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► To cite this version:

Maximilien Génard-Walton, Charline Warembourg, Solène Duros, Martine Ropert-Bouchet, Tiphaine Lefebvre, et al.. Heavy metals and diminished ovarian reserve: single-exposure and mixture analyses amongst women consulting in French fertility centres. *Reproductive BioMedicine Online*, 2023, pp.103241. 10.1016/j.rbmo.2023.05.013 . hal-04165967

HAL Id: hal-04165967

<https://hal.science/hal-04165967>

Submitted on 21 Jul 2023

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Highlights

- Research on the effect of metals on the ovarian reserve is limited.
- Cd and Cr were non-significantly associated with diminished ovarian reserve.
- Metal mixture analyses showed a non-significant increased risk of DOR.
- There was no evidence of interaction between metals in our BKMR analysis.

Journal Pre-proof

Heavy metals and diminished ovarian reserve: single-exposure and mixture analyses amongst women consulting in French fertility centres

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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 ≤ 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.

Introduction

The ovarian reserve is defined as the number of primordial follicles in both ovaries (Richardson et al., 2014). It is constituted in utero and decreases at birth and thereafter until women reach menopause (Hansen et al., 2008). The rate at which the follicle pool decreases can vary between women but the term diminished ovarian reserve (DOR) is used when a threshold for the number of follicles has been reached (Ferraretti et al., 2011). This threshold implies a reduced potential to reproduce (Tal and Seifer, 2017). The most accurate indirect methods to measure the ovarian reserve, and consequently DOR, are the antral follicle count (AFC) and anti-Müllerian hormone (AMH) levels. DOR is considered to be a marker for a poor response to ovarian stimulation (Yun et al., 2017) and is possibly associated with recurrent pregnancy loss (Bunnewell et al., 2020). It is of particular concern, especially because the average age at first conception in high-income countries is increasing (Schmidt et al., 2012), and because couples are therefore increasingly resorting to assisted reproduction technology to increase chances to procreate (Sunderam et al., 2022; Wyns et al., 2021).

Factors that have been previously associated with decreased AMH or AFC include clinical factors (age, body mass index (BMI) and history of chemotherapy and radiotherapy) and lifestyle factors such as tobacco usage (de Angelis et al., 2020; Moslehi et al., 2018; Spears et al., 2019). Recently, particulate matter (PM) of various diameters, sulfur dioxide (SO₂), and nitrous dioxide (NO₂), have been associated with 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). These markers of the ovarian reserve have also been inconsistently associated with persistent organic pollutants (Björvang et al., 2021) and non-persistent endocrine-disrupting chemicals (EDCs) (Mínguez-Alarcón and Gaskins, 2017).

Among the different chemicals with potential impacts on the ovarian reserve, we focussed specifically on heavy metals. These substances are generally referred to as metals with high densities. The human body requires low amounts of some heavy metals - the essential metals such as iron, zinc and chromium (Cr) - for a variety of biochemical and physiological functions but these metals can be toxic at higher concentrations or under different forms. Non-essential metals, such as lead (Pb), mercury (Hg) and cadmium (Cd), on the other hand, are potentially toxic even at very low concentrations (Briffa et al., 2020). Metals can be found in the air, food, water, indoor dust, cigarette, alcohol or cosmetics (Jin et al., 2020), as a result of natural and anthropogenic activities such as agriculture, mining, manufacturing, and the burning of fossil fuels (Agency for Toxic Substances and Disease Registry, 2020, 1999; Streets et al., 2017). They are partially stored in different organs or tissues including the bones and kidneys, but can also reach female reproductive organs (Agency for Toxic Substances and Disease Registry, 2020, 2012a). Cd has notably 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).

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). Exposure to metals has been inconsistently associated with a delayed time-to-pregnancy (Buck Louis et al., 2012; Cole et al., 2006) and unfavorable IVF outcomes (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. 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. 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

The AROPE case-control study (« Altération de la réserve ovarienne : étude du rôle de l'exposition aux perturbateurs endocriniens persistants et aux solvants organiques ») recruited 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.

Women consulting for infertility in these centres underwent a standardized endovaginal AFC by a trained gynaecologist between days 2 and 4 of a spontaneous menstrual cycle (Broekmans et al., 2010) and as well as an AMH measurement using an automated Roche Elecsys immunoassay system in all centres excepted for Rennes University Hospital where the automated Beckman-Coulter Access Dxi assay was used. AFC and AMH levels were either recorded at inclusion when participants consulted for the first time or within the year prior to the inclusion for women who had already started consulting in the clinics.

In the AROPE study, cases of DOR were defined using threshold values for the AFC and AMH levels proposed by the European Society of Human Reproduction and Embryology (ESHRE) Bologna criteria consensus (Ferraretti et al., 2011). Thus, cases were women presenting at least one of the following characteristics: a number of antral follicles < 7 and/or an AMH blood level ≤ 1.1 ng/mL. Controls were frequency matched by five-year age group and centre and were women with normal evaluations (AFC ≥ 7 and AMH between 1.1 and 5 ng/mL), no genital malformations, and a menstrual cycle between 26 and 35 days.

Women with 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 ≥ 35 kg / m²), or subjected to legal protection or deprived of their liberty were not included in the study.

During the inclusion process, a gynaecologist collected medical information on the reproductive history (regularity and length of menstrual cycles, infertility duration, previous ovarian stimulations) and fertility indicators (AFC, and circulating hormone concentrations including AMH and steroids) of the participants, and these completed a self-administered questionnaire covering social and demographic characteristics, personal habits and lifestyle information. Finally, whole blood was also collected at inclusion to measure the concentrations of heavy metals.

In total, the AROPE study included 149 cases and 161 controls with a participation rate of 65% for cases and 75% for controls.

Assessment of exposure

Heavy metals measurements were carried out on the 139 cases and 153 controls for whom there were sufficient quantities of whole blood. Pb, Hg, Cd, and Cr were measured at inclusion 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). Blood samples were diluted 1:30 by addition of a diluent solution consisting of 0.5% (v/v) HNO₃ (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). A more

detailed description of the analysis method is provided in Supplementary Material S1. The lower detection limits were 0.5 µg/L for Pb, 0.1 µg/L for Hg, 0.02 µg/l for Cd, and 0.2 µg/L for Cr. 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. One chromium concentration value was deleted due to a sample contamination.

Statistical analyses

Heavy metals were analysed both as terciles based on control concentrations in order to better represent exposure in the general population, and as continuous variables (log-10 transformed). Concentrations below the limit of detection (LOD) were replaced by combining the results of five imputations using maximum likelihood estimations (Cole et al., 2009). Associations between metals and DOR were studied using unconditional logistic regression (Kuo et al., 2018; Pearce, 2016). Logistic regression results are presented as odds ratio (OR) with their corresponding 95% confidence intervals (CI).

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. Specifically, quantile g-computation (Keil et al., 2020) is a method that can be used to estimate the effect of simultaneously increasing all exposures within a mixture by one quantile. BKMR is a non-parametric Bayesian method (Bobb et al., 2015) which can flexibly model the association between multiple exposures and an outcome using a kernel function and can accommodate for non-linear associations and detect potential interactions between pollutants.

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 (± 4.0) whilst the mean age in the control group was 32.4 years (± 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 µg/L), followed by Hg (1.5 µg/L), Cr (1.1 µg/L) and Cd (0.1 µg/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 25th percentile to the 75th 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 9.1µg/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, Cr 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°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 explained 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 they 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.

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).

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.

Funding

The study was funded by the Fondation de France (grant number 2014-50537 and 00110196) and the French Biomedicine Agency (2016).

Ethics approval

The study was approved by the relevant ethical committee for studies involving human subjects (Comité de Protection des Personnes, authorisation n°2016-A00307-44), and the French national security agency for medicine and health products (ANSM). All participants provided written informed consent for their participation in the study.

Acknowledgements

The authors are grateful to the participants of the study and the staff of the fertility centres that included patients. We wish to thank the NUMECAN team for the metal concentration analyses, Violaine Benoit, the project manager of the Rennes University Hospital, and the public health doctoral network coordinated by the EHESP.

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Figure legends

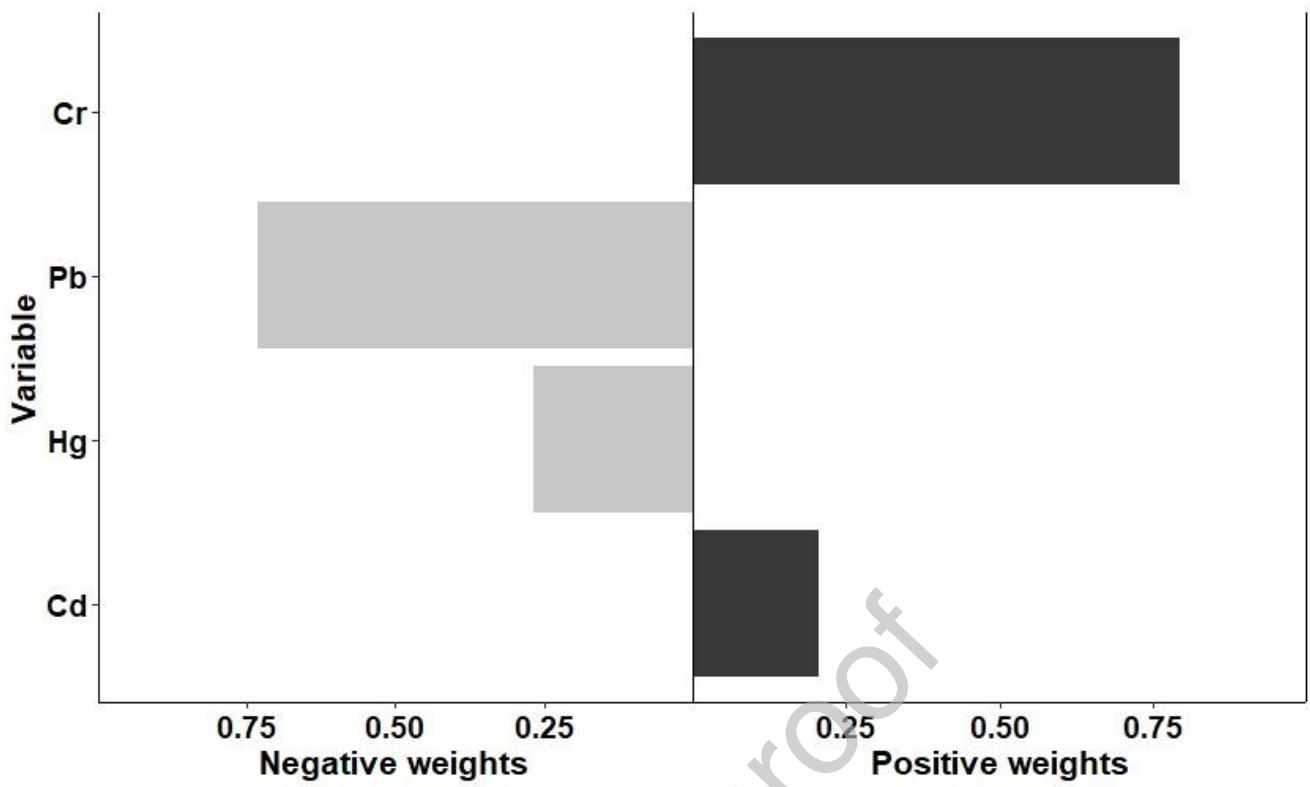


Figure 1. Title: Quantile g-computation weights of heavy metals on the metal mixture effect on diminished ovarian reserve (AROPE study: N = 139 cases and 153 controls)

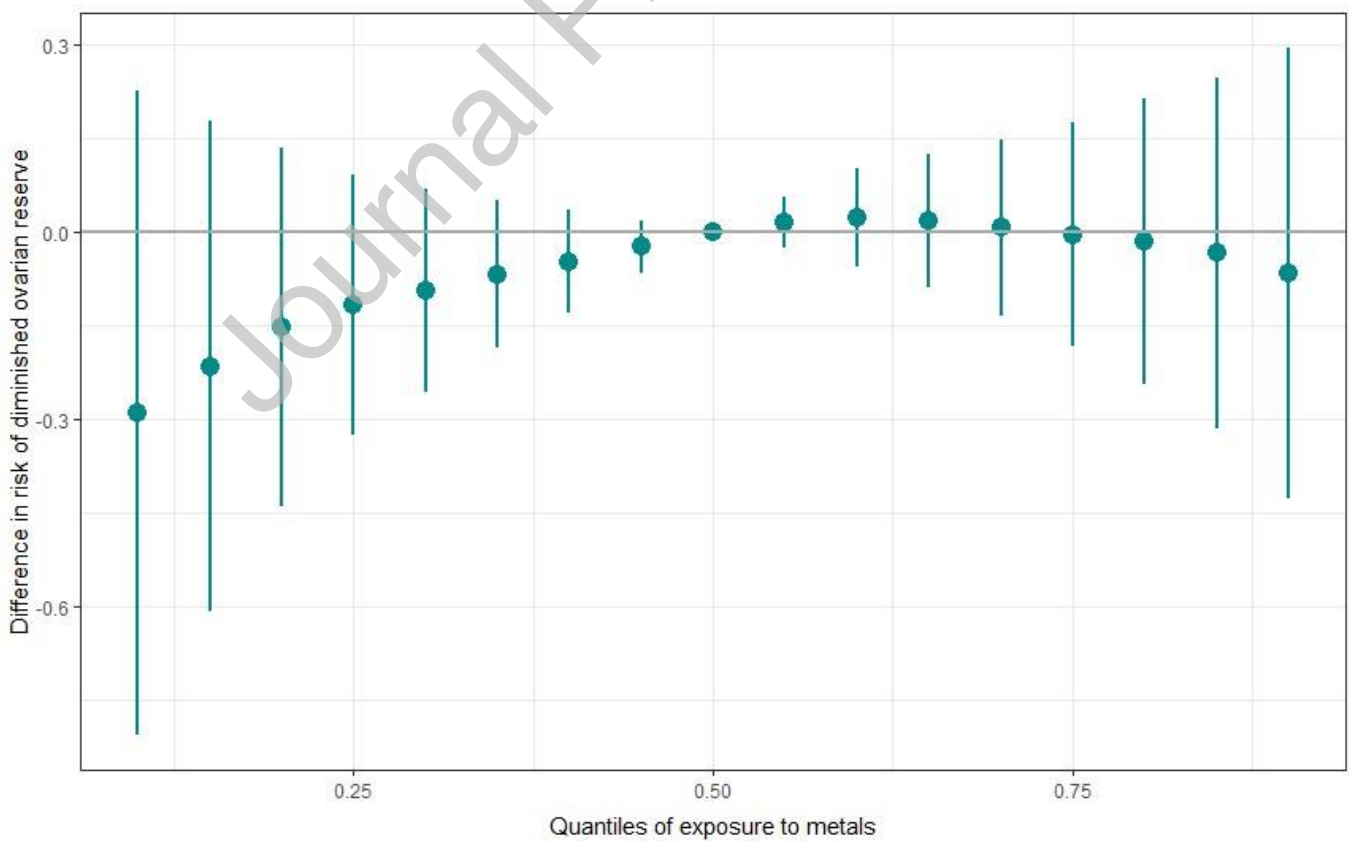


Figure 2. Title: Mixture effect of heavy metals on diminished ovarian reserve at different percentiles of exposure compared to the 50th percentile

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.

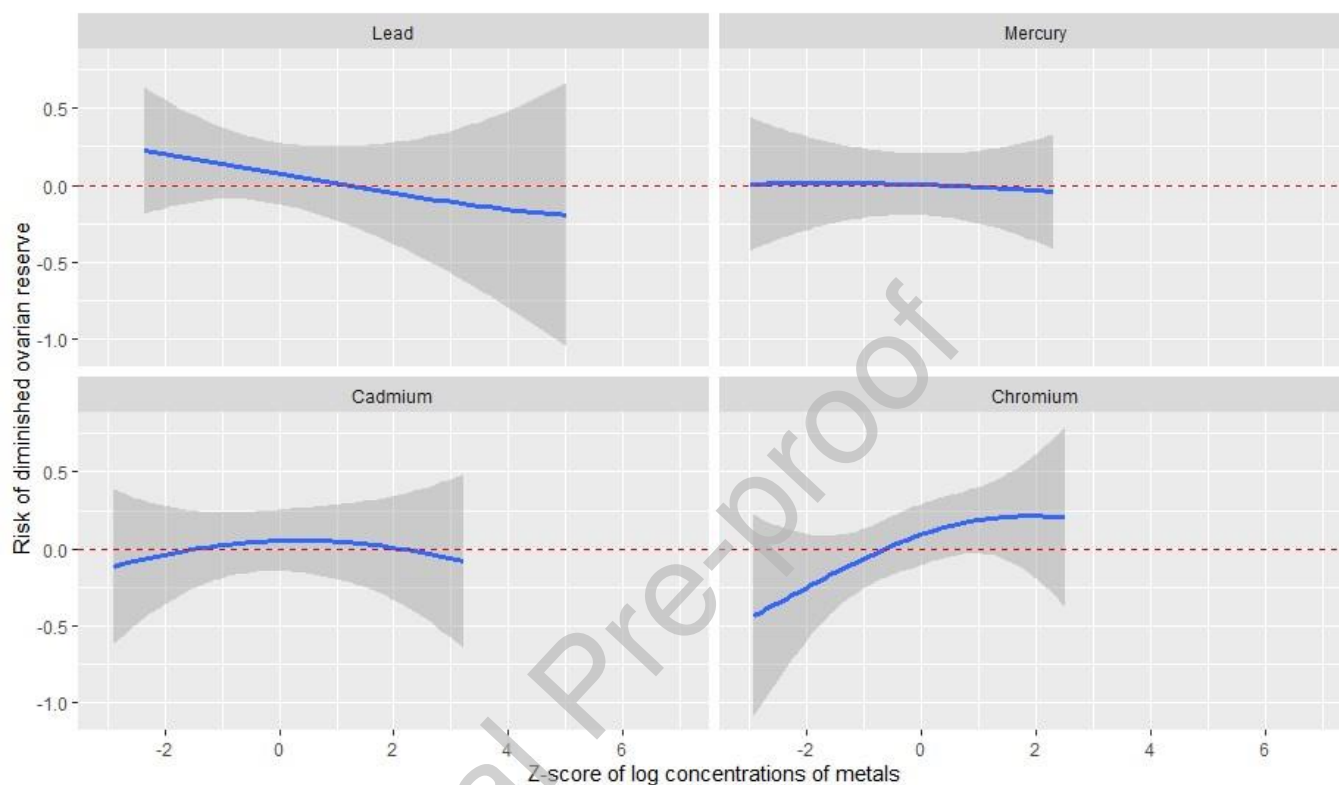


Figure 3. Title: Mixture analysis of the exposure-response relationships between individual heavy metals and diminished ovarian reserve

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.

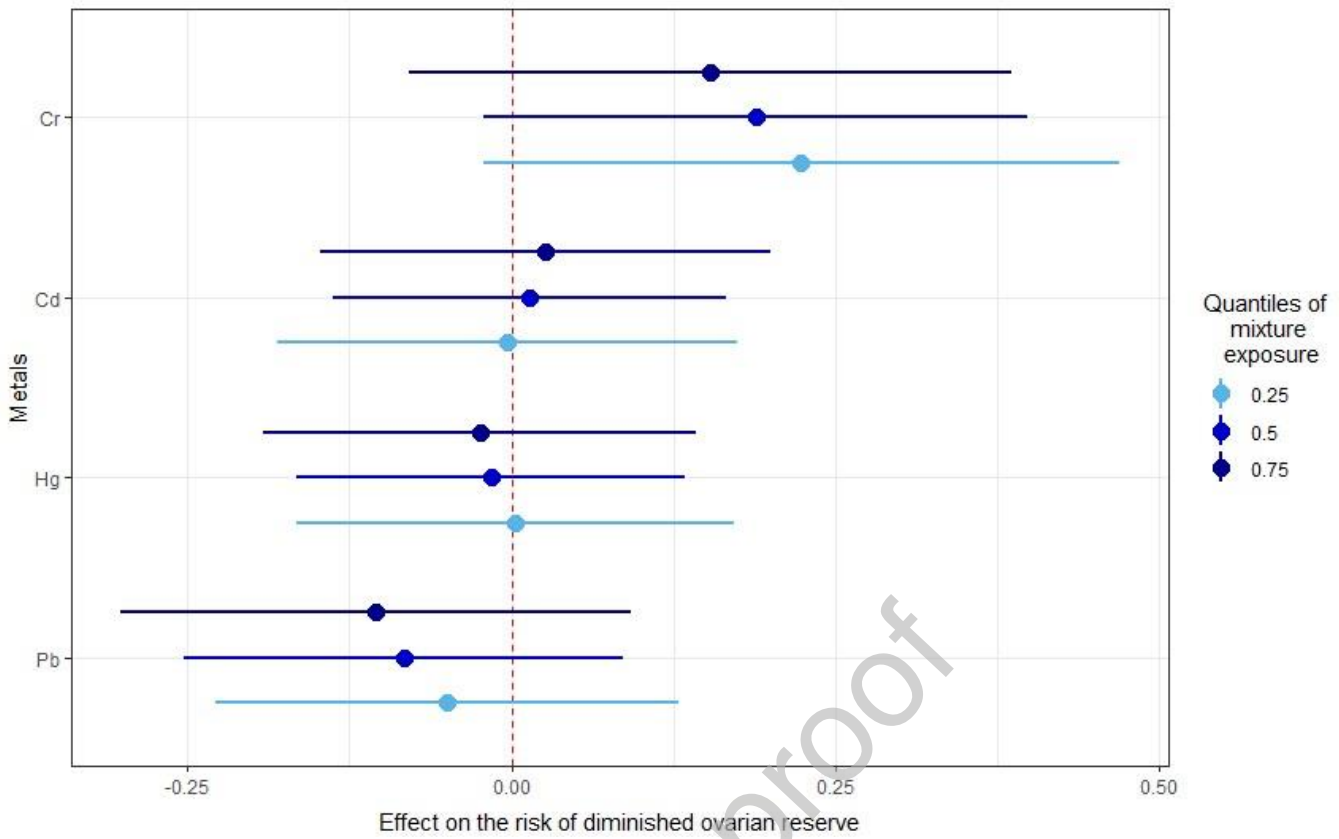


Figure 4. Title: Effects of heavy metals on the risk of diminished ovarian reserve when the mixture exposure is fixed at different quantiles

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

Characteristic - N (%)	Cases (N = 139)	Controls (N = 153)
Centre		
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)
Season		
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)
Age		
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)
Ethnic background		
Europe	112 (87.5)	128 (90.8)
Outside Europe	16 (12.5)	13 (9.2)
Missing data	11	12
Education		
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
Body-Mass Index (kg/m²)		
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
Infertility status		
Primary	85 (61.2)	90 (58.8)
Secondary	54 (38.8)	63 (41.2)
Parity		
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)
Anti-müllerian hormone levels (ng/L)		
Mean \pm SD	0.61 \pm 0.34	2.93 \pm 1.00
Antral follicle count		
Mean \pm SD	8 \pm 5	21 \pm 9
Age at menarche		
Mean \pm SD	12.91 \pm 1.57	12.96 \pm 1.61
Missing data	17	20
Smoking status		

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
Cigarette pack-years		
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
Alcohol (number of drinks per week)		
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

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

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

Metal	LOD (µg/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.

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

Diminished Ovarian Reserve 139 cases and 153 controls							
Metal	Concentration (ug/L) ²	Number of cases	Number of controls	Univariate logistic regression		Multivariate ¹ logistic regression	
				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

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

² All metals were log₁₀-transformed when treated as continuous variables.

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

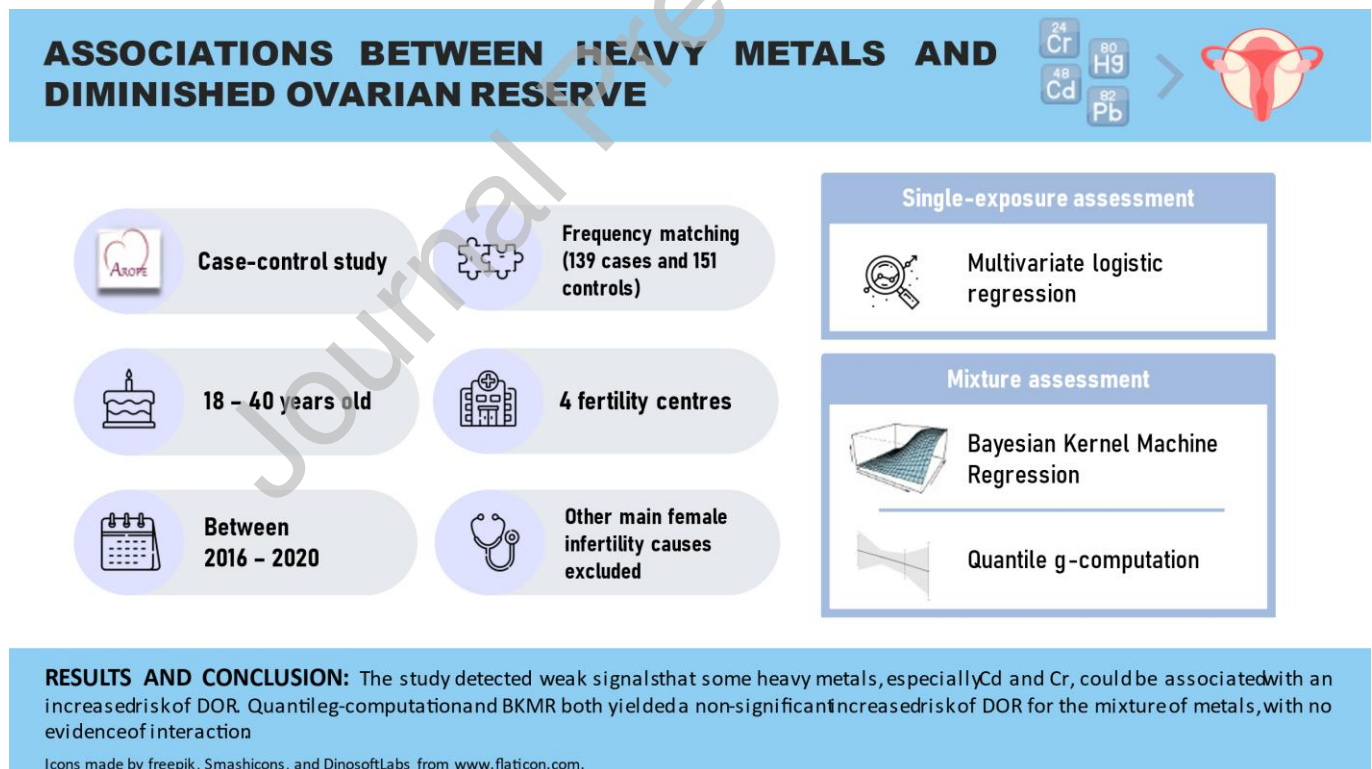
Table 4. Posterior inclusion probabilities for conditional inclusion in the BKMR model (AROPE study: N = 138 cases and 153 controls)

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

Key message

Some heavy metals could be associated with an increased risk of diminished ovarian reserve, one of the most frequently diagnosed pathology in women consulting for infertility. Future research should focus on exploring different windows of susceptibility, and on better understanding the potential biological mechanisms connecting metals and female fertility.

Graphical abstract



Vitae

Maximilien Génard-Walton is a final-year PhD student from the Institut de recherche en santé, environnement et travail and the University of Rennes. His research primarily focuses on the impacts of endocrine disrupting chemicals on female fertility.



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