0.73 - 6.19
	> 18.91	24	50	2.33	0.68 - 8.03
PCB 183	Continuous	51	151	1.36	0.77 - 2.40
	≤ 0.94	10	51	Ref.	Ref.
	]0.94-1.52]	18	51	1.54	0.58 - 4.09
	> 1.52	23	49	1.78	0.62 - 5.05
PCB 187	Continuous	51	151	1.41	0.80 - 2.48
	≤ 2.06	15	52	Ref.	Ref.
	]2.06-3.54]	12	52	0.84	0.29 - 2.41
	> 3.54	24	47	2.09	0.78 - 5.62
PCB 194	Continuous	51	151	1.40	0.91 - 2.15
	≤ 1.23	10	52	Ref.	Ref.
	]1.23-2.38]	19	49	1.46	0.52 - 4.08
	> 2.38	22	50	1.53	0.47 - 4.96
PCB 196+203 <sup>3</sup>	Continuous	51	151	1.41	0.83 - 2.40
	≤ 1.12	9	51	Ref.	Ref.
	]1.12-1.89]	18	51	1.80	0.62 - 5.20
	> 1.89	24	49	2.36	0.73 - 7.67
	Continuous	51	151	1.49	0.86 - 2.58

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>Log2-transformed. Continuous analyses were only performed when linearity was respected.

<sup>3</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material SIV. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – Cases = AFC < 7 (N = 55 cases & 151 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 55)	Number of controls (N = 151)	Diminished Ovarian Reserve Multivariate <sup>1</sup> logistic regression	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 22.39	11	51	Ref.	Ref.
	]22.39-40.74]	23	50	1.49	0.60 - 3.68
	> 40.74	21	50	1.42	0.55 - 3.67
	Continuous	55	151	1.43	1.05 - 1.94
p,p'-DDT	< LOD	22	51	Ref.	Ref.
	≥ LOD	33	100	0.93	0.43 - 1.98
cis-Heptachlore epoxyde	≤ 1.71	18	50	Ref.	Ref.
	]1.71-2.42]	18	51	0.77	0.33 - 1.81
	> 2.42	19	50	0.92	0.40 - 2.11
	Continuous	55	151	1.17	0.47 - 2.92
HCB	≤ 6.43	11	51	Ref.	Ref.
	]6.43-8.42]	19	50	1.28	0.49 - 3.32
	> 8.42	25	50	1.64	0.62 - 4.33
	Continuous	55	151	1.51	0.67 - 3.40
β-HCH	≤ 3.62	15	51	Ref.	Ref.
	]3.62-5.77]	26	50	1.35	0.59 - 3.10
	> 5.77	14	50	0.47	0.18 - 1.26
	Continuous	55	151	0.57	0.34 - 0.98
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.41	17	49	Ref.	Ref.
	]0.41-0.60]	15	51	0.76	0.22 - 2.62
	> 0.60	23	51	1.36	0.55 - 3.38
	Continuous	55	151	0.94	0.58 - 1.52
PCB 74	≤ 1.65	12	51	Ref.	Ref.
	]1.65-2.52]	20	49	1.18	0.47 - 2.94
	> 2.52	23	51	1.26	0.50 - 3.20
	Continuous	55	151	0.98	0.54 - 1.81
PCB 99	≤ 1.82	13	51	Ref.	Ref.
	]1.82-2.69]	18	50	1.26	0.52 - 3.06
	> 2.69	24	50	1.71	0.71 - 4.13
	Continuous	55	151	1.46	0.71 - 3.00
PCB 118	≤ 3.45	8	51	Ref.	Ref.
	]3.45-5.50]	24	50	2.98	1.09 - 8.11
	> 5.50	23	50	2.54	0.91 - 7.07
	Continuous	55	151	1.18	0.72 - 1.96

PCB 138+163 <sup>3</sup>	≤ 3.71	13	51	Ref.	Ref.
	]3.71-6.44]	16	50	1.07	0.42 - 2.73
	> 6.44	26	50	1.31	0.45 - 3.82
	Continuous	55	151	1.08	0.63 - 1.86
PCB 153	≤ 16.36	10	51	Ref.	Ref.
	]16.36-26.21]	22	50	1.94	0.74 - 5.11
	> 26.21	23	50	1.72	0.60 - 4.93
	Continuous	55	151	1.21	0.67 - 2.16
PCB 170	≤ 11.34	11	51	Ref.	Ref.
	]11.34-16.43]	14	50	1.07	0.40 - 2.82
	> 16.43	30	50	2.24	0.85 - 5.90
	Continuous	55	151	1.13	0.65 - 1.95
PCB 180	≤ 10.85	9	51	Ref.	Ref.
	]10.85-18.91]	22	50	2.00	0.73 - 5.49
	> 18.91	24	50	1.76	0.54 - 5.72
	Continuous	55	151	1.22	0.70 - 2.13
PCB 183	≤ 0.94	10	51	Ref.	Ref.
	]0.94-1.52]	22	51	1.85	0.72 - 4.74
	> 1.52	23	49	1.67	0.60 - 4.65
	Continuous	55	151	1.56	0.70 - 3.47
PCB 187	≤ 2.06	16	52	Ref.	Ref.
	]2.06-3.54]	14	52	0.89	0.33 - 2.40
	> 3.54	25	47	1.91	0.74 - 4.94
	Continuous	55	151	1.47	0.80 - 2.70
PCB 194	≤ 1.23	11	52	Ref.	Ref.
	]1.23-2.38]	22	49	1.49	0.56 - 3.98
	> 2.38	22	50	1.28	0.41 - 4.00
	Continuous	55	151	1.46	0.69 - 3.09
PCB 196+203 <sup>3</sup>	≤ 1.12	9	51	Ref.	Ref.
	]1.12-1.89]	21	51	2.03	0.72 - 5.70
	> 1.89	25	49	2.41	0.76 - 7.68
	Continuous	55	151	1.55	0.71 - 3.36

<sup>1</sup>Adjusted for age, body mass-index, centre, education level, and season of inclusion.

<sup>2</sup>Log2-transformed. Continuous analyses were only performed when linearity was respected.

<sup>3</sup>These POPs could not be distinguished during the measurement process.

Abbreviations: OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material SV. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – Cases = AMH ≤ 1.1 ng/ml (N = 134 cases & 151 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 134)	Number of controls (N = 151)	Diminished Ovarian Reserve Multivariate <sup>1</sup> logistic regression	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 22.39	28	51	Ref.	Ref.
	]22.39-40.74]	49	50	1.53	0.77 - 3.04
	> 40.74	57	50	1.90	0.96 - 3.76
	Continuous	134	151	1.39	1.09 - 1.77
p,p'-DDT	< LOD	51	51	Ref.	Ref.
	≥ LOD	83	100	1.14	0.67 - 1.96
cis-Heptachlore epoxyde	≤ 1.71	52	50	Ref.	Ref.
	]1.71-2.42]	49	51	0.84	0.46 - 1.53
	> 2.42	33	50	0.63	0.33 - 1.17
	Continuous	134	151	0.82	0.52 - 1.30
HCB	≤ 6.43	38	51	Ref.	Ref.
	]6.43-8.42]	51	50	0.82	0.52 - 1.29
	> 8.42	45	50	1.03	0.54 - 1.94
	Continuous	134	151	0.92	0.46 - 1.84
β-HCH	≤ 3.62	49	51	Ref.	Ref.
	]3.62-5.77]	49	50	0.74	0.40 - 1.36
	> 5.77	36	50	0.47	0.24 - 0.93
	Continuous	134	151	0.63	0.44 - 0.89
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.41	39	49	Ref.	Ref.
	]0.41-0.60]	44	51	0.99	0.44 - 2.25
	> 0.60	51	51	1.32	0.66 - 2.63
	Continuous	134	151	1.02	0.72 - 1.43
PCB 74	≤ 1.65	38	51	Ref.	Ref.
	]1.65-2.52]	46	49	0.99	0.52 - 1.89
	> 2.52	50	51	0.94	0.48 - 1.83
PCB 99	Continuous	134	151	0.85	0.62 - 1.17
	≤ 1.82	34	51	Ref.	Ref.
	]1.82-2.69]	41	50	1.26	0.65 - 2.44
PCB 118	> 2.69	59	50	1.62	0.84 - 3.12
	Continuous	134	151	1.04	0.72 - 1.51
	≤ 3.45	33	51	Ref.	Ref.

	]3.45-5.50]	52	50	1.43	0.75 - 2.71
	> 5.50	49	50	1.26	0.64 - 2.49
PCB 138+163 <sup>3</sup>	Continuous	134	151	0.95	0.65 - 1.38
	≤ 3.71	32	51	Ref.	Ref.
	]3.71-6.44]	40	50	1.09	0.54 - 2.20
	> 6.44	62	50	1.51	0.68 - 3.35
PCB 153	Continuous	134	151	0.99	0.67 - 1.48
	≤ 16.36	35	51	Ref.	Ref.
	]16.36-26.21]	46	50	1.05	0.53 - 2.09
	> 26.21	53	50	1.11	0.52 - 2.34
PCB 170	Continuous	134	151	1.04	0.67 - 1.60
	≤ 11.34	35	51	Ref.	Ref.
	]11.34-16.43]	30	50	0.70	0.35 - 1.40
	> 16.43	69	50	1.68	0.83 - 3.39
PCB 180	Continuous	134	151	1.03	0.68 - 1.56
	≤ 10.85	31	51	Ref.	Ref.
	]10.85-18.91]	42	50	1.11	0.55 - 2.26
	> 18.91	61	50	1.43	0.64 - 3.19
PCB 183	Continuous	134	151	1.04	0.69 - 1.56
	≤ 0.94	30	51	Ref.	Ref.
	]0.94-1.52]	47	51	1.36	0.69 - 2.69
	> 1.52	57	49	1.57	0.75 - 3.27
PCB 187	Continuous	134	151	1.23	0.82 - 1.84
	≤ 2.06	39	52	Ref.	Ref.
	]2.06-3.54]	39	52	1.00	0.49 - 2.03
	> 3.54	56	47	1.49	0.73 - 3.04
PCB 194	Continuous	134	151	1.09	0.79 - 1.50
	≤ 1.23	29	52	Ref.	Ref.
	]1.23-2.38]	56	49	1.47	0.72 - 3.00
	> 2.38	49	50	1.02	0.43 - 2.40
PCB 196+203 <sup>3</sup>	Continuous	134	151	1.11	0.75 - 1.64
	≤ 1.12	29	51	Ref.	Ref.
	]1.12-1.89]	50	51	1.46	0.70 - 3.01
	> 1.89	55	49	1.51	0.66 - 3.45
	Continuous	134	151	1.19	0.80 - 1.78

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>Log2-transformed. Continuous analyses were only performed when linearity was respected.

<sup>3</sup>These POPs could not be distinguished during the measurement process.

Abbreviations: OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material SVI. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – Cases = AFC < 5 and/or AMH ≤ 0.7 ng/ml (N = 90 cases & 151 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 90)	Number of controls (N = 151)	Diminished Ovarian Response Multivariate <sup>1</sup> logistic regression	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 22.39	18	51	Ref.	Ref.
	]22.39-40.74]	37	50	1.61	0.75 - 3.48
	> 40.74	35	50	1.78	0.80 - 3.97
	Continuous	90	151	1.45	1.11 - 1.90
p,p'-DDT	< LOD	30	51	Ref.	Ref.
	≥ LOD	60	100	0.89	0.47 - 1.68
cis-Heptachlore epoxyde	≤ 1.71	35	50	Ref.	Ref.
	]1.71-2.42]	29	51	0.73	0.37 - 1.45
	> 2.42	26	50	0.71	0.35 - 1.41
	Continuous	90	151	0.93	0.56 - 1.54
HCB	≤ 6.43	25	51	Ref.	Ref.
	]6.43-8.42]	29	50	0.90	0.44 - 1.87
	> 8.42	36	50	1.09	0.51 - 2.34
	Continuous	90	151	1.03	0.54 - 1.95
β-HCH	≤ 3.62	33	51	Ref.	Ref.
	]3.62-5.77]	35	50	0.76	0.38 - 1.51
	> 5.77	22	50	0.38	0.17 - 0.82
	Continuous	90	151	0.50	0.32 - 0.76
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.41	25	49	Ref.	Ref.
	]0.41-0.60]	29	51	1.07	0.39 - 2.92
	> 0.60	36	51	1.41	0.63 - 3.14
	Continuous	90	151	1.03	0.70 - 1.50
PCB 74	≤ 1.65	22	51	Ref.	Ref.
	]1.65-2.52]	33	49	1.19	0.57 - 2.48
	> 2.52	35	51	1.12	0.52 - 2.41
	Continuous	90	151	0.90	0.63 - 1.29
PCB 99	≤ 1.82	22	51	Ref.	Ref.
	]1.82-2.69]	30	50	1.44	0.68 - 3.01
	> 2.69	38	50	1.65	0.78 - 3.50
	Continuous	90	151	1.05	0.69 - 1.59
PCB 118	≤ 3.45	18	51	Ref.	Ref.
	]3.45-5.50]	38	50	1.84	0.88 - 3.86
	> 5.50	34	50	1.51	0.70 - 3.28

PCB 138+163 <sup>3</sup>	Continuous	90	151	0.96	0.63 - 1.46
	≤ 3.71	24	51	Ref.	Ref.
	]3.71-6.44]	23	50	0.81	0.37 - 1.78
	> 6.44	43	50	1.30	0.54 - 3.13
PCB 153	Continuous	90	151	1.03	0.67 - 1.58
	≤ 16.36	22	51	Ref.	Ref.
	]16.36-26.21]	32	50	1.22	0.57 - 2.62
	> 26.21	36	50	1.15	0.49 - 2.67
PCB 170	Continuous	90	151	1.08	0.67 - 1.74
	≤ 11.34	21	51	Ref.	Ref.
	]11.34-16.43]	26	50	1.03	0.48 - 2.21
	> 16.43	43	50	1.65	0.74 - 3.68
PCB 180	Continuous	90	151	1.05	0.67 - 1.66
	≤ 10.85	21	51	Ref.	Ref.
	]10.85-18.91]	28	50	1.04	0.47 - 2.30
	> 18.91	41	50	1.28	0.51 - 3.19
PCB 183	Continuous	90	151	1.10	0.71 - 1.70
	≤ 0.94	20	51	Ref.	Ref.
	]0.94-1.52]	33	51	1.46	0.68 - 3.11
	> 1.52	37	49	1.45	0.63 - 3.33
PCB 187	Continuous	90	151	1.21	0.77 - 1.89
	≤ 2.06	27	52	Ref.	Ref.
	]2.06-3.54]	29	52	1.06	0.48 - 2.35
	> 3.54	34	47	1.38	0.61 - 3.09
PCB 194	Continuous	90	151	1.11	0.78 - 1.58
	≤ 1.23	21	52	Ref.	Ref.
	]1.23-2.38]	35	49	1.20	0.54 - 2.65
	> 2.38	34	50	0.97	0.38 - 2.48
PCB 196+203 <sup>3</sup>	Continuous	90	151	1.16	0.76 - 1.77
	≤ 1.12	20	51	Ref.	Ref.
	]1.12-1.89]	33	51	1.30	0.58 - 2.92
	> 1.89	37	49	1.39	0.56 - 3.47
	Continuous	90	151	1.22	0.79 - 1.89

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>Log2-transformed. Continuous analyses were only performed when linearity was respected.

<sup>3</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material SVII. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – No prior stimulation treatment (N = 97 cases & 97 controls)**

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N = 97)	Number of controls (N = 97)	Diminished Ovarian Reserve Multivariate <sup>1</sup> logistic regression	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 21.98	22	33	Ref.	Ref.
	]21.98-37.32]	33	32	1.25	0.56 - 2.80
	> 37.32	42	32	1.71	0.76 - 3.83
	Continuous	97	97	1.55	1.14 - 2.11
p,p'-DDT	< LOD	37	36	Ref.	Ref.
	≥ LOD	60	61	1.20	0.62 - 2.33
cis-Heptachlore epoxyde	≤ 1.75	39	33	Ref.	Ref.
	]1.75-2.37]	31	31	0.65	0.30 - 1.39
	> 2.37	27	33	0.59	0.28 - 1.26
	Continuous	97	97	0.66	0.37 - 1.16
HCB	≤ 6.69	31	33	Ref.	Ref.
	]6.69-8.70]	33	32	0.80	0.37 - 1.74
	> 8.70	33	32	0.75	0.33 - 1.68
	Continuous	97	97	0.85	0.41 - 1.73
β-HCH	≤ 3.52	31	33	Ref.	Ref.
	]3.52-5.66]	38	32	0.76	0.35 - 1.64
	> 5.66	28	32	0.45	0.19 - 1.06
	Continuous	97	97	0.68	0.44 - 1.03
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.42	29	36	Ref.	Ref.
	]0.42-0.59]	28	28	0.96	0.39 - 2.33
	> 0.59	40	33	1.40	0.63 - 3.11
	Continuous	97	97	1.27	0.80 - 2.03
PCB 74	≤ 1.61	25	33	Ref.	Ref.
	]1.61-2.52]	32	31	0.98	0.43 - 2.24
	> 2.52	40	33	0.96	0.42 - 2.21
	Continuous	97	97	0.88	0.58 - 1.35
PCB 99	≤ 1.81	28	33	Ref.	Ref.
	]1.81-2.66]	27	31	0.92	0.40 - 2.08
	> 2.66	42	33	1.30	0.58 - 2.93
	Continuous	97	97	0.99	0.62 - 1.59
PCB 118	≤ 3.31	18	33	Ref.	Ref.
	]3.31-5.32]	43	32	1.91	0.87 - 4.22
	> 5.32	36	32	1.37	0.59 - 3.17



PCB 138+163 <sup>3</sup>	Continuous	97	97	0.91	0.58 - 1.44
	≤ 3.67	22	33	Ref.	Ref.
	]3.67-6.30]	29	32	0.94	0.40 - 2.24
	> 6.30	46	32	1.16	0.42 - 3.20
PCB 153	Continuous	97	97	1.00	0.61 - 1.64
	≤ 15.87	23	33	Ref.	Ref.
	]15.87-26.21]	35	32	1.00	0.43 - 2.34
	> 26.21	39	32	1.01	0.39 - 2.57
PCB 170	Continuous	97	97	1.01	0.59 - 1.72
	≤ 11.06	24	33	Ref.	Ref.
	]11.06-16.43]	22	32	0.70	0.30 - 1.63
	> 16.43	51	32	1.60	0.67 - 3.80
PCB 180	Continuous	97	97	1.00	0.60 - 1.67
	≤ 10.91	20	33	Ref.	Ref.
	]10.91-18.13]	28	32	1.04	0.43 - 2.56
	> 18.13	49	32	1.35	0.47 - 3.84
PCB 183	Continuous	97	97	1.07	0.65 - 1.78
	≤ 0.93	23	33	Ref.	Ref.
	]0.93-1.39]	20	32	0.69	0.29 - 1.67
	> 1.39	54	32	1.91	0.77 - 4.71
PCB 187	Continuous	97	97	1.36	0.82 - 2.27
	≤ 2.05	27	35	Ref.	Ref.
	]2.05-3.50]	29	32	0.99	0.42 - 2.31
	> 3.50	41	30	1.31	0.53 - 3.20
PCB 194	Continuous	97	97	1.14	0.77 - 1.71
	≤ 1.20	21	32	Ref.	Ref.
	]1.20-2.22]	30	32	0.93	0.37 - 2.31
	> 2.22	46	33	1.09	0.37 - 3.20
PCB 196+203 <sup>3</sup>	Continuous	97	97	1.15	0.70 - 1.89
	≤ 1.10	19	32	Ref.	Ref.
	]1.10-1.78]	35	33	1.32	0.52 - 3.31
	> 1.78	43	32	1.34	0.45 - 4.04
	Continuous	97	97	1.33	0.80 - 2.22

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>Log2-transformed. Continuous analyses were only performed when linearity was respected.

<sup>3</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: OR, odds ratio; CI, confidence interval; Ref, reference.

Supplementary Material SVIII. Sensitivity analysis – Associations between serum persistent organic pollutants and diminished ovarian reserve in the AROPE study – Participants with outcomes measured at inclusion (N = 50 cases & 48 controls)

Persistent organic pollutant	Concentration (ng/g lipid base) <sup>2</sup>	Number of cases (N=50)	Number of controls (N=48)	Diminished Ovarian Response	
				OR	CI
<b>Organochlorine pesticides</b>					
p,p'-DDE	≤ 21.33	9	16	Ref.	Ref.
	]21.33-35.63]	15	16	1.21	0.31 - 4.73
	> 35.63	26	16	2.60	0.72 - 9.43
	Continuous <sup>2</sup>	50	48	1.36	0.83 - 2.24
p,p'-DDT	< LOD	17	13	Ref.	Ref.
	≥ LOD	33	35	1.44	0.49 - 4.27
cis-Heptachlore-Epoxyde	≤ 1.75	19	16	Ref.	Ref.
	]1.75-2.48]	22	16	0.66	0.22 - 1.98
	> 2.48	9	16	0.51	0.14 - 1.83
	Continuous	50	48	0.98	0.39 - 2.42
HCB	≤ 6.52	14	16	Ref.	Ref.
	]6.52-7.99]	12	16	0.61	0.18 - 2.08
	> 7.99	24	16	1.09	0.34 - 3.55
	Continuous	50	48	1.48	0.43 - 5.15
Beta-HCH	≤ 3.64	13	16	Ref.	Ref.
	]3.64-6.41]	26	16	1.88	0.59 - 6.01
	> 6.41	11	16	0.60	0.15 - 2.51
<b>Polychlorinated biphenyls</b>					
PCB 28	≤ 0.39	13	16	Ref.	Ref.
	]0.39-0.53]	21	16	2.27	0.45 - 11.33
	> 0.53	16	16	1.96	0.39 - 9.94
	Continuous	50	48	1.06	0.55 - 2.04
PCB 74	≤ 1.61	10	16	Ref.	Ref.
	]1.61-2.90]	25	17	2.57	0.71 - 9.31
	> 2.90	15	15	1.34	0.34 - 5.28
PCB 99	≤ 1.81	8	16	Ref.	Ref.
	]1.81-2.50]	15	16	1.26	0.35 - 4.54
	> 2.50	27	16	2.47	0.73 - 8.33
	Continuous	50	48	1.33	0.65 - 2.72
PCB 118	≤ 3.31	8	16	Ref.	Ref.
	]3.31-5.01]	19	16	2.90	0.76 - 11.08
	> 5.01	23	16	3.83	0.99 - 14.87
	Continuous	50	48	1.37	0.69 - 2.72
PCB 138 + 163 <sup>3</sup>	≤ 11.68	9	16	Ref.	Ref.
	]11.68-15.43]	14	16	1.30	0.35 - 4.83

	> 15.43	27	16	2.33	0.65 - 8.33
	Continuous	50	48	1.53	0.67 - 3.52
PCB 153	≤ 17.15	10	16	Ref.	Ref.
	]17.15-24.18]	12	16	0.69	0.18 - 2.64
	> 24.18	28	16	1.80	0.49 - 6.57
PCB 170	≤ 4.23	14	16	Ref.	Ref.
	]4.23-6.38]	13	16	0.60	0.16 - 2.28
	> 6.38	23	16	1.20	0.31 - 4.71
	Continuous	50	48	1.26	0.54 - 2.91
PCB 180	≤ 12.81	14	16	Ref.	Ref.
	]12.81-18.86]	16	16	0.72	0.20 - 2.67
	> 18.86	20	16	1.00	0.25 - 3.97
	Continuous	50	48	1.13	0.48 - 2.69
PCB 183	≤ 0.94	8	16	Ref.	Ref.
	]0.94-1.34]	12	16	0.99	0.25 - 3.96
	> 1.34	30	16	2.55	0.68 - 9.54
	Continuous	50	48	1.82	0.80 - 4.15
PCB 187	≤ 2.07	12	16	Ref.	Ref.
	]2.07-3.31]	18	15	1.41	0.37 - 5.40
	> 3.31	20	17	1.75	0.45 - 6.79
	Continuous	50	48	1.55	0.77 - 3.13
PCB 194	≤ 1.40	13	16	Ref.	Ref.
	]1.40-2.37]	22	16	1.48	0.43 - 5.14
	> 2.37	15	16	0.81	0.18 - 3.67
PCB 196 + 203 <sup>3</sup>	≤ 1.19	13	16	Ref.	Ref.
	]1.19-1.88]	20	16	0.74	0.21 - 2.63
	> 1.88	17	16	0.79	0.19 - 3.37

<sup>1</sup>Adjusted for age, body-mass index, centre, education level, and season of inclusion.

<sup>2</sup>All POPs were log<sub>2</sub>-transformed when treated as continuous variables.

<sup>3</sup>These POPS could not be distinguished during the measurement process.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

**Appendix 2. Supplementary material for article 2 “Heavy metals and diminished ovarian reserve: single-exposure and mixture analyses amongst women consulting in French fertility centres”.**

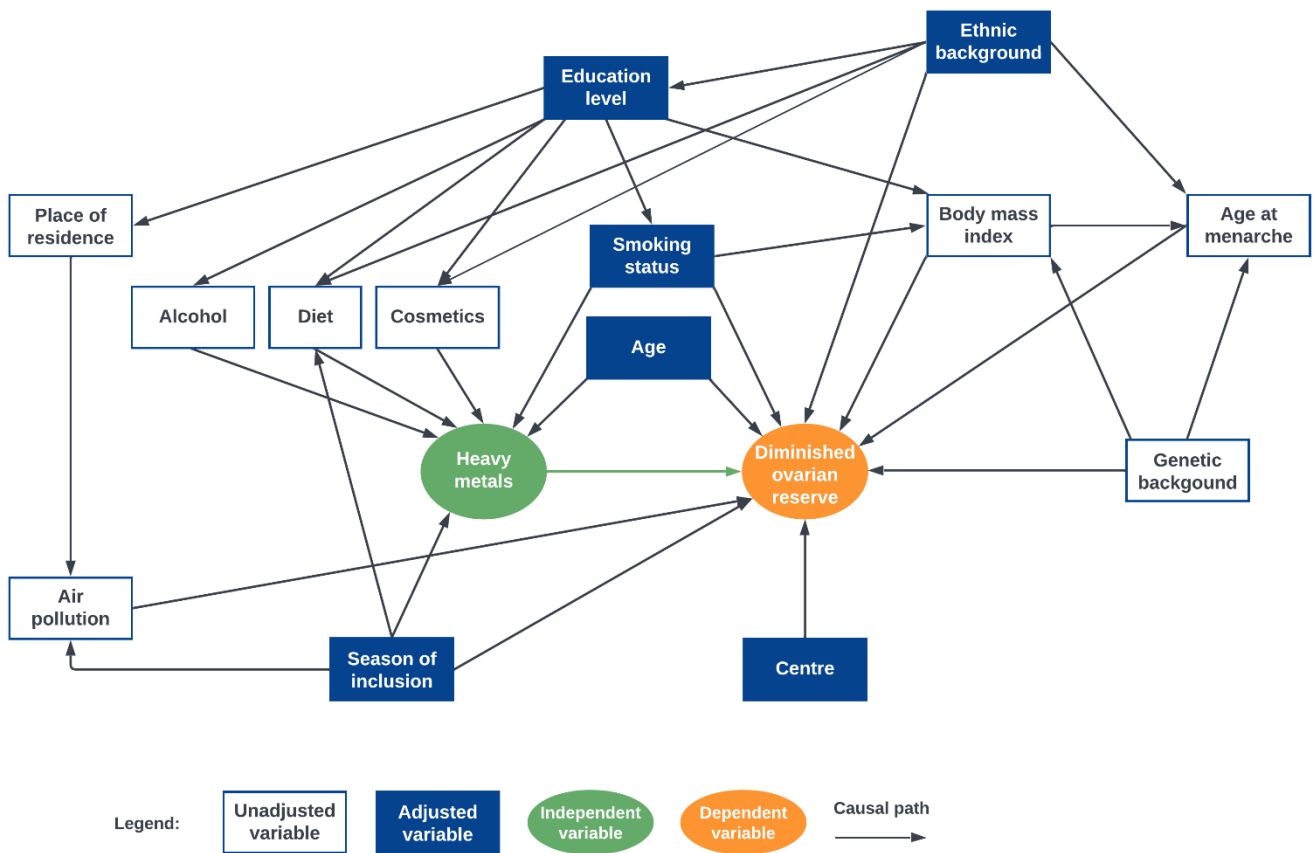
**Supplementary Material S1. Detailed heavy metal measurement methods**

Lead (Pb), Mercury (Hg), cadmium (Cd), and chromium (Cr) were measured in whole blood by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), on an ICAP-TQ from Thermo Scientific® equipped with collision cell technology (Platform AEM2, University of Rennes 1 / Biochemistry Laboratory, Rennes University Hospital). The source of plasma was argon (Messer®) with high degree of purity (>99.999%). The collision/reaction cell used was pressurized with helium (Messer®). All samples were handled with care in order to avoid environmental contamination during their manipulation. All rinse, diluent, and standards were prepared with  $\geq 18$  M $\Omega$  cm ultrapure water using a Millipore® MilliQ Advantage A10 water station. Blood samples, stored at -80°C, were diluted 1:30 by addition of a diluent solution consisting of 0.5% (v/v) HNO<sub>3</sub> (Optima Grade Fischer Chemical®) and 0.01% (v/v) Triton X100 (Merck®). The internal standard used was rhodium (Fisher Scientific®). Calibration ranges preparation was carried out using a multi-element calibrator solution (SCP Science® Plasma Cal). Calibration and verification of instrument performance were realized using multi-element solutions (Thermo®). Quality control was Clincheck controls for trace elements (Recipe).

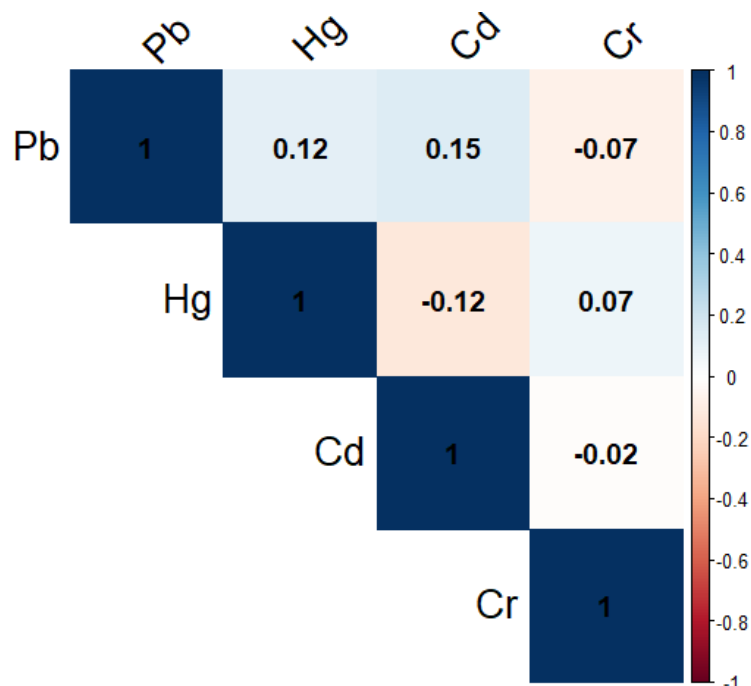
The accuracy of the ICP-MS assay method for the selected metals was verified through participation in two External Quality Assessment Schemes (EQAS); first organized by OELM, European organizer of EQAS in Occupational and Environmental Laboratory Medicine and the second by INSTAND e.V., a German EQAS appointed by the German Medical Association.

The within-run and between-run precision had a coefficient of variation (CV %) of <5% for Pb, <6% for Hg, <4.5% for Cd, and <6% for Cr. The lower detection and quantification limits were, respectively, 0.5 and 2.5  $\mu\text{g/l}$  for Pb, 0.1 and 0.5  $\mu\text{g/l}$  for Hg, 0.02 and 0.05  $\mu\text{g/l}$  for Cd, and 0.2 and 0.5  $\mu\text{g/l}$  for Cr.

**Supplementary Material S2. Directed acyclic graph between heavy metals and diminished ovarian reserve**



**Supplementary Material S3. Pearson correlation plot for heavy metals in the control population (N = 153)**



**Supplementary Material S4. Sensitivity analysis – Associations between heavy metals and diminished ovarian reserve in the AROPE study – Cases = AFC < 7 & AMH ≤ 1.1 ng/ml (N = 51 cases & 153 controls)**

Metal	Concentration <sup>2</sup> (ug/L)	Number of cases	Number of controls	Diminished Ovarian Reserve	
				OR	CI
Pb	≤ 7.40	16	51	Ref.	Ref.
	]7.40-11.47]	23	51	1.16	0.50 - 2.70
	> 11.47	12	51	0.59	0.23 - 1.53
	Continuous	51	153	0.55	0.11 - 2.86
Hg	≤ 1.09	10	51	Ref.	Ref.
	]1.09-1.96]	13	51	1.35	0.54 - 3.39
	> 1.96	28	51	1.84	0.72 - 4.70
	Continuous	51	153	1.75	0.61 - 5.01
Cd	≤ 0.10	14	51	Ref.	Ref.
	]0.10-0.24]	20	51	1.05	0.39 - 2.86
	> 0.24	17	51	0.72	0.22 - 2.38
	Continuous	51	153	0.58	0.23 - 1.45
Cr	≤ 0.72	15	51	Ref.	Ref.
	]0.72-1.71]	18	51	0.77	0.29 - 2.04
	> 1.71	17	51	0.90	0.28 - 2.82
	Continuous	50	153	0.41	0.10 - 1.67

<sup>1</sup>Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

<sup>2</sup>All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material S5. Sensitivity analysis – Associations between heavy metals and diminished ovarian reserve in the AROPE study – Cases = AFC < 7 (N = 55 cases & 153 controls)**

Metal	Concentration <sup>2</sup> (ug/L)	Number of cases	Number of controls	Diminished Ovarian Reserve	
				OR	CI
Pb	≤ 7.40	17	51	Ref.	Ref.
	]7.40-11.47]	26	51	1.30	0.58 - 2.91
	> 11.47	12	51	0.57	0.22 - 1.44
	Continuous	55	153	0.48	0.10 - 2.40
Hg	≤ 1.09	12	51	Ref.	Ref.
	]1.09-1.96]	15	51	1.36	0.57 - 3.25
	> 1.96	28	51	1.59	0.65 - 3.93
	Continuous	55	153	1.59	0.59 - 4.29
Cd	≤ 0.10	16	51	Ref.	Ref.
	]0.10-0.24]	21	51	1.04	0.39 - 2.73
	> 0.24	18	51	0.77	0.26 - 2.30
	Continuous	55	153	0.59	0.26 - 1.36
Cr	≤ 0.72	17	51	Ref.	Ref.
	]0.72-1.71]	18	51	0.75	0.30 - 1.90
	> 1.71	19	51	0.95	0.32 - 2.81
	Continuous	55	153	0.41	0.11 - 1.49

<sup>1</sup>Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

<sup>2</sup>All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material S6. Sensitivity analysis – Associations between heavy metals and diminished ovarian reserve in the AROPE study – Cases = AMH  $\leq$  1.1 ng/ml (N = 135 cases & 153 controls)**

Metal	Concentration <sup>2</sup> (ug/L)	Number of cases	Number of controls	Diminished Ovarian Reserve	
				OR	CI
Pb	$\leq$ 7.40	47	51	Ref.	Ref.
	]7.40-11.47]	54	51	0.94	0.51 - 1.70
	> 11.47	34	51	0.63	0.33 - 1.20
	Continuous	135	153	0.59	0.18 - 1.92
Hg	$\leq$ 1.09	39	51	Ref.	Ref.
	]1.09-1.96]	39	51	0.89	0.49 - 1.64
	> 1.96	57	51	1.11	0.58 - 2.11
	Continuous	135	153	0.94	0.46 - 1.90
Cd	$\leq$ 0.10	35	51	Ref.	Ref.
	]0.10-0.24]	61	51	1.98	1.01 - 3.87
	> 0.24	39	51	1.27	0.58 - 2.77
	Continuous	135	153	1.06	0.57 - 1.98
Cr	$\leq$ 0.72	22	51	Ref.	Ref.
	]0.72-1.71]	50	51	1.29	0.67 - 2.50
	> 1.71	62	51	1.82	0.86 - 3.86
	Continuous	135	153	1.70	0.66 - 4.37

<sup>1</sup>Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

<sup>2</sup>All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.



**Supplementary Material S7. Sensitivity analysis – Associations between heavy metals and diminished ovarian reserve in the AROPE study – Cases = AFC < 5 and/or AMH ≤ 0.7 ng/ml (N = 90 cases & 153 controls)**

Metal	Concentration <sup>2</sup> (ug/L)	Number of cases	Number of controls	Diminished Ovarian Reserve	
				OR	CI
Pb	≤ 7.40	29	51	Ref.	Ref.
	]7.40-11.47]	39	51	1.19	0.60 - 2.33
	> 11.47	22	51	0.68	0.32 - 1.44
	Continuous	90	153	0.58	0.16 - 2.10
Hg	≤ 1.09	25	51	Ref.	Ref.
	]1.09-1.96]	24	51	0.98	0.49 - 1.97
	> 1.96	41	51	1.23	0.60 - 2.54
	Continuous	90	153	1.10	0.51 - 2.41
Cd	≤ 0.10	26	51	Ref.	Ref.
	]0.10-0.24]	40	51	1.43	0.67 - 3.03
	> 0.24	24	51	0.72	0.28 - 1.83
Cr	≤ 0.72	18	51	Ref.	Ref.
	]0.72-1.71]	32	51	1.08	0.51 - 2.30
	> 1.71	39	51	1.46	0.65 - 3.24

<sup>1</sup>Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

<sup>2</sup>All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

**Supplementary Material S8. Sensitivity analysis – Associations between heavy metals and diminished ovarian reserve in the AROPE study in women with no prior ovarian stimulation – Cases = AFC < 7 and/or AMH ≤ 1.1 ng/ml (N = 107 cases & 110 controls)**

<b>Diminished Ovarian Reserve</b>					
<b>Multivariate logistic regression<sup>1</sup></b>					
<b>Metal</b>	<b>Concentration<sup>2</sup> (ug/L)</b>	<b>Number of cases</b>	<b>Number of controls</b>	<b>OR</b>	<b>CI</b>
Pb	≤ 7.40	37	39	Ref.	Ref.
	(7.40-11.47]	45	34	1.22	0.61 - 2.43
	> 11.47	25	37	0.61	0.28 - 1.32
Hg	≤ 1.09	32	37	Ref.	Ref.
	(1.09-1.96]	30	39	0.75	0.37 - 1.50
	> 1.96	45	34	1.19	0.56 - 2.54
Cd	Continuous	107	110	0.89	0.36 - 2.22
	≤ 0.10	24	33	Ref.	Ref.
	(0.10-0.24]	48	40	1.48	0.68 - 3.22
Cr	> 0.24	35	37	1.16	0.48 - 2.78
	≤ 0.72	20	34	Ref.	Ref.
	(0.72-1.71]	40	39	0.78	0.36 - 1.72
	> 1.71	46	37	1.26	0.51 - 3.09
	Continuous	106	110	1.50	0.45 - 5.05

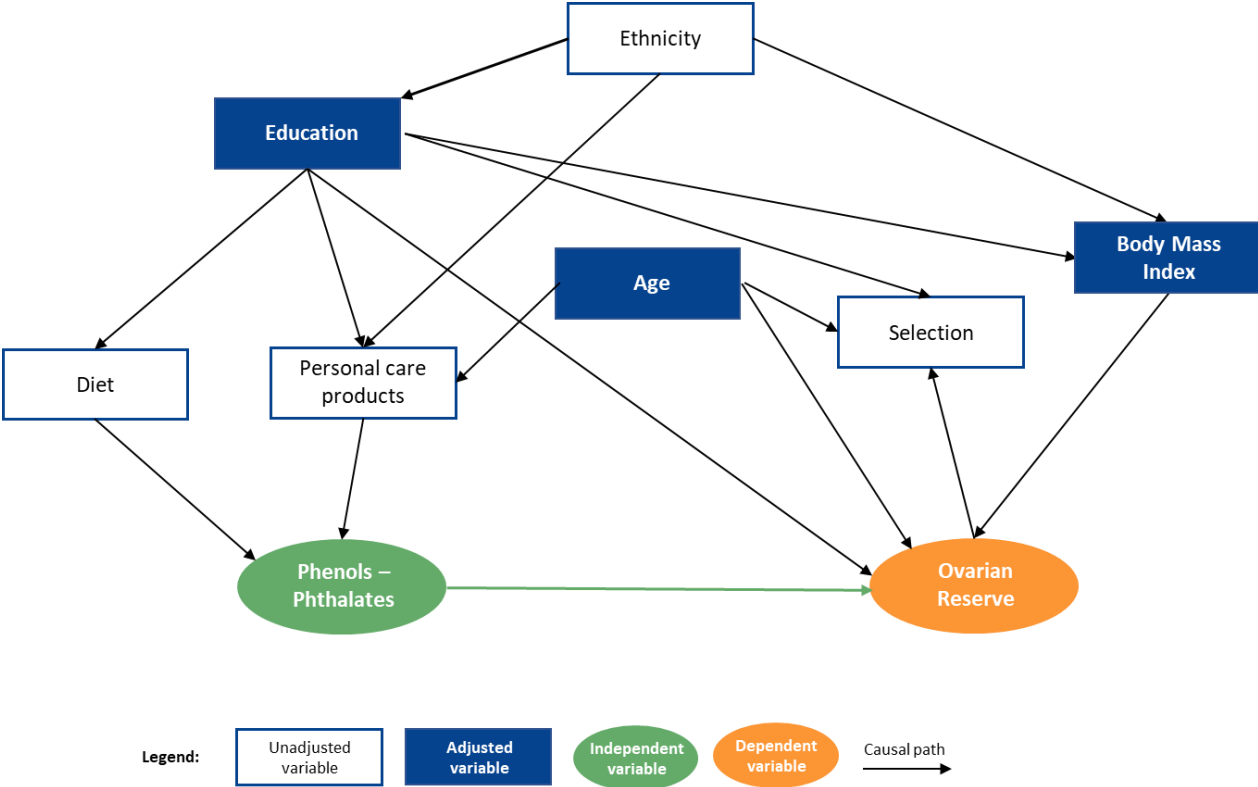
<sup>1</sup>Adjusted for age, centre, education level, ethnic origin, smoking status and season of inclusion.

<sup>2</sup>All metals were log<sub>10</sub>-transformed when treated as continuous variables.

Abbreviations: N, number of subjects; OR, odds ratio; CI, confidence interval; Ref, reference.

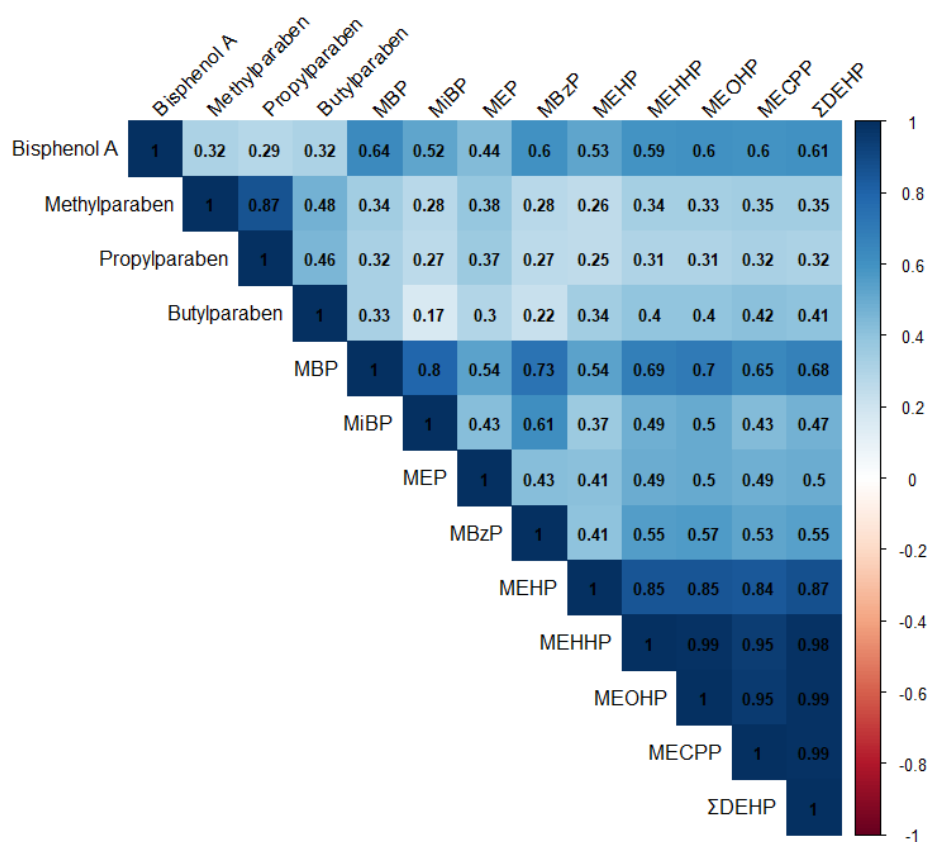
**Appendix 3. Supplementary material for article 3 “Mixtures of urinary concentrations of phenols and phthalate biomarkers in relation to the ovarian reserve among women attending a fertility clinic”.**

**Supplemental Material 1. Directed acyclic graph between the mixture of phenols and phthalates and the ovarian reserve**



Selection: Probability of being selected into the study

**Supplemental Material 2. Spearman correlation plot for all phenol and phthalate biomarkers (N = 271)**



N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; ΣDEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

**Supplemental Material 3. Associations between a quartile increase in mixture exposure biomarker concentrations and ovarian reserve markers in quantile g-computation**

<b>Lower antral follicle count (N = 262)</b>			
	<b>OR</b>	<b>95% CI</b>	<b>p-value</b>
<b>Chemical mixture</b>	1.10	0.52 - 2.30	0.806

<b>Day-3 follicle stimulating hormone (N = 261)</b>			
	<b>Mean difference</b>	<b>95% CI</b>	<b>p-value</b>
<b>Chemical mixture</b>	0.30	-0.32 - 0.93	0.343

<b>Diminished ovarian reserve (N = 271)</b>			
	<b>OR</b>	<b>95% CI</b>	<b>p-value</b>
<b>Chemical mixture</b>	1.02	0.51 - 2.05	0.950

Abbreviations: N, number; CI, credible interval.

**Supplemental Material 4. Associations between a change in mixture exposure biomarker concentrations from the 25<sup>th</sup> to the 75<sup>th</sup> percentile and ovarian reserve markers in Bayesian Kernel Machine Regression**

<b>Lower antral follicle count (N = 262)</b>		
	<b>Risk difference</b>	<b>95% CI</b>
<b>Chemical mixture</b>	-0.00	-0.08 - 0.08

<b>Day-3 follicle stimulating hormone (N = 261)</b>		
	<b>Mean difference</b>	<b>95% CI</b>
<b>Chemical mixture</b>	0.05	-0.24 - 0.35

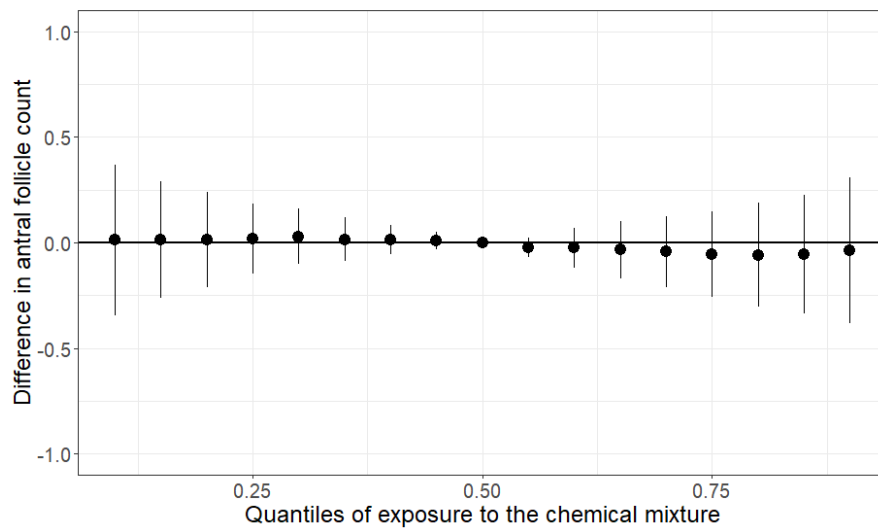
  

<b>Diminished ovarian reserve (N = 271)</b>		
	<b>Risk difference</b>	<b>95% CI</b>
<b>Chemical mixture</b>	-0.01	-0.08 - 0.07

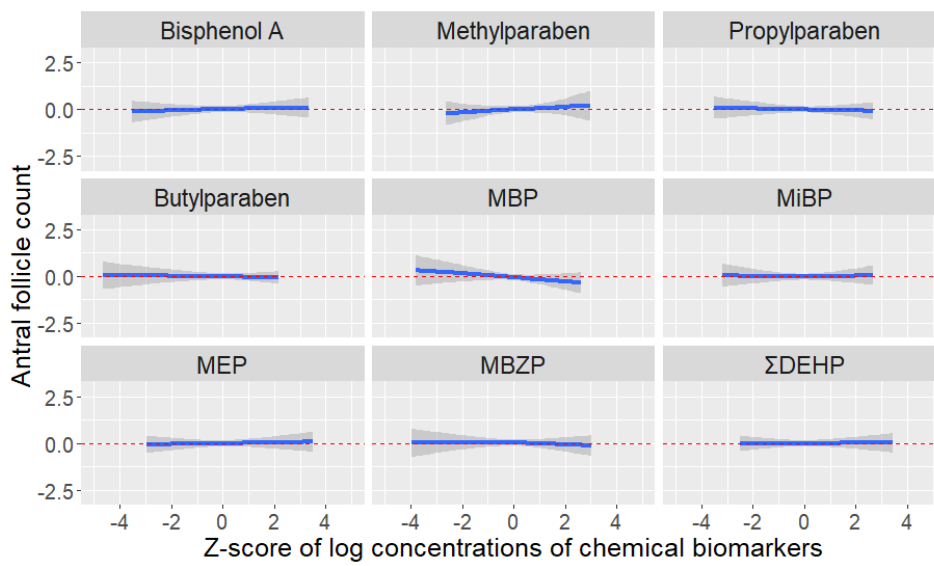
Abbreviations: N, number; CI, credible interval.

**Supplemental Material 5. BKMR mixture associations of phenol and phthalate biomarkers and antral follicle count (N = 262)**

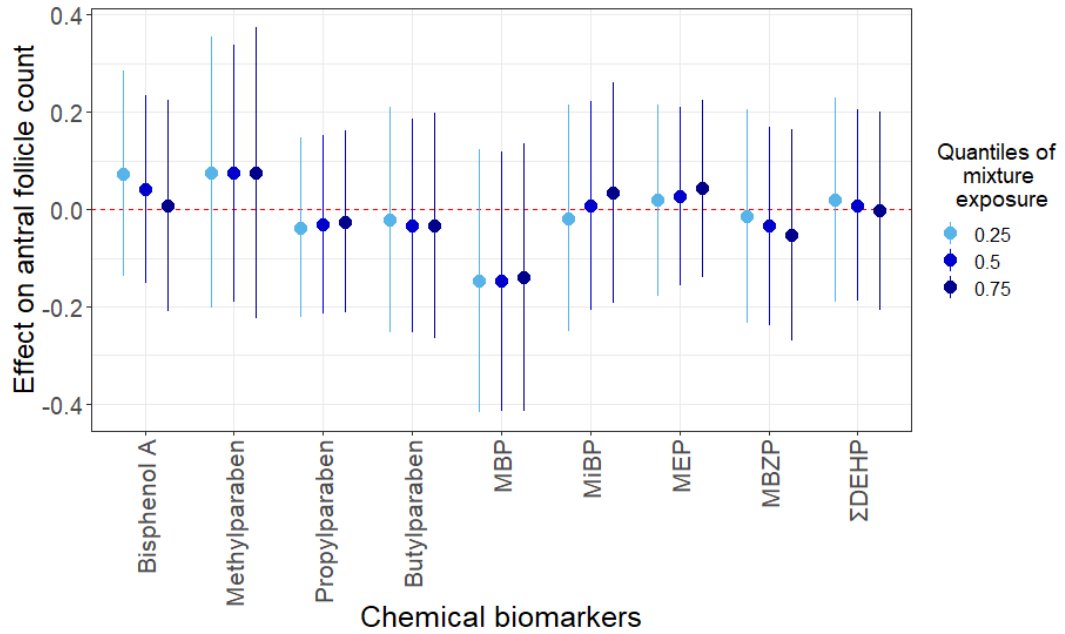
**A.**



**B.**



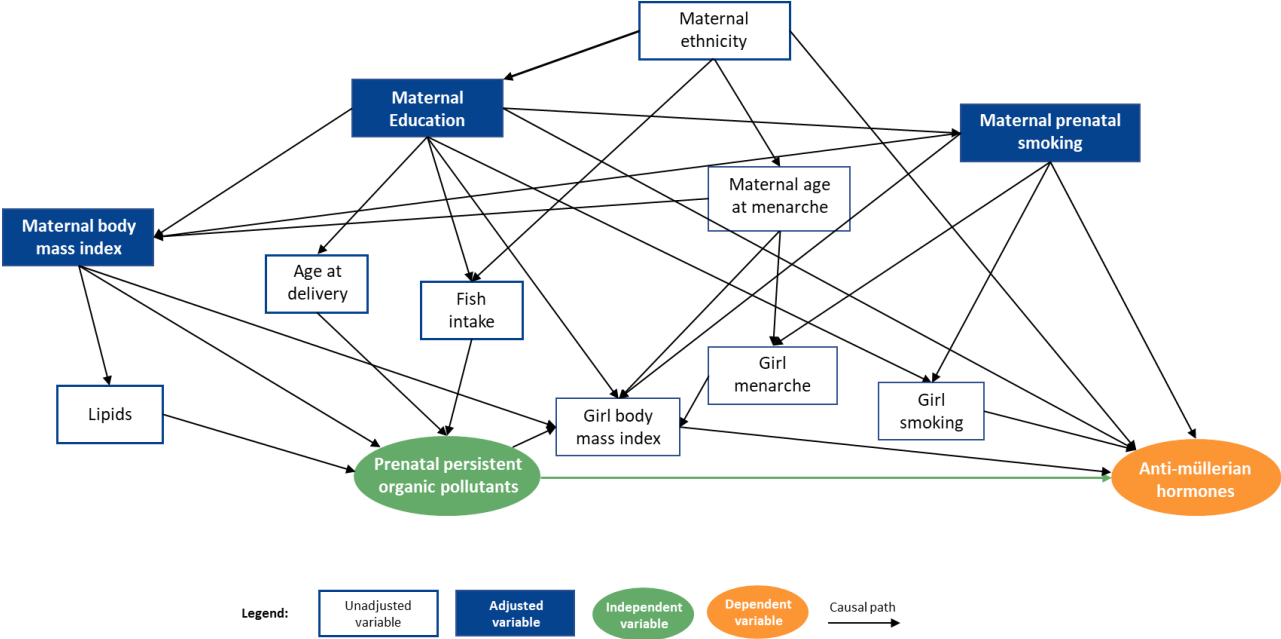
C.



Adjusted for age, body mass index, education level and specific gravity. **A.** Overall association of the chemical biomarker mixture at different concentration percentiles compared to the 50th percentile and their 95% credible intervals. **B.** Exposure-response relationships for each chemical biomarker while holding all other biomarkers at their median concentrations. **C.** Mean difference comparing the 75<sup>th</sup> to 25<sup>th</sup> percentile of each exposure (estimates and 95% credible intervals) when all the other biomarker concentrations are fixed at the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles. N: number of participants; MBP: mono-n-butyl phthalate; MiBP: mono-isobutyl phthalate; MEP: monoethyl phthalate; MBzP: monobenzyl phthalate; MEHP: mono(2-ethylhexyl) phthalate; MEHHP: mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP: mono(2-ethyl-5-oxohexyl) phthalate; MECPP: mono(2-ethyl-5-carboxypentyl) phthalate; ΣDEHP: molar sum of di(2-ethylhexyl) phthalate metabolites.

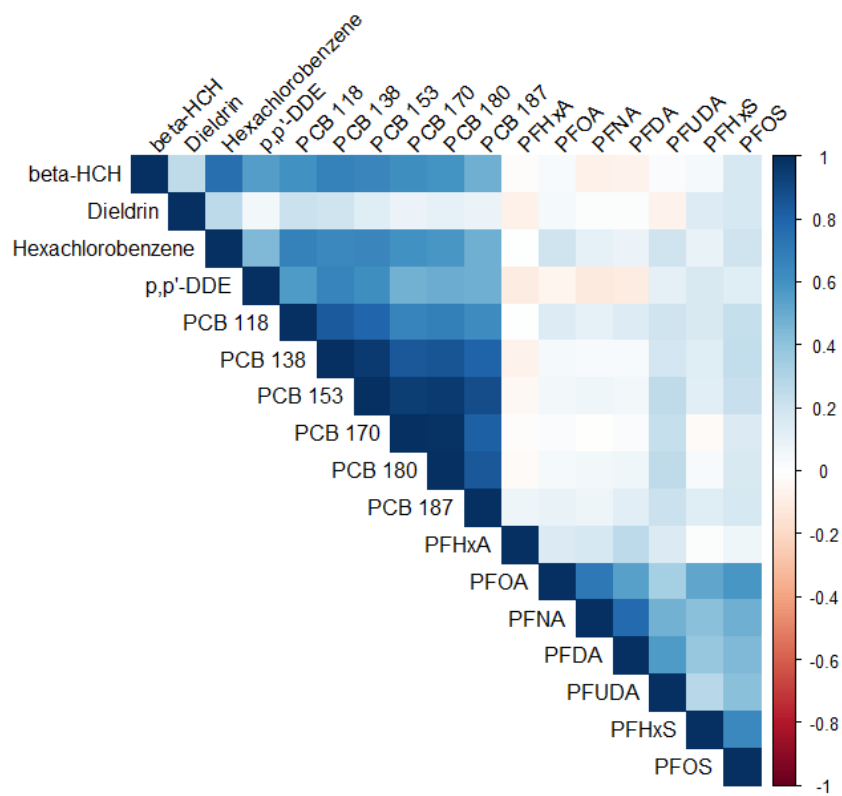
**Appendix 4. Supplementary material for article 4 “Prenatal exposure to persistent organic pollutants and its impact on the ovarian reserve in the PELAGIE mother-child cohort”.**

**Supplemental Material 1. Directed acyclic graph between the prenatal persistent organic pollutants and the ovarian reserve**





**Supplemental Material 2. Spearman correlation plot for all persistent organic pollutant biomarkers**



**Titre :** Environnement et fertilité féminine : étude du rôle des perturbateurs endocriniens sur la réserve ovarienne.

**Mots clés :** Réserve Ovarienne ; Infertilité féminine ; Exposition Environnementale ; Perturbateurs Endocriniens ; Mélanges.

**Résumé :** Les perturbateurs endocriniens (PEs) sont soupçonnés d'affecter le système reproducteur féminin. Cependant, peu d'attention a été accordée à leur impact potentiel sur la réserve ovarienne, un élément clé de la fertilité féminine.

L'objectif principal de cette thèse était d'étudier l'impact des PEs sur la réserve ovarienne. Plus précisément, nous avons examiné les effets de quatre familles de PEs (53 polluants organiques persistants (POPs), 4 métaux lourds, 4 phénols et 8 phtalates), en tant qu'expositions individuelles et en mélange, sur différents indicateurs de la réserve ovarienne, dont les hormones anti-Müllériennes (AMH), le nombre de follicules antraux (AFC), les hormones folliculostimulantes (FSH) et la diminution de la réserve ovarienne (DOR). De plus, nous avons exploré si l'exposition in utero aux POPs était associée aux niveaux d'AMH chez les adolescentes. Nous avons mené notre recherche auprès de deux populations de femmes en recherche d'aide à la procréation dans deux pays (France et États-Unis) et dans la cohorte mère-enfant française PELAGIE.

Dans l'ensemble, nous avons trouvé peu d'associations entre PEs et les marqueurs de la réserve ovarienne ou la DOR, individuellement ou en mélange. Dans l'étude cas-témoins française AROPE, le p,p'-DDE était significativement associé à un risque accru de DOR, tandis que le  $\beta$ -HCH était significativement associé à un risque réduit de DOR. De plus, nous avons observé des risques non significatifs accrus de DOR pour le cadmium et le chrome, avec une association proche de la significativité pour le deuxième tercile de cadmium. Nous avons également observé que des niveaux accrus de PCB 180 mesurés dans le sang du cordon augmentaient significativement les niveaux d'AMH chez les adolescentes de la cohorte PELAGIE. Dans la cohorte américaine EARTH, les phénols et les phtalates étaient associés à aucun indicateur de la réserve ovarienne (AFC, FSH ou DOR).

Ce travail comprend les premières études sur les associations entre les POP et les métaux lourds, et la DOR. D'autres études sont nécessaires pour valider nos résultats et explorer l'influence des PEs pendant différentes fenêtres de susceptibilité sur la réserve ovarienne à différentes étapes de la vie.

**Title:** The environment and female fertility: a study of the role of endocrine disruptors on the ovarian reserve.

**Keywords:** Ovarian Reserve; Female Infertility; Environmental Exposure; Endocrine Disrupting Chemicals; Mixtures.

**Abstract:** Endocrine disrupting chemicals (EDCs) are suspected to affect the female reproductive system. However, little attention has been given to their potential impact on the ovarian reserve, a key component of female fertility.

The overarching aim of this thesis was to study the impact of EDCs on the ovarian reserve. Specifically, we looked at the effects of four families of EDCs (53 persistent organic pollutants (POPs), 4 heavy metals, 4 phenols, and 8 phthalates), as single-exposure analysis and as mixtures, on different indicators of the ovarian reserve including anti-Müllerian hormones (AMH), antral follicle count (AFC), follicle-stimulating hormones (FSH) and diminished ovarian reserve (DOR). In addition, we explored whether in utero exposure to POPs were associated with AMH in teenage girls. We conducted our research among two populations of women seeking reproductive assistance at fertility centres in two countries (France and the United States) and in the French mother-child PELAGIE cohort.

Overall, we found limited evidence that EDCs were associated with markers of the ovarian reserve or with DOR, both individually and as mixtures. In the French AROPE case-control study, p,p'-DDE was significantly associated with an increased risk of DOR, whereas  $\beta$ -HCH was significantly associated with a decreased risk of DOR. Furthermore, we observed non-significant increased risks of DOR for cadmium and chromium with a close to significant association for the second tercile of cadmium. We also observed that increased PCB 180 measured in cord blood significantly increased AMH levels in teenage girls in the PELAGIE cohort. In the American EARTH cohort, phenols and phthalates were not associated with various ovarian reserve markers (AFC, FSH or DOR).

This work includes the first studies on the associations between POPs and heavy metals, and DOR. Further studies are necessary to validate our results and explore the influence of EDCs during various susceptibility windows on the ovarian reserve at different life stages.