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The Relation of IVF Success as Assessed by Oocyte Retrieval to Environmental Exposures to Endocrine Disrupting Chemicals

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Abstract

The Relation of IVF Success as Assessed by Oocyte Retrieval to Environmental Exposures to Endocrine Disrupting Chemicals

By Tamara Opila

Introduction: Near ubiquitous exposure to phthalates and bisphenol A (BPA) in consumer products has recently become a growing concern for human health and reproduction. While most measurements of BPA and phthalate exposure have been previously studied in matrices such as urine or saliva, the novel matrix of follicular fluid (FF) was sampled to investigate the effects of measurable concentrations of these endocrine disruptors on target tissue (i.e. ovarian follicle). The aim of this study was to quantify levels of high-molecular weight phthalate (HMWP) and low-molecular weight (LMWP) metabolites in FF samples, compare metabolite concentrations in FF to levels previously published in urine and determine any relation between phthalate metabolite concentrations in FF and number of oocytes retrieved for IVF patients.

Methods: Samples were collected over several IVF cycles from a cohort of Israeli women and analyzed using HPLC/MS ($n=86$). Concentrations of HMWP and LMWP metabolites (ng/mL) were standardized using a molar sum procedure. Paired t-tests and intraclass correlation coefficients (ICC) were employed for determining repeat measure reliability and study subject interfollicular variation. Multiple linear regression was used to investigate the association between the number of oocytes retrieved and increasing quartiles of HMWP and LMWP.

Discussion: E2 levels were positively correlated with the number of oocytes retrieved ($r=0.29$, $p<0.05$). The greatest decline in estradiol (E2) was associated with the second quartile of LMWP, which closely approaches statistical significance ($p=0.08$). Conversely, the greatest appreciation in E2 levels was correlated with the unadjusted model of HMWP ($p<.05$). A non-linear response in oocytes retrieved was observed for both HMWP and LMWP. ICC for phthalate metabolites ranged from 0.47 to 0.89 and minimal interfollicular variation was observed among follicle pairs.

Conclusions: Repeat measure reliability and method development provided additional evidence supporting the validity of study design, however FF maximum concentration observed for all analytes were approximately 100x less than those observed in urine. Phthalates and BPA act additively as endocrine disruptors and result in a synergistic response, therefore additional analysis of all samples for detected BPA concentrations may elucidate why a linear-dose response was not observed among quartiles of increasing concentrations for phthalate monoesters.

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I. INTRODUCTION

Background

The effects of endocrine disruptors were first realized in the mid- 20th century, through the advent of diethylstilbestrol (DES), a widely prescribed medication for managing pregnancy complications in women. Concerns have been mounting for similar effects from ubiquitous exposure to bisphenol A (BPA, Figure 1) and phthalates in consumer products that include cosmetics, food packaging and plastics. These modern-use chemicals are currently being investigated for their relation to infertility, precocious puberty, and low testosterone from anti-androgen action (i.e. testes malformation). To date, most measurements of BPA and phthalate exposure have been made in convenience matrices such as urine or saliva and these measurements have been used to link exposure to effect. However, target tissue measurements provide more relevant information about how much of the chemical actually reaches the target tissue to induce its effect, and thus are more meaningful and most proximal when trying to link exposures to effects (Meeker et al., 2009). For reproductive success, a more relevant matrix for exposure assessment may be follicular fluid (FF). The measurement of BPA and phthalate metabolite concentrations in FF is a novel matrix for determining reproductive success, a factor for oocyte viability (i.e. maturation), and also a consideration among women desiring to become pregnant (Souter et al., 2013). A source population undergoing in vitro fertilization (IVF) is optimal for analysis of FF corresponding to the mature follicles retrieved prior to fertilization of the oocyte; FF also serves as a target matrix for chronic, long-term exposures. The goal of the present study is to determine whether FF concentrations of phthalate metabolites and BPA are negatively associated with IVF success as estimated by the number of oocytes retrieved.

Health Effects of Endocrine Modulation

The endocrine system comprises glands and hormone receptors of cells and organs, specifically controlling the production of estrogen, testosterone, androgens and estrogenic precursors and indirectly controlling receptor expression for these respective hormones. BPA is similar to estradiol in its ligand binding activity, and although it lacks the basic four-ring structure of a steroid hormone, promiscuous binding with the estrogen receptor (ER) has been demonstrated in previous studies (Witorsch, 2002). This induced fit mechanism is a key feature of DES in mimicking estradiol. While the activity of phthalates continues to be debated, BPA appears to have circumvented the lack of affinity for the both ERα and ERβ, and targets membrane estrogen receptors to promote steroid-induced modulation (Nadal et al., 2000). The non-competitive allosteric mechanisms of these compounds for endocrine targets such as the pancreas and brain have far-reaching impacts for chronic disease including glucose dysregulation, learning disabilities and reduced IQ.

Perhaps the most clinically reported negative outcomes for endocrine disruptors relate to embryonic development, sexual maturation and fertility. Reduced anogenital distance, spermatogenesis and sperm motility have been studied across species to gain a better understanding of the impact upon the fertility for the male sex. Development of a male phenotype (masculinization) is driven by three hormones from the fetal testis: anti-Müllerian hormone, insulin-like factor 3, and testosterone (Sharpe, 2006). These hormones direct the development of reproductive and other organs from female to male and also play a role in testis development. An example of this phenomenon is enzymatic

activity and genetic control in the testicular biosynthesis of testosterone from cholesterol and further metabolism to the potent androgen, dihydrotestosterone (DHT). Female reproductive issues specific to monoovulators concerns the ability to initiate folliculogenesis, whereby the most mature follicle is selected to continue the ovulation cycle while the less developed experience atresia and resorption (Uzumcu & Zachow, 2007). Masculinization is a hormone-dependent process, which is inherently vulnerable to disruption by factors that interfere with hormone synthesis, bioavailability, and metabolism (Sharpe, 2006); in contrast the female reproductive system is more susceptible to epigenetic disorders including abnormal ovulation (Uzumcu et al., 2012).

Normal Follicle Development

The development of an immature follicle is dependent upon conversion of cholesterol into progesterone and an addition of an aromatic ring to form estrogen, especially the predominant type, estradiol (E2, Figure 1), from testosterone and androgen precursors. The presence of androgen precursors (E2 and progesterone), associated with theca cells surrounding the granulosa cells is integral for the maturation process of preovulatory follicles, culminating in the development of large antral follicles during the luteal phase of folliculogenesis. Cell differentiation is mediated by the luteinizing hormone (LH). Theca and granulosa cells are both steroid-dependent; this synergistic relationship is evidenced by granulosa cells' positive effects on differentiation, which is proposed to occur via growth factors. In this positive feedback loop, theca cells respond to LH by producing androgens, which diffuse into granulosa cells and are used as substrates for E2 production. Typically, monoovulating species undergo atresia to select

a single follicle, however for in vitro fertilization (IVF) therapy, gonadotropin releasing hormone (GnRH) agonist and antagonists disrupt this process to promote polymaturation for oocyte retrieval (Uzumcu & Zachow, 2007).

BPA: Exposure Routes and Mechanisms

BPA (Figure 1) is a chemical used in monomeric form in the production of polycarbonate and epoxy resins for plastic bottles (Le, 2008). Most exposure to BPA results from dietary exposure from its use in canned goods or from residual emissions in water. Nearly everyone in the US population has some low-level exposure to BPA (Calafat et al., 2008). BPA is a xenoestrogen that exerts biological activity by binding to and activating the ER. Although it has lower affinity for genomic ER than E2, circulating concentrations outcompete E2 and are 'within a biologically active range' (Nagel & Bromfield, 2013). Decreased pregnenolone production results in impaired antral folliclular growth, inhibition of steroid synthesis, and downregulation of rate-limiting enzymes in the E2 biosynthesis pathway. Data from a previous study indicates that BPA affects steroid production in the granulosa and theca cells, beginning by downregulating the rate-limiting step of the E2 biosynthesis pathway and later, affecting intercellular communication resulting from altered hormone diffusion (Peretz et al., 2010).

Phthalates Exposure Routes and Mechanisms

High-molecular weight phthalates (HMWPs) are added to plastics to make them soft and flexible, including vinyl building materials, children's toys and medical devices,

whereas low-molecular weight phthalates (LMWPs) are a vehicle for adding fragrances and texture to cosmetics, lotions and nail polish and many other daily products (Figure 2). LMWPs include diethylphthalate (DEP), dibutylphthalate (DBP) and benzylbutyl phthalate (BBzP) which form monoester metabolites mono-ethyl phthalate (MEP), mono-n-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP) and monobenzyl phthalate (MBzP). HMWPs include di-2-ethylhexyl phthalate (DEHP) which produce monoester, oxidative and elimination metabolites including mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxyhexyl) phthalate (MEOHP), and mono-2-ethylhexyl phthalate (MEHP). Exposure to both HMWP and LMWP can occur through ingestion, inhalation, dermal absorption and parenteral routes. Due to the prevalence of LMWPs in beauty products, women may have a unique exposure profile to these phthalates. A CDC research study demonstrated 1.5 times greater urinary concentrations of phthalate metabolite MBP, among women of reproductive age (20-40 years old) when compared to women of other age groups, demonstrating a potential vulnerability among this population (Blount et al., 2000). In a separate study, murine granulosa cells were cultured and MEHP inhibited FSH-stimulated cAMP and progesterone production, resulting in anovulation and reduced oocyte production (Lovekamp-Swan & Davis, 2003).

Potential measurements and methods: Urine and follicular fluid as a matrix

Biomarkers in urine are measurements of toxicants not reabsorbed during glomerular filtration and may be influenced by associations with other proteins and competition for transporters. Regardless, urine is the typical matrix used to assess

individual or population exposure to BPA and phthalates because of their transient biological half-lives of 12-24 hours (Townsend, 2013). An inherent limitation of urinary biomarker measurements is the variable urine dilution of typical spot urine samples and the narrow window for capturing exposure. Normalizing urinary analyte concentrations is not always universal due to varying excretory mechanisms of byproducts and other related chemicals (Hauser et al., 2004). Methods to adjust for variations in dilution include determining urinary creatinine concentration and specific gravity (Barr et al., 2005). Despite the limited half-life of BPA, urinary measurement may reflect steady state concentrations resulting from low-level daily exposures; making the measurement more interpretable. Phthalate metabolite measurements are more variable. In addition, a measurement in an elimination product may not accurately reflect what is occurring at the target tissue (Benedict et al., 2011).

In most instances, target tissue is nearly impossible to obtain; however, when studying IVF success and BPA or phthalates exposure, FF, the target matrix may be readily available as a by-product of the IVF procedure. For example, previous studies investigating the concentration of cotinine, a nicotine metabolite, have demonstrated a weak intra-patient correlation with urine samples. Despite the urinary biomarkers' accuracy in estimating exposure, FF measures are not necessarily correlated and better reflect individual pharmacodynamics variations that may alter what and how the chemical is deposited at the target tissue (Benedict et al., 2011)

FF is the target tissue matrix for IVF (e.g., fertilization of oocyte, implantation of embryo) and thus may be the most physiologically-relevant sampling matrix for measuring phthalate metabolites and BPA among other anti-estrogen environmental

contaminants (Benedict et al., 2011). FF is comprised of blood-plasma that crosses the gradient of the follicle via passive transport (Bloom et al., 2010); gap junctions of the thecal and granulosa cells allow passive transport up to 1 kDa molecular mass. The measurement of BPA and phthalate metabolites in FF would represent direct exposure of a developing oocyte to these analytes. The timing of sample collection during oocyte retrieval is also favorable for measuring bioaccumulation, due to the short half-life of BPA and phthalates. Sampling multiple ovaries for individual cycles of IVF allows for calculating the intraclass correlation coefficient (ICC) and increasing study reliability by examining the variance of repeated measurements within an individual study subject. For a longitudinal study, the proportion of total variance in the outcome that is attributable to intrafollicular variation is also necessary in ascertaining the need for replicate sampling (Zenzes & Reed, 1998).

Aims and Hypotheses

Aim 1: Quantify levels of MEP, MBzP, MBP, MiBP, MEHP, MEHHP, MEOHP, and MECPP in 86 FF samples using high performance liquid chromatography-tandem mass spectrometry

Aim 2: Compare metabolite concentrations in FF to levels previously published in more traditional biological matrices (e.g. urine, saliva).

Aim 3: Determine the relation, if any, phthalate metabolite concentrations in FF and number of oocytes retrieved.

I hypothesize that phthalate metabolites will be detected in FF, biomarker concentrations will be similar to those found in urine and increasing levels of exposure

will be inversely associated with IVF success as estimated by a reduced number of oocytes.

II. METHODS

Study Population and Enrollment

Israel has the highest use of IVF technology per person among all countries in the world (at 28,000 cycles annually) and Sheba Medical Center has one of the largest IVF units in Israel, performing >1000 IVF cycles a year. There are few requirements that preclude potential IVF treatment in patients, with the exception that women must be $\leq$ 45 years old. Patients are not excluded based upon sexual preference, religion, or socioeconomic status and the treatments are heavily subsidized by the nationalized health system, at an estimated cost of $3,500 per cycle (Kraft, 2011).

Every citizen is entitled to primary, secondary and tertiary care via the National Health Insurance Law, whereby the Israeli government mandated compulsory participation with a health maintenance organization for provision of services (Jotkowitz et al., 2006). Universal provision of IVF allows for unlimited cycles for up to two live births. The current proportion of births attributed to IVF in Israel totals 4%, in contrast to 1% of births for the United States.

Study subjects currently undergoing IVF were recruited from Sheba Medical Center in Tel Aviv, Israel. There were various indications for IVF therapy among the study population; female infertility diagnoses were comprised of ‘mechanical,’ ‘poor response’, ‘no ovulation’ and ‘unexplained’. Mechanical female infertility refers to barriers for oocyte travel and implantation due to cysts, adhesions fibroids and

endometriosis. Poor response denotes a poor stimulation or implantation outcome from a previous implantation cycle. No ovulation is defined as irregular menstrual cycles resulting from polycystic ovary syndrome (PCOS) and other hormonal conditions. The remaining diagnoses were independent of female reproductive issues, including male (i.e. obstructive azoospermia), single status of female patient and preimplantation genetic diagnosis (PGD). The high proportion of PGD screenings among IVF patients is specific to the Israeli population resulting from the increased prevalence among those with a Jewish and European ancestry for a number of genetically inherited conditions such as Gaucher disease and Tay-Sachs. The latter instances of disease have no impact on follicular integrity and oocyte development (i.e. oogenesis).

FF Collection

Three IVF protocols for ovulation induction were administered to participants as indicated by patient history; these included the use of GnRH antagonist and both short and long agonist cycles for study participants. The antagonist cycle is sustained for approximately 4 weeks and is initiated in the follicular phase of a new cycle; the GnRH antagonist ensures a coordinated response among follicles to exogenous gonadotropins, promoting simultaneous maturation (Depalo et al., 2012). GnRH-antagonist protocol blocks FSH and LH, thus suppressing the production of E2. The agonist short cycle lasts 2-3 weeks and only uses stimulating hormone, without the down-regulating, antagonist therapy. In contrast the long agonist therapy is initiated during the preceding luteal phase and may continue for up to 6 weeks. A human chorionic gonadotropin hormone (hCG)

injection to promote final oocyte maturation was administered to all patients 36 hours prior to oocyte retrieval regardless of the type of IVF protocol.

The dominant follicle is visualized and selected for aspiration of FF via ultrasound, with a minimum required size of 17mm. Each follicle was aspirated by the method in which a needle is inserted to extract FF into the syringe and then gently pushed back into the same follicle multiple times (with a minimum 0.5 mL collected) before moving to the next follicle. After collection, the sample is centrifuged and the supernatant is isolated, aliquoted and stored in -70C freezer. Double lumen-retrieval needles capable of flushing ovarian follicles have been developed to overcome the potential for oocyte retention within ovarian follicles, however no flushing was performed to ensure pure FF. Samples were examined for blood and any samples visually contaminated with plasma were replaced or compared with additional recurrent cycle samples of the same patient. All retrieval and collection materials were tested for phthalate contamination prior to use and all were found to be analyte-free.

Chemicals and Materials

BPA-free water was prepared using Strata C18-E 10g/60mL Giga Cartridge (Part No. 8B-S001-MFF), conditioned with MilliQ water and methanol and purchased from Phenomenex (Torrance, CA). Sigma Life Sciences β-glucuronidase from Helix pomatia was used for FF deconjugation and incubation (St. Louis, MO). Internal standard (ISTD) solutions for $^{13}C_4$ isotopically labeled phthalate metabolites were purchased from Cambridge Isotopes Laboratories (Cambridge, MA, USA). These standards were

obtained as individual solutions in HPLC-grade acetonitrile; the Low (QCL) and high (QCH) concentration quality control solutions were preparations of 1-5ng/mL and 10-25ng/mL, respectively. ABS Elut-NEXUS 3cc cartridges were used for extraction of phthalate metabolites and purchased from Agilent Technologies (Santa Clara, CA). The GC glass vials and non-reactive glass inserts were purchased from Agilent Technologies (Santa Clara, CA).

Analytical Chemistry Methods

i. FF Method Development

Phthalate metabolites are typically glucuronide- or sulfate-bound in biological media such as blood or urine. Previous studies have demonstrated the efficacy of β-glucuronidase/sulfatase deconjugation by spiking all samples with 4-methylumbelliferone and observing the levels released from this conjugated standard (Kato et al., 2003) without taking into account the different kinetics of the reaction between the surrogate standard and phthalates. Thus, current phthalate methods do not quantitatively liberate the bound metabolites prior to analysis resulting in concentrations that are biased low. In order to optimize the yield of phthalate metabolite during the deconjugation step of the method, we evaluated the incubation time and enzyme amount needed for maximum yield. Response rate (RR) and concentration (ng/mL) were used to evaluate the effectiveness of each experimental condition. Urine was used, as the matrix in this experimental approach as it is more readily available than FF and the methodology has been cross-validated for both matrices. Experimental parameters altered were enzyme volumes for 2-hour incubations (250, 500 and 1000µL) and a 4-hour incubation (500µL).

For all detected phthalate metabolites, the 4:1 ratio of β-glucuronidase to unknown sample with a 2-hour incubation period was determined to produce the greatest yield among 250μL urine samples (Table 1), and for MiBP and MBP metabolites this increased the calculated concentration by 42% and 23% respectively. No significant additional yield was observed when incubation was increased from 2 to 4 hours; these results were in agreement with previously conducted research demonstrating that the time of incubation (~90 min) was adequate of the amount of glucuronide used (Silva et al. 2008).

ii. Phthalate Extraction

Aliquots (100μL) of unknown FF samples were analyzed concurrently with calibration standards, a blank sample and high and low quality control materials to ensure data validity. Samples were spiked with isotopically labeled internal standards and the samples were mixed. 1000uL of enzyme was added to the samples and they were incubated for 2 hours at 37 C. The deconjugation step was terminated by the addition of 1.5mL phosphate buffer (pH 2). ABS-Elut Nexus 3cc cartridges were conditioned with 2mL acetonitrile (ACN) and phosphate buffer. After loading samples, the columns were washed with 2mL 0.1 M formic acid and BPA-free water, respectively. The analyte-containing sample fractions were collected following elution with 1.5mL acetonitrile; the eluates were collected with an additional 1.5mL ethyl acetate. The samples were brought to total dryness in the evaporator at 57°C at 15psi air. Subsequent, reconstitution with 100mL BPA-free water followed by centrifugation at 1500rpm ensured sample clarity and optimal readings for injection.

iii. Preventing Contamination During Analysis

Several measures were taken to prevent contamination though analysis of sample collection methods, laboratory equipment contamination and deionized water. Using a quality-control blank is integral for determining contamination of samples from ubiquitous compounds such as BPA and has identified contamination via sample collection and laboratory equipment as a factor for researching BPA and phthalates. For example, the lining of blood collection bags has contributed BPA to serum samples in previous studies (Markham et al., 2010). The contamination of laboratory equipment was prevented for all incubation and extraction protocol by using glass laboratory apparatus in place of plastic. Also, the use of deionized water has contaminated samples via leaching from a polyethersulfone filter has been documented; thus all samples are prepared with BPA-free water via Phenomenex Strata C18-E 10g/60mL Giga Cartridge filtration (Ye et al., 2013). Samples of purified water were analyzed in triplicate and provided validation that all controls were effective for preventing contamination; results documented levels below quantification for all (8) phthalate metabolites.

Data Analysis

For all analyses, a full set of ten calibration standards, a blank sample and high and low quality control materials were analyzed in succession, with unknown samples to ensure proficiency of data collection. Quantification was achieved using isotope dilution calibration plots. In order to be positively identified as the target analyte, the analyte had

to possess the correct quantifying and confirmatory ions with signal-to-noise ratios > 3, these ions had to quantify results within ±20% (signal-to-noise ratios) of each other and each ion had to co-elute chromatographically with its isotopically labeled standard.

For an analytic run to be considered valid, calibration standards require 100±20% relative recovery and quality control materials must be within 20% of the expected target value, for defining the lowest concentration where a single measurement is calculated. After blank subtraction, the method limit of quantification (LOQ) was set as 3x the standard deviations (SD) of the blank (which ensures 99% certainty that the reported value is originating from the sample), taking into consideration the volume of the analyzed sample (Petro et al., 2012). Calibration curves were selected as either linear or quadratic based on the criteria: $R^2 > 0.995$ (Figure 3 and 4).

The functional sensitivity (LOQ) for phthalate metabolites in FF samples is (in ng/mL): MCPP: 0.2, MEP: 0.5, MECPP: 0.5, MEHHP: 0.2, MBP 0.5, MiBP 0.25, MEOHP: 0.2, MCMHP: 0.2, MBzP: 0.1, MEHP: 1.0 (Table 2).

Data processing was performed using Agilent Technologies Mass Hunter Workstation software for mass spectrometry. All statistical procedures were performed in SAS 9.3 for ANOVA, paired t-test, intraclass correlation coefficient (ICC) and linear regression; graphs, tables and figures were prepared in Microsoft Excel. For the individual phthalate metabolites detected, we standardized the compounds that represent similar sources and similar biologic activity using a molar sum procedure (Figure 5). The sum of low-molecular weight phthalates MEP, MiBP, MnBP were converted to molar concentration then summed then converted back into MEP equivalents (Molecular Weight: 194 g/mol); similarly, the HMWP, MECPP was converted to a molar

concentrations then reconverted into MEHP equivalents (Molecular Weight: 278 g/mol). (Teitelbaum et al, 2012).

III. RESULTS

Demographic Data

The average age of participants in the study was 32.6 years old and the majority were non-smokers (Table 4). The number of IVF attempts ranged from one to six; 42% of study subjects had a history of successful live birth and 33% had an infertility-related medical diagnosis. Day-3 FSH levels were obtained for measuring the baseline ovarian reserve of each woman prior to initiating follicular hyperstimulation. The FSH for normally ovulating women has been established as 5.9 ± 2.1mIU/mL (Siemens, 2010) and our study group had an average of 6.7 ± 2.4mIU/mL, with higher levels indicating a lower ovarian reserve. Our participants had an average E2 level of 5553 ± 2599pg/mL, which fall within the 75th percentile for similar studies. Previous research has documented that high E2 levels appeared to be associated with improved treatment outcomes (Chen et al., 2003).

Metabolites were measurable in all FF samples obtained from Sheba Medical Center; the majority of metabolites were detected in MECPP, MEP, MiBP and MBP, respectively (Table 3). Imputation for values below the level of quantification was conducted for all categories of the (4) metabolites using the formula: LOQ/√2. MEP was the metabolite detected with the highest concentration (median = 1.49ng/mL), followed by MiBP, MBP MECPP. MEP was also the analyte with the highest intraindividual variation with levels ranging from 0.13 to 411ng/mL. The maximum concentration

observed for all analytes was approximately 100x less than concentrations observed in urine.

Exploratory Analysis of Predictor Variables

We investigated the correlation of continuous and categorical covariates, including the number of previous IVF cycles, reproductive history (i.e.gravida, para, spontaneous abortion, artificial abortion), ΣHMWP and ΣLMWP metabolite concentration, age, BMI, birth history, IVF protocol, infertility diagnosis, smoking status, peak E2 (pg/mL) and Day 3 FSH (IU/mL). The association of smoking to retrieval of fewer oocytes was investigated via a Student's t-test; comparison of number of oocytes retrieved by smoking status revealed significantly fewer mean oocytes retrieved in smokers (mean: 6.85 +/- 3.4 std. dev), when compared to non-smokers (mean: 9.8 +/- 5.1 std. dev) and significant at $p<0.048$ (Figure 6).

BMI was also investigated as a confounding factor and may serves as a surrogate measure of obesity and increased waist circumference, which were determined as indicators by previous studies. Our analysis included the comparison of patient metabolite quartiles of LMWPs with HMWPs and mean BMI within each subgroup. Three metabolite groups: MECPP, MEP and MBP demonstrated a positive slope for phthalate quartile vs. continuous BMI: 0.56, 0.07 and 0.34, respectively (Figure 7), An ANOVA calculation for multiple comparisons concluded that the variances are not significantly different across groups. Overweight status has been investigated because of the proposed action of HMWPs with lipids. The activity of endocrine disruptors is proposed to delay metabolism, increase half-life and produce elevated biomarkers for chronic exposure; increasing BMI has also been associated adverse IVF outcomes

(Trasande et al., 2013; Dokras et al., 2006).

An exploratory analysis for age was conducted to investigate the impact of increasing age on oocyte retrieval. Study participants ranged in age from 23.3 to 43.6 years of age and a Pearson's correlation analysis showed a significant negative correlation between oocytes retrieved and age ($r=-0.22$, $p<0.05$); Figure 8 displays decreasing mean oocytes for 5-year age increments. Additionally, the analysis verified that elevated E2 levels were positively correlated with the number of oocytes retrieved ($r=0.29$, $p<0.05$); while Day 3FSH levels were negatively correlated with E2 levels.

FF Associations: Intrafollicular Agreement among Metabolites

The levels of LMWP and HMWP metabolites were significantly correlated with a Spearman correlation coefficient obtained for $r \geq 0.55$, 0.53 and 0.96 MEP, MBP and MiBP, respectively at $p < 0.05$ (Figure 9). Data points with standardized residuals greater than an absolute value of 3 were excluded as a conventional method for determining outliers. These values may indicate that a patient with a high level of LMWP are also likely to be exposed to HMWP due to multiple sources of exposure.

In order to determine the between- and within-person variability in FF sampling, the ICC and 95% confidence intervals were calculated for phthalate metabolites concentrations of follicle pairs using SAS PROC MIXED. ICC is a measure of the reliability of repeat measures over time for continuous variables and frequently reported when using complex instrumentation for biomarker studies; it is defined as the ratio of between-subject variance to total variance. Values range from zero to one indicating poor

and excellent reliability, respectively (Rosner, 2000); ICCs for the various phthalate metabolites ranged from 0.47 to 0.89 in our study (Table 5).

FF Associations: Interfollicular Variation for Follicle Pairs

A paired t-test was conducted to determine interfollicular variation of HMWP metabolite concentrations for arithmetic means among ovary pairs. Table 6 displays mean values for (2) measurements for each follicle pair (A & B). Nineteen pairs were available for sampling among the study population, and several had to be excluded from serial testing due to low sample volume. All samples were retrieved from patients without history of female reproductive complications; results indicated difference in means approaching significance among (1) study subject (t-score: 7.75, $p<0.08$).

A scattergram for FF(A) vs. FF(B) illustrates that the majority of values lie close to the line, indicating no difference between HMWP concentrations within participants (Figure 10). One outlying pair indicates a substantial difference, which may be attributed to a number of potential issues including measurement error and anatomical issues including differences in follicle size or vasculature resulting from hyperstimulation (Zenzes & Reed, 1998). Overall the concentrations of HMWP among two follicle samples for patients are highly correlated at 0.92 ($p<.001$).

Multivariate Linear Regression Model

Multiple linear regression was employed for investigating correlation of continuous and categorical covariates to the number of oocytes retrieved; this method was selected due to the continuous nature of the dependent variable. Cut points were established for summed HMW and LMW phthalates quartiles, and each increasing

phthalate quartile was compared using Q1 as the reference group. We stratified on variables including type of IV protocol, since the protocol will be selected based on previous IVF success. For example, those who have not had optimal results with agonist therapy may transition to antagonist therapy for recurrent cycles; thus the IVF protocol may serve as potential intermediate on the causal pathway between urinary phthalate concentration and implantation failure model (Ehrlich et al., 2012). Age, BMI, smoking status and Day 3 FSH were all included in the final model due to their clinical relevance and statistical significance. Results were presented for both adjusted models and unadjusted models to display phthalates effect on E2 and oocytes retrieved and investigate the association of MLW and HMW phthalates with overall ovarian response (Table 7).

The unadjusted model does not demonstrate a linear relationship between either phthalates group; the greatest decline in E2 is associated with the second quartile of LMW phthalates and closely approaches statistical significance ($p=0.08$). Conversely, the greatest appreciation in E2 levels is correlated with the unadjusted model of HMW phthalates; significant at the $p<0.05$, with an increase of 1459pg/mL for the second quartile. This relationship remained constant when compared with the adjusted model. Age and Day 3 FSH are determined as significantly correlated; when age is removed from the model the effect of FSH on E2 is significant ($p<0.05$).

Increased concentrations of phthalates groups and decreased oocytes do not exhibit a linear relationship for either the adjusted or unadjusted model (Table 8). When controlling for all other predictor variables, IVF patients demonstrate a 0.22 decrease in

oocytes for each additional year of age. Smoking was also inversely correlated with the number of eggs retrieved, and demonstrates a borderline significant trend ($p=0.09$).

IV. DISCUSSION

Measurement Reliability

Although this study did not produce significant results substantiating a linear dose-response relationship among phthalate monoester metabolites, it did provide additional evidence supporting the validity of the study design. Minimum acceptable values of an ICC have previously established the range of 0.40 to 0.75 ‘fair to good’ and greater than 0.75 as ‘exceptionally reproducible’ (Townsend et al., 2013). Our results for all (4) metabolite compounds are within this range and demonstrate acceptable levels of measurement reliability.

Studies investigating cotinine concentrations for patients with acute exposures, documented significantly different levels detected among follicles ($\alpha < 0.05$). While these findings supported obtaining multiple aliquots for averaging concentrations, we did not observe significant variance for interfollicular HMW phthalate levels. This may be attributed to the non-persistent chemical nature of phthalates (i.e. short half-life), with the bulk of phthalate monoesters being excreted in the first 24-hour period (Anderson, et al. 2001). Thus, we do not expect the reliability of a measurement to be compromised when using a single aliquot with calibrated HPLC/MS-MS. These findings also indicate that pooling multiple follicle samples in order to have a greater sampling volume may be

optimal. Additional considerations for reducing variability during sample collection include obtaining FF from similarly sized, mature oocytes (mean diameter $\geq$17mm).

Findings from our regression models were contrary to the hypothesis set forth, in investigating the association of LMW and HMW phthalates concentrations with E2 levels and the number of oocytes retrieved. In the unadjusted model for LMW concentrations' effect on E2, Quartile 2 and Quartile 4 are negatively correlated with estradiol levels, however Quartile 3 is positively correlated. Additionally, all quartiles of HMW phthalates are positively correlated with increasing E2 levels. When investigating the association of phthalate groups and number of oocytes retrieved, both the adjusted and unadjusted models fail to exhibit an accelerating decrease in eggs for comparison of phthalate quartiles with a reference group. Previous meta-analysis has documented instances of a non-monotonic, dose-response curve for BPA and other endocrine disruptors. While these *in vitro* studies are informative for *in vivo* endpoints, this specific exposure data is not available for analytical comparisons in our study.

Limitations

Detected concentrations of FF were much lower when compared to urine, at a 100-fold average difference. The majority of samples were detected below LOQ for MEHHP, MEOHP, MEHP and MBzP; only MECPP concentrations were available for calculating molar sum equivalents of HMW phthalates. In order to provide repeat measurements for producing reliability estimates, we were limited by total volume and this may have impacted the sensitivity of our analysis. While FF is the target matrix for

IVF outcomes, urine samples are more readily accessible, non-invasive and available in greater volume.

The relationship between oocyte retrieval and fertilization success is also difficult to estimate, due to the parabolic nature of oocytes retrieved, and use as a surrogate measure for positive predictive reproductive outcomes. The range for oocyte retrieval is considered to be a minimum of 10, with the optimal range of 13-17 oocytes depending upon patient age (van der Gaast et al., 2006). In treating the outcome variable as continuous, we did not fit the model for association with multiple discrete outcomes and instead relied upon an inverse correlation for ordinal counts of oocytes retrieved. Conducting a multinomial logistic regression would serve to establish cut points supported by previous studies for poor, sub-optimal, and optimal categories, to account for decreased fertilization beyond 13-17 oocytes, after which the integrity of immature eggs may be compromised. Sample size conditions for the maximum likelihood equation were violated for estimating parameters, and could not be attempted for the purposes of this study.

V. CONCLUSIONS AND FUTURE DIRECTION

While overall this study presented null results, the next steps for the project will include analysis of all samples for detected BPA concentrations. Phthalates and BPA act additively as endocrine disruptors and result in a synergistic, antagonist cellular response to estrogen; additional research may provide a missing piece of the puzzle as to why a linear-dose response was not observed among the quartiles of phthalate monoesters.

Results will be disseminated for BPA concentration and additional reproductive outcomes, including oocyte cohort maturation, embryo quality and fertilization rates.

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VII. TABLES AND FIGURES

Metabolite	250 µL, 2 hr		500 µL, 2 hr		1000 µL, 2 hr		500 µL, 4 hr	
	Average							
	RR	Calc. Conc.	RR	Calc. Conc.	RR	Calc. Conc.	RR	Calc. Conc.
MBzP	0.13	1.71	0.14	1.81	0.13	1.72	0.13	1.72
MEHP	0.32	2.56	0.30	2.22	0.30	2.08	0.27	1.60
MEOHP	0.17	2.86	0.20	3.33	0.19	3.23	0.19	3.19
MiBP	0.57	3.06	0.73	3.99	0.81	4.40	0.78	4.23
MBP	0.64	16.56	0.78	20.39	0.79	20.58	0.78	20.23
MEHHP	0.24	4.12	0.28	4.84	0.26	4.45	0.27	4.63
MEP	0.13	1.71	0.14	1.81	0.13	1.72	0.13	1.72
MECPP	0.30	4.90	0.31	5.09	0.29	4.71	0.29	4.81

Table 1. Phthalate Extraction Method Development β-Glucuronidase Ratio and Incubation Time

Analyte	Calibration	LOQ (ng) in 100uL	STD Level
MEP	Linear	0.5	S2
MECPP	Linear	0.5	S3
MEHHP	Linear	0.2	S1
MBP	Linear	0.5	S2
MiBP	Linear	0.25	S2
MEOHP	Linear	0.2	S1
MBzP	Quadratic	0.1	S1
MEHP	Linear	1	S3

Table 2. Limit of Quantification for Phthalate Metabolites in FF

	25% Q1 (ng/mL)	50% Median (ng/mL)	75% Q3 (ng/mL)	Mean (ng/mL)	Std. Deviation
ΣHMWP (MECPP)	0.48	0.66	0.91	0.922	±1.048
ΣLMWP	2.02	5.94	10.45	12.531	±33.693
MiBP	0.31	0.31	4.56	6.332	±27.618
MBP	0.15	0.27	1.85	1.677	±3.359
MEP	0.35	1.49	4.19	4.161	±10.556

Table 3. Selected quartiles of phthalate metabolite concentrations in FF

Demographic characteristics		(n=86)
Age (years):		
Mean ± SD		32.6 ± 5.2
Range		23.3-43.6
BMI (kg/m2):		
Mean ± SD		23.6 ± 4.4
Range		16.8-36.2
Smoking ((n(%)):		
Smoker		13(15)
Non-smoker		71(85)
Day 3 FSH (IU/L):		
Mean ± SD		6.6 ± 2.4
Range		1.7-12.8
Peak E2 (pmol/mL):		
Mean ± SD		5534 ± 2611.1
Range		357.0-11317.0
IVF attempt (n(%)):		
	1	26 (31)
	2	21 (24)
	3	20(23)
	4	12(14)
	5	5(6)
	6	2(2)
Infertility diagnosis (n(%)):		
Mechanical		5(6)
Poor Response		3(4)
No ovulation		1(1)
Unexplained		14(17)
Other IVF indication(n(%)):		
Pre-implantation genetic diagnosis (PGD):		32(37)
Single		4(5)
Male		27(31)
Type of IVF Therapy (n(%)):		
Short agonist		4(5)
Long agonist		18(21)
Antagonist		64(74)
Oocytes retrieved		
Mean ± SD		9.36 ± 4.9
Range		1.0-25.0

Table 4. Demographics of Sheba Medical Center IVF study participants

Phthalate Metabolite	ICC and confidence limits	
Low-molecular weight	**ICC**	**CI (95%)**
MEP	0.89	(0.81-0.97)
MBP	0.47	(0.17, 0.75)
MiBP	0.49	(0.21,0.77)
High-molecular weight	**ICC**	**CI (95%)**
MECPP	0.68	(0.46, 0.89)

Table 5. Intraclass Correlation Coefficients for all Metabolites

Patient ID	FFA (mean)	FFB (mean)	t-score	df	p<
1	0.87	0.84	0.14	3	0.92
2	0.37	1.35	7.75	3	0.08
3	0.82	0.80	0.19	3	0.88
4	0.78	0.45	2.12	3	0.28
5	0.73	0.63	0.87	3	0.54
6	0.75	0.71	1.17	3	0.45
7	0.74	0.59	2.20	3	0.27
8	1.08	1.22	2.32	3	0.26
9	0.72	0.48	4.17	3	0.15
10	0.64	0.58	4.04	3	0.15
11	0.36	0.31	0.81	3	0.57
12	0.59	0.68	3.34	3	0.19
13	1.46	1.38	1.99	3	0.30
14	0.40	0.37	0.80	3	0.57

Table 6. Within-person differences for Mean FF of High Molecular Weight Concentrations (2) Follicles (n=14)

Covariates		LMW Phthalates			HMW Phthalates		
		Effect Estimate	SEM	*P*-value	Effect Estimate	SEM	*P*-value
Unadjusted Models	Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
	Quartile 2	-1272.0	726.2	0.08	1459.6	710.7	0.04
	Quartile 3	212.9	708.7	0.76	841.2	710.7	0.24
	Quartile 4	-354.9	717.1	0.62	1042	719.9	0.15
Adjusted Models	Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
	Quartile 2	-725.6	761.7	0.34	1239.8	738.9	0.09
	Quartile 3	698.3	792.4	0.38	586.6	727.9	0.43
	Quartile 4	242.1	778.6	0.75	1256.7	773.1	0.11
	Age	-61.1	55.0	0.27	-48.5	52.94	0.36
	BMI	-67.9	68.3	0.32	-93.3	67.4	0.18
	Day 3 FSH [&]	-202.8	117.8	0.09	-209.9	116.5	0.08
	IVF Protocol (antag)	Ref	Ref	Ref	Ref	Ref	Ref
	IVF Protocol (short)	474.4	1250.7	0.71	128.2	1244.2	0.92
	IVF protocol (long)	554.4	774.2	0.48	641.4	768.7	0.41
	Smoker (yes v. no)_	-390.9	802.0	0.67	-190.1	750.8	0.80

Table 7. Effect of Phthalates and covariates on peak E2, among 86 women undergoing IVF
(Ref=Reference)

Covariates		LMW Phthalates			HMW Phthalates		
		Effect Estimate	SEM	*P*-value	Effect Estimate	SEM	*P*-value
Unadjusted Models	Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
	Quartile 2	-0.60	1.55	0.70	-1.27	1.51	0.40
	Quartile 3	-0.09	1.53	0.95	1.49	1.51	0.33
	Quartile 4	0.73	1.55	0.64	0.59	1.51	0.70
Adjusted Models	Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
	Quartile 2	-0.36	1.59	0.82	-1.18	1.56	0.45
	Quartile 3	0.89	1.65	0.59	0.89	1.54	0.57
	Quartile 4	2.52	1.62	0.13	1.08	1.63	0.51
	Age	-0.22	0.11	0.05	-0.15	0.11	0.17
	BMI	-0.06	0.14	0.67	-0.04	0.14	0.77
	Day 3 FSH	-0.20	0.25	0.42	-0.20	0.25	0.42
	IVF Protocol (antag)	Ref	Ref	Ref	Ref	Ref	Ref
	IVF Protocol (short)	-3.71	2.61	0.16	-2.90	2.63	0.27
	IVF protocol (long)	1.97	1.62	0.23	2.02	1.63	0.22
	Smoker (yes v. no)_	-2.84	1.67	0.09	-2.58	1.59	0.11

Table 8. Effect of Phthalates and covariates on oocytes, among 86 women undergoing IVF
(Ref=Reference)

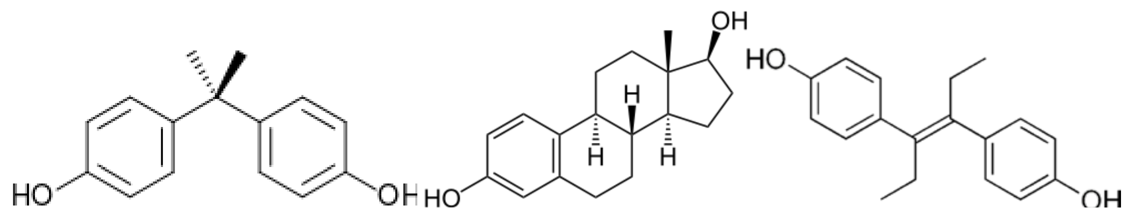


Figure 1. BPA, E2, and DES structures show similar three-dimensional configurations

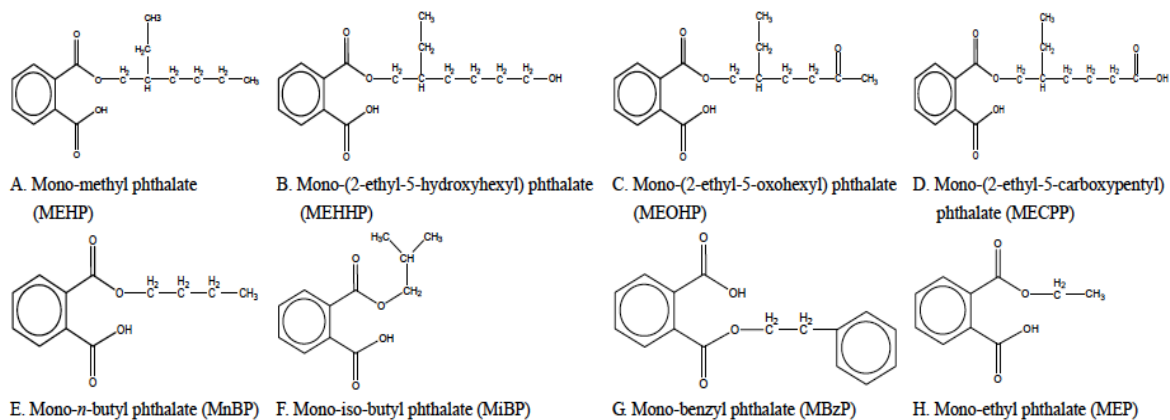


Figure 2. Phthalate metabolite general structure

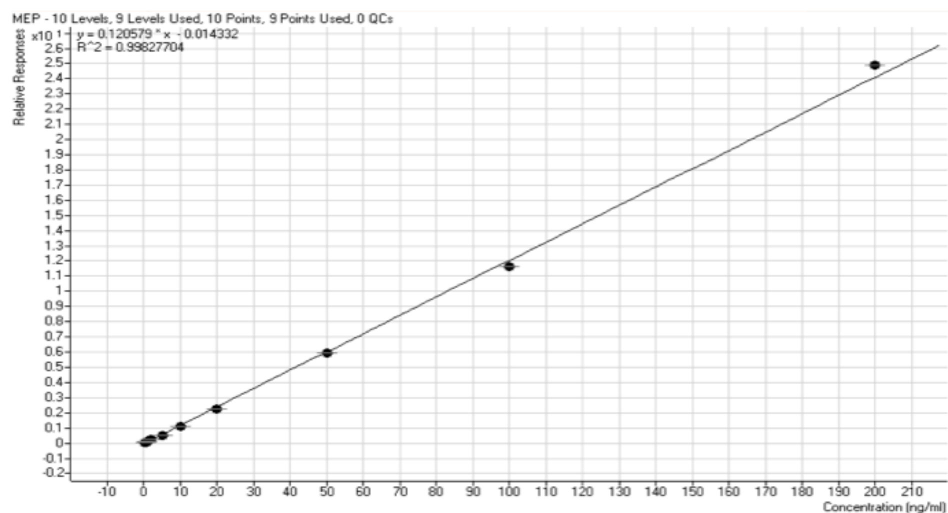


Figure 3. MEP Linear Calibration Curve: (R^2=0.998)

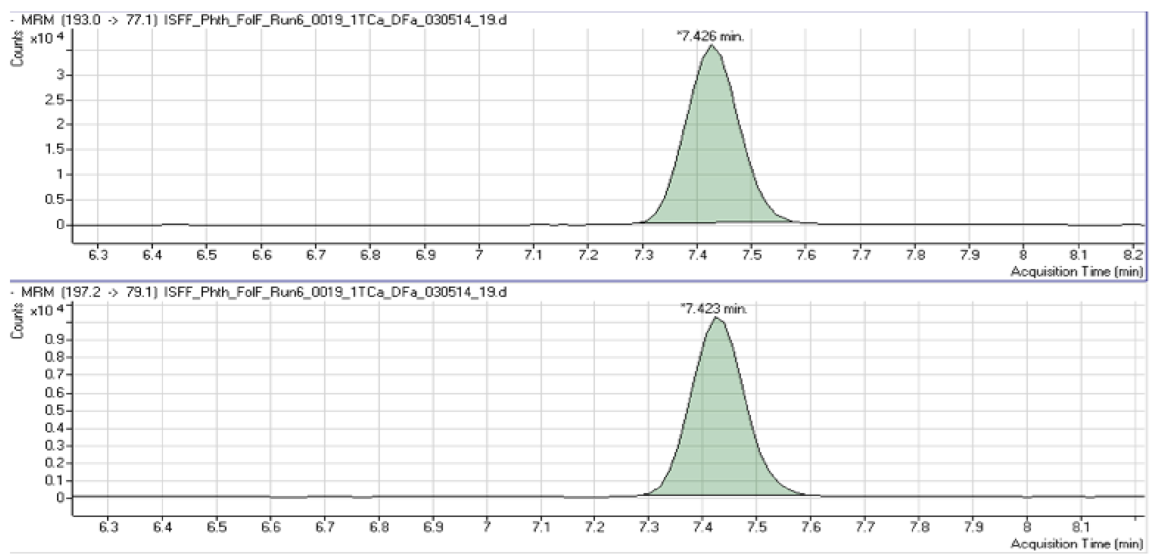


Figure 4. Analyte Integration for MEP (28.4ng/mL)

Low-Molecular Weight (MEP) Phthalate Molar Sum Equation:

Σ MEP+MiBP+MnBP

MEP=194g/mol

$$\frac{\text{Sample conc. (ng/mL)}}{194} = \frac{\text{nmole}}{1\text{mL}} \times \frac{1000\text{mL}}{1\text{L}} = \frac{\text{ng/mL}}{\text{ng/nmole}}$$

$$\text{MEP} = \frac{\text{Sample conc.}}{194} \times 1000 = \text{nM}$$

$$\text{MiBP} = \frac{\text{Sample conc.}}{222} \times 1000 = \text{nM}$$

$$\text{MBP} = \frac{\text{Sample conc.}}{222} \times 1000 = \text{nM}$$

High-Molecular Weight (MEHP) Phthalate Molar Sum Equation:

MECPP

MEHP=278g/mol

$$\frac{\text{Sample conc. (ng/mL)}}{278} = \frac{\text{nmole}}{1\text{mL}} \times \frac{1000\text{mL}}{1\text{L}} = \frac{\text{ng/mL}}{\text{ng/nmole}}$$

$$\text{MECPP} = \frac{\text{Sample conc.}}{303} \times 1000 = \text{nM}$$

Figure 5. MEP and MEHP Molar Sum Equivalent Formulas

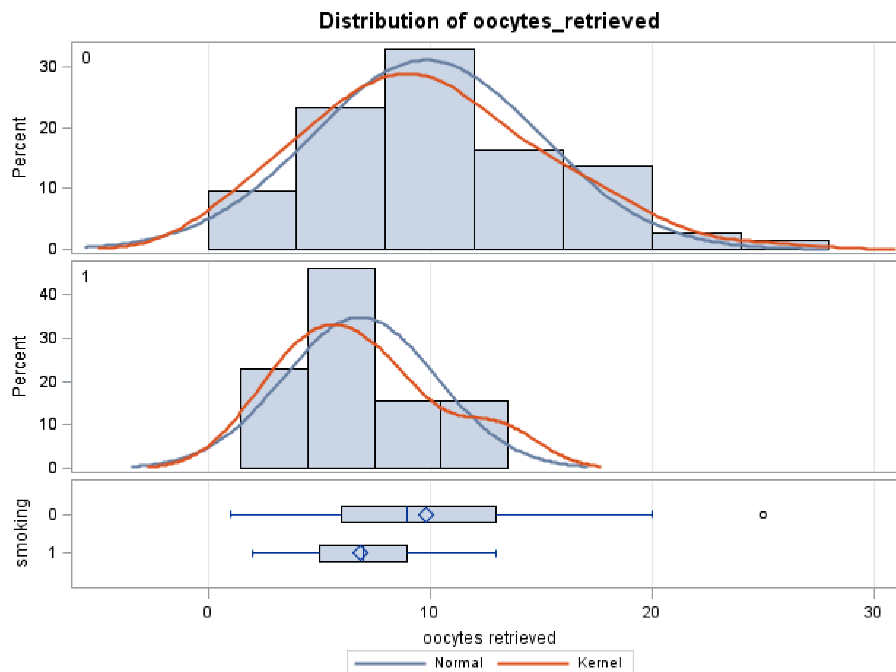


Figure 6. Decreased oocytes retrieved among smokers

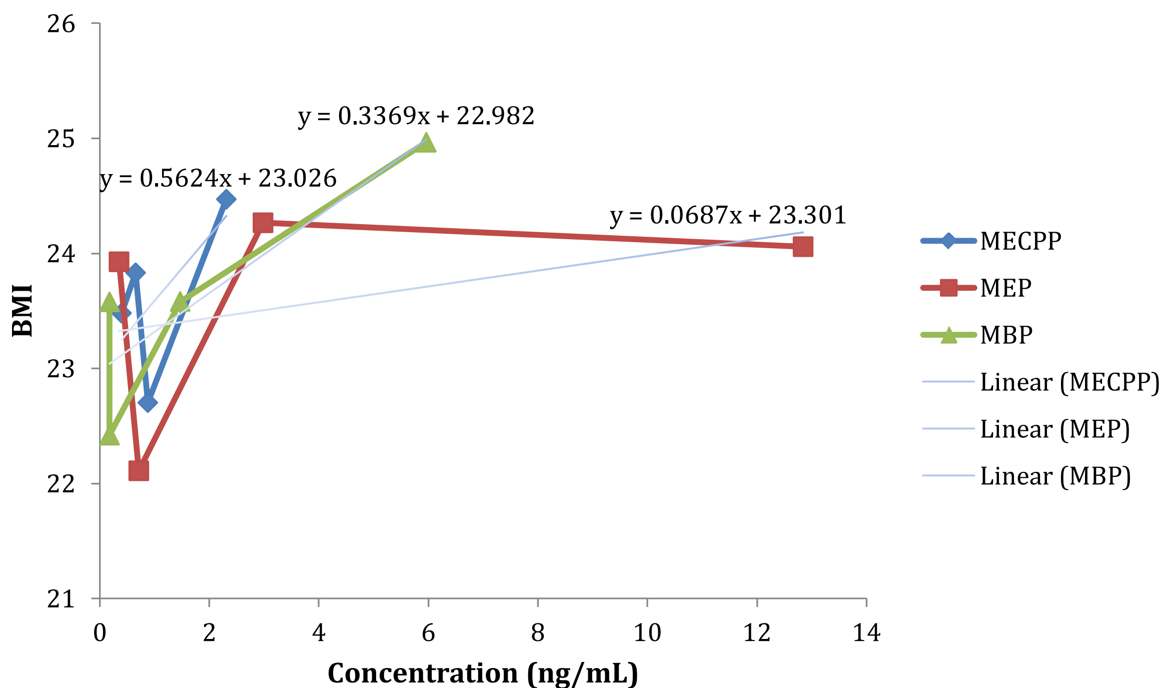
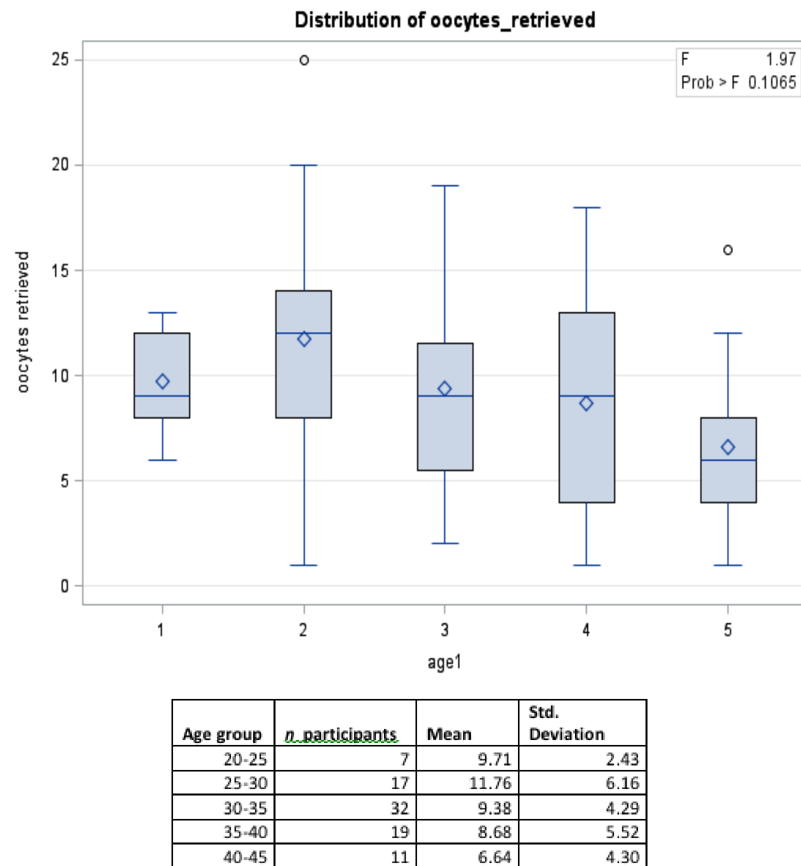


Figure 7. Body Mass Index (BMI) means in relation to urinary phthalate metabolite quartile concentrations for MECPP, MEP and MBP



Age group	*n* participants	Mean	Std. Deviation
20-25	7	9.71	2.43
25-30	17	11.76	6.16
30-35	32	9.38	4.29
35-40	19	8.68	5.52
40-45	11	6.64	4.30

Figure 8. ANOVA comparison of oocytes retrieved among age groups

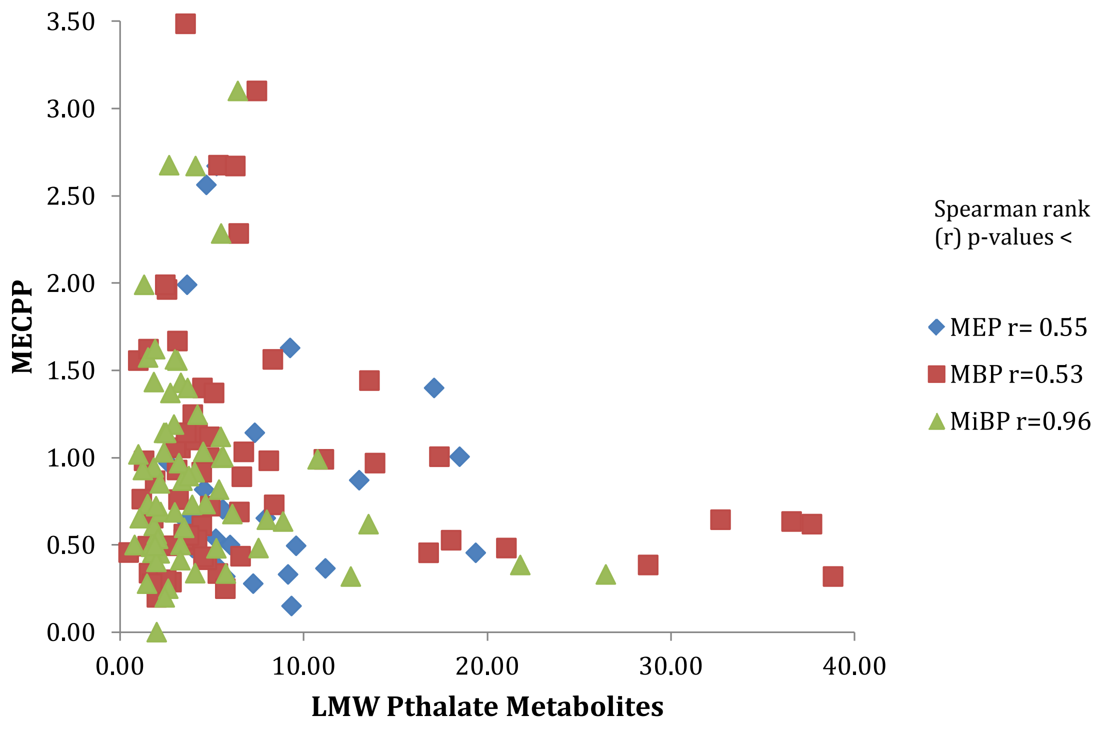


Figure 9. Urinary concentration of MECPP versus MEP, MBP and MiBP (n=86) for ng/mL

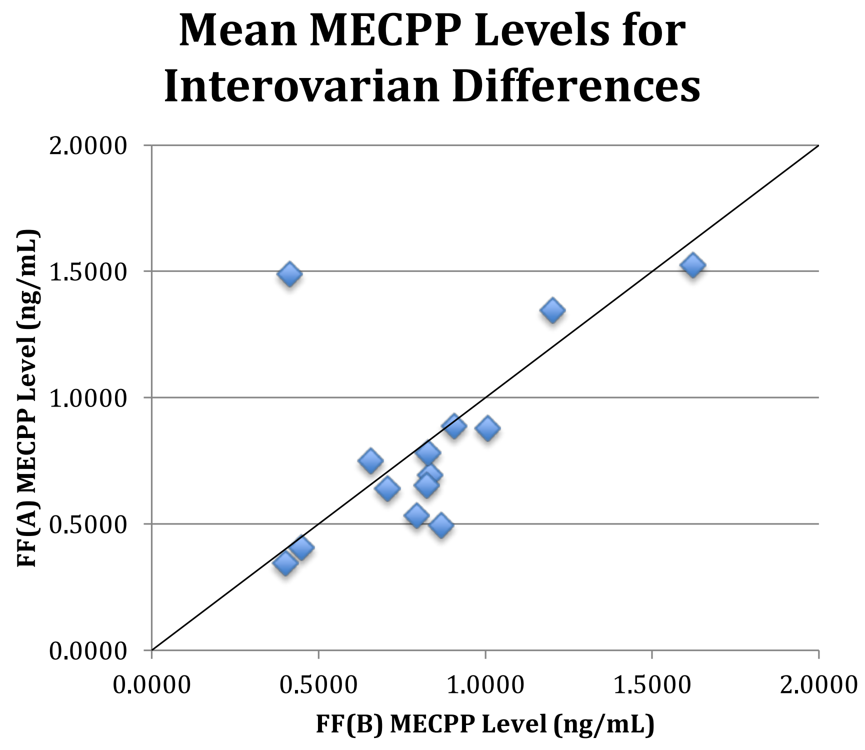
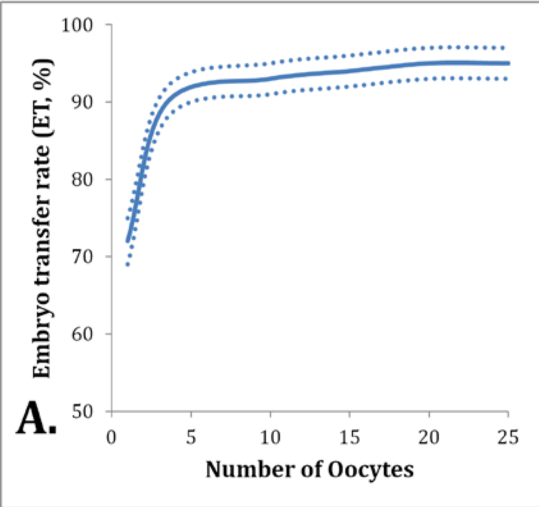


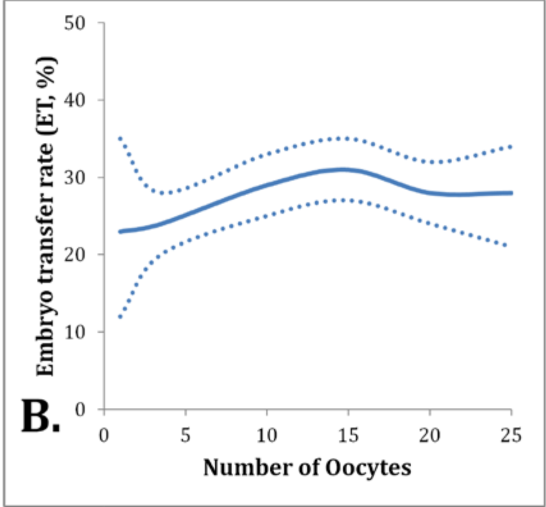
Figure 10. Scattergram of mean interfollicular (HMW) phthalate level

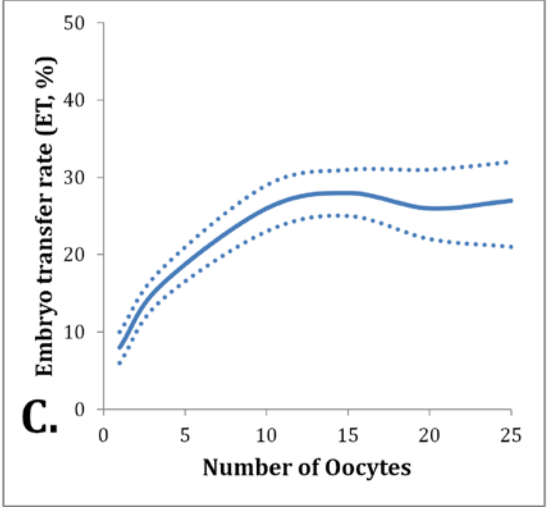
VII. APPENDICES

Appendix A:



The number of retrieved oocytes (mean with 95% CI) in relation to embryo transfer rate (A); pregnancy rate per embryo transfer (B); pregnancy rate per started IVF cycle (C). The optimal number of obtained oocytes to conceive is 13.





Monotonic curve: oocytes retrieved vs. fertilization success
Adapted from: van der Gaast et al., 2006

Appendix B:

EMORY

ROLLINS SCHOOL OF PUBLIC HEALTH

Department of Environmental Health

23 October 2013

RE: IRB approval for Tamara Opila's Capstone Project

To whom it may concern:

Having previously served on CDC's Institutional Review Board (IRB) of human subjects research evaluation, I am well versed in the Code of Federal Regulations Title 45 Public Welfare, Part 46 Protection of Human Subjects. According to 45 CFR 46.102 (b)(4), the thesis project proposed by MPH candidate Tamara Opila and titled "Environmental Exposure to BPA and Phthalates, Measured in Human Follicular Fluid, as a Determinant for in vitro Fertilization Success" is exempt from IRB approval because all data will be anonymized. Although the study itself has IRB approval in Israel where participant enrollment/sample collection takes place, Ms. Opila will only have decoded, anonymized data and will have no interaction with subjects and no subject identifiers.

Sincerely,

Dana B. Barr

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