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ORIGINAL ARTICLE

Toxic elements in follicular fluid adversely influence the likelihood of pregnancy and live birth in women undergoing IVF

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STUDY QUESTION: Are follicular fluid (FF), arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) concentrations associated with IVF outcomes among women undergoing IVF?

SUMMARY ANSWER: There was a non-linear association between higher FF Hg concentration and a lower likelihood of biochemical pregnancy and live birth. Higher FF Pb concentration was also associated with a lower probability of live birth.

WHAT IS KNOWN ALREADY: Previous research suggests that toxic elements may affect fertility among couples conceiving with and without assistance. However, the results have been inconsistent, possibly related in part to exposure misclassification. Very few studies have used ovarian FF to measure toxic elements, as it requires an invasive collection procedure, yet it may offer a more accurate estimate of a biologically effective dose than blood or urine.

STUDY DESIGN, SIZE, DURATION: This is a prospective study of 56 women undergoing IVF, from October 2015 to June 2017. FF was collected for analysis on the day of oocyte retrieval.

PARTICIPANTS/MATERIALS, SETTING, METHODS: As, Cd, Hg and Pb were determined in 197 FF specimens, using inductively coupled plasma tandem mass spectrometry. FF glutathione peroxidase, glutathione reductase, total glutathione-S-transferase, superoxide dismutase, arylesterase and paraoxonase (PON1p) activities were measured using kinetic enzyme assays.

MAIN RESULTS AND THE ROLE OF CHANCE: Non-linear associations were detected, in which the probabilities of biochemical pregnancy ($P = 0.05$) and live birth ($P = 0.05$) were lower in association with FF Hg greater than ~0.51 μg/l Hg, adjusted for age, race, cigarette smoking and recent seafood consumption. Higher FF Pb was also associated with a lower likelihood of live birth (relative risk (RR) = 0.68, 95% CI: 0.46, 1.00; $P = 0.05$). We also found a suggestive, although imprecise, antagonizing mediating effect of PON1p activity on the association between FF Pb and live birth (−28.3%; 95% CI: −358%, 270%).

LIMITATIONS, REASONS FOR CAUTION: The results should be interpreted judiciously given the limited sample size and difficulty accounting for correlated data in generalized additive models and mediation analyses. Additionally, women undergoing IVF are highly selected with respect to age and socioeconomic status, and so the generalizability of the results may be limited.

WIDER IMPLICATIONS OF THE FINDINGS: Overall, the results suggest that FF Hg was associated with a lower likelihood of biochemical pregnancy and live birth, with a potential threshold effect, and that higher FF Pb was associated with a lower probability of live

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birth. These results may help to guide clinical recommendations for limiting the exposure of patients to Hg and Pb and ultimately improve IVF success rates.

STUDY FUNDING/COMPETING INTEREST(S): This work was funded in part by the National Institute of Environmental Health Sciences (NIEHS), grant number 1R56ES023886-01, to the University at Albany (M.S.B.), and in part by the National Institute of Environmental Health Sciences (NIEHS), grant number 1U2CES026542-01, to the Wadsworth Center (P.J.P.). The authors declare no competing interests.

TRIAL REGISTRATION NUMBER: N/A

Key words: antioxidant enzymes / biomarkers / follicular fluid / IVF / lead / live birth / mercury / pregnancy / toxic elements

WHAT DOES THIS MEAN FOR PATIENTS?

This study examines whether exposure to four widely distributed toxic elements (arsenic, mercury, cadmium and lead) is associated with a woman's chance to become pregnant and have a live birth after IVF treatment. It also looks at whether antioxidant enzymes (proteins protective against oxidative stress) are important in the association.

Previous studies measured toxic elements in blood and urine, but only a few studies have looked at the levels in follicular fluid (FF). As FF surrounds the developing oocyte, it may better represent exposure to toxic elements than blood or urine. With the growing use of IVF, clinicians and patients have great interest in learning about factors that may improve their chances of a live birth.

This research included women receiving IVF treatment at an academic medical centre over 2 years. High mercury concentrations in FF were related to a lower chance of becoming pregnant and a lower chance of a live birth. Higher lead concentrations in FF were also related to a lower chance of a live birth. One antioxidant enzyme may protect against the negative relationship between lead levels in FF and the chance of having a live birth, but this needs to be researched further.

The study results are consistent with previous studies that have found a relationship between toxic elements and IVF outcomes. These results may inform women and their doctors to work together to modify exposures to toxic elements when looking to start IVF treatment.

Introduction

Infertility, defined as the inability to achieve a clinical pregnancy within 12 months of intercourse (ASRM 2013), affects ~16% of US women (Thoma *et al.*, 2013). Accordingly, there has been a dramatic increase in the use of ART, primarily IVF, over the past 30 years (Sunderam *et al.*, 2019). A total of 81 478 live US births, 1.9% of all live births, were attributed to ART in 2018 (CDC, 2020). However, IVF can cost upwards of $15 715 per cycle (Crawford *et al.*, 2016) and is infrequently reimbursed by US health insurance (Zagadailov *et al.*, 2020). Additionally, women and couples seeking IVF experience high levels of psychosocial stress (often seeking treatment after continued difficulty conceiving) contending with societal and familial pressures (Malina and Pooley, 2017). Multiple IVF cycles may also carry health risks (Gallos *et al.*, 2017). Considering the substantial financial and emotional costs, clinicians and patients have a vested interest in identifying modifiable risk factors contributing to IVF outcomes.

While delayed childbearing, genetics, obesity, sexually transmitted infections and lifestyle factors play important roles in infertility (Homan *et al.*, 2007), widespread and ongoing exposures to environmental contaminants, including toxic metals and metalloids, may also be important (Kumar, 2004; Al-Gubory, 2014). Women are exposed to arsenic (As), a metalloid, and the toxic heavy metals mercury (Hg), cadmium (Cd) and lead (Pb) through diet (Fujimoto *et al.*, 2009; Kim *et al.*, 2013; Nachman *et al.*, 2013; Butts *et al.*, 2020) and tobacco smoke (Satarug *et al.*, 2003). Although the results have been inconsistent, exposure to As, Hg, Cd and Pb has been associated with diminished fecundity in couples conceiving with (Choy *et al.*, 2002; Chang *et al.*, 2006; Silberstein *et al.*, 2006; Al-Saleh *et al.*, 2008; Bloom *et al.*, 2010, 2011, 2012a,b; Dickerson *et al.*, 2011; El Mohr *et al.*, 2020; Wu *et al.*, 2020) and without (Cole *et al.*, 2006; Louis *et al.*, 2012) assistance. Arsenic, Hg, Cd and Pb increase oxidative stress (OS) (Agarwal *et al.*, 2003; Al-Gubory, 2014): an imbalance of reactive oxygen species and antioxidant (AOX) enzyme activities. OS damages proteins, lipids and nucleic acids (Ercal *et al.*, 2001), potentially impacting the developmental competence of an oocyte (Agarwal *et al.*, 2012 ; Lu *et al.*, 2018) and subsequently conception.

Most previous epidemiologic investigations have used blood and urine as biomarkers of exposure to toxic elements (Choy *et al.*, 2002; Chang *et al.*, 2006; Bloom *et al.*, 2010, 2011, 2012a; Dickerson *et al.*, 2011; Louis *et al.*, 2012), while few have employed ovarian follicular fluid (FF) as a biomarker of exposure (Younglai *et al.*, 2002; Silberstein *et al.*, 2006; Al-Saleh *et al.*, 2008; Bloom *et al.*, 2012b; El Mohr *et al.*, 2020; Wu *et al.*, 2020). Ovarian FF surrounds the oocyte, directly reflecting its developmental microenvironment (Gosden *et al.*, 1988). Hence, concentrations of toxic elements in FF may provide more accurate indicators of the biologically effective doses that influence reproductive outcomes, in comparison to blood or urine (Butts *et al.*, 2021). Additionally, toxic elements may have non-monotonic or threshold-type effects on IVF outcomes (Zhu *et al.*, 2020), which have been under-investigated.

The primary aim of this study was to investigate associations between As, Hg, Cd and Pb in ovarian FF and IVF outcomes. In a secondary, hypothesis-generating aim, we explored AOX enzyme

activities as mediators of the associations. As one of only a few studies exploring FF toxic element exposures in association with IVF outcomes, and the first to investigate AOX enzyme activity as a potential mechanism, the findings from this study may be useful in developing clinical interventions to improve IVF outcomes and in guiding future studies.

Materials and methods

Study sample and clinical protocol

Couples and women undergoing IVF treatment at the University of California at San Francisco (UCSF) were enrolled into the Study of Metals and Assisted Reproductive Technologies (SMART) from October 2015 to June 2017. Approximately 895 emails (some duplicates) were sent to couples and women initiating IVF cycles at UCSF. There were 598 'read' receipts and 125 replies received, including 55 refusals. Of the remaining 70 women, 65 enrolled in the study, 50 with a male partner. For the remaining 15 women, the male partner either declined (n = 8), was ineligible (n = 1 sperm aspiration) or was unavailable (n = 6). Informed consent was obtained for all participants prior to study enrolment. Nine women were excluded for reasons which included cancelled cycles (n = 3), conversion to IUI (n = 1), or not having FF collected (n = 5). As described by Supplementary Fig. S1, the current analysis includes n = 56 women who contributed follicles analysed for toxic elements (197 follicles) and AOX enzymes (195 follicles).

Women received a baseline infertility examination, as part of the usual clinical IVF protocol, and completed an infertility questionnaire to collect demographic information and medical and reproductive histories. Study participants also completed an exposure questionnaire on the day of or shortly before oocyte retrieval, which captured lifestyle factors and consumption of seafood and other potential sources of exposure to toxic elements.

Women initiating IVF treatment underwent gonadotropin-induced ovarian stimulation according to standard clinic protocols. Follicular maturation and endometrial development were monitored by serum estradiol levels and transvaginal ultrasound. hCG was administered when a sufficient number of follicles ≥17 mm in diameter developed. Oocytes were retrieved by transvaginal fine needle aspiration within 36 h of hCG administration. Between 1.0 and 10 ml of undiluted FF was collected from the two largest individual follicles in each ovary (i.e. up to four follicles per participant), following recovery of the oocyte for clinical use. Four follicles were collected for the majority of participants (n = 42; 75.0%), while five (8.9%), seven (12.5%) and two (3.6%) women contributed three, two and one follicle/s, respectively. A new needle was used for each woman and aspiration needles were rinsed with saline before each individual puncture. However, the follicle itself was not rinsed. FF was examined for evidence of blood contamination (Levay *et al.*, 1997), then centrifuged and aliquoted into 1.8 ml cryovials, before freezing at −80°C until shipment to the laboratory for analysis. FF storage containers were acid washed prior to use and all laboratory disposables were pre-screened for contamination.

Collected oocytes were fertilized by conventional IVF or ICSI. For ICSI, oocytes were denuded of the cumulus mass and evaluated for maturity as being in metaphase II arrest (MII-arrest), prior to injection with a single sperm collected from the male partner or a donor. For conventional IVF, oocytes were cultured with sperm from the male partner or a donor, individually or as a co-culture with other oocytes from the same woman. Oocytes were individually tracked for outcomes in cases where they were not co-cultured.

Within 16–18 h, fertilization was confirmed by the presence of two pronuclei. Embryos were cultured through to the day of transfer to the recipient's uterus, individually or as a co-culture with other embryos from the same woman.

Embryos were transferred on days 2, 3 or 5 following fertilization, contingent on clinical factors and grading according to cleavage rate (measured by embryo cell number (ECN)), embryo fragmentation score (EFS) and embryo symmetry score (ESS). Higher ECN and greater symmetry (ESS) are positive predictors of pregnancy and live birth, while higher fragmentation (EFS) is a negative predictor (Fujimoto *et al.*, 2011; Racowsky *et al.*, 2011). ECN was defined as the number of blastomeres in each embryo at the time of transfer, and EFS was characterized by an ordinal grade reflecting the degree of cytoplasmic fragmentation (Grade 1, 0% fragmentation; Grade 2, 1–10% fragmentation; Grade 3, 11–25% fragmentation; Grade 4, 26–50% fragmentation; Grade 5, ≥51% fragmentation). ESS characterizes the degree of blastomere symmetry in a cleaved embryo, with scores of 1, 2 and 3 denoting fully symmetric, slightly symmetric and highly asymmetric blastomeres, respectively. We defined better/poorer embryo-level outcomes a priori based on previously reported associations between embryo grading criteria and live birth rate (Racowsky *et al.*, 2011) and experience at our clinic, as ECN ≥6/<6, EFS ≤grade 2/>grade 2 and ESS = 1 (full symmetry)/>1.

A serum hCG test was completed 2 weeks after embryo transfer, indicating a successful implantation or biochemical pregnancy, and clinical pregnancy was confirmed by ultrasound visualization of a gestational sac 2 weeks later. Birth outcomes were collected from the patients by mail.

Toxic elements analysis

One FF aliquot from each sample (n = 197) was analysed for As, Hg, Cd and Pb, at the Laboratory of Inorganic and Nuclear Chemistry, Wadsworth Center, New York State Department of Health (Albany, NY, USA). The analytical method, described in detail in a previous publication (Galusha *et al.*, 2019), was developed on an Agilent 8900 inductively coupled plasma tandem mass spectrometer (ICP-MS/MS) (Agilent Technologies, Santa Clara, CA, USA). Briefly, specimens were prepared along with standards and quality control samples (QCs), diluted into polypropylene autosampler tubes and analysed by ICP-MS/MS. The limits of detection (LODs) were defined in a manner consistent with ISO/IUPAC harmonized guidelines: 3× the standard deviation of the analyte measured in an FF matrix pool (Thompson *et al.*, 2002) and were 0.04 µg/l, 0.03 µg/l, 5.60 ng/l and 0.03 µg/l for As, Hg, Cd and Pb, respectively. All instrument measured values were employed for statistical analysis irrespective of LODs (Richardson and Ciampi, 2003; Schisterman *et al.*, 2006).

Antioxidant enzyme activity analysis

A second FF aliquot (n = 195) was shipped to the Oxidative Stress Laboratory at the University at Buffalo, State University of New York

(Buffalo, NY, USA) for analysis of AOX enzyme activities. Glutathione peroxidase (GPx), glutathione reductase (GR), total glutathione-S-transferase (GST), superoxide dismutase (SOD) (Pippenger et al., 1998), arylesterase (PON1a) and paraoxonase (PON1p) (Browne et al., 2007) activities were quantified by kinetic enzyme assay using a Cobas Fara II automated chemistry analyser (Roche Diagnostics, Indianapolis, IL, USA) (Browne et al., 2008b). GPx and GR assays were conducted using reagent assay kits from ZeptoMetrix, Corp. (Buffalo, NY, USA).

The PON1 Q192R polymorphism phenotype (QQ, QR or RR) governs PON1 activity (Humbert et al., 1993). PON1 activities, which include PON1a and PON1p, were determined as described in detail elsewhere (Browne et al., 2007). Briefly, PON1a and PON1p activities were measured by the rate of formation of phenol using phenyl acetate as the substrate, and the rate of formation of p-nitrophenol using paraoxon as the substrate, respectively. The phenotypic ratios of PON1a and PON1p activities correspond to PON1 Q192R genotype with 100% accuracy (Browne et al., 2007). All samples for a single participant were analysed simultaneously and in duplicate, with controls for quality assurance and QC purposes.

Statistical analyses

Descriptive analysis

We first inspected distributions of demographic and clinical variables as well as toxic elements. We then estimated bivariate associations of demographic and clinical variables with toxic elements and IVF outcomes, including MII-arrest, fertilization, ECN, EFS, ESS, biochemical pregnancy and live birth. All IVF outcomes were dichotomized (yes/no), by comparing a favourable to a less favourable outcome ('better' vs. 'poorer'). One fresh embryo transfer cycle per participant was included in the analyses and there were no missing covariate data.

Multivariable analysis

We first employed generalized additive models (GAMs), which are used to assess the linearity of associations, to estimate dose–response relations between four toxic elements with IVF outcomes, using a Poisson distribution with a natural logarithm link function, and including a thin plate regression spline term for each element (Wood, 2003) . For IVF outcomes without a non-linear association (i.e. without a statistically significant spline), including MII-arrest, fertilization, ECN, EFS and ESS, we constructed modified Poisson regression models using generalized estimating equations (GEE) (Zou and Donner, 2013), adjusted for race (Asian vs. 'other'), smoking (having smoked more than 100 cigarettes in a lifetime vs. not) and seafood consumption (consuming 1–5 lbs. within the last week vs. <1 lb.) as confounding variables. For biochemical pregnancy and live birth, which had statistically significant spline terms indicating non-linear associations, we repeated the GAMs including the spline terms, adjusting for race, smoking and seafood consumption as confounders, and adjusted for follicle, to control in part for the effect of multiple follicles per woman.

Mediation analysis

We conducted an exploratory analysis to evaluate AOX enzyme activities as intervening variables in toxic element-IVF outcome associations. Prior to mediation, we assessed whether (i) a toxic element (predictor) was associated with the IVF outcome; (ii) a toxic element predicted AOX enzyme activity (mediator); and (iii) an AOX enzyme (mediator) had an effect on the IVF outcome of interest. Given the exploratory nature of this mediation analysis, we had no a priori expectations regarding which AOX enzymes may act as mediators. Only the predictors, mediators and outcomes that satisfied all three criteria were included in the mediation analysis.

Ultimately, we adopted a model-based causal mediation approach using the 'mediation' package in the R statistical software package (Tingley et al., 2014). In the first step, we specified two models, one estimating the associations between the toxic element predictor (T_i) and the mediator (M_i), adjusting for covariates (X_i) and the second estimating associations for T_i and M_i with the outcome (Y_i), also adjusting for X_i (Tingley et al., 2014). In the second step, we estimated the average causal mediation effect (ACME), the average direct effect (ADE), the total effect of the predictor and mediator on the outcome, and the proportion of the total effect attributed to the mediator. We employed the 'mediate' function to produce non-parametric bootstrap confidence intervals for the effect estimates based on up to 1000 bootstrap samples. The ACME is the effect of the predictor on the outcome that passes through the mediator and the ADE is the effect of the predictor on the outcome that does not. We included spline terms in the mediation analysis to accommodate non-linear associations between toxic elements and IVF outcomes, and adjusted for race, smoking, seafood consumption and follicle as covariates.

Because we were unable to accommodate the correlated nature of IVF outcomes within a woman during the mediation analysis, we assessed possible inaccuracies in the standard errors. We compared the results of a Poisson model using GEE to a Poisson model without GEE, but incorporating follicle as fixed effects, in other words, an approach that ignores the clustering. This allowed us to assess how much of the total variance was due to correlated data within a woman by comparing the estimated standard errors.

We used SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and R 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) for all statistical analyses. Statistical significance was defined as $P < 0.05$ for main effects (i.e. modified Poisson models) and $P < 0.10$ for non-linear (i.e. GAM) and mediation effects, using two-tailed hypothesis tests.

Ethical approval

The study protocol was approved by the UCSF Committee for Human Research, and the Institutional Review Boards at the University at Albany and the New York State Department of Health.

Results

Distribution of demographic and clinical factors and bivariate analyses

We described the distributions of demographic variables and toxic elements in Table I. Approximately 34% of the participants were Asian, with the remainder being mostly white. Median FF concentrations were 0.34 μg/l for As, 0.34 μg/l for Hg, 18.1 ng/L for Cd and 0.06 μg/l for Pb. As summarized in Supplementary Table SI, we previously reported that the majority of FF As, Hg and Cd variability was

Table I Distribution of demographic and clinical variables and toxic elements As, Hg, Cd and Pb in FF among n = 56 women undergoing IVF[a]

Variables	n	Mean ± SD	Min	25th %tile	Median	75th %tile	Max	LOD	% > LOD
Age	56	38.3 ± 3.3	30.0	36.0	38.0	41.0	44.0	–	–
BMI (kg/m^2)	56	24.2 ± 5.0	18.2	1.6	22.7	25.7	39.5	–	–
Race (%):									
Other	37	66.1%	–	–	–	–	–	–	–
Asian	19	33.9%	–	–	–	–	–	–	–
Smoked >100 cigarettes (%):									
Yes	14	25.0%	–	–	–	–	–	–	–
No	42	75.0%	–	–	–	–	–	–	–
Primary diagnosis (%):									
Diminished ovarian reserve	22	39.3%	–	–	–	–	–	–	–
Unexplained	16	28.6%	–	–	–	–	–	–	–
Tubal/male/non-ovary related	15	26.8%	–	–	–	–	–	–	–
Ovarian disorder	3	5.4%	–	–	–	–	–	–	–
Stimulation protocol (%):									
Lupron down-regulated	13	23.2%	–	–	–	–	–	–	–
Gonadotropin antagonist	34	60.7%	–	–	–	–	–	–	–
Flare	9	16.1%	–	–	–	–	–	–	–
Peak E_2 (pg/ml)	56	2610.88	1359.46	506.30	1658.00	2592.50	3279.50	6241.00	2610.88
Intended treatment (%):									
ICSI	44	78.6%	–	–	–	–	–	–	–
IVF	12	21.4%	–	–	–	–	–	–	–
Toxic elements in FF[b]									
As (μg/l)	56	0.57 ± 0.67	0.04	0.16	0.34	0.58	3.47	0.04	98.2
Hg (μg/l)	56	0.42 ± 0.32	0.01	0.18	0.34	0.58	1.44	0.03	91.1
Cd (ng/l)	56	22.66 ± 13.29	8.01	14.34	18.09	27.34	80.08	5.60	100.0
Pb (μg/l)	56	0.07 ± 0.04	−0.01	0.05	0.06	0.09	0.30	0.03	94.6

[a] n = 197 follicular fluid samples.
[b] Mean and SD of individual averages.
FF, follicular fluid; LOD, limit of detection; Min, minimum observed value; Max, maximum observed value, SD, standard deviation.

attributed to sources between women, while most FF Pb variability was attributed to sources between follicles (Butts *et al.*, 2021).

The distributions of IVF outcomes are displayed in Table II. On average 12.8 oocytes were collected from each woman, and women produced an average of 7.6 embryos, and mostly had transfers on Day 3 (40.6%). There were a total of 16 biochemical pregnancies (29.6%) and 9 live births (16.7%), excluding n=2 undergoing fertility preservation.

Figure 1 shows differences in FF toxic element levels (μg/l) by demographic and clinical factors. Participants' average FF Pb levels (in up to four follicles each) were correlated with age ($r = 0.31$, $P = 0.02$) and BMI ($r = -0.28$, $P = 0.03$). We did not detect statistically significant differences between peak estradiol and FF trace elements (data not shown). Women eating more than 1 lb. of seafood (~0.5 kg) in the past week had higher FF Hg ($P = 0.04$) than women eating less. The unadjusted associations between IVF outcomes and covariates are described in Supplementary Table SII.

The distributions of FF AOX enzymes and their correlations are described in Supplementary Table SIII and SIV, respectively, and associations with covariates are described in Supplementary Table SV. Asians had higher mean FF GPx ($P = 0.05$) and PON1p ($P = 0.001$) than other races, and PON1p was lower among women with biochemical pregnancies ($P = 0.04$) and live births ($P = 0.04$). Approximately 52% of women had an R+ PON phenotype (QR or RR), which was more common among Asians ($P = 0.001$).

Associations between toxic elements, AOX enzyme activities and IVF outcomes

We did not detect evidence of non-linear associations for toxic elements with oocyte maturity, fertilization, ECN, EFS or ESS using GAMs (data not shown). Similarly, there were no associations between FF toxic elements and oocyte maturity, fertilization, ECN, EFS or ESS using modified Poisson regression models (Table III).

In contrast, we detected statistically significant non-linear splines for FF Hg with biochemical pregnancy ($s = 3.22$, $P = 0.05$) and live birth ($s = 4.12$, $P = 0.05$), adjusted for other toxic elements and covariates, as displayed in Fig. 2. There appeared to be no meaningful association

Table II Distribution of clinical outcomes among n = 56 women undergoing IVF.[a]

Variables	n	Mean ± SD	Min	25th %tile	Median	75th %tile	Max
Total oocytes collected	56	12.8 ± 7.4	0.0	8.0	11.0	17.0	31.0
Total embryos produced	56	7.6 ± 5.5	0.0	4.0	6.5	9.0	22.0
Proportion of mature oocytes[b]	43	0.72 ± 0.22	0.0	0.67	0.74	0.85	1.0
Proportion of fertilized oocytes[c]	55	0.65 ± 0.21	0.14	0.50	0.67	0.80	1.0
Mean ECN	48	7.5 ± 1.2	4.7	6.9	7.4	8.3	12.0
Mean EFS	47	2.0 ± 0.46	1.1	1.6	1.9	2.3	3.0
Mean ESS	47	1.8 ± 0.27	–	–	–	–	–
Day 2 embryo transfer (%)	8	25.0%	–	–	–	–	–
Day 3 embryo transfer (%)	13	40.6%	–	–	–	–	–
Day 5 embryo transfer (%)	11	34.4%	–	–	–	–	–
Biochemical pregnancy (%)[d]	16	29.6%	–	–	–	–	–
Live births (%)[d]	9	16.7%	–	–	–	–	–

[a] Pooled outcomes.
[b] Defined as the average proportion of oocytes retrieved in metaphase II arrest, excludes n = 12 women receiving IVF and n = 1 woman with cycle cancelled after retrieval.
[c] Defined as proportion of fertilized oocytes with the presence of two pronuclei, excludes n = 1 woman with cycle cancelled after retrieval.
[d] n = 2 undergoing fertility preservation excluded.
Min, minimum observed value; Max, maximum observed value; SD, standard deviation.

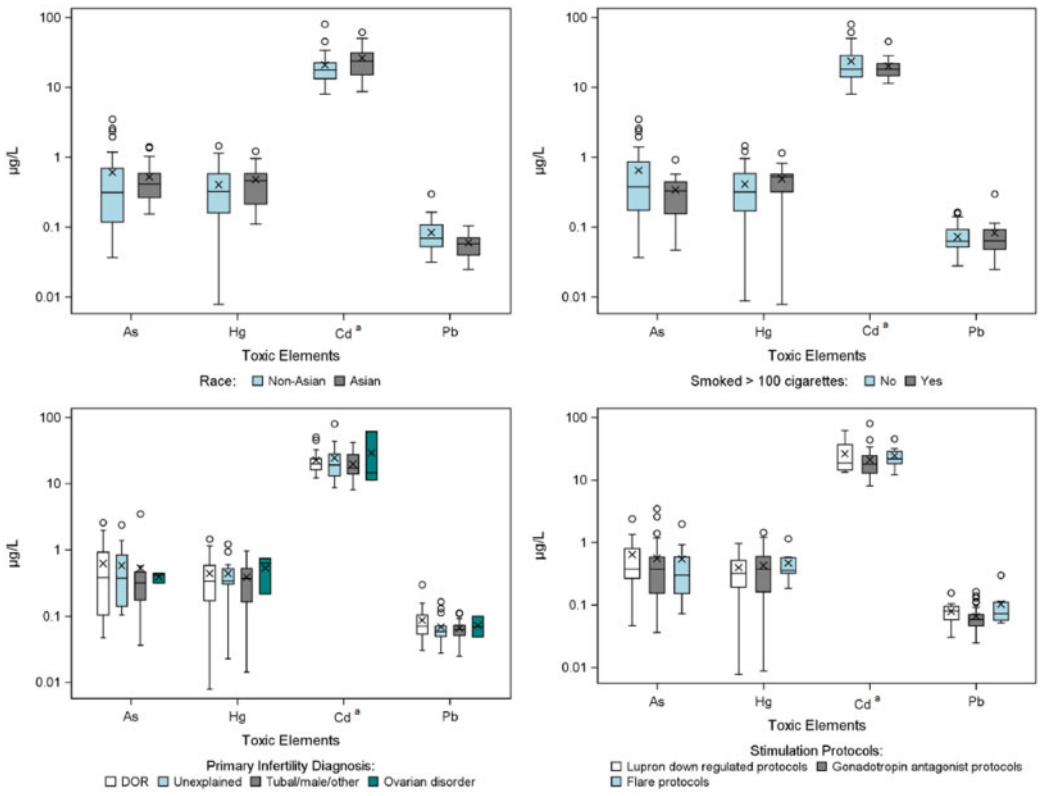

Figure 1 Distributions of follicular fluid (FF) arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) levels (µg/l) by demographic and clinical factors among n = 56 women undergoing IVF. Individual follicles were tracked to individual oocytes and embryos using a 'one follicle-one oocyte' approach for oocyte and embryo outcomes. The boxplots show the mean as 'x', the median as the central horizontal line, the upper and lower quartiles as the top and bottom of the box, respectively, 1.5 times the interquartile range as the whiskers, and outlying values as circles beyond the whiskers. DOR, diminished ovarian reserve. [a]Cadmium (Cd) in ng/l. [b]n = 2 undergoing fertility preservation excluded; *$P < 0.05$ for difference between groups.

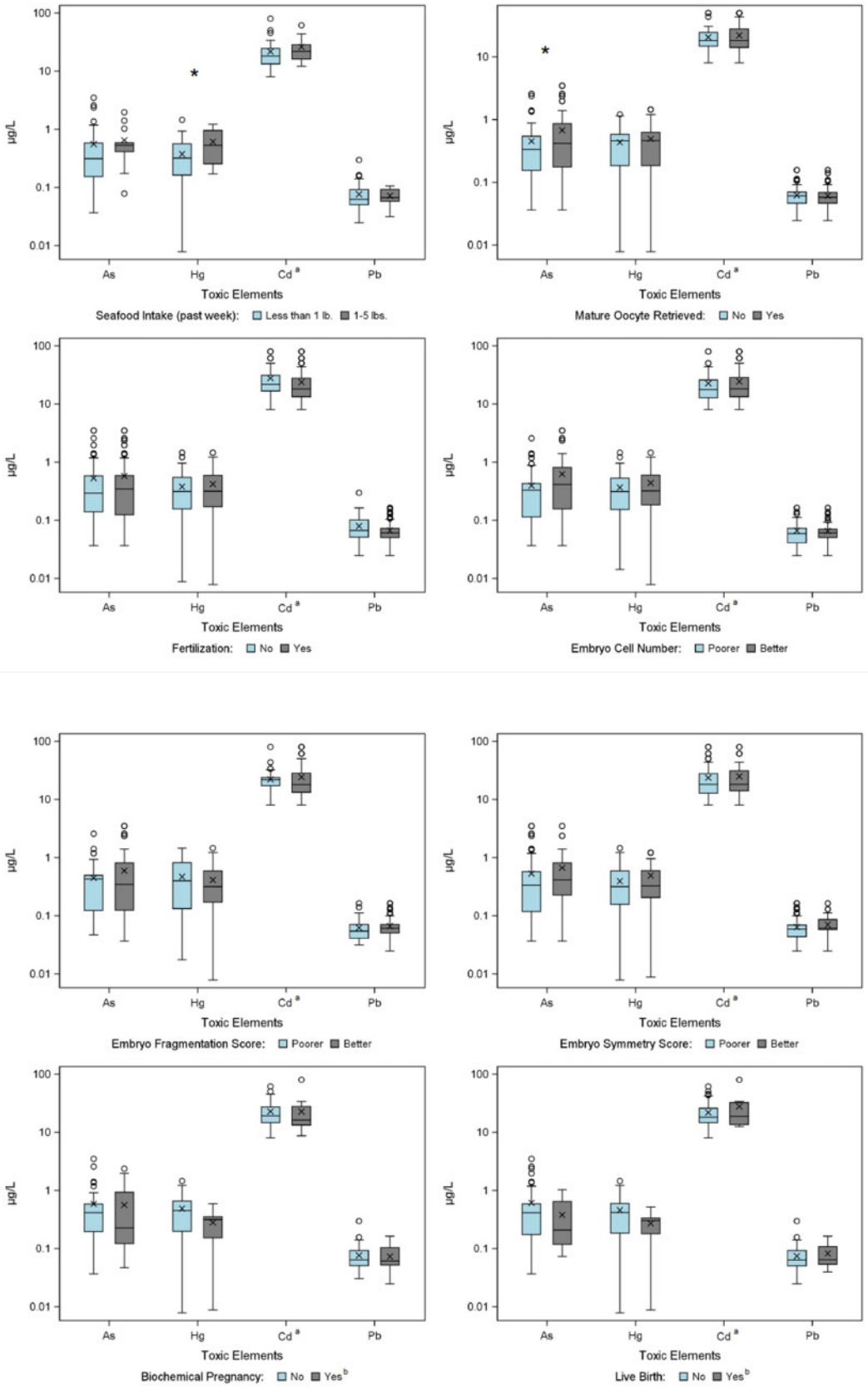

Figure 1 (Continued)

Table III Multi-element modified Poisson models of FF As, Hg, Cd and Pb with clinical outcomes among n = 56 women undergoing IVF.[a]

Toxic elements	RR	95% CI	*P*-value
MII oocyte[b] (92)			
As	1.03	0.95, 1.10	0.50
Hg	1.07	0.95, 1.22	0.27
Cd	1.10	0.93, 1.29	0.25
Pb	0.95	0.74, 1.20	0.65
Fertilization[b] (113)			
As	1.07	0.90, 1.28	0.45
Hg	0.98	0.83, 1.15	0.78
Cd	0.87	0.71, 1.07	0.20
Pb	1.15	0.94, 1.41	0.17
ECN[c] (68)			
As	1.01	0.88, 1.16	0.87
Hg	1.05	0.92, 1.20	0.50
Cd	1.10	0.93, 1.30	0.26
Pb	1.21	0.86, 1.70	0.27
EFS[c] (66)			
As	0.95	0.83, 1.09	0.45
Hg	1.01	0.91, 1.13	0.80
Cd	0.91	0.79, 1.06	0.24
Pb	1.21	0.90, 1.63	0.20
ESS[c] (66)			
As	1.03	0.46, 2.30	0.95
Hg	1.19	0.73, 1.94	0.49
Cd	1.11	0.43, 2.89	0.82
Pb	1.24	0.24, 6.51	0.80

Value in parentheses is the number of observations.
[a] Natural log-transformed values adjusted for age (As, Hg, Cd and Pb), race (As, Hg, Cd and Pb), cigarette smoking (As and Cd) and seafood consumption (As, Hg, Cd and Pb);
[b] Models predicted positive outcome (i.e. oocyte retrieved in MII-arrest, oocyte fertilized);
[c] Models predicted 'better' embryo quality as outcome.
CI, confidence interval; ECN, embryo cell number; EFS, embryo fragmentation score; ESS, embryo symmetry score; MII oocyte, mature oocyte retrieved/in metaphase II arrest; fertilization, fertilized oocytes denoted presence of two pronuclei; RR, relative risk.

for FF Hg with biochemical pregnancy until ~0.51 µg/l FF Hg, above which the likelihood of biochemical pregnancy declined with greater FF Hg. While the GAM for FF Hg with live birth indicated a slightly greater probability of live birth between ~0.05 and 0.35 µg/l Hg, it too declined with greater FF Hg above ~0.51 µg/l FF Hg. We also found suggested negative linear associations between FF Pb (relative risk (RR) = 0.79, 95% CI: 0.57, 1.08; $P = 0.14$) and biochemical pregnancy, and for FF As (RR = 0.66, 95% CI: 0.40, 1.10; $P = 0.11$) and FF Pb with live birth (RR = 0.68, 95% CI: 0.46, 1.00; $P = 0.05$), in GAMs adjusted for other toxic elements and covariates.

The confounder-adjusted associations between AOX enzymes and IVF outcomes are described in Supplementary Table SVI. Greater FF GST was associated with retrieving a mature oocyte (RR = 1.18, 95% CI: 1.04, 1.34; $P = 0.01$). However, greater PON1p was associated with a lower likelihood of biochemical pregnancy (RR = 0.48, 95% CI: 0.27, 0.86; $P = 0.01$) and live birth (RR = 0.36, 95% CI: 0.17, 0.73; $P = 0.01$).

Mediation analysis

We assessed PON1p, which met the three a priori criteria, as a mediator of the associations between FF Hg and biochemical pregnancy, and FF Hg and Pb with live birth, as shown in Table IV. Most of the FF Hg biochemical pregnancy association was direct, with ~2.9% (95% CI: −0.80%, 16.0%) mediated by PON1p. Similarly, the majority of the FF Hg live birth association was also direct, with ~2.6% (95% CI: −0.88%, 15.0%) mediated by PON1p. In contrast, ~28.3% (95% CI: −358%, 270%) of the Pb live birth association was mediated by PON1p, although the estimate was imprecise.

Discussion

In this prospective investigation of women undergoing IVF, we examined associations between FF concentrations of four widely distributed toxic elements and a spectrum of IVF outcomes, from oocyte maturity to live birth. Greater FF Hg was associated with a lower likelihood for biochemical pregnancy and live birth in a non-linear fashion, and greater FF Pb was associated with a lower probability for a live birth. Our results suggest that FF Hg and FF Pb may impact IVF success. We recently reported seafood-heavy diets as an important source of FF Hg in this study population (Butts *et al.*, 2020). Hence, our results may have important clinical implications, as diet is a potentially modifiable behaviour to help limit exposures to toxic elements, not undervaluing the importance of nutrients in fish, such as omega-3 polyunsaturated fatty acids (Gaskins and Chavarro, 2018). Our mediation analysis suggested that GPx, GR, GST and SOD activities were not important mechanisms driving the associations between FF Hg, biochemical pregnancy and live birth. Yet, PON1p played a potentially important mechanistic role in the inverse association between FF Pb and live birth.

Comparison to previous studies

Our results are consistent with previous literature exploring the impact of toxic elements in FF and IVF outcomes. Similar to the results of a smaller, previous UCSF cohort (Bloom *et al.*, 2012b), no associations were suggested between FF Hg, Cd or Pb and oocyte maturity among IVF patients in the current study. Like an earlier Saudi Arabian cohort (Al-Saleh *et al.*, 2008), no associations were found with FF Hg and oocyte fertilization. However, our results suggested a negative association between FF Pb and biochemical pregnancy, similar to a study that reported higher median FF Pb levels among non-pregnant compared to pregnant participants (Silberstein *et al.*, 2006). The previous UCSF cohort reported lower probabilities of biochemical (−25%) and clinical (−56%) pregnancies with greater FF Hg, though not statistically significant (Bloom *et al.*, 2012b). Another study also reported no association of FF Hg concentrations and pregnancy, but a significant negative association for blood Hg and pregnancy (Al-Saleh *et al.*, 2008). A study from Egypt found no difference of FF As, Cd and Pb levels in women with and without a pregnancy (El Mohr *et al.*, 2020). In contrast, a

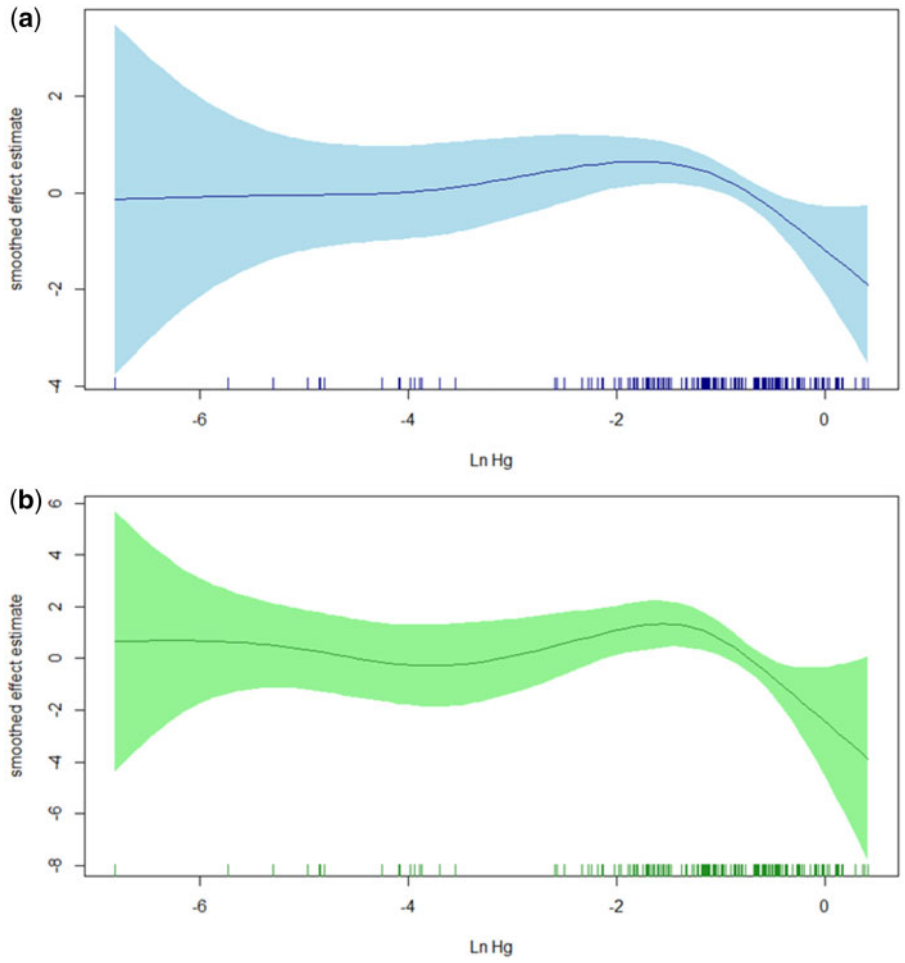

Figure 2 Generalized additive models (GAM) describing non-linear associations of follicular fluid (FF) mercury (Hg) with (a) biochemical pregnancy and (b) live birth, among n = 54 women undergoing IVF. The *x*-axis represents natural log-transformed FF Hg concentrations (μg/L) and the *y*-axis represents effect estimates for the association between log FF Hg concentration and the likelihood of pregnancy or live birth using a smoothing function. Models were adjusted for age, race, cigarette smoking and recent seafood consumption. Both graphs suggest a threshold effect at ∼0.51 μg/l Hg.

recent study from China reported lower likelihoods of implantation, pregnancy and live birth with greater FF Cd measured in a single FF specimen (Wu *et al.*, 2020). However, the median FF Cd concentration of 60 ng/l in that study was more than three times greater than 18.09 ng/l in this study, which may account in part for the different results.

Although earlier investigations did not report associations for FF Hg with IVF outcomes, our GAM-based approach allowing for non-linear associations may have uncovered previously undetected relationships. In fact, a study of reproductive age women using a nationally representative 2013–2016 US data set, reported a non-linear dose–response association for blood Hg concentrations and clinical infertility, with a threshold of ∼5.3 μg/l (Zhu *et al.*, 2020). Our results are particularly concerning given that the FF Hg (Al-Saleh *et al.*, 2008; Bloom *et al.*, 2012b; Tolunay *et al.*, 2016) and Pb (Paksy *et al.*, 2001; Silberstein *et al.*, 2004; Al-Saleh *et al.*, 2008; Bloom *et al.*, 2012b; Tolunay *et al.*, 2016; Wdowiak *et al.*, 2018) concentrations in our study population are lower than reported by other studies.

The results from our bivariate analyses were consistent with previous studies, lending credibility to our results. Older women were less likely to conceive a pregnancy and to deliver a live birth (Hull *et al.*, 1996), and cigarette smokers were less likely to have mature oocytes retrieved than non-smokers (Freour *et al.*, 2012). Surprisingly, smoking was not correlated with the other IVF outcomes, but that may be in part a reflection of response bias or our crude measure as a lifetime history of smoking more than 100 cigarettes. Still, cigarette smoking was a statistically significant predictor of poorer IVF outcomes in the regression models, suggesting that our variable captured smoking information in part. Non-white women, including Asians, typically have lower IVF success rates than white women in the USA (Fujimoto *et al.*, 2010; McQueen *et al.*, 2015). Similar to previous reports (Purcell *et al.*, 2007; Bloom *et al.*, 2011), Asian women in our study

Table IV Mediation analysis employing paraoxonase (PON1p) as a mediator, toxic elements as the predictor, and biochemical pregnancy and live birth as the outcomes, among n = 54 women undergoing IVF.

Toxic elements	β	95% CI	*P*-value
Biochemical pregnancy with FF Hg			
Total effect	−0.06	−0.09, 0.00	0.01
ACME	−0.002	−0.01, 0.00	0.11
ADE	−0.05	−0.08, 0.00	0.01
Proportion mediated	2.9%	−0.80%, 16.0%	0.12
Live birth with FF Hg[a]			
Total effect	−0.01	−0.03, 0.00	0.01
ACME	−0.0002	−0.003, 0.00	0.10
ADE	−0.01	−0.03, 0.00	0.01
Proportion mediated	2.6%	−0.88%, 15.0%	–
Live Birth with FF Pb[a,b]			
Total effect	−0.02	−0.03, 4.36	0.35
ACME	0.01	0.001, 2.02	0.01
ADE	−0.02	−0.06, 2.37	0.22
Proportion mediated	−28.3%	−358%, 270%	0.41

ACME, average causal mediation effects or the indirect effect of the treatment on the outcome that goes through the mediator; ADE, average direct effect or the direct effect of the treatment on the outcome; total effect, represents both the direct and indirect effects of the treatment on the outcome; CI, confidence interval.
[a] Simulations truncated due to model instability;
[b] Employed 500 simulations to calculate non-parametric bootstrap CIs.

tended to have more favourable embryo outcomes, yet had lower chances of fertilization than other races.

We also found a higher probability of retrieving a mature oocyte and better ECN in association with greater FF GST and PON1p activities, respectively. While FF PON1a, which reflects the arylesterase activity of the paraoxonase enzyme, was positively associated with ECN (OR = 1.09, 95% CI: 1.01–1.17) in a previous IVF cohort, no associations were detected for PON1p (Browne *et al.*, 2008a). In a study from Turkey, serum PON1a and PON1p levels were lower among women with early pregnancy failure (Toy *et al.*, 2009). Yet, we found an association between higher PON1p and lower chances of biochemical pregnancy and live birth. Notably, women in the Turkish study were younger compared to this study and were not seeking fertility treatment, and some underlying factors associated with infertility were exclusion factors (Brugo-Olmedo *et al.*, 2001), which may account in part for the discordant results.

Mediation results

The results of the mediation analysis provided little evidence for mediating activities by the measured AOX enzymes on associations between FF toxic elements and IVF outcomes. Based on the a priori criteria for mediation, we did not assess GPx, GR, GST, SOD or PON1a. Our PON1p results raised the possibility for modest mediation of FF Hg associations with biochemical pregnancy and live birth. While the ACME was stronger for the association between FF Pb and live birth, the results were highly imprecise. When the ACME and ADE are opposite signs (i.e. negative and positive, respectively), referred to as 'inconsistent mediation', there may be a suppression of effects (MacKinnon *et al.*, 2000). Pb directly produces singlet oxygen, H_2O_2 and hydroperoxides (Jomova and Valko, 2011), potent reactive oxygen species that increase OS, while PON1 facilitates the hydrolysis of hydroperoxides (Dias *et al.*, 2014), mitigating OS, and so this result warrants further investigation.

Similar to previous reports (Li *et al.*, 2006; Kim *et al.*, 2017), we found more PON1 Q192R R+ phenotypes in Asian than in other women as described in Supplementary Table SVII, and higher PON1p activity among R+ women compared to QQ women (Humbert *et al.*, 1993), shown in Supplementary Table SVIII. Greater PON1p activity was associated with lower blood Hg and Pb in prior work (Ginsberg *et al.*, 2014; Li *et al.*, 2006), with a more pronounced association among PON Q192R RR homozygotes (Li *et al.*, 2006). Still, we did not find an interaction between Pb and PON1 Q192R phenotype (data not shown), although the limited sample precluded a stratified analysis.

Overall, the mediation results for PON1p and Hg with pregnancy and live birth reflect a very minor role, suggesting that other pathways, such as endocrine disruption (Gore, 2007; Iavicoli *et al.*, 2009), may be a more relevant mechanism.

Strengths and limitations

Although our work offers several strengths, the results should be interpreted with caution given a number of limitations. First, the small sample size may have undermined statistical power to detect modest differences in associations between FF toxic elements and IVF outcomes. Furthermore, the unstable mediation analysis estimates may be due in part to the small number of live births, coupled to the limited distributions of FF Hg and Pb. However, we leveraged the collection of up to four follicles from each woman to increase study power, modelling each follicle as the unit of observation. Notably, not all women contributed four follicles. This may in part be related to age, which is associated with diminished ovarian reserve (Wallace and Kelsey, 2004). The average age in this study population (38.3 years) and most frequent primary infertility diagnoses (DOR, 39.3%), introduces the possibility of heterogeneity by diagnosis. As such, these results require confirmation in a larger future investigation that includes a stratified analysis.

Second, although we incorporated a random intercept using linear models to account for multiple follicles in each woman, we did not account for correlated outcomes among women in GAMs and the mediation analysis. Models were adjusted for follicle as a fixed effect to partially accommodate clustering within woman. However, we found a modest bias in the standard errors for FF Hg associated with biochemical pregnancy (2.4% to 10.1%) and live birth (−8.8% to 9.7%), and for Pb associated with live birth (−8.0% to 9.7%), with and without PON1p included as a mediator in the model (data not shown). These results suggest that bias due to correlated outcomes was likely modest and that our results may underestimate the effects of Hg and Pb with live birth.

Additionally, as exposures do not occur in isolation, it is important to consider methods to model exposure mixtures (Hernández and Tsatsakis, 2017). Although we simultaneously modelled toxic elements as predictors of IVF outcomes, we did not account for their potential

interactions and so a larger future investigation will be necessary to more definitively assess the simultaneous impact of FF toxic elements. However, our FF exposure biomarker may allow for a closer approximation of a biologically effective dose than blood and urine, and our collection of up to four individual follicles offers an advantage over the more frequently employed pooled follicle approach, which may be subject to exposure measurement error (Butts *et al.*, 2021). We also adjusted for seafood consumption, to reduce residual confounding by diet, the primary non-occupational exposure source for Hg.

IVF populations are highly selected with respect to age, marital status and socioeconomic class, which may limit the generalizability of our study results (Datta *et al.*, 2016; Farland *et al.*, 2016). This creates the possibility for selection bias, over- or underestimating effects, if participants have different exposures and outcome risks than infertile women in general, non-responders or non-participants. However, As, Hg, Cd and Pb exposure is widespread (CDC, 2019) and the mechanisms by which they exert effects are unlikely to differ across these groups. Furthermore, we have no reason to believe that participation in the study would be related to exposure levels.

Conclusions

Our results suggest that Hg is associated with biochemical pregnancy and live birth rates, and Pb is associated with live birth rates in women undergoing IVF treatment. Additionally, AOX enzyme activity does not appear to be a major pathway through which toxic elements impact IVF outcome. While these findings warrant corroboration in a larger study, this investigation offers unique insight into the biology underlying the relationship between toxic element exposures and IVF outcomes and may ultimately help IVF patients improve their chances of a live birth.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

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Authors' roles

C.D.B.: Conceptualization, Methodology, Formal analysis, Data curation and Writing: Original Draft and Visualization; M.S.B.: Conceptualization, Methodology, Writing: Review and Editing, Supervision, Project administration and Funding acquisition; A.M.: Validation, Investigation and Writing: Review and Editing; N.L.: Validation, Investigation and Writing: Review and Editing; R.W.: Validation, Investigation and Writing: Review and Editing; E.M.-L.: Resources, Writing: Review and Editing and Supervision; P.J.P.: Conceptualization, Resources, Writing: Review and Editing, Supervision and Funding acquisition; A.L.G.: Validation, Investigation and Writing: Review and Editing; R.M.Y.: Formal analysis and Writing: Review and Editing; B.J.F.: Writing: Review and Editing; R.W.B.: Conceptualization and Writing: Review and Editing; V.Y.F.: Conceptualization, Resources, Writing: Review and Editing, Supervision and Project administration.

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Conflict of interest

The authors have no conflict of interest to declare in relation to this work.

Data availability

The data underlying this article cannot be shared publicly to ensure the privacy of individuals who participated in the study. The data will be shared on reasonable request to the corresponding author.

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