



Follicular fluid and blood levels of persistent organic pollutants and reproductive outcomes among women undergoing assisted reproductive technologies

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ABSTRACT

Persistent organic pollutants (POPs) are industrial chemicals resistant to degradation and have been shown to have adverse effects on reproductive health in wildlife and humans. Although regulations have reduced their levels, they are still ubiquitously present and pose a global concern. Here, we studied a cohort of 185 women aged 21–43 years with a median of 2 years of infertility who were seeking assisted reproductive technology (ART) treatment at the Carl von Linné Clinic in Uppsala, Sweden. We analyzed the levels of 9 organochlorine pesticides (OCPs), 10 polychlorinated biphenyls (PCBs), 3 polybrominated diphenyl ethers (PBDEs), and 8 perfluoroalkyl substances (PFASs) in the blood and follicular fluid (FF) samples collected during ovum pick-up. Impact of age on chemical transfer from blood to FF was analyzed. Associations of chemicals, both individually and as a mixture, to 10 ART endpoints were investigated using linear, logistic, and weighted quantile sum regression, adjusted for age, body mass index, parity, fatty fish intake and cause of infertility. Out of the 30 chemicals, 20 were detected in more than half of the blood samples and 15 in FF. Chemical transfer from blood to FF increased with age. Chemical groups in blood crossed the blood-follicle barrier at different rates: OCPs > PCBs > PFASs. Hexachlorobenzene, an OCP, was associated with lower anti-Müllerian hormone, clinical pregnancy, and live birth. PCBs and PFASs were associated with higher antral follicle count and ovarian response as

Abbreviations: AFC, Antral follicle count; AMH, anti-Müllerian hormone; ART, assisted reproductive technologies; BMI, body mass index; FF, follicular fluid; FSH, follicle-stimulating hormone; GC-MS/MS, gas chromatography-triple quadruple mass spectrometry; GM, geometric mean; GV, germinal vesicle; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; ICSI, intracytoplasmic sperm injection; IS, internal standard; IV, intravenous; IVF, *in vitro* fertilization; IQR, interquartile range; LA, lipid-adjusted; LC-MS/MS, liquid chromatography-triple quadruple mass spectrometry; LOD, Limit of detection; LOQ, limit of quantification; MI, metaphase I; MII, metaphase II; NHANES, National Health and Nutrition Examination Survey; OCP, organochlorine pesticide; OPU, ovum pick-up; OSI, ovarian sensitivity index; p,p'-DDT, dichlorodiphenyltrichloroethane; p,p'-DDE, dichlorodiphenyldichloroethylene; PBDE, polybrominated diphenyl ether; PCB, polychlorinated biphenyl; PeCB, pentachlorobenzene; PFAS, perfluoroalkyl substance; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFHpA, perfluoroheptanoic acid; PFHxS, perfluorohexane sulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFUnDA, perfluoroundecanoic acid; POP, persistent organic pollutant; SD, standard deviation; TC, total cholesterol; TG, triglycerides; WQS, weighted quantile sum.

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measured by ovarian sensitivity index, but also with lower embryo quality. As a mixture, similar findings were seen for the sum of PCBs and PFASs. Our results suggest that age plays a role in the chemical transfer from blood to FF and that exposure to POPs significantly associates with ART outcomes. We strongly encourage further studies to elucidate the underlying mechanisms of reproductive effects of POPs in humans.

1. Introduction

Infertility is a global issue with one in six couples experiencing involuntary childlessness at some point in their life. The current prevalence for this is 8–12% worldwide for women aged 20–44 (European Society of Human Reproduction and Embryology, 2020). Causes of infertility can be explained by male factor in 20–30% of cases, female factor in 20–35% of cases, and both male and female factors in 25–40% of cases. However, in 10–20% of cases, it cannot be explained by currently known factors (European Society of Human Reproduction and Embryology, 2020). In addition to biological factors, several lifestyle factors have been shown to be associated with infertility including age (Crawford and Steiner, 2015; Nugent and Balen, 2001; Society of Obstetricians and Gynecologists of Canada, 2012), smoking (American Society for Reproductive Medicine, 2006), and body mass index (Gesink Law et al., 2007). Exposure to air pollution and endocrine-disrupting chemicals have also raised concerns of susceptibility of the reproductive function to such exposures (Bergman et al., 2013; Conforti et al., 2018). In particular, we and others have shown that persistent organic pollutants (POPs) are associated with lower biomarkers of ovarian reserve, longer time-to-pregnancy, and higher odds for infertility (Björvang et al., 2020, 2021a; Buck Louis et al., 2013; Cohn et al., 2011; Pan et al., 2019).

POPs are halogenated industrial chemicals that are highly stable, resistant to biodegradation, bioaccumulative and toxic to wildlife and humans (Bergman et al., 2013). Examples of POPs are organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), which are lipophilic and accumulate in adipose tissue (Mustieles and Arrebola, 2020), as well as perfluoroalkyl substances (PFASs), which are amphiphilic and predominantly bound to plasma proteins such as albumin (Forsthuber et al., 2020). Consumption of contaminated food such as fish is the dominating source of exposure to POPs (Guo et al., 2019). Other routes of exposure are through inhalation and skin absorption, and for the fetus, placental transfer. OCPs, PCBs, and PBDEs were already banned decades ago, where OCPs and PCBs were included as part of the Dirty Dozen when the Stockholm Convention came into force in 2004 (Stockholm Convention, 2008). For PFASs, only perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are currently regulated. Although these restrictions have reduced their levels, they are still ubiquitously present in the environment, humans and wildlife, and continue to be of global concern.

Currently, assisted reproductive technologies (ART) provide a venue for collecting patient information and samples, a valuable tool to measure a wide range of endpoints from ovarian function to clinical outcomes. Ovarian reserve in ART patients is estimated using biomarkers such as anti-Müllerian hormone (AMH) and basal antral follicle count (AFC) (Broekmans et al., 2006; Broer et al., 2014; La Marca and Sunkara, 2014). Ovarian response to stimulation is measured as ovarian sensitivity index (OSI) (Huber et al., 2013). Fertilization rate and embryo quality are reproductive outcomes unique to ART that would otherwise be difficult to assess in a normal pregnancy. Clinical outcomes include clinical pregnancy and live birth. ART also allows investigation of the follicular fluid (FF), which is the environment to which the oocytes are directly exposed during maturation. The FF that fills the antrum of the growing follicle is derived from both the blood constituents that cross the blood-follicle barrier as well as products of the follicle structures (Rodgers and Irving-Rodgers, 2010). It is a complex mixture of endogenous substances with accumulation of hormones, lipoproteins and

metabolites but also anthropogenic substances (Hallberg et al., 2021b; Pantasri et al., 2015). While the low molecular weight components are similar between FF and serum, larger molecules are found at lower concentrations in FF than in plasma (Rodgers and Irving-Rodgers, 2010). Although there are several epidemiological studies linking POPs with a wide set of ART endpoints (Lefebvre et al., 2021), more research is needed in larger cohorts and with a wider set of chemicals. Moreover, studies involving clinical pregnancies as well as live births from frozen transfers are limited (Lefebvre et al., 2021). In our study, we investigated 30 chemicals in the blood and FF in a cohort of women undergoing ART. We analyzed how POPs in the blood reflect the oocyte chemical exposure, and how the transfer from blood to FF is related to age. We also studied associations between these chemicals and an extensive range of ART outcomes from ovarian function to clinical outcomes including clinical pregnancy and live birth endpoints to include up to four frozen transfers from 2016 to 2021.

2. Materials and methods

2.1. Study population and sample collection

Women undergoing ART at the Carl von Linné Clinic in Uppsala, Sweden were recruited from April to June 2016. Out of 244 women, 185 were enrolled to the study (75.8% participation rate). They received written and oral information about the study from the nurse and signed a written consent form in accordance with the Declaration of Helsinki. On the day of their ovum pick-up (OPU), an intravenous (IV) catheter was inserted for administration of drugs during the procedure. At the time of the placement of the IV catheter, blood was collected in serum and plasma tubes. The first tube was discarded to minimize contaminants from the catheter. Blood tubes were centrifuged within 30 min at 1400×g. Serum and plasma were aspirated from their respective tubes, aliquoted and stored at –80 °C prior to analysis. OCPs, PCBs, and PBDEs were measured in the serum, and PFASs in the plasma. For simplicity, both sample types are referred to as “blood” in this paper. After oocyte retrieval, clear FF with no visible blood contamination was collected on ice, discarding the first aliquot for possible contamination with wash media used in the OPU-tubing system. All FF samples from one patient were pooled and centrifuged at 500×g. The supernatant was stored at –80 °C prior to analysis. Women were also asked to answer a short questionnaire on lifestyle and habits at the time of recruitment. Clinical data were collected from the medical records. The cohort was followed for frozen transfers for five years until June 2021. All biological samples and data were pseudonymized with random codes. The data were handled according to relevant regulations (the Swedish data protection law PUL and the General Data Protection Regulation GDPR). This study was approved by the Swedish Ethical Review Authority (Dnr 2015/798-31/2, 2016/360-32, and 2016/1523-32).

2.2. Clinical protocols and ART endpoints

Clinical protocols were performed as previously described (Brodin et al., 2013; Holte et al., 2007; Lind et al., 2018; Vaegter et al., 2017). Briefly, all women and their male partners underwent infertility investigation where cause of infertility was determined by a reproductive endocrinologist as male factor (i.e., poor semen quality/quantity or structural problems), female factor (i.e., endometriosis, anovulation, diminished ovarian reserve, fallopian tube damage/blockage, among others), both male and female factor, or unexplained. For the women,

Table 1

Demographic, lifestyle, and reproductive characteristics of women undergoing ART (n = 185).

Characteristics	n	Median (min-max) or %
Age (years)	185	35 (21–43)
BMI (kg/m ²)	184	23 (17.4–34.2)
Parity	185	0 (0–2)
Smoking		
Never smoked	162	87.6
Former smoker	11	5.9
Current smoker	12	6.5
Fatty fish intake		
Daily/Weekly	88	49.3
Monthly	71	40.0
Seldom/Never	19	10.7
Infertility duration (years)	172	2 (0.5–12)
Infertility diagnosis		
Female Factor	54	29.2
Male Factor	44	23.8
Both	14	7.5
Unexplained	73	39.5
Previous IVF treatments	185	1 (0–7)
Children from previous IVF treatments	185	0 (0–2)
Menstrual cycle		
Regular	160	86.5
Irregular	25	13.5
AMH (ng/ml)	185	2.28 (0.05–15.3)
Basal antral follicle count (AFC)	164	16 (2–60)
Treatment protocol		
GnRH agonist	152	82
GnRH antagonist	34	18
Total FSH dose (IU)	185	2400 (525–5850)
Number of oocytes retrieved	185	10 (1–39)
Number of M2 oocytes	185	9 (1–36)
ICSI/IVF		
ICSI	80	43.2
IVF	91	49.2
Both	14	7.6

baseline serum concentration of AMH was measured. Basal AFC, which is the total number of follicles with a 2–10 mm size, was determined via ultrasound before starting stimulation. The women underwent either a GnRH agonist protocol (82% of women) using Suprecur (Cheplapharm) or Synarela (Pfizer) where the pituitary is desensitized starting the luteal phase, or a GnRH antagonist protocol (18% of women) where GnRH antagonist Orgalutran (Organon) was given on day 6 of menses. To stimulate follicle growth and egg maturation, follicle-stimulating hormone (FSH) (Gonal-F, Fostimon, Bemfola) and/or human menopausal gonadotropin (Menopur) were given starting on day 3 of menses. Once

there were at least three follicles of ≥ 17 mm, human chorionic gonadotropin was given. After 36–37 h, oocytes were retrieved through transvaginal ultrasound-guided ovarian puncture and classified as a germinal vesicle (GV), metaphase I (MI), metaphase II (MII) or empty zonas/degenerated oocytes. The oocytes were then fertilized using *in vitro* fertilization (IVF) or injected with sperm after denudation for intracytoplasmic sperm injection (ICSI). Fertilization was confirmed 16–20 h later with the presence of two pronuclei. The embryos were evaluated on day 2 based on blastomere number, fragmentation, blastomere size variation, symmetry of cleavage and mononuclearity in the blastomeres, with a score ranging from 1 to 10 (Holte et al., 2007). Embryos were then transferred on day 2, 3, 5 or 6, or frozen and transferred later. Clinical pregnancy was denoted by the presence of a gestational sac via ultrasound at around 6–7 weeks of gestation. Live birth was defined as a birth of a live baby with at least 24 weeks of gestation. ART endpoints analyzed in this study were as follows: To estimate ovarian reserve, AMH and basal AFC were used. To measure ovarian response to stimulation, OSI was calculated using the formula:

$$OSI = \log \left(\frac{\text{number of oocytes retrieved}}{\text{total FSH dose (IU)}} \times 1000 \right)$$

To illustrate intermediate ART outcomes, we investigated fertilization rate which is calculated by the number of embryos with two pronuclei divided by the number of MII oocytes. In addition, average embryo quality score and presence of at least one top-quality embryo (yes/no) (defined as having a score ≥ 9.1 by Holte et al. (2007)) were also assessed. For clinical outcomes, clinical pregnancy (yes/no) as well as live birth (yes/no) were considered for fresh transfers only as well as for fresh and frozen transfers.

2.3. Quantification of persistent organic pollutants in blood and FF

Concentrations of nine organochlorine pesticides (OCPs) [pentachlorobenzene (PeCB), hexachlorobenzene (HCB), isomers of hexachlorocyclohexane (α -HCH, β -HCH, γ -HCH), oxychlorodane, transnonachlor, dichlorodiphenyltrichloroethane (p,p'-DDT), dichlorodiphenyldichloroethylene (p,p'-DDE), ten polychlorinated biphenyl (PCB) congeners (PCB 74, 99, 118, 138, 153, 156, 170, 180, 183 and 187) as well as polybrominated diphenyl ethers (PBDE 47, 99, and 153) were analyzed in serum and FF using an accredited methods as described before (Koponen et al., 2013). Briefly, 200 μ l ethanol and 400 pg of ¹³C-labeled internal standards (IS) of each compound in 100 μ l of toluene were added to serum (200 μ l) or tissue (200 mg) samples in test tubes and mixed for 4 min at 1800 rpm to equilibrate IS and to

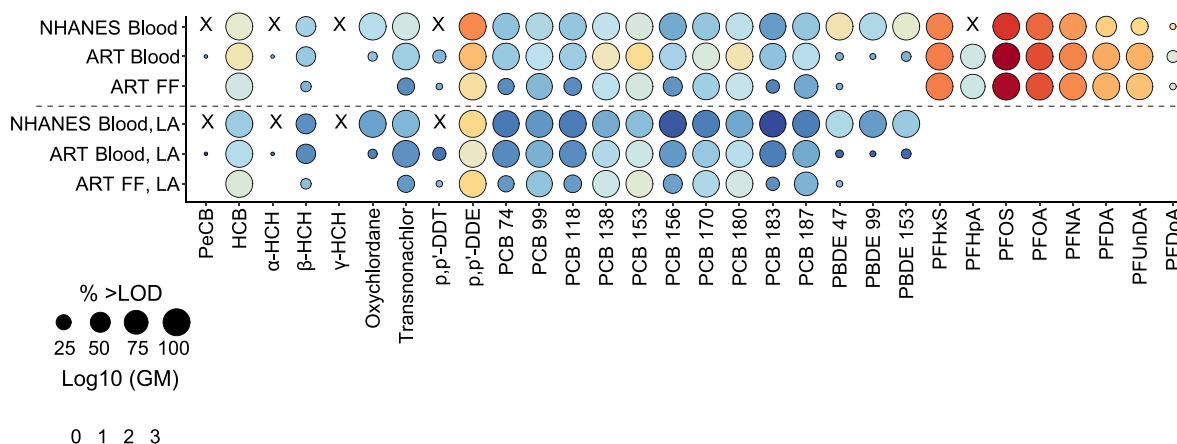


Fig. 1. Comparison of POP exposure of women in Sweden and USA. Crude and LA concentration, and occurrence of 30 POPs in blood and FF of the Sweden cohort (ART in figure) collected in 2016 alongside chemicals in blood of women aged 20–39 years in the USA cohort (NHANES 2015–2016). Size of the dot denotes percent of samples above limit of detection (LOD) while the color represents log₁₀ of geometric mean (GM) of the chemical concentration (pg/ml). X means it was not analyzed in the sample.

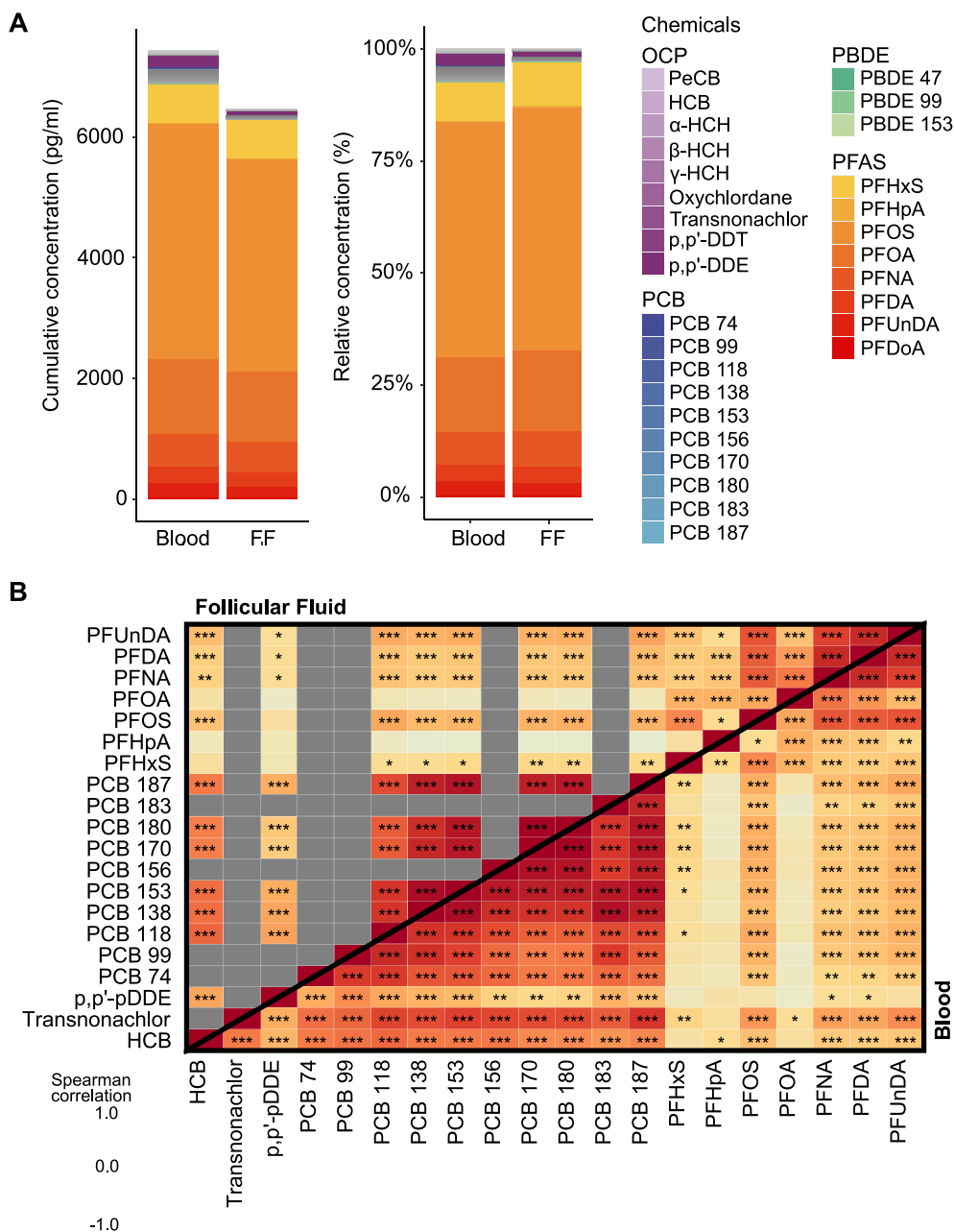


Fig. 2. Total chemical burden and correlation of chemicals with each other. A) Cumulative burden was calculated by adding the medians of chemicals in each matrix while the relative proportion was determined by dividing the median of the chemical by the cumulative burden, multiplied by 100. B) Correlation of chemicals in each matrix with more than half of the samples above the limit of detection (LOD). Upper triangle denotes correlation for 15 chemicals in the FF while lower triangle for 20 chemicals in the blood. Spearman's correlation with rho ranging from -1 (dark blue) to 1 (dark red). Grey means it was not analyzed. * indicates p-value <0.05, ** <0.01 and ***<0.001.

precipitate the proteins. Dichloromethane-hexane (1:4) was added for extraction followed by activated silica to bind the sample water, ethanol and precipitate. Dichloromethane-hexane from the top was poured to multilayer silica column containing 44% sulfuric acid silica, 10% AgNO₃-silica and sodium sulfate-silica mixture (1:2, v/v) to clean the extracts. Extraction of solid residue was repeated three times and eluate from cleanup column was concentrated to 15–20 μL for gas chromatography – tandem mass spectrometry (GC-MS/MS) analysis (Agilent 7010 GC-MS/MS system, Wilmington, DE, USA) using a DB-5MS UI GC column (J&W Scientific, 20 m, ID 0.18 mm, 0.18 μm). Limits of detection (LOD) of our samples ranged from 2 pg/mL to 15 pg/mL while limits of quantification (LOQ) ranged from 5 pg/mL to 40 pg/mL (Supplementary Table 1). LOD and LOQ were calculated as the concentration corresponding to 3 and 10 times the standard deviation (SD) of the in-house low-concentration control sample, respectively.

Concentrations of eight amphiphilic PFASs (perfluorohexane sulfonic acid (PFHxS), perfluoroheptanoic acid (PFHpA), perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA),

perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoA)) were measured in plasma and FF using a modified method described previously (Lindh et al., 2012). Briefly, samples were prepared by adding labeled IS of all measured PFAS and proteins were precipitated using acetonitrile under vigorous shaking for 30 min then centrifuged. The analysis was conducted with 4 μL of obtained supernatant using liquid chromatography system (LC-MS/MS, LCUFCLXR, Shimadzu Corporation, Kyoto, Japan) coupled to triple quadrupole mass spectrometry (QTRAP 5500; AB Sciex, Framingham, MA, USA). LOD ranged from 10 pg/mL to 20 pg/mL while LOQ ranged from 30 pg/mL to 60 pg/mL (Supplementary Table 1). LOD and LOQ were defined as the concentration corresponding to 3 and 10 times the SD of the ratio of the peak at the same retention time as the analyzed compounds and the corresponding IS divided by the slope of the calibration line and determined in the chemical blank samples.

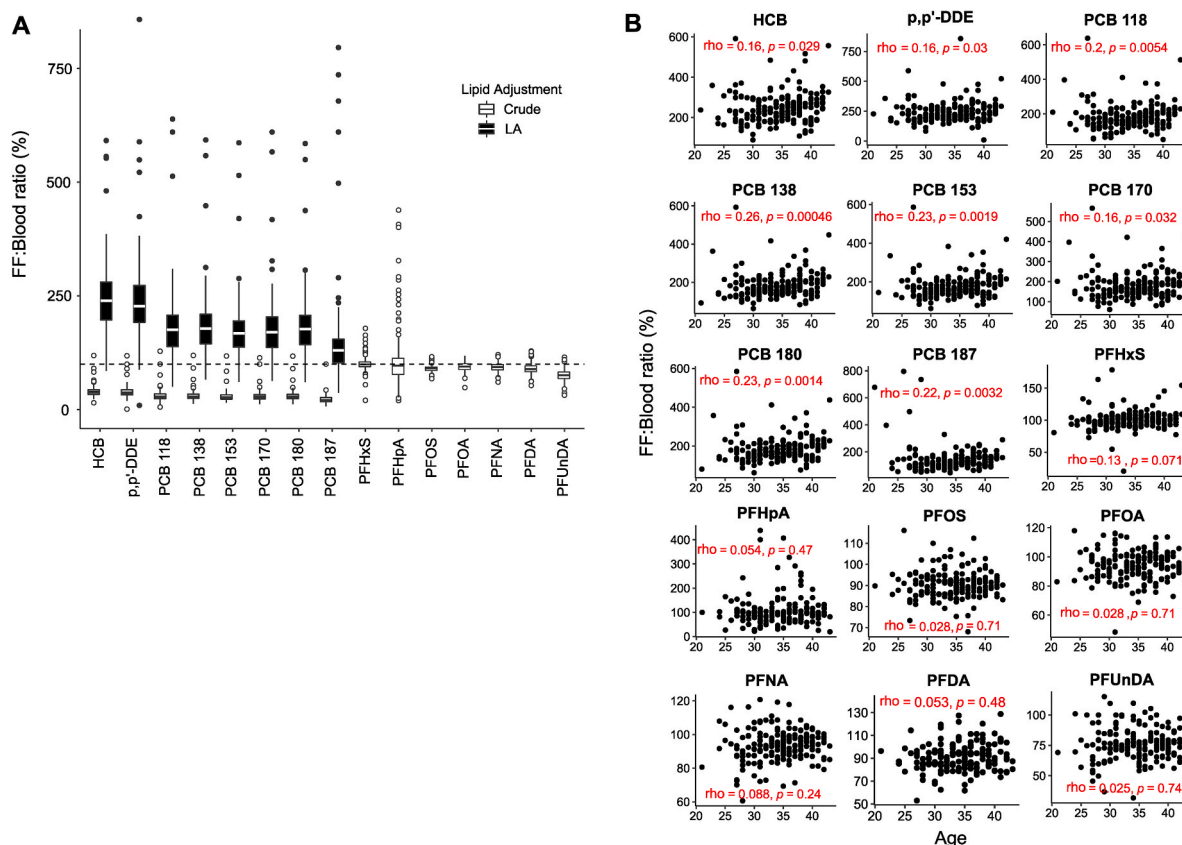


Fig. 3. Chemical transfer from blood to FF and their relationship with age. A) FF:Blood ratio (%) was computed by dividing the chemical concentration in FF by that in the blood and multiplied by 100. This was performed for both crude and LA OCPs and PCBs. Dashed line marks 100% where concentration in FF is equivalent to that of the blood. Boxplots consist of the interquartile range (IQR) and the median, and whiskers are $1.5 \times \text{IQR}$. Outliers are defined as values greater than $1.5 \times \text{IQR}$ and depicted with dots. B) Scatter plot of FF:Blood ratio and age, showing spearman correlation rho and p values. A and B include only 15 chemicals that were detected in more than half of the samples in both matrices.

2.4. Analyses of lipids in the blood and FF

Total lipid levels were determined to adjust the concentrations of the lipophilic POPs. Enzymatic colorimetric kits were used to measure the total cholesterol (TC) and triglycerides (TG) in the samples, namely Cholesterol CHOD-PAP and Triglycerides GPO-PAP, respectively (Cobas, Roche Diagnostics GmbH, Mannheim, Germany). The TC kit has a measurement range of 3–800 mg/dL and CV 0.8% and the TG kit has a measurement range of 4–1000 mg/dL and CV 1.5%. Briefly, 10 μL serum was diluted with 10 μL water (or 20 μL undiluted FF), where 100 μL of each specific reagent was added. After incubating the mixture for 30 min at room temperature, absorbance was measured at 492 nm using a microplate spectrophotometer (Tecan Infinite F500, Tecan group Ltd., Männedorf, Switzerland). Color intensity was directly proportional to TC and TG concentration. To determine the standard curve, Precinorm U (Cobas, Roche Diagnostics, GmbH, Mannheim) was used. Internal control reference used was a pooled serum sample from 8 healthy volunteers, who signed the informed consent form in adherence to the Declaration of Helsinki. This sample was prepared at the Div. of Clinical Chemistry, Dept. of Laboratory Medicine, Karolinska Institutet at Huddinge University Hospital, Stockholm, Sweden. To calculate the total lipid concentration, we used the following formula (Bernert et al., 2007):

$$\text{total lipid} = 2.27 \times \text{TC} + \text{TG} + 62.3 \text{ mg/dl}$$

2.5. Statistical analyses

Continuous variables were described by medians, minimum and

maximum values, and categorical variables were described by numbers and percentages. Chemicals that were detected in more than half of the samples were included in further analyses, where concentrations below LOD were replaced with $\text{LOD}/2$. Out of the 30 chemicals measured, there were 20 chemicals included for the blood while there were 15 chemicals included for the FF. Spearman correlation coefficients between chemicals in each matrix were assessed. The lipophilic POPs were considered both as crude and lipid-adjusted (LA) by dividing the chemical with the total lipids. For missing data on BMI (0.5%), fatty fish intake (3.2%), as well as lipids in blood (36.4%) and FF (0.5%), multiple imputation by chained equations was done. To compare exposure patterns between Sweden and the United States, we utilized chemical concentrations in the serum of women aged 20–39 years in the National Health and Nutrition Examination Survey (NHANES) in 2015–2016 conducted by Centers for Disease Control and Prevention in the United States (Centers for Disease Control and Prevention (CDC) and National Center for Health Statistics (NCHS), 2016). Total chemical burden was determined for blood and FF by adding the median chemical concentrations (pg/ml) of all 30 measured chemicals.

To determine transfer of chemicals from the blood to the FF, we calculated their ratio using the formula:

$$\text{FF : Blood ratio (\%)} = \frac{\text{FF}_{\text{concentration}}}{\text{Blood}_{\text{concentration}}} \times 100$$

Spearman correlation coefficients between the FF:Blood ratio and age were assessed for the 15 chemicals that were above LOD in more than half of the samples for both blood and FF. LogKow, which is the log of the octanol-water partition coefficient of a chemical denoting its lipophilicity, was used to estimate transfer of chemicals through passive

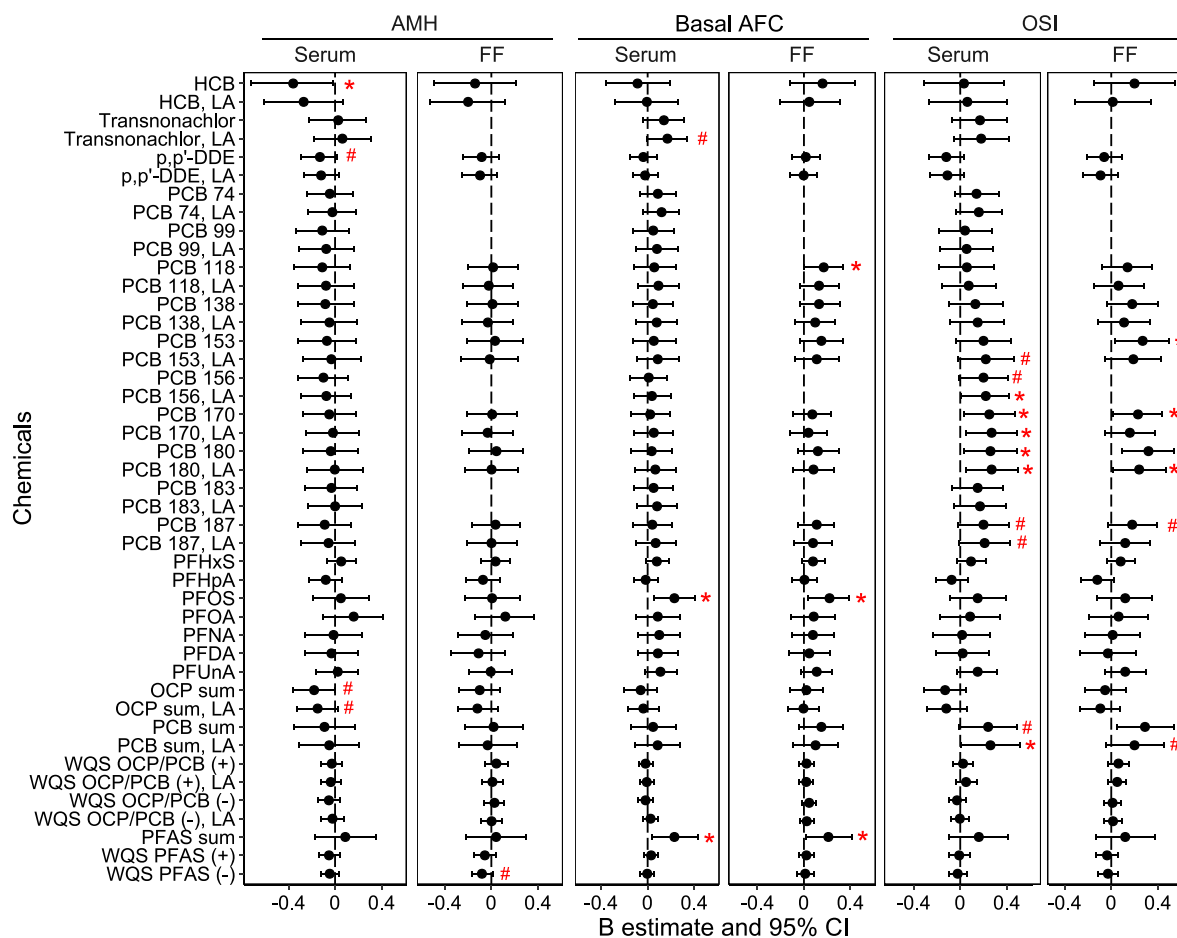


Fig. 4. Associations between chemical concentrations in blood and FF, and ART endpoints depicting ovarian function. Dot shows β -estimate with 95% confidence interval. Dashed vertical line corresponds to estimate = 0. Linear and weighted quantile sum (WQS) regressions were adjusted for age, BMI, parity, fatty fish intake and cause of infertility. AMH, basal AFC and chemicals were log₁₀-transformed. WQS analyzed for positive (+) and negative (-) associations. Lipophilic chemicals were analyzed as crude concentrations (pg/mL) and lipid-adjusted (LA) concentrations (ng/g lipid). Only those chemicals that were above the LOD in more than half of the women were included for each matrix. * indicated for p-value < 0.05 while # for p-value between 0.05 and 0.1.

diffusion. Medians of log₁₀ of FF:blood ratio were plotted as a function of logKow, wherein Spearman correlation coefficient was calculated. The ratio of indicator PCBs (PCB 118, 138, 153, and 180) to the sum of PCBs in the serum were compared to that of the follicular fluid using Wilcoxon rank sum test.

We assessed the associations between chemicals and ART endpoints using both individual chemical and chemical mixture approaches. The statistical models were controlled for covariates of fertility determined *a priori*, namely age, body mass index (BMI), parity and cause of infertility (male, female, both, unexplained). Additionally, fatty fish intake was also taken into account as an exposure source for the chemicals (daily/weekly, monthly, seldom/never). Smoking was not included because a majority of the women (87.5%) were non-smokers. We used linear and logistic regression model to study associations of individual chemicals to AMH, basal AFC, OSI, fertilization rate, average embryo quality score, presence of top-quality embryo (yes/no), clinical pregnancy from fresh transfer (yes/no) and from fresh/frozen transfer (yes/no), as well as live birth from fresh transfer (yes/no) and from fresh/frozen transfer (yes/no). Chemical concentrations, AMH, basal AFC, fertilization rate+1, and average embryo quality were log₁₀-transformed to reduce skewness. Chemical mixture analyses were carried out in two ways. First, by summing up the chemicals in the same group (e.g. OCPs together). Second, we used a strategy suitable for highly correlated datasets called weighted quantile sum (WQS). This is used for estimating empirical weights for a weighted sum of quartiles associated with health outcomes (Carrico et al., 2015; Czarnota et al., 2015). Chemicals of concern were

identified with a threshold of 7.7% and 12.5% (100% divided by 13 and 8 chemicals for blood and FF, respectively) for lipophilic POP mixture (both crude and LA), and 14.3% (100% divided by 7 chemicals for both blood and FF) for PFAS mixture. The dataset was split into 40% training set and 60% validation set. Similarly, models were adjusted for age, BMI, parity, cause of infertility, and fatty fish intake. Both positive and negative associations were tested. A machine learning algorithm Boruta was utilized to see if chemicals will be considered as important variables in relation to the studied outcomes. Boruta is a wrapper algorithm that finds relevant features by comparing original attributes' importance with importance achieved at random, which is estimated using their shadows as shuffled copies (Kursa and Rudnicki, 2010). In all analyses, significance was defined as $p < 0.05$, and tendencies $0.05 < p < 0.10$ were also denoted in figures. All statistical analyses were performed using the R programming language (R Core Team, 2018) through RStudio (RStudio Team, 2016) with the packages ggplot2 (Wickham, 2009), ggpubr (Kassambara, 2017), reshape2 (Wickham, 2007), mice (van Buuren and Groothuis-Oudshoorn, 2011), dichromat (Lumley, 2013), gWQS (Renzetti et al., 2018), and Boruta (Kursa and Rudnicki, 2010).

3. Results and discussion

3.1. Cohort characteristics

Our study cohort consists of 185 women undergoing ART treatment

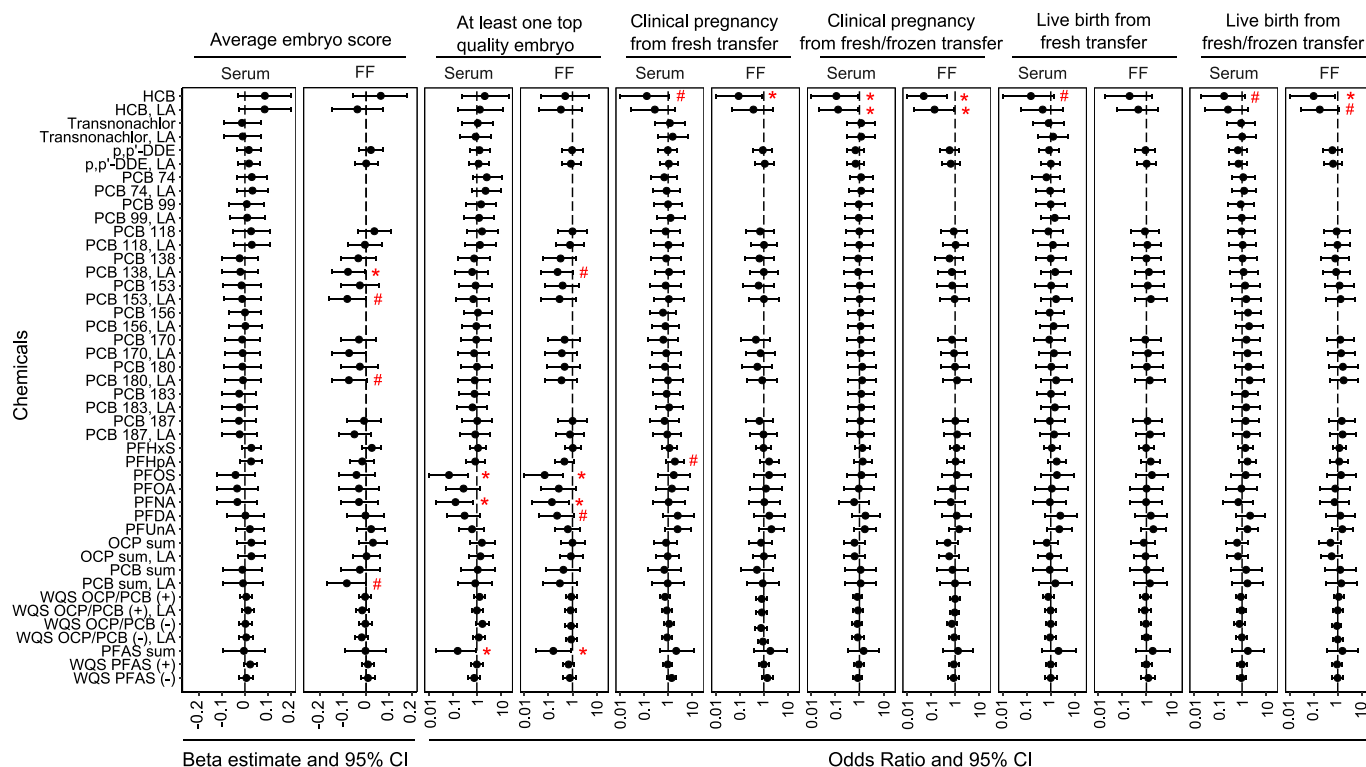


Fig. 5. Associations between chemical concentrations in blood and FF, and post-fertilization ART endpoints. Dot shows β -estimate or odds ratio with 95% confidence interval. Dashed vertical line corresponds to estimate = 0 or odds ratio = 1. Linear, logistic, and weighted quantile sum (WQS) regressions were adjusted for age, BMI, parity, fatty fish intake and cause of infertility. Average embryo score and chemicals were log10-transformed. WQS analyzed for positive (+) and negative (-) associations. Lipophilic chemicals were analyzed as crude concentrations (pg/mL) and lipid-adjusted (LA) concentrations (ng/g lipid). Only those chemicals that were above the LOD in more than half of the women were included for each matrix. * indicated for p-value < 0.05 while # for p-value between 0.05 and 0.1.

at the Carl von Linné Clinic in Uppsala, Sweden. They were mostly nulliparous, had a median age of 35 years, and BMI of 23 kg/m². The cause of infertility was 29.2% female factor, 23.8% male factor, 7.5% both male and female, and 39.5% unexplained. Table 1 shows demographic, lifestyle, and reproductive characteristics of the cohort at the time of recruitment. Fertilization rate was 66.7% with an average embryo score of 7.3 and 73% having at least one top-quality embryo. There were 157 fresh transfers, resulting in 66 clinical pregnancies (42%) and 59 live births (38%). There were 90 frozen transfers (range 0–4 frozen transfers per woman), resulting in 39 clinical pregnancies (43%) and 30 live births (33%).

3.2. Concentrations of POPs in blood and FF are significantly correlated

We measured 30 POPs, namely OCPs, PCBs, PBDEs, and PFASs, in blood and FF. While the OCPs, PCBs, and PBDEs are extensively regulated by the Stockholm Convention, the PFASs are not yet as extensively controlled (Stockholm Convention, 2008). Despite regulation for several years, which has led to reduced concentrations in humans and wildlife, OCPs, PCBs and PBDEs are still commonly detected in contemporary biological samples. In our samples, PFASs had highest concentrations, followed by OCPs, and PCBs (Fig. 1). This is probably due to the amphiphilic natures of PFASs, leading to accumulation in the blood compared to the lipophilic OCPs, PCBs, and PBDEs, which are likely higher in the adipose tissue compared to the serum (Forsthuber et al., 2020; Mustieles and Arrebola, 2020).

Twenty chemicals were above the LOD for more than half of the blood samples (Supplementary Table 1). Fifteen out of the 20 were also detected in more than half of the FF samples (Supplementary Table 2). The five chemicals that were present in over half of the blood samples but in less than half of the FF samples were transnonachlor and PCBs 74,

99, 156 and 183. FF is the environment in which the oocytes grow and mature. It affects the quality of the oocyte as well as influences the reproductive function of women (Broi et al., 2018). The presence of POPs in the FF implies that the chemicals pass through the blood-follicle barrier, exposing the maturing oocytes. Experimental animal studies directly exposing oocytes *in vitro* to POPs have shown negative effects on oocyte developmental competence, viability and maturation, as well as altered steroidogenesis and intercellular communication with granulosa cells (Domínguez et al., 2016; Hallberg et al., 2019, 2021a, 2021b; Wójtowicz et al., 2004). In humans, POPs are often found in FF but associations to ART outcomes such as number of mature oocytes retrieved, high-quality embryos, basal AFC, and clinical pregnancy have been inconsistent (Björvang and Damdimopoulou, 2020; Lefebvre et al., 2021). The inconsistencies could depend, for instance, on co-occurring chemicals because POPs are not the only chemicals present in human FF and mixture effects are possible. A recent non-target analysis of FF samples from this same cohort revealed thousands of features, out of which hundreds correlated with embryo quality (Hallberg et al., 2021b). It will be an important task to identify what features are derived from man-made chemicals in order to properly control the use of chemicals with reprotoxic properties.

We compared the chemical concentrations in our cohort with those of similarly aged women in the United States (Fig. 1). Although the detection patterns of OCPs, PCBs and PFASs were similar in both groups of women, a noticeable difference was the high detection of PBDEs in the American cohort compared to the Swedish cohort. This difference may be due to various factors such as flame-retarded products available in the market, diet, and local regulations. Nonetheless, women were still exposed to an extensive of mixture of POPs in both countries.

We examined the total burden of chemicals in the blood and FF. The sum of the median of chemical concentrations in the blood was 7425 pg/

ml. For FF, the median was 6468 pg/ml. For both matrices, the majority of the chemicals were composed of PFASs (97% in blood, 92% in FF) (Fig. 2a). The chemicals were significantly correlated with each other in each matrix, except for PFHxS, PFHpA, and PFOA, which were only correlated with other PFASs but not with OCPs or PCBs (Fig. 2b). The degree of correlation was still stronger in chemicals belonging to the same group (i.e., PCBs as a group and long-chained PFASs as a group). This suggests the co-occurrence of POPs, such that exposure to one chemical may predict exposure to other chemicals as well. To know whether the concentrations in the blood reflected the concentration in the FF, we calculated the Spearman correlation between the two matrices. There was a significant positive correlation between the blood and FF for the 15 chemicals (ρ 0.64–0.99, $p < 0.001$ for all) (Supplementary Figure 1). This suggests that the blood provides a good estimate of the chemicals present in the FF and may be used as a surrogate marker for oocyte exposure.

3.3. Chemical group and age affects chemical transfer from blood to FF

Chemical transfer from blood to FF was studied based on the 15 chemicals that were detected in more than half of the samples for both matrices. For the OCPs and PCBs, the ratios were around 50% before LA, suggesting low transfer to FF. However, after LA, the medians of FF: blood ratio were above 100%. For the PFASs, the FF and blood levels were at similar concentrations (i.e., around 100%). Overall, the chemical groups transferred at different efficiencies – OCPs > PCBs > PFASs (Fig. 3a). In our earlier study on human fetuses, we observed a similar trend for transfer efficiency from mother to the child (Björvang et al., 2021b). To know whether passive diffusion could explain this behavior, we assessed the correlation of the FF: blood ratio with $\log K_{ow}$, a measure of lipophilicity of chemicals. $\log K_{ow}$ was negatively correlated with the chemicals (Supplementary Figure 2). This indicates that lipophilicity alone cannot explain the partitioning of the chemical from blood to FF. Hence, other physicochemical parameters or molecular descriptors would be needed. In addition, we studied possible selectivity of chemical transfer by calculating ratios for each indicator PCB to the sum of PCBs in each matrix. Ratios of PCBs 138 and 180 were higher in the FF compared to the blood, suggesting that certain selectivity for chemicals could be involved in the transfer from blood to FF (Supplementary Figure 3). These results encourage further studies on the structure-activity relationships of these chemicals to understand how physicochemical properties can affect transfer efficiency through the blood-follicle barrier.

To study whether biological factors such as age may affect the FF: blood ratios, we performed correlation analyses. Age was significantly positively correlated with LA OCP and PCB ratios but not with the PFAS ratios (Fig. 3b). These results imply that age may be a factor in the transfer of lipophilic chemicals from blood to FF. It also suggests that the transfer mechanisms may differ between different types of chemicals.

It is widely known that fertility decreases with increasing age in women. There is a steady decrease of fertility until the age of 35 years, followed by an accelerated decline (ESHRE Capri Workshop Group, 2005; Faddy et al., 1992; Silber et al., 2017). This reduced fertility can be due to the decline in total number of oocytes and their quality, as well as changes in the surrounding environment such as the FF. Previous studies have shown that possible age-related mechanisms include changes in the antioxidant defences and lipid metabolism in the FF (Luddi et al., 2020; Tatone et al., 2008; Zhang et al., 2020). Oxidative stress alters protein structures (Luddi et al., 2020; Tatone et al., 2008), which may in turn allows more chemicals to pass through the blood-follicle barrier. Moreover, altered lipid metabolism may also explain our findings on OCPs and PCBs, which are lipophilic in nature. Nevertheless, more studies are encouraged to further investigate the possible increasing leakage of the aging follicle.

3.4. Chemicals are associated with ART endpoints

We evaluated the associations between chemical exposures and ART endpoints, both as individual chemicals and as a mixture. AMH, basal AFC and OSI depicted ovarian function. Crude HCB in the blood was associated with lower AMH (Fig. 4). Crude PCB 118 in the FF as well as PFOS in the blood and FF were associated with higher basal AFC (Fig. 4). We also found positive associations between OSI and PCB 156, 170, 180 in the blood and PCB 153, 170, 180 in the FF (Fig. 4). Higher basal AFC and OSI are typically regarded as positive predictors of ART treatment success. However, we did not find positive associations between chemicals and treatment outcomes. Instead, there was a negative association between average embryo score and PCB 138 in FF. PFOS and PFNA in blood and FF were significantly associated with lower odds for having at least one top-quality embryo (Fig. 5). The chemicals were not significantly associated to fertilization rate. In addition, crude and LA HCB in the blood and FF were associated with lower odds for clinical pregnancy from fresh/frozen transfers, and crude HCB in blood with lower odds for clinical pregnancy from fresh transfer and live birth from fresh/frozen transfers (Fig. 5).

Humans are not exposed to a single chemical but a complex mixture of many chemicals. It is then important that the chemicals are also assessed as a mixture. However, combination effects of chemicals still present as a challenge in human risk assessment. While there is no single approach that outperforms the others in chemical mixture assessment (Taylor et al., 2016), we have utilized two approaches in this study – additive approach and use of WQS modelling. When taken as a mixture, the sum of the PCBs were associated with higher OSI (Fig. 4). Sum of PFASs were associated with higher basal AFC but lower odds for having at least one top-quality embryo (Figs. 4 and 5). None of the mixtures through WQS modelling was significant although PFASs were suggestive of possible association with lower AMH (Fig. 4).

Overall, PCBs and PFASs were linked to higher biomarkers of ovarian reserve and ovarian response but lower quality of embryos while HCB had a negative association with ovarian reserve as well as clinical pregnancy and live birth. Our results support previous POPs and ART studies where HCB was associated with lower implantation rate (Mahalingaiah et al., 2012), and PCBs and PFASs with lower embryo quality (Bloom et al., 2017; Ma et al., 2020; Petro et al., 2012).

We also used a machine learning algorithm called Boruta to identify important features in our dataset in relation to the studied outcomes. This feature-selection algorithm compares the importance of the original variables with the importance of the shuffled variables to determine if importance was achieved at random. As expected, biological factors such as age and BMI were among the important factors (Supplementary Table 3). Interestingly, PCBs and PFASs also appear as important variables for ART outcomes, especially for AMH, basal AFC, OSI, fertilization rate, embryo quality, and clinical pregnancy (Supplementary Table 3). However, no chemicals were important for live birth. It is also important to acknowledge that this algorithm can only identify important features, but not the direction of the effect. Nonetheless, these results show that chemicals may be linked with ART endpoints and more studies are encouraged to explore further the relationship of chemicals and reproductive outcomes.

4. Strengths and limitations

To the best of our knowledge, this is the first study to extensively investigate several ART endpoints in a relatively large cohort with a long follow-up period of five years to include up to four frozen transfers from the same OPU. Moreover, we assessed a wide range of POPs, comprising of 30 chemicals in 4 subgroups. We also explored the relationship of age and chemical transfer through the blood-follicle barrier. Likewise, we explored a wide range of ART endpoints, from those depicting ovarian function to clinical pregnancy outcomes. We studied the association of the chemicals to ART endpoints, both as an individual chemical and as a

mixture. As there are many ways to assess mixtures, we utilized two methods, namely as a sum and through WQS modelling. In addition, we used machine learning to determine the importance of these chemicals relative to the different ART outcomes.

This study has limitations. ART endpoints are influenced by both male and female factors. In our study, we have mostly focused on the female and did not have any data from the male partners aside from the cause of infertility. The female factor as cause of infertility was also grouped as one despite its heterogeneity (i.e., anovulation, endometriosis, uterine factor, fallopian tube factor). Furthermore, the cohort is comprised of women undergoing ART, which may have a different biological picture compared to the general population. Additionally, the machine learning algorithm Boruta can only identify the important variables, but not the direction of the effect of these variables on the outcomes. Finally, our relatively large cohort still has limited power and the findings need repetition in independent larger studies.

5. Conclusions

Despite decades since restrictions in Sweden, lipophilic POPs are still widely detected in blood and FF of women living in Sweden. They are accompanied by PFASs, a large family of extremely resistant chemicals that have not yet been efficiently regulated. The measured POP blood levels reflect well the levels in FF, suggesting that blood can be used as a reliable surrogate sample for FF. Chemical group and age affect the transfer of POPs from blood to FF. Exposure of women to POPs were associated with various ART endpoints. Specifically, PCBs and PFASs, both as individual chemicals and as a mixture, were associated with higher basal AFC and OSI but lower embryo quality. HCB was associated with lower AMH, and lower odds for clinical pregnancy and live birth. Our results highlight the adverse reproductive outcomes that may be related to exposure to POPs, and that ageing might augment the oocyte exposure. Our findings urge further studies to unravel mechanisms that may explain these relationships. Details on mechanisms are important as they will help to amend regulatory guideline assays for chemical safety testing to include key events that are targeted by chemical toxicants relevant to humans and associated to adverse outcomes. Finally, this study advocates the importance of immediate action on regulations to prevent further exposure to these chemicals.

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CRedit author statement

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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.

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Appendix A. Supplementary data

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