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Variability of essential and non-essential trace elements in the follicular fluid of women undergoing in vitro fertilization (IVF)

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Abstract

Both essential and non-essential elements have been associated with female reproductive function in epidemiologic investigations, including among IVF populations. To date, most investigators have used blood or urine to assess biomarkers of exposure, with few employing ovarian follicular fluid (FF). FF may offer a more direct “snapshot” of the oocyte microenvironment than blood or

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CRediT authorship contribution statement

Celeste D. Butts: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Visualization. **Michael S. Bloom:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Alexandra McGough:** Validation, Investigation, Writing - review & editing. **Nikolaus Lenhart:** Validation, Investigation, Writing - review & editing. **Rebecca Wong:** Validation, Investigation, Writing - review & editing. **Evelyn Mok-Lin:** Resources, Writing - review & editing, Supervision. **Patrick J. Parsons:** Conceptualization, Resources, Writing review & editing, Supervision, Funding acquisition. **Aubrey L. Galusha:** Validation, Investigation, Writing - review & editing. **Recai M. Yucel:** Formal analysis, Writing - review & editing. **Beth J. Feingold:** Writing review & editing. **Richard W. Browne:** Conceptualization, Writing review & editing. **Victor Y. Fujimoto:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111733.

urine, however previous studies report follicle-to-follicle variability in FF constituents that may contribute to exposure misclassification. Our objectives were to investigate sources of trace element variability, to estimate FF biomarker reliability among women undergoing IVF (n = 34), and to determine the minimum number of follicles required to estimate subject-specific mean concentrations. We measured As, Hg, Cd, Pb, Cu, Mn, Se, and Zn in FF samples using inductively coupled plasma tandem mass spectrometry. Inter-subject (between-women) variability contributed most of the variability in FF element concentrations, with ovarian, follicular, and analytical as smaller sources of variability. The proportion of variability attributable to sources between-follicles differed by age, body mass index (BMI), race, and cigarette smoking for Cu, Se, and Zn, by BMI and cigarette smoking for As, by primary infertility diagnosis for Hg, Cu, Se, and Zn, and by ovarian stimulation protocol for Mn and Se. Four to five individual follicles were sufficient to estimate subject-specific mean Cu, Se, and Zn concentrations, while >14 were necessary for As, Hg, Cd, Pb, and Mn. Overall, our results suggest that FF is a suitable source of biomarkers of As and Hg exposure in ovarian follicles. Although limited in size, our study offers the most comprehensive exploration of biological variation in FF trace elements to date and may provide guidance for future studies of ovarian trace element exposures.

Keywords

Biologic variability; Biomarkers; Follicular fluid; In vitro fertilization; Reliability; Trace elements

1. Introduction

Essential elements, such as copper (Cu), selenium (Se), manganese (Mn), and zinc (Zn), are critical to human development at trace concentrations (Al-Fartusie and Mohssan, 2017; Fraga, 2005). Non-essential elements, including arsenic (As), mercury (Hg), cadmium (Cd), and lead (Pb) are ubiquitous in the environment, and may be toxic even at trace (i.e., low μg/L) levels of exposure (Jaishankar et al., 2014; Zahir et al., 2005). Exposure to trace elements, occurs mainly through diet and lifestyle-related behaviors (Kim et al., 2013; Nachman et al., 2013; Satarug et al., 2003). Essential and non-essential trace elements have been associated with female infertility and subfecundity at low and moderate levels (<10 μg/L) through non-occupational exposure sources (Cole et al., 2006; Louis et al., 2012), including among populations undergoing in vitro fertilization (IVF) (Al-Saleh et al., 2008; Bloom et al., 2010, 2011, 2012a, 2012b; Chang et al., 2006; Choy et al., 2002; Ingle et al., 2017; Silberstein et al., 2006; Singh and Rana, 2007; Skalny et al., 2018; Sun et al., 2017; Tolunay et al., 2016; Younglai et al., 2002).

While most previous observational studies of reproductive function used blood or urine as sources of biomarkers of trace element exposure, other studies have more recently employed ovarian follicular fluid (FF) (Al-Saleh et al., 2008; Bloom et al., 2012b; Ingle et al., 2017; Silberstein et al., 2006; Singh et al., 2013; Sun et al., 2017; Tolunay et al., 2016; Younglai et al., 2002). Follicular fluid, composed of granulosa and theca cell secretions, and filtered blood plasma, (Edwards, 1974; Shalgi et al., 1973), is routinely collected and discarded during IVF, but can be retained for analysis. Compared to more easily obtained blood and urine specimens, FF presumably provides a better estimate of exposures that might impact

reproductive outcomes, as it more closely reflects the microenvironment surrounding the developing oocyte. However, previous research suggests that there may be substantial follicle-to-follicle, or biologic variability in FF constituents (Bloom et al., 2012b; Silberstein et al., 2009; Zenzes and Reed, 1998). Thus, use of single or pooled FF biomarkers could misclassify trace element exposures in study participants and undermine statistical power to detect modest effects in observational studies. Therefore, it is important to investigate the sources of and contributing factors to FF trace element variability, and to determine the minimum number of study follicles necessary to accurately estimate mean concentrations for a given study participant (i.e., subject-specific means).

We previously reported sources of variability in urinary trace elements among women undergoing IVF (Kim et al., 2011), and described sources of exposure to FF trace elements (Butts et al., 2020). Here, we characterized sources of biologic variability for essential and non-essential trace elements in FF, including As, Hg, Cd, Pb, Cu, Mn, Se, and Zn concentrations, estimated differences by demographic and clinical factors, and calculated the number of independent specimen collections necessary to estimate subject-specific means. Collectively, these data will enhance our understanding of the physiology of trace elements within the ovary and be useful in the design of future studies using FF biomarkers.

2. Methods

2.1. Study population

We recruited women undergoing IVF at the University of California at San Francisco (UCSF) between October 2015 and June 2017 as part of the Study of Metals and Assisted Reproductive Technologies (SMART). Women who planned a fresh embryo transfer with non-donor oocytes were eligible for participation. Patients initiating IVF cycles at UCSF completed an infertility questionnaire, which collected demographic information and medical and reproductive histories. Additionally, study participants completed a detailed exposure questionnaire prior to or on the day of oocyte retrieval. The questionnaire collected information about participants' recent (within the past week) and annual (on average per week over the past year) consumption of specific foodstuffs (seafood, poultry, organ meats, and rice), associated with exposure to toxic trace elements.

Participants, after being informed about the objectives of the study, gave their consent. Per clinic protocol, women were treated with gonadotropin-induced ovarian stimulation utilizing Lupron down regulated (DR), gonadotropin antagonist, or flare protocols for up to two weeks prior to retrieval. After development of a sufficient number of follicles ≥ 17 mm in diameter, human chorionic gonadotropin (hCG) was administered, and oocytes were retrieved within 36 h via transvaginal fine needle aspiration.

While sixty-five women consented to participate, nine were excluded due to conversion to intrauterine insemination (IUI) (n = 1), in which sperm is injected directly into the uterus, cycle cancellation (n = 3), and missing FF (n = 5). We collected undiluted FF from the two leading follicles in each ovary for the remaining 56 women, not exceeding four study specimens from each participant, within the usual clinical context of IVF. On the day of oocyte retrieval, study follicles were individually punctured with a fresh saline-rinsed

aspiration needle and the laterality documented. Remaining follicles were collected according to standard practice. The FF specimens were visually assessed for blood contamination (Levay et al., 1997) and subsequently centrifuged, aliquoted into 1.8 mL cryovials, and frozen (−80 °C) until shipped to the laboratory for analysis. 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.

2.2. Laboratory analysis

FF specimens were shipped on dry ice to the Laboratory of Inorganic and Nuclear Chemistry, Trace Elements Section at the Wadsworth Center, New York State Department of Health (Albany, NY USA) and stored at −80 °C until analysis. A panel of trace elements including As, Hg, Cd, Pb, Cu, Mn, Se, and Zn were measured in FF utilizing a method optimized and validated for the Agilent 8900 inductively coupled plasma tandem mass spectrometer (ICP-MS/MS; Agilent Technologies, Santa Clara, CA USA) (Galusha et al., 2019). All specimen collection and storage materials were pre-screened for contamination and FF storage containers were acid-washed at the laboratory before shipment to the study site. Because Clinical Laboratory Improvement Amendments (CLIA) prohibit washing materials that contact oocytes directly, we assessed pre-analytic contamination using random samples of 2–3 items from each lot of aspiration needles and vacuum tubing, collection tubes, and petri dishes employed during oocyte retrieval.

Specimens were analyzed across five analytical batches as described in detail by a previous publication (Galusha et al., 2019). Briefly, FF samples were thawed at room temperature, mixed on a rocker, and diluted 1 + 24 using a reagent solution containing 0.5% (v/v) HNO_3, 1000 μg/L Au (Inorganic Ventures, Christiansburg, VA USA), and 0.005% (v/v) Triton X-100™ (Sigma Aldrich Co., St. Louis, MO USA); 0.5 μg/L Ir, Y, Rh, and Ga were added to the solution as internal standards (single elements stocks, High Purity Standards, Charleston, SC USA) into autosampler tubes. Analytes were quantified using Agilent MassHunter software (Agilent Technologies, Inc.), after calibration of the ICP-MS/MS instrument against multielement standards. As part of the internal quality control (QC) practices, independent duplicate analyses (i.e., analyzed in a separate analytical run) were randomly conducted for approximately 30% of samples, generating n = 34 women with 2–6 values for each FF specimen, and comprising the subset of specimens that we used in this study. The limit of detection (LOD) was defined for each element as three times the standard deviation of a low level FF matrix sample and were reported as: 0.04 μg/L for As, 0.03 μg/L for Hg, 5.60 ng/L for Cd, 0.03 μg/L for Pb, 53.0 μg/L for Cu, 0.05 μg/L for Mn, 7.0 μg/L for Se, and 21.0 μg/L for Zn. We used all instrument-reported values for the statistical analysis irrespective of LODs, to prevent potential bias introduced by imputing data below the LOD (Richardson and Ciampi, 2003; Schisterman et al., 2006).

2.3. Statistical analysis

The statistical analysis included n = 34 with repeated FF analyses. The sampling strategy is described in detail in Fig. 1. We characterized the distributions of demographic and clinical variables as well as FF trace element concentrations. We employed linear mixed models using restricted maximum likelihood (Spilke et al., 2005), to generate variability

components as: $Y_{ijkl} = \mu + (woman)_i + (ovary)_{j(i)} + (follicle)_{k(ji)} + e_{l(ijk)}$. Y_{ijkl} represents the trace element concentrations collected from the i^{th} woman (i = 1,…, 34), in the k^{th} follicle (k = 1, …, 4) of the j^{th} ovary (j = 1, 2), where l denotes repeated measures (l = 1,…, 6). The overall mean value for each trace element concentration is denoted by (μ), $woman_i$ characterizes the random effect of the i^{th} woman on the overall mean, $ovary_{j(i)}$ characterizes the random effect of the j^{th} ovary within the i^{th} woman, $follicle_{k(ji)}$ characterizes the random effect of the k^{th} follicle nested within the j^{th} ovary within the i^{th} woman, and $e_{l(ijk)}$ characterizes the random effect of the l^{th} determination nested within the k^{th} follicle within the j^{th} ovary of the i^{th} woman. Following the conventional distributional assumptions, all of these random-effects are assumed to be distributed as normal with zero mean and corresponding variance component. This approach accounted for the unbalanced study design, in which women had varied numbers of ovary, follicle, and repeated FF measures. We included *ovary* in the model as a fixed effect for trace elements with negative or inestimable variance components.

We used variance components to characterize total FF trace element variabilities (σ^2_{Tot}), and proportions of variabilities due to sources between-women ($\%\sigma^2_{Wom}$), between-ovaries ($\%\sigma^2_{Ova}$), between-follicles ($\%\sigma^2_{Fol}$), and attributed to analytical factors ($\%\sigma^2_{Ana}$). We calculated ratios of variability ($\%\sigma^2_{Fol}$: $\%\sigma^2_{Wom}$), to estimate in situ follicle regulation of trace element concentrations, and intraclass correlation coefficients (ICCs), to describe the repeatability of repeated measurements within woman. ICCs were calculated as the proportion of between-woman variance contributing to the total variance ($\sigma^2_{Wom}/\sigma^2_{Tot}$), with corresponding 95% confidence intervals (Lachin, 2004). We also calculated ICCs to describe the correlation of observations made between ovaries in the same woman ($\%\sigma^2_{Ova}/(\%\sigma^2_{Ova} + \%\sigma^2_{Fol} + \%\sigma^2_{Ana})$), with corresponding 95% confidence intervals.

We then compared $\%\sigma^2_{Fol}$ according to age dichotomized at the median (≤38 vs. >38 years), body mass index (BMI) dichotomized as normal/underweight vs. overweight/obese (<25 vs. ≥25 kg/m^2), race (Asian or non-Asian), smoking status (≤100 vs. >100 cigarettes in a lifetime), primary infertility diagnosis, and IVF stimulation protocol. We tested differences between groups by inspecting overlapping 84% confidence intervals, which corresponds to a hypothesis test of $\alpha = 0.05$ (Julious, 2004). Additionally, we calculated the number of specimens needed to estimate the subject-specific mean within |10%| of the “true” value, as $m_{10\%} = \left[1.96 \times (CV_A^2 + CV_I^2)^{1/2}/10\right]^2$ (Fraser, 2001). Analytical variation (CVA), was calculated by dividing the square root of the variance due to analytic factors ($\sqrt{\sigma^2_{Ana}}$) by the mean value for each element and CVI, the average within-subject variation, was calculated by averaging the subject-specific CVs (i.e., standard deviation/mean) for each element

We also estimated the unadjusted differences in mean FF trace element concentrations according to age, BMI, race, history of cigarette smoking, and primary infertility diagnosis by one-way analysis of variance (ANOVA) or Student t-test. Finally, we estimated least squares mean FF trace element concentrations according to diagnosis in a hypothesis-generating exploratory analysis, adjusting for age, race, and smoking (As and Cd). All statistical analyses were completed in SAS 9.4 (SAS Institute, Inc. Cary, NC USA). Statistical significance was determined as $p < 0.05$ for a two-tailed test in our main analyses

and $p < 0.10$ for a two-tailed test in the hypothesis-generating exploratory analysis of associations between FF trace elements and infertility diagnosis.

3. Results

3.1. Demographic and clinical factors

The distributions of demographic and clinical factors of the 34 women with replicate FF analyses are summarized in Table 1. Women were 38.7 years of age on average with a mean BMI of 24.0 kg/m^2. Approximately 68.0% of women were non-Asian, most of whom were white, and approximately one third (32.3%) were Asian. Seven (20.6%) women reported a lifetime history of smoking more than 100 cigarettes. The leading primary infertility diagnosis was diminished ovarian reserve (DOR) (45.5%, n = 15) and proportions of unexplained infertility and tubal, male factor, or other non-ovary related diagnoses were comparable at 30.3% (n = 10) and 24.2% (n = 8), respectively. The majority of study participants (58.8%) were treated with a gonadotropin antagonist ovarian stimulation protocol (n = 20). We present the distributions of demographic and clinical factors for all 56 SMART participants in Supplemental Table S1.

3.2. Characteristics of biologic variability for FF trace elements

Table 2 describes the distributions of FF trace element concentrations as well as sources of biologic variability (i.e., between-follicles) for FF trace elements. In models for As, Cu, Mn, Se, and Zn, which included ovary as a random effect, we found smaller between-ovary variabilities relative to between-follicle variabilities, with the exception of Cd, for which we found greater variability between-ovaries (30.01%) than between-follicles (13.94%). Manganese was the only element with a greater proportion of variability between-ovaries (28.22%) than between-women (18.97%).

Including ovary as a fixed effect (because random effects were inestimable), we found a substantially greater proportion of Hg variability between-women (97.77%) than between-follicles (1.47%), and conversely greater variability between-follicles (63.53%) than between-women (9.57%) for Pb. Analytical factors contributed the smallest percentage of total variability (<4.00%) for all trace elements with the exception of Pb (26.90%), for which higher background concentrations may have misclassified some participants. The ratio of variabilities between-follicle to between-women was 6.64 for Pb, suggesting greater follicular than individual variability.

The distributions of ICCs are described in Table 2 and in Fig. 2. ICCs varied from a low of 0.10 for Pb to highs of 0.97 and 0.98 for As and Hg, respectively. Also described in Table 2 are the numbers of specimens required to estimate subject-specific mean concentrations with no more than 10% error ($m_{10\%}$). There were 4–5 individual follicles required for the essential trace elements Cu, Se, and Zn. For the non-essential trace elements and Mn, which is essential at very low levels, at least 14 individual FF specimens were required. However, when relaxing the level of error to 15% ($m_{15\%}$), fewer individual follicle specimens were necessary to estimate subject-specific means, although Pb still required the greatest number of specimens (>50). The distribution of ICCs according to ovary are presented in

Supplemental Fig. S1. ICCs for measurements within ovary varied from a low of 0.23 for Zn to a high of 0.63 for Cd and were generally lower than ICCs for measurements within woman overall, with the exception of Cd and Mn.

3.3. Biologic variability according to demographic and clinical factors

Table 3a, 3b, 3c describes the proportions of FF trace element variabilities due to sources between-follicles according to demographic and clinical factors. Women 38 years of age and younger tended to have smaller proportions of variability between-follicles for Cu, Se, and Zn than women more than 38 years of age. Overweight and obese women (BMI ≥ 25 kg/m^2), tended to have greater proportions of total As, Cu, Se, and Zn variabilities attributed to sources between-follicles than normal/underweight weight women (BMI < 25 kg/m^2). Most notably, more than 80.0% of total FF Cu, Se, and Zn variabilities were attributed to sources between-follicles among women with higher BMIs compared to <6.50% among women less than 25 kg/m^2.

Compared to other races, Asian women tended to have a greater proportion of Cu variability attributed to sources between-follicles, yet smaller proportions of between-follicle variability for Se and Zn. We found the largest difference in relative between-follicle variability for FF Zn in Asians compared to other races (35.64% vs. 43.02%, respectively). We also found greater relative variabilities from sources between-follicles for As, Cu, Se, and Zn among women with a history of cigarette smoking. Women diagnosed as unexplained infertility tended towards smaller proportions of total Cu, Se, and Zn variability attributed to sources between-follicles than women with DOR and tubal/male factor/non-ovary related diagnoses. However, women diagnosed as tubal/male factor/non-ovary related had a greater proportion of total Hg variability attributed to sources between-follicles (5.33%) than women with DOR (0.51%) and unexplained infertility (0.72%). We found a smaller proportion of total Mn variability attributed to sources between-follicles among women receiving Lupron down regulated protocols (63.34%) than for gonadotropin antagonist protocols (72.70%). Selenium also varied across ovarian stimulation protocols, with sources between-follicles contributing 67.66%, 38.35%, and 10.83% of the total variability among those receiving Lupron down regulated, gonadotropin antagonist, and flare protocols, respectively.

3.4. Associations between FF trace elements and demographic and clinical factors

Unadjusted FF trace element concentration differences by demographic and clinical factors are displayed in Table 4. Women > 38 years of age had greater FF Pb concentrations, although lower FF Zn, Cu, and Se concentrations. We also found greater FF Cd, Pb, and Mn concentrations among overweight/obese than for normal/underweight women. Women with higher BMIs tended to have greater FF Cu concentrations compared to normal/underweight women and FF As, Hg, and Cd concentrations tended to be greater in Asian women than in other races. Women receiving flare protocols had greater FF Se compared to other protocols and FF Cu concentration was highest among women receiving gonadotropin antagonist treatment. Lower Zn and greater Mn were also suggested for women receiving Lupron down regulated compared to other treatment protocols.

3.5. Associations between trace elements and primary infertility diagnosis

Least squares geometric mean FF trace element concentrations, adjusted for age, race, and cigarette smoking, are presented in Table 5, according to primary infertility diagnosis. Women with unexplained infertility tended to have greater FF As and Hg concentrations than other women. Yet, women with tubal/male factor/non-ovary related diagnoses tended to have greater FF Mn concentrations than women with unexplained infertility.

4. Discussion

In this biomarker reliability study, we found differences in the sources of variability for FF As, Hg, Cd, Pb, Cu, Mn, Se, and Zn concentrations among women undergoing IVF. We also found that sources between-women made the greatest relative contributions to the total variance of FF As, Hg, Cd, Cu, Se, and Zn concentrations, whereas sources between-follicles made the largest contributions to the total variability of FF Pb and Mn.

An ICC ≥ 0.8 provides an acceptable level of reliability for repeated biomarker measurements in epidemiological studies, with lower levels potentially biasing effect estimates due to exposure misclassification and undermining study power (Lachin, 2004). FF As and Hg had substantially higher ICCs than the other analytes in our study, suggesting comparably greater utility as biomarkers for epidemiologic studies and that the levels may reflect blood plasma concentrations. Unfortunately, blood plasma concentrations were unavailable and so we were unable to investigate this hypothesis, though notably plasma trace elements tend to have low reliability. We plan to analyze blood trace elements in a future study. ICCs according to ovary were <0.80 for all trace elements, suggesting that FF concentrations were not necessarily representative of concentrations from contralateral ovaries.

Our results suggested differences in biologic (i.e., between-follicle) trace element variabilities by age, BMI, race, cigarette smoking, primary infertility diagnosis, and ovarian stimulation protocol, although caution is warranted given the small sample sizes for some groups. With the exception of FF Mn, the essential trace elements required fewer independent follicle collections to estimate subject-specific means (i.e., ≥5 specimens for Cu, Se, and Zn) than the toxic trace elements. Thus, a “one follicle-one oocyte” design may better capture associations between toxic trace element exposures and IVF endpoints than more commonly used “pooled” follicle approaches. However, we found the highest CVs, largest value ranges, and substantially lower concentrations of FF As, Hg, and Cd compared to those for Cu, Se, and Zn. So, it is also possible that this finding may be a function of their very different concentrations.

4.1. Comparison to previous studies of FF trace element variabilities

Few previous papers have characterized sources of FF trace element variability. Earlier studies include a previous investigation of women undergoing IVF at UCSF (Bloom et al., 2012b), where variability was assessed in seven women with two repeated FF measures. Similar to the current results, the greatest proportion of measured variation for FF Hg was from sources between-women (62.2%). Sources between-women (58.0%) made the greatest

contribution in the previous study, whereas in the current study most FF Pb variation (63.5%) was attributed to sources between-follicles. The discordant results may be due in part to different analytical methodology or potentially to inadvertent contamination, some of which could be due to pre-analytical contamination, for example in clinical supplies or from airborne exposures. In the current study, we employed an ICP-MS/MS method optimized for sensitivity, achieving lower detection limits (for As, Hg, Cd, and Mn), and high accuracy at lower concentrations (Galusha et al., 2019), compared to the former study that used High Resolution (HR-) Sector Field-ICP-MS (Kruger et al., 2012). For example, only 54.3% (Cd) and 93.5% (Pb) of 46 FF values exceeded the detection limit in our earlier work using HR-ICP-MS compared to 100% (Cd) and 96.6% (Pb) of FF values in this study using ICP-MS/MS. Detection limits for As, Hg, Cd, and Mn were 0.2, 0.2, 0.02, and 0.1 μg/L in the previous study using HR-ICP-MS compared to ICP-MS/MS LODs of 0.04, 0.03, 5.60 ng/L, and 0.05 μg/L in the current study, respectively.

An earlier IVF study (n = 23) reported significant inter-follicle FF Se correlations within women ($r = 0.70$; $p = 0.001$), but without a significant difference across women (Paszkowski et al., 1995). Another clinical study reported variation in FF trace element concentrations as a function of follicle maturity (Silberstein et al., 2009). With greater follicle size, toxic trace element levels more closely resembled blood concentrations. Though we did not adjust for follicular volume, our collection of the four largest follicles in each ovary, each at least 17 mm diameter, reduced the likelihood of exposure misclassification related to follicle diameter in the current study.

Ionic mimicry, in which non-essential elements exhibit characteristics similar to essential elements (Bridges and Zalups, 2005), may in part account for the substantial follicle-to-follicle variability we found for Pb and Cd. In contrast to the high ICCs for As and Hg, the low ICCs for Pb and Cd might be attributed in part to active follicular regulation, given ionic similarities between calcium and Pb (Simons, 1986) and Cd and Zn (Brzoská and Moniuszko-Jakoniuk, 2001), which also may compete to bind metallothionein within the oocyte (Ménézo et al., 2011). Animal studies report intrafollicular regulation of Zn and Pb, including lower Zn (Bhardwaj and Sharma, 2011) and higher Pb (Taupeau et al., 2001) concentrations in atretic compared to non-atretic ovine and murine follicles, respectively. Some toxic trace elements may be regulated in situ within the follicle and therefore biologically effective doses impacting reproductive outcomes would be potentially misclassified using blood and urine to quantify exposure.

4.2. Differences in FF trace element variabilities by demographic and clinical factors

Our results suggested that the between-follicle, or biologic variabilities of Cu, Se, and Zn, essential elements, were associated with demographic and clinical predictors of IVF success, including age, race, BMI, and cigarette smoking (Freour et al., 2008, 2012; Fujimoto et al., 2010; Hull et al., 1996; McQueen et al., 2015; Nelson and Lawler, 2011; Purcell et al., 2007; Rittenberg et al., 2011; Wellons et al., 2012). It is tempting to speculate that poorer IVF outcomes reported among older women, Asian women, reported to have higher toxic trace element exposures from greater consumption of seafood and use of traditional herbal medicines than other groups (Butts et al., 2020; Garvey et al., 2001; Harris et al., 2011; Liu

et al., 2018; Saper et al., 2008; Wang et al., 2019), obese women, and smoking women, may in part be related to lower in situ regulation of trace elements important to cellular anti-oxidative stress activities. Oxidative stress can be detrimental to reproductive function (Agarwal et al., 2005), and Cu, Mn, Zn, and Se may be important to the progression and maintenance of a pregnancy. Copper, Mn, and Zn are components of the antioxidant (AOX) superoxide dismutase (SOD) metalloenzyme (Zidenberg-Cherr and Keen, 1991), which neutralizes superoxide radicals ($O_2^{\bullet -}$) and prevents formation of more reactive and harmful free radical species (Al-Gubory, 2014). SOD activity was previously linked to endometrial function and early pregnancy (Sugino et al., 1996), and inversely associated with fertilization (Sabatini et al., 1999). Selenium is a component of glutathione peroxidase (GPx), which detoxifies hydrogen peroxide (Al-Gubory, bory, 2014). Among 112 Irish IVF patients, Se/GPx levels were higher in fertilized oocytes than in unfertilized oocytes (Paszkowski et al., 1995). Additionally, smokers and women with higher BMIs tended to have greater relative As variability between-follicles in our study; As inhibits the activity of several AOX enzymes (Jomova and Valko, 2011). A future study of FF AOX enzyme activities as a mediator of associations with FF trace elements is necessary to help clarify the role of FF trace elements in IVF outcomes.

Our results also suggested differences in the between-follicle variabilities for Mn and Se by ovarian stimulation protocol. A Turkish study reported lower FF Se and Zn among women undergoing IVF compared to a group not undergoing treatment (Özkaya et al., 2011). While ovarian stimulation may impact FF levels of essential trace elements, possibly leading to exposure misclassification in epidemiologic studies investigating IVF outcomes, a larger investigation will be necessary to characterize the association more clearly.

4.3. Exploratory analysis of associations between trace elements and primary infertility diagnosis

In our hypothesis-generating analysis of potential associations between FF trace elements and primary infertility diagnosis, we found lower Mn and suggested greater As and Hg among women with unexplained fertility compared to those diagnosed with tubal/male factor/ non-ovary related disorders. While these results might indicate an association between FF trace element and infertility diagnosis, we are unable to ascertain causality given the cross-sectional nature of the analysis. It is also possible that this was a chance finding, given the large number of statistical tests and small strata. As this was a secondary and exploratory analysis, we wanted to maximize sensitivity for detecting plausible associations for future confirmation and did not adjust for multiple comparisons (Goldberg and Silbergeld, 2011; Rothman, 1990). A larger and more comprehensive investigation will be necessary for a more definitive interpretation of these preliminary results.

4.4. Strength s and limitations

While novel, our results should be interpreted judiciously given several limitations. First, our small sample size, including having repeated measurements for only 34 of 56 women contributing follicles to the SMART, may have been insufficient to detect modest differences in the relative contributions of biologic variability sources across subgroups. In fact, we experienced difficulty estimating the variability components for infertility diagnosis (Mn)

and stimulation protocol (Cd and Zn) in stratified models due to sparse strata. Additionally, it is important to note that variance estimates are typically sensitive to sample size. Still, we used a mixed modeling approach to leverage multiple FF samples per woman and maximize the available statistical power.

We were unable to isolate the FF Hg and Pb variabilities due to sources between-ovaries from sources between-women for most models, given limited variability. Additionally, with the exception of the age-stratified models, we encountered difficulty estimating the variability components for Pb. Potential reasons for this limitation include sparse data across strata and the very low FF Pb levels in our study population. The low FF Pb levels may reflect, in part, the highly selected nature of our study population, and so our results should be generalized to other infertile populations with caution. IVF coverage is not mandated by California health insurance regulations and so women undergoing IVF procedures tend to be more educated and more affluent than women residing in states with mandated coverage (Hammoud et al., 2009; Henne and Bundorf, 2008). However, human exposure to the trace elements explored in this analysis is ubiquitous. Additionally, we have no reason to believe the biology would differ between women included in this investigation compared to other IVF populations. Our findings offer insight into factors that may impact biologic variation, which could be particularly relevant for women with similar levels of exposure to trace elements. Overall, despite its limitations, our study offers the most comprehensive exploration of variability sources in FF trace elements to date.

5. Conclusion

To conclude, our study results suggest that most of the variability in measured FF trace elements occurred between-women, although, ovarian, follicular, and analytical variability were also important in some cases. Our analysis of demographic and clinical variables also suggested plausible differences in the biological, or between-follicles variability of FF trace elements by age, BMI, race, cigarette smoking, infertility diagnosis, and ovarian stimulation protocol, although these results require replication in a larger study. In general, studies employing FF as an exposure biomarker may ultimately enhance our understanding of the impact of trace elements on reproductive function given their proximity to the developing oocyte. Our findings contribute important information to help elucidate the impact of trace element exposures on IVF outcomes and inform the design of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

σ^2_{Tot}	total trace element variability
$\%\sigma^2_{Wom}$	variability between-women
σ^2_{Ova}	variability between-ovaries
σ^2_{Fol}	variability between-follicles
σ^2_{Ana}	analytical variability
CV	coefficient of variation
CV_A	analytical coefficient of variation
CV_I	individual coefficient of variation
FF	follicular fluid
ICC	intraclass correlation coefficient

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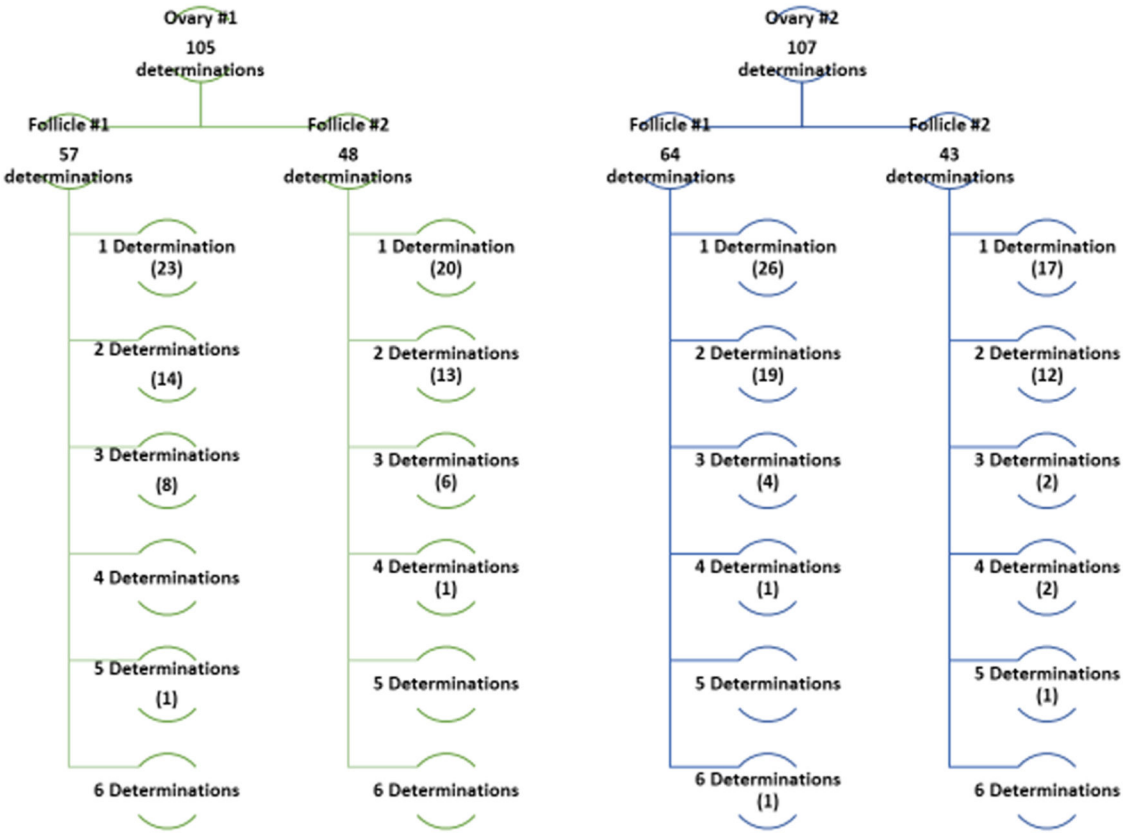

Fig. 1.

Sampling strategy for follicular fluid (FF) trace elements for 212 determinations collected from contralateral follicles of in vitro fertilization (IVF) patients, (data from 34 women with replicate FF).

Note: Each woman contributed up to four follicles with up to six determinations (repeat runs). Counts displayed are based on maximum number of repeat measures which were for mercury. Other totals were as follows: 211 for arsenic, 117 for cadmium, 104 for lead, 212 for copper, 202 for manganese, 211 for selenium, and 209 for zinc. Numbers in parentheses represent total samples with that specified number of determinations.

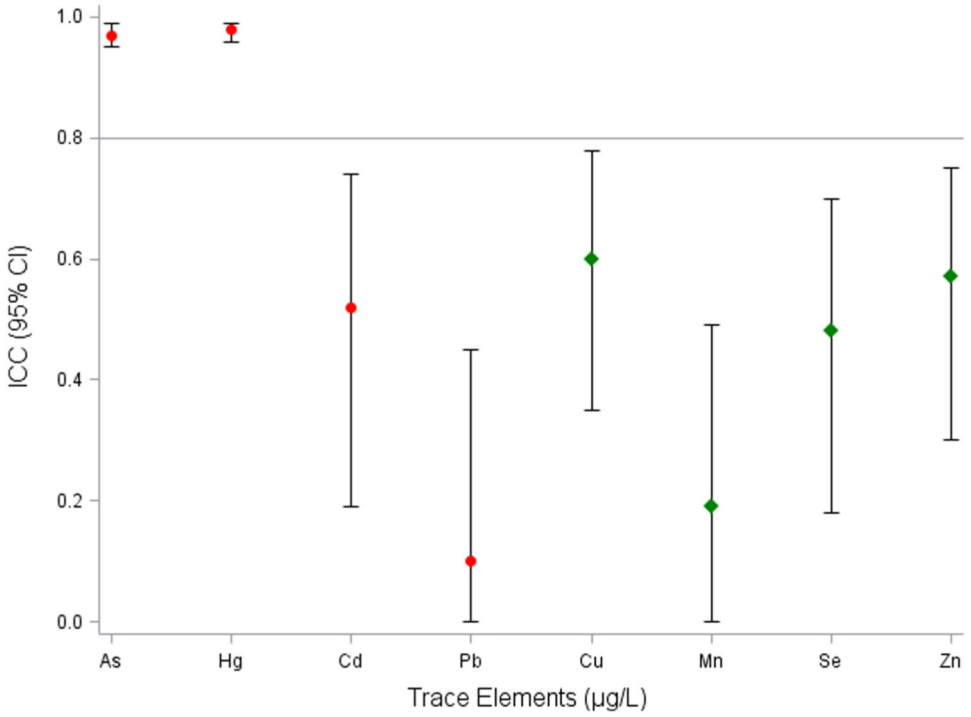

Fig. 2.

Intraclass correlation coefficients with 95% confidence intervals (CI) for follicular fluid trace elements (µg/L) measured in n = 34 in vitro fertilization (IVF) patients, describing the correlations between repeat measures from follicles within the same woman.

As- arsenic, Hg- mercury, Cd- cadmium, Pblead, Cu- copper, Mn- manganese, Se- selenium, and Zn- zinc measured in FF specimens collected from contralateral follicles; Cd in ng/ L.

Table 1

Distribution of demographic and clinical factors for in vitro fertilization (IVF) patients (data from 34 women with replicate follicular fluid).

Factors	n	Mean± SD/(%)	Min	25th %tile	Median	75th %tile	Max
Age	34	38.7 ± 3.3	30.0	37.0	39.0	41.0	44.0
BMI (kg/m^2)	34	24.0 ± 5.0	18.5	20.1	22.7	25.8	39.5
Race (%):							
Non-Asian	23	(67.7)	–	–	–	–	–
Asian	11	(32.3)	–	–	–	–	–
Smoked > 100 cigarettes (%):							
No	27	(79.4)	–	–	–	–	–
Yes	7	(20.6)	–	–	–	–	–
Primary diagnosis (%)[a]:							
Diminished ovarian reserve (DOR)	15	(45.5)	–	–	–	–	–
Unexplained	10	(30.3)	–	–	–	–	–
Tubal/ male factor/non-ovary related	8	(24.2)	–	–	–	–	–
Stimulation protocol (%):							
Lupron down regulated	8	(23.5)	–	–	–	–	–
Gonadotropin antagonist	20	(58.8)	–	–	–	–	–
Flare	6	(17.7)	–	–	–	–	–

Min, minimum observed value; Max, maximum observed value; SD, standard deviation.

[a] n = 1 participant with polycystic ovary syndrome (PCOS), an endocrine disorder that disrupts ovulation, excluded.

Table 2

Characteristics of biologic variability for trace element concentrations in follicular fluid (FF) (μg/L) sampled from in vitro fertilization (IVF) patients (data from 34 women with replicate FF)[a].

Elements	n	GM	GSD	GCV %	CV_I	σ^2_{Tot}	$\%\sigma^2_{Wom}$	$\%\sigma^2_{Ova}$	$\%\sigma^2_{Fol}$	$\%\sigma^2_{Ana}$	$\%\sigma^2_{Fol}$: $\%\sigma^2_{Wom}$	ICC	95% CI	$m_{10\%}$	$m_{15\%}$
As	34	0.28	3.58	203	11.8	1.65	97.17	0.89	1.41	0.53	0.01	0.97	0.95, 0.99	14	6
Hg	34	0.25	3.03	156	17.2	1.26	97.77	_[b]	1.47	0.76	0.02	0.98	0.96, 0.99	34	10
Cd (ng/L)[c]	27	17.9	2.22	94.0	24.1	0.61	52.09	30.01	13.94	3.96	0.27	0.52	0.19, 0.74	22	15
Pb	26	0.07	1.59	42.9	40.7	0.34	9.57	_[b]	63.53	26.90	6.64	0.10	0.00, 0.45	<50	<50
Cu	34	843	1.70	57.7	10.2	0.28	60.38	15.07	23.70	0.86	0.64	0.60	0.35, 0.78	4	2
Mn	34	0.82	1.72	58.7	31.1	0.36	18.97	28.22	50.16	2.66	0.56	0.19	0.00, 0.49	41	18
Se	34	59.4	1.65	53.6	11.5	0.25	48.10	19.87	31.23	0.81	0.65	0.48	0.18, 0.70	5	2
Zn	34	428	1.48	41.3	10.3	0.16	56.81	9.95	32.15	1.09	0.57	0.57	0.30, 0.75	4	2

Note: GM-geometric mean; GSD-geometric standard deviation; GCV-geometric coefficient of variation describing the between subject variation; CV_I- coefficient ofvariation describing the average within subject variation; σ^2_{Tot}-total variability measured; σ^2_{Wom}-variability between-women; σ^2_{Ova}-variability between-ovaries; σ^2_{Fol}-variability between-follicles; σ^2_{Ana}-variability attributed to analytical factors; ICC-Intraclass correlation coefficient; CI-confidence interval; $m_{10\%}$- number of specimens needed to estimate the mean within 10% of the “true” value; $m_{15\%}$- number of specimens needed to estimate the mean value within 15% of the “true” value.

[a]Each woman contributed up to four follicles for totals of 211 for As, 212 for Hg, 117 for Cd, 104 for Pb, 212 for Cu, 202 for Mn, 211 for Se, and 209 for Zn.

[b]Ovary was included as a fixed effect in mixed models.

[c]Excluding 1 follicle with a Cd value that was an extreme outlier.

Table 3 a

Characteristics of biologie variability for concentrations of trace elements in follicular fluid (FF) (μg/L) from in vitro fertilization (IVF) patients (data from 34 women with replicate FF).

	Age (years)							
	≤38 (n = 16)				>38 (n = 18)			
	$\%\sigma^2_{Wom}$	$\%\ \sigma^2_{Ova}$	$\%\ \sigma^2_{Fol}$	$\%\ \sigma^2_{Ana}$	$\%\ \sigma^2_{Wom}$	$\%\ \sigma^2_{Ova}$	$\%\ \sigma^2_{Fol}$	$\%\ \sigma^2_{Ana}$
As	98.06	-	1.45[a]	0.49	96.91	-	2.55[a]	0.54
Hg	97.24	-	2.32[a]	0.44	98.15	-	0.94[a]	0.91
Cd	16.57	55.31[a]	21.39[a]	6.73	71.11	15.46[a]	11.06[a]	2.39
Pb	6.94	-	67.85[a]	25.21	20.48	-	50.34[a]	29.18
Cu	71.83	-	26.01[a]	2.16	58.08	-	41.25[b]	0.68
Mn	26.32	30.81[a]	39.14[a]	3.73	18.39	25.07[a]	54.56[a]	1.99
Se	60.25	-	36.96[a]	2.79	47.92	-	51.62[b]	0.47
Zn	64.01	-	32.73[a]	3.27	48.34	-	50.95[b]	0.70
	BMI (kg/m^2)							
	<25 (n = 24)				≥25 (n = 10)			
	$\%\sigma^2_{Wom}$	$\%\ \sigma^2_{Ova}$	$\%\ \sigma^2_{Fol}$	$\%\ \sigma^2_{Ana}$	$\%\ \sigma^2_{Wom}$	$\%\ \sigma^2_{Ova}$	$\%\ \sigma^2_{Fol}$	$\%\ \sigma^2_{Ana}$
As	98.77	-	0.75[a]	0.47	96.07	-	3.40[b]	0.54
Hg	98.30	-	1.20[a]	0.50	97.40	-	1.68[a]	0.91
Cd	58.96	17.31[a]	18.02[a]	8.92	15.56	72.95[a]	15.56[a]	2.58
Pb	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
Cu	94.95	-	4.38[a]	0.66	10.51	-	88.46[b]	1.03
Mn	19.79	18.52[a]	58.10[a]	3.58	16.29	42.19[a]	39.75[a]	1.77
Se	93.12	-	6.21[a]	0.66	15.28	-	84.11[b]	0.62
Zn	92.61	-	6.50[a]	0.90	10.93	-	87.95[b]	1.13

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Note: σ^2_{Wom}-variability between-women; σ^2_{Ana}-variability between-ovaries; σ^2_{Fol} -variability between-follicles; σ^2_{Ana} -variability attributed to analytical factors; % σ^2_{Ova} -where blank, ovary was included as a fixed effect; n = 7 missing for Cd, n = 8 missing for Pb; Cd values in ng/L; Cd models exclude 1 follicle with a value that was an extreme outlier.

^{a,b}Superscripts, where different, denote $p < 0.05$ for differences across groups.

^{c}Inestimable.

Table 3b

Characteristics of biologic variability for concentrations of trace elements in follicular fluid (FF) (μg/L) from in vitro fertilization (IVF) patients (data from 34 women with replicate FF).

	Race							
	Non-Asian (n = 23)				Asian (n = 11)			
	% σ^2_{Wom}	%σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}
As	97.50	0.73 [a]	1.24[a]	0.53	94.63	1.94[a]	2.89[a]	0.54
Hg	97.97	-	1.19[a]	0.84	94.34	-	5.17[a]	0.49
Cd	59.33	18.36[a]	17.44[a]	4.88	0.98	81.29[a]	14.29[a]	3.44
Pb	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
Cu	60.43	14.05[a]	24.87[a]	0.65	60.03	7.44[a]	29.44[b]	3.09
Mn	27.62	29.14[a]	40.28[a]	2.97	1.26	46.06[a]	50.37[a]	2.31
Se	54.49	-	44.89[a]	0.62	56.78	-	41.72[b]	1.50
Zn	55.99	-	43.02[a]	0.99	62.15	-	35.64[b]	2.20
	Smoked > 100 cigarettes							
	No (n = 27)				Yes (n = 7)			
	% σ^2_{Wom}	%σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}
As	98.54	-	1.12[a]	0.34	92.62	-	6.00[b]	1.38
Hg	98.17	-	1.13[a]	0.69	96.83	-	2.35[a]	0.82
Cd	85.06	-	11.42[a]	3.52	9.19	-	85.37[a]	5.43
Pb	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
Cu	93.40	-	5.97[a]	0.63	19.62	-	79.43[b]	0.95
Mn	0.29	-	67.37[a]	3.13	51.09	-	47.37[a]	1.54
Se	89.27	-	10.02[a]	0.71	22.85	-	76.60[b]	0.55
Zn	89.71	-	9.21[a]	1.08	26.42	-	72.97[b]	0.60

Note: σ^2_{Wom} -variability between-women; σ^2_{Ova} -variability between-ovaries; σ^2_{Fol} -variability between -follicles; σ^2_{Ana} -variability attributed to analytical factors; % σ^2_{Ova} -where blank, ovary was included as a fixed effect; n = 7 missing for Cd, n = 8 missing for Pb; Cd values in ng/L; Cd models exclude 1 follicle with a value that was an extreme outlier.

a,b Superscripts, where different, denote $p < 0.05$ for differences across groups.

c Inestimable.

Table 3 c

Characteristics of biologie variability for concentrations of trace elements in follicular fluid (FF) (μg/L) from in vitro fertilization (IVF) patients (data from 34 women with replicate FF).

	Primary infertility diagnosis											
	Diminished ovarian reserve (n = 15)				Unexplained (n = 10)				Tubal/male factor/ non-ovary related (n = 8)			
	%σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}
As	97.84	-	1.62[a]	0.54	99.36	-	0.10[a]	0.54	95.10	-	4.50[a]	0.40
Hg	98.52	-	0.51[a]	0.97	99.00	-	0.72[a]	0.28	94.17	-	5.33[b]	0.50
Cd	*c*	*c*	*c*	*c*	78.86	-	15.88[a]	5.26	63.35	-	26.74[a]	9.92
Pb	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
Cu	64.82	-	34.60[a]	0.59	88.46	-	6.62[b]	4.91	37.68	-	60.27[a]	2.05
Mn	50.00	-	47.54[a]	2.46	30.43	-	64.26[a]	5.30	*c*	*c*	*c*	*c*
Se	60.58	-	38.95[a]	0.47	88.57	-	7.77[b]	3.66	20.16	-	77.84[a]	2.00
Zn	54.13	-	45.28[a]	0.60	78.46	-	14.33[b]	7.20	62.84	-	35.34[a]	1.82
	Lupron down regulated (n = 8)				Stimulation protocol Gonadotropin antagonist (n = 20)				Flare (n = 6)			
	%σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}	% σ^2_{Wom}	% σ^2_{Ova}	% σ^2_{Fol}	% σ^2_{Ana}
As	96.62	-	2.60[a]	0.78	98.29	-	1.34[a]	0.38	98.17	-	0.95[a]	0.88
Hg	97.53	-	1.46[a]	1.01	98.06	-	1.29[a]	0.65	94.42	-	4.20[a]	1.38
Cd	73.21	17.76[a]	7.64[a]	1.39	31.06	39.59[a]	21.11[a]	8.24	*c*	*c*	*c*	*c*
Pb	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*c*
Cu	*c*	*c*	*c*	*c*	62.64	-	33.94[a]	3.42	89.04	-	9.35[a]	1.61
Mn	35.00	-	63.34[a]	1.66	22.63	-	72.70[b]	4.67	54.26	-	44.65[a,b]	1.08
Se	32.03	-	67.66[a]	0.31	59.18	-	38.35[a]	2.48	87.72	-	10.83[b]	1.45
Zn	*c*	*c*	*c*	*c*	73.15	-	24.74[b]	2.11	81.15	-	17.41[a]	1.44

Note: σ^2_{Wom} -variability between-women; σ^2_{Fol} -variability between-follicles; σ^2_{Ova} -variability between-ovaries; σ^2_{Ana} -variability attributed to analytical factors

σ^2_{Ova} -where blank, ovary was included as a fixed effect; n = 7 missing for Cd, n = 8 missing for Pb; Cd values in ng/L; Cd models exclude 1 follicle with a value that was an extreme outlier.

a,b Superscripts, where different, denote $p < 0.05$ for differences across groups.

c Inestimable.

Table 4

Differences in geometric mean (SD) follicular fluid (FF) trace element levels (μg/L) by demographic and clinical variables (data from 34 women with replicate FF)[a,b].

Factors	n[c]	As	Hg	Cd[d]	Pb	Cu	Mn	Se	Zn
Age:									
≤38	16	0.29 (3.7)	0.25 (2.6)	19.7 (1.4)	0.06 *(1.5)	946 (1.3)	0.74 (1.5)	64.9 (1.3)	484 *(1.2)
>38	18	0.27 (3.7)	0.25 (3.5)	18.0 (2.6)	0.08 *(1.6)	737 (2.0)	0.79 (1.7)	52.9 (2.0)	375 *(1.6)
BMI (kg/m^2):									
<25	24	0.29 (3.4)	0.26 (2.9)	15.6 **(1.9)	0.06 *(1.6)	772 (1.8)	0.70 *(1.5)	57.3 (1.8)	408 (1.3)
≥25	10	0.25 (4.4)	0.23 (3.8)	27.4 **(1.9)	0.08 *(1.5)	982 (1.4)	0.96 *(1.9)	60.5 (1.4)	460 (1.3)
Race (%):									
Non-Asian	23	0.24 (4.3)	0.19 (3.5)	13.7 **(2.1)	0.07 (1.5)	786 (1.9)	0.78 (1.5)	55.7 (1.8)	411 (1.6)
Asian	11	0.38 (2.2)	0.40 (1.8)	27.5 **(1.9)	0.05 (1.7)	926 (1.3)	0.75 (1.9)	64.0 (1.3)	448 (1.2)
Smoked > 100 cigarettes (%):									
No	27	0.31 (3.7)	0.24 (3.1)	18.7 (2.2)	0.06 (1.6)	851 (1.8)	0.73 (1.6)	58.9 (1.7)	425 (1.5)
Yes	7	0.18 (3.1)	0.29 (3.3)	19.4 (1.3)	0.09 (1.6)	748 (1.6)	0.91 (1.7)	55.6 (1.7)	414 (1.5)
Primary diagnosis (%)[e]:									
Diminished ovarian reserve	15	0.22 (4.2)	0.20 (3.7)	13.8 (2.2)	0.08 (1.6)	664 (2.1)	0.71 (1.6)	53.4 (2.1)	382 (1.7)
Unexplained	10	0.37 (3.0)	0.31 (2.6)	16.0 (2.1)	0.06 (1.5)	1016 (1.2)	0.67 (1.5)	68.4 (1.2)	496 (1.2)
Tubal/male factor/non-ovary related	8	0.30 (3.7)	0.27 (2.8)	22.1 (1.7)	0.06 (1.6)	939 (1.3)	0.91 (1.7)	55.3 (1.3)	412 (1.4)
Stimulation protocol (%):									
Lupron down regulated	8	0.26 (4.4)	0.17 (3.3)	16.8 (3.9)	0.08 (2.0)	561 *(2.6)	0.94 (1.9)	40.9 *(2.5)	330 (2.0)
Gonadotropin antagonist	20	0.30 (3.8)	0.26 (3.4)	17.1 (1.8)	0.06 (1.4)	952 *(1.3)	0.71 (1.5)	63.3 *(1.3)	455 (1.3)
Flare	6	0.23 (2.7)	0.34 (1.6)	19.1 (1.1)	0.06 (1.5)	879 *(1.5)	0.75 (1.7)	70.4 *(1.5)	462 (1.4)

[a] Geometric mean (standard deviation).

[b] One-way ANOVA or Student t-test.

* $p < 0.10$ for difference in trace element concentrations between categories.

** $p < 0.05$.

[c] n = 7 missing for Cd, n = 8 missing for Pb.

[d] Cd values in ng/L.

[e] n = 1 woman with polycystic ovary syndrome (PCOS), an endocrine disorder that disrupts ovulation, not included in analysis.

Table 5

Adjusted means for follicular fluid (FF) trace element levels (μg/L) by diagnosis in single element models (data from 33 women with replicate FF)[a,b].

		95% CI				95% CI	
	Least squares means	**low**	**high**		**Least squares means**	**low**	**high**
As				Cu			
Diminished ovarian reserve	0.16	0.06	0.41	Diminished ovarian reserve	726	506	1042
Unexplained	0.39	0.15	0.99	Unexplained	1035	723	1481
Tubal/male factor/non-ovary	0.25	0.08	0.77	Tubal/male factor/non-ovary	1018	658	1575
Hg				Mn			
Diminished ovarian reserve	0.16	0.07	0.34	Diminished ovarian reserve	0.78	0.57	1.06
Unexplained	0.38	0.18	0.82	Unexplained	0.66*	0.48	0.90
Tubal/male factor/non-ovary	0.24	0.09	0.60	Tubal/male factor/non-ovary	0.99*	0.68	1.45
Cd (ng/L)[c]				Se			
Diminished ovarian reserve	18.3	10.63	31.6	Diminished ovarian reserve	58.4	40.7	83.7
Unexplained	16.6	10.42	26.3	Unexplained	68.5	47.9	97.9
Tubal/male factor/non-ovary	29.5	15.5	56.0	Tubal/male factor/non-ovary	58.1	37.6	89.9
Pb[d]				Zn			
Diminished ovarian reserve	0.07	0.05	0.10	Diminished ovarian reserve	406	307	537
Unexplained	0.06	0.04	0.08	Unexplained	488	368	645
Tubal/male factor/non-ovary	0.06	0.04	0.09	Tubal/male factor/non-ovary	415	296	583

Note: Tubal/male factor/non-ovary includes tubal disorders, primary infertility diagnosis is male factor, and diagnoses unrelated to ovary function.

[a] Natural log-transformed values adjusted for maternal age, cigarette smoking (in As and Cd models), and race.

[b] n = 15 diminished ovarian reserve, n = 10 unexplained, n = 8 tubal/male factor/non-ovary; n=1 woman with PCOS, an endocrine disorder that disrupts ovulation, not included in analysis.

[c] n = 7 missing.

[d] n = 8 missing.

* $p < 0.10$ for difference from diminished ovarian reserve as the reference category.