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Using follicular fluid metabolomics to investigate the association between air pollution and oocyte quality

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Using follicular fluid metabolomics to investigate the association between air pollution and oocyte quality

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2018

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An abstract of
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Abstract

Using follicular fluid metabolomics to investigate the association between air pollution and oocyte quality

By Sueyoun Hwang

BACKGROUND AND AIM: Our objective was to use metabolomics in a toxicological-relevant target tissue to gain insight into the biological processes that may underlie the negative association between air pollution exposure and oocyte quality.
METHODS: Our study included 125 women undergoing *in vitro* fertilization at an academic fertility center in Massachusetts, US (2005-2015). A follicular fluid sample was collected during oocyte retrieval and untargeted metabolic profiling was conducted using liquid chromatography with ultra-high-resolution mass spectrometry. Daily exposure to nitrogen dioxide (NO_2), ozone, fine particulate matter, and black carbon was estimated at the women's residence using spatiotemporal models and averaged over the period of ovarian stimulation (2-weeks). Multivariable linear regression models were used to evaluate the associations between the air pollutants, number of mature oocytes, and metabolic feature intensities. A meet-in-the-middle approach was used to identify overlapping features and metabolic pathways.
RESULTS: Of the air pollutants, NO_2 exposure had the largest number of overlapping metabolites (C18: 105, HILIC: 91) and biological pathways (C18: 3; HILIC: 6) with number of mature oocytes. Key pathways of overlap included vitamin D3 metabolism (both columns), bile acid biosynthesis (both columns), C21-steroid hormone metabolism (HILIC), androgen and estrogen metabolism (HILIC), vitamin A metabolism (HILIC), carnitine shuttle (HILIC), and prostaglandin formation (C18). Three overlapping metabolites were annotated with level-1 evidence. For example, hypoxanthine, a metabolite that protects against oxidant-induced cell injury, was positively associated with NO_2 exposure and negatively associated with number of mature oocytes. Minimal overlap was observed between the other pollutants and the number of mature oocytes.
CONCLUSIONS: Higher exposure to NO_2 during ovarian stimulation was associated with many metabolites and biologic pathways involved in endogenous vitamin metabolism, hormone synthesis, and oxidative stress that may mediate the observed associations with lower oocyte quality.

KEYWORDS: Air pollution, Fertility, Metabolomics, Ovary

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Introduction

Air pollution is considered a major threat to public health due to its contribution to various adverse health outcomes affecting individuals across the lifespan.[1] Exposure to ambient air pollution has long been associated with several adverse birth outcomes such as preterm birth and low birth weight.[2,3] Recently, studies have suggested a negative association between exposure to ambient air pollution and reduced fertility.[4–6]

While the association between air pollution and semen quality is well documented, less is known about the harmful effect of periconception air pollution on female fertility, and more specifically ovarian function. Recent studies found evidence of ovarian dysfunction in mice following exposure to fine particulate matter ($PM_{2.5}$), a type of air pollution, shown by exacerbated ovarian oxidative stress and inflammation[7] and apoptosis of ovarian granulosa cells and oocytes.[8] Two studies – the first among subfertile American women[9] and the second among subfertile Korean women[10] – have also documented an association between higher exposure to outdoor air pollution and lower ovarian reserve. While these human studies support the animal literature suggesting that air pollution has a specific detrimental effect on ovarian function, the potential biological mechanisms remain unclear.

Studies evaluating the association between air pollution and female fertility are often conducted among women utilizing assisted reproductive technology (ART) as it allows for the direct measure of markers of fertility (e.g. oocyte quality), can document important early reproductive events (e.g. embryo development), and can define exact windows of air pollution exposure for critical stages of reproduction. Integrating metabolomic markers into these types of studies may also help us better understand the important mediators underlying the causal pathways between air pollution and human reproduction. A recent study of 200 women who underwent a fresh autologous ART cycle identified several pre-conception serum metabolic features and pathways as potential mediators of the negative association between air pollution and live birth[4]. While this study focused on serum metabolites, previous work has suggested that

the primary target of air pollution may be the ovary. Therefore, studying the follicular fluid, which is the immediate microenvironment surrounding the oocyte and its constituents,[11] may represent the ideal matrix for measuring and identifying metabolites of interest.[12] Measuring metabolites in the follicular fluid will also allow for the investigation of early biomarkers of maternal exposure to air pollution that may be unique to the ovary. Second, while our previous study focused on the clinically relevant outcome of live birth, a more temporally and biologically relevant outcome may be oocyte quality which is a critical component of female fertility and is often influenced by external factors including maternal exposure to air pollution.[13]

Building on this previous research, our objective was to use untargeted high-resolution metabolomics to identify metabolites and pathways in the follicular fluid associated with periconceptional exposure to air pollution, including nitrogen dioxide (NO_2), black carbon (BC), $PM_{2.5}$, and ozone (O_3), and oocyte quality among a prospective cohort of women undergoing fresh autologous ART. Findings from this study will help improve our understanding of the biological relationship between ambient air pollution and female fertility.

Methods

Study design and participants

Women included in our analysis were a sub-set of participants in the Environment and Reproductive Health (EARTH) study, a prospective cohort designed to evaluate environmental and nutritional determinants of fertility among couples presenting for infertility treatment and evaluation at the Massachusetts General Hospital Fertility Center (2005-2019). The EARTH study was approved by the Human Studies Institutional Review Boards of the MGH and the Harvard T. H. Chan School of Public Health. In brief, 135 women from the EARTH study who underwent a fresh assisted reproductive technology (ART) cycle and provided a follicular fluid sample during oocyte retrieval were eligible for our analysis. We then excluded 10 women who did not provide a follicular fluid sample from their dominant follicle to reduce variability due to this factor. This left

125 women for our analysis. A further description of our study flow is provided in Supplemental Figure 1.

Air pollution assessment

At enrollment, all women in our study provided their residential address for reimbursement purposes. These addresses were geocoded using ArcGIS and linked to several existing spatio-temporal models to derive daily ambient air pollution exposures at the woman's address starting 3 months prior to the date of oocyte retrieval. Daily $PM_{2.5}$ and NO_2 concentrations were modeled at a 1 km^2 resolution using satellite remote sensing data in combination with land use terms.[14,15] Daily O_3 concentrations were also modelled at a 1 km^2 resolution using chemical transport models, O_3 vertical profiles, meteorological variables, and other atmospheric compounds.[16] Daily BC exposure was estimated at the home address using support vector machine regression models based on ambient measurements collected across New England as well as several spatial and temporal predictors.[17] In this analysis, we focused on average ambient air pollution exposure in the two weeks prior to oocyte retrieval as it represents both a clinically (e.g. during controlled ovarian stimulation) and biologically (e.g. during the final stages of oocyte maturation) relevant time window.

Outcome assessment

Women in our study underwent one of three controlled ovarian stimulation protocols as clinically indicated: luteal-phase GnRH agonist, GnRH-antagonist protocol, or a follicular phase GnRH-agonist protocol. During gonadotropin stimulation, women were monitored to ensure follicular development including serum estradiol, follicle size measurements and counts, and endometrial thickness. Once 3 or more lead follicles (≥16 mm in diameter) were visualized and the estradiol level was >600 pmol/L, hCG was administered to induce oocyte maturation and 35-37 hours later oocyte retrieval was performed using a transvaginal ultrasound guided aspiration.

During oocyte retrieval, a follicular fluid sample was taken from women's first three follicles with a 16 G needle. Each sample was collected in a separate tube prepared with 1 ml of flushing media. Once the oocytes were removed, the follicular fluid was centrifuged to separate the supernatant and pellet. The resulting aliquots were then stored at -80°C. Embryologists classified the retrieved oocytes as germinal vesicle, metaphase I, metaphase II (MII) or degenerated. Total oocyte yield was defined as the sum of all oocytes retrieved regardless of type. Mature oocyte yield was the sum of all MII oocytes.

Metabolomic assessment

Follicular fluid supernatant samples were shipped overnight, on dry ice, to Emory University for metabolomics analysis. Once received, samples were randomized prior to analysis to minimize batch effects. Follicular fluid samples were analyzed using liquid chromatography with high resolution mass spectrometry (LC-HRMS; Dionex Ultimate 3000 RSLCnano; Thermo Orbitrap Fusion). To facilitate greater feature detection, two chromatography columns were used: the C18 hydrophobic chromatography column with negative electrospray ionization (ESI) and the hydrophilic interaction chromatography (HILIC) column with positive ESI. We used two quality control samples, NIST 1950[18] and pooled human plasma (Equitech Bio). We used ProteoWizard to covert raw data files to .mzML files using apLCMS and xMSanalyzer.[19–21] Unique features were characterized based on mass-to-charge ratio (m/z), retention time, and ion intensity. To filter out the noise signals and optimize the metabolomics data quality, only metabolic features detected in >10% of the follicular fluid samples with median coefficient of variation (CV) among technical replicates <30% and Pearson correlation >0.7 were included in further analyses. Following quality assessment, the median intensity was taken across replicate samples and these intensities were natural log transformed for analysis.

Statistical analysis

We analyzed the associations between air pollutant exposure in the two weeks prior to oocyte retrieval and metabolic features in the follicular fluid using multivariable linear regression models adjusted for age, BMI, smoking status, education, and average temperature. Separate models were conducted for each metabolic feature detected in each chromatography chrome (i.e., C18 column with negative ESI and HILIC column with positive ESI). Similar multivariable models adjusted for age, BMI, smoking status, education, protocol were used to examine the association between total number of MII oocytes retrieved and metabolic features. Multiple comparison correction was conducted using the Benjamini-Hochberg false discovery rate (FDR_{B-H}) procedure, a widely used procedure in MWAS studies, at a 5% false positive threshold.

We conducted pathway enrichment analysis using all the metabolic features significant at $p<0.05$ by utilizing mummichog (v. 1.0.10), a bioinformatics platform that infers and categorizes functional biological activity directly from mass spectrometry output, without prior metabolite validation[22]. An adjusted p-value for each pathway was calculated from resampling the reference input file in mummichog using a gamma distribution, which penalizes pathways with fewer reference hits, and assigning greater significance to pathways with more reference hits.[22] We conducted pathway analysis separately for each of the air pollutants and for each chromatography column. Heat maps were used to display the associations with the top metabolic pathways. We used an in-house database of previously confirmed metabolites to annotate the significant features identified in our analyses. This annotation was based on a comparison of adduct, m/z, retention time, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) spectra to analytical standards or database spectra to confirm level 1 and level 2 evidence based on the Metabolomics Standards Initiative criteria.[23,24]

Results

The 125 women included in our analysis had a mean age of 34.7 years and BMI of 24.0 kg/m^2 (**Supplemental Table 1**). The majority were White (86%), never smokers (77%), with a

college degree or higher (93%). Most women had been diagnosed with unexplained infertility (43%) and were treated with a luteal phase agonist protocol (72%). The mean (standard deviation) number of mature oocytes retrieved was 9.7 (4.8). The median (25^{th}, 75^{th} percentile) exposure to air pollution in the 2 weeks prior to oocyte retrieval was 22.3 (15.3, 36.0) ppb for NO_2, 32.2 (26.1, 42.0) ppb for O_3, 8.4 (7.3, 10.1) μg/m³ for $PM_{2.5}$, and 0.5 (0.4, 0.7) μg/m³ for BC (**Supplemental Table 2**). The air pollutants were weakly to moderately correlated with one another (ρ = 0.07 for NO_2 and $PM_{2.5}$ to 0.39 for NO_2 and BC) (**Supplemental Table 3**).

From the C18 and HILIC chromatography columns, 14,394 and 17,161 metabolic features were extracted from the follicular fluid, respectively. Across the two columns, thousands of metabolic features were associated with each air pollutant at a raw p-value<0.05 (**Table 1**); however, after FDR correction (<0.20), only higher exposure to NO_2 and O_3 in the two weeks prior to oocyte retrieval was associated with 96 and 47 feature(s), respectively. Using the features with raw p-value <0.05 as inputs, there were 5, 1, 2, and 1 metabolic pathways in the C18 chromatography column associated with exposure to NO_2, O_3, $PM_{2.5}$, and BC exposure, respectively (**Figure 1A**). In the HILIC chromatography column, 7, 5, 6, and 5 metabolic pathways were associated with NO_2, O_3, $PM_{2.5}$, and BC exposure, respectively (**Figure 1B**). Only four pathways were associated with more than one pollutant- Vitamin D3 (cholecalciferol) metabolism (NO_2 and $PM_{2.5}$), Vitamin A (retinol) metabolism (NO_2 and $PM_{2.5}$), C21-steroid hormone biosynthesis and metabolism (NO_2 and BC), and Bile acid biosynthesis (NO_2, O_3 and BC).

There were 897 and 1046 metabolic features in the follicular fluid that were associated with total mature oocytes at a raw p-value <0.05 (**Table 1**). After FDR correction (P<0.20), only three features remained significant. Using the features with raw p-value <0.05 as inputs into the pathway analysis, 7 and 9 pathways were significantly associated with total mature oocytes in the C18 and HILIC chromatography columns, respectively (**Figure 1A & B**). Top hits in the C18 chromatography column included bile acid biosynthesis, vitamin D3 (cholecalciferol) metabolism, prostaglandin formation from dihomo gama-linoleic acid, urea cycle/amino group metabolism,

ascorbate (Vitamin C) and aldarate metabolism, polyunsaturated fatty acid biosynthesis, and caffeine metabolism. Top hits in the HILIC chromatography column included carnitine shuttle, C21-steroid hormone biosynthesis and metabolism, vitamin D3 (cholecalciferol) metabolism, vitamin A (retinol) metabolism, tyrosine metabolism, androgen and estrogen biosynthesis and metabolism, bile acid biosynthesis, propanoate metabolism, and squalene and cholesterol biosynthesis.

We found 105 and 91 overlapped metabolic features with raw p-value<0.05, associated with both NO_2 exposure and number of mature oocytes retrieved from the C18 and HILIC chromatography columns. Most of the pathways that were associated with the air pollutants and mature oocytes were non-overlapping in both chromatography columns (**Figure 1A & B**). However, there was three pathways in the C18 chromatography column and six pathways in the HILIC chromatography column that were shared between at least one air pollutant exposure and number of mature oocytes retrieved. In C18 chromatography column, metabolites in the prostaglandin formation from dihomo gama-linoleic acid, vitamin D3 (cholecalciferol) metabolism, bile acid biosynthesis were altered with higher exposure to NO_2 and varied according to number of mature oocytes. In HILIC chromatography column, dysregulation of Vitamin D3 (cholecalciferol) metabolism and Vitamin A (retinol) metabolism was shared between NO_2 and $PM_{2.5}$ exposure and mature oocytes and a dysregulation in the C21-steroid hormone biosynthesis and metabolism was shared between NO_2 and BC exposure and mature oocytes. Androgen and estrogen biosynthesis and metabolism and Carnitine shuttle were shared between NO_2 exposure and mature oocytes. Bile acid biosynthesis were shared among NO_2, O_3, BC exposure and mature oocytes.

We further confirmed 8 metabolites with level-1 evidence (**Table 2**). Metabolites that were associated with both NO_2 exposure and number of mature oocytes included hypoxanthine, d-lactose, and caffeine. While the metabolites that were associated with BC or $PM_{2.5}$ were negatively associated with the pollutants – being higher among women with lower exposure to air pollution,

we found the opposite pattern for NO_2 as the annotated metabolites were higher among women with higher exposure to air pollution.

Discussion

We applied untargeted high-resolution metabolomics to follicular fluid, a toxicologically relevant target tissue, to lend insight into the potential biological mechanisms underlying the relationship between ambient air pollution exposure and oocyte quality among women undergoing ART. Of the air pollutants examined, NO_2, which tends to be a marker of vehicle emissions, had the largest number of overlapping metabolites and metabolic pathways with the number of mature oocytes retrieved while the other air pollutants, O_3, $PM_{2.5}$, and BC had limited overlap. Our study provides novel mechanistic insight into the potential biological pathways such as endogenous vitamin metabolism, hormone synthesis, and oxidative stress and the specific metabolites, such as hypoxanthine, that may be mediating the negative association between NO_2 exposure and lower oocyte quality in women.

The finding that NO_2 exposure had the largest number of overlapping metabolites and pathways with mature oocyte yield is in line with previous epidemiological studies suggesting that women with higher exposure to traffic-related air pollution have lower fertility as measured by high incidence of infertility,[25] longer time to pregnancy,[26] decreased success with ART,[27] and higher risk of pregnancy loss.[28] Moreover, it suggests that compromised oocyte quality could be a primary mediator. An experimental study in mice found a significant reduction in the number of ovarian antral follicles following traffic-generated PM exposure.[29] PM-induced ovarian damages are also demonstrated as an inflammatory response in ovarian tissues, ovarian oxidative stress, apoptosis, and abnormal ultrastructural alterations in mice.[30] The findings from the animal literature can thus provide biological plausibility to why we observed NO_2 as the strongest pollutants being associated with mature oocyte yield.

Three key pathways that were shared between NO_2 exposure and the number of mature oocytes retrieved included vitamin D3 metabolism, vitamin A metabolism, and bile acid biosynthesis. Vitamin D and A have long been implicated in human reproduction. Vitamin D signaling is directly involved in the expression of the anti-Mullerian hormone (AMH), which is produced by the ovarian granulosa cells and known to play a role in the regulation of follicular recruitment and selection. Therefore, vitamin D deficiency in females may contribute to impairment in ovarian physiology via disrupted AMH signaling.[31] Given that enzymes known to be involved in retinoid synthesis are found in the ovary, it is plausible that vitamin A deficiency can lead to the deterioration of oocyte quality.[32] Emerging evidence also suggests that air pollution may directly (through reduced UVB exposure) and indirectly (through less time spent outdoors) lessen the cutaneous production of vitamin D3[33] and reduce levels of the vitamin A precursor, β carotene (a potent antioxidant), in the body.[34] It is also interesting that bile acid biosynthesis was implicated as the absorption of lipid-soluble vitamins (such as vitamins D and A) from the diet requires bile acids and high levels of these vitamins may also repress bile acid synthesis to protect against potentially toxic levels of lipid-soluble vitamins in the diet.[35] Traffic-related air pollution has been implicated in altered bile acid homeostasis[36] and has been commonly found as a dysregulated pathway in studies on air pollution and the blood metabolome.

Other overlapping pathways associated with both NO_2 and oocyte quality included C21-steroid hormone metabolism, androgen and estrogen metabolism, and prostaglandin formation. Steroid hormones are considered critical elements in reproductive outcomes and the composition of these hormones in the follicular environment is an important determinant of oocyte quality.[37] Environmental pollutants are known to interfere with steroid hormone metabolism through disruption of hydroxysteroid dehydrogenases, a group of steroidogenic enzymes, resulting in impaired reproductive functions.[38] Diesel exhaust, in particular, contains a variety of substances including polycyclic aromatic hydrocarbons with documented estrogenic, anti-estrogenic, and anti-androgenic properties that can affect gonadal steroidogenesis and gametogenesis.[39–42] The

last overlapping pathway that might also mediate the association between NO_2 and oocyte quality is the carnitine shuttle. L-carnitine plays an important role in female reproduction, more specifically, oocyte development and quality enhancement. It acts as an antioxidant by promoting β-oxidation in oocytes, attenuating oxidative damage, and preventing apoptosis.[43,44] Metabolites in carnitine metabolism such as acyl-carnitines have also been reported to be affected by traffic-related air pollutants most notably in association with NO_2. [45]

Among the three overlapping metabolites we confirmed with level-1 evidence, hypoxanthine, a purine derivative that protects against oxidant-induced cell injury,[46] appears to be the most intuitive potential mediator as it was increased with higher exposure to NO_2 and was increased among women with fewer mature oocytes retrieved. Corroborating this finding include multiple studies which found this specific metabolite to be associated with air pollution in the plasma metabolome[47–49] and multiple experimental studies which showed that hypoxanthine plays a critical role in inhibiting the nuclear maturation of oocytes.[50,51] While we also observed two other metabolites, d-lactose and caffeine, to be associated with both NO_2 exposure and number of mature oocytes retrieved, their role as potential mediators was less intuitive based on previous research and biological hypotheses.

In a previous paper from our group, we identified 13 metabolic pathways in serum preconception samples that were significantly associated with NO_2 exposure and probability of live birth following ART.[4] Interestingly, none of these pathways overlapped with the follicular fluid pathways associated with air pollution and mature oocyte yield. While three follicular fluid pathways associated with mature oocyte yield- bile acid biosynthesis, urea cycle/amino group metabolism, and ascorbate (vitamin C) and aldarate metabolism- were also found in the serum to be associated with probability of live birth following ART, almost none of the serum pathways associated with NO_2 exposure overlapped with the follicular fluid pathways associated with NO_2 exposure. This is likely due to a combination of several factors. First, the follicular fluid is a distinct biological fluid from blood and may represent a more toxicologically relevant biofluid for

investigating the impacts of air pollution on ovarian function. Second, NO_2 exposure may have a specific negative impact on the ovary, which may be better captured by studying follicular fluid as opposed to blood.

While the findings from this study expand our understanding of the biological mechanism underlying the negative association between air pollution exposure and oocyte quality, they should also be considered in the context of its limitations. First, given the large number of metabolic features we identified and multiple comparisons made between different air pollutants, there is an increased probability of false-positive and Type 1 errors. Although we reported the number of significant metabolic features at different levels of p-value including FDR_{B-H} correction, we had to use a cut-off of raw p-value <0.05 for our pathway analyses to allow for meaningful interpretation. Considering our small sample size, we used less stringent criteria for statistical significance to decrease the false-negative rates. Second, a proxy to estimate ambient air pollution exposure using participants' residential addresses may not be perfect, which could decrease the precision of our effect estimates. However, the prospective design of this study made it less likely for this uncertainty in exposure assessment to be influenced by our outcome. Third, most of our sample were White and highly educated, which may limit the generalizability of our findings. The women in our study's exposure to air pollution also tended to be low. Therefore, it is possible we may have underestimated or missed associations that could be present in other, more highly polluted regions.

In summary, we successfully identified metabolites and pathways in the follicular fluid that are overlapping between periconceptional exposure to air pollution and oocyte quality using untargeted high-resolution metabolomics and a 'meet-in-the-middle' approach. These results provide valuable information to the investigation of how air pollutants, in particular those due to traffic, may negatively impact oocyte quality.

Table 1. Significant metabolic features associated with NO_2, O_3, $PM_{2.5}$, and black carbon exposure and mature oocyte among 125 women in the EARTH study.

	C18 Negative (N=14394)					HILIC Positive (N=17161)				
	Raw <0.05	Raw <0.005	Raw <0.0005	FDR <0.20	FDR <0.05	Raw <0.05	Raw <0.005	Raw <0.0005	FDR <0.20	FDR <0.05
NO_2	1317	128	21	8	5	1648	263	55	88	19
O_3	521	59	9	0	0	1138	196	44	47	13
$PM_{2.5}$	1166	90	12	0	0	1040	119	11	0	0
BC	599	55	6	0	0	762	74	9	0	0
MII Oocytes	897	111	15	3	1	1046	117	9	0	0

(NO_2 = Nitrogen Dioxide; O_3 = Ozone; $PM_{2.5}$ = Fine Particulate Matter; BC = Black Carbon; MII oocytes = total number of Mature oocytes)

Figure 1. Metabolic pathways associated with air pollution and mature oocytes in the C18 negative (Panel A) and HILIC positive (Panel B) platforms.

A.

Metabolic Pathways	Number of metabolites in pathway	C18 Negative Mode (praw<0.05)				
		NO_2	O_3	$PM_{2.5}$	Black Carbon	Mature Oocyte
Prostaglandin formation from dihomo gama-linoleic acid	3	100%				67%
Vitamin D3 (cholecalciferol) metabolism	16	44%				31%
Bile acid biosynthesis	38	32%				29%
Histidine metabolism	22	32%				
Androgen and estrogen biosynthesis and metabolism	47	26%				
Hexose phosphorylation	20		25%			
Glycine, serine, alanine and threonine metabolism	32			25%		
Vitamin A (retinol) metabolism	25			24%		
Electron transport chain	2				100%	
Urea cycle/amino group metabolism	45					20%
Ascorbate (Vitamin C) and Aldarate Metabolism	33					21%
Polyunsaturated fatty acid biosynthesis	4					50%
Caffeine metabolism	10					30%

B.

Metabolic Pathways	Number of metabolites in pathway	HILIC Positive Mode (praw<0.05)				
		NO_2	O_3	$PM_{2.5}$	Black Carbon	Mature Oocyte
Vitamin D3 (cholecalciferol) metabolism	10	70%		40%		50%
C21-steroid hormone biosynthesis and metabolism	53	38%			21%	34%
Androgen and estrogen biosynthesis and metabolism	24	46%				33%
Vitamin A (retinol) metabolism	22	41%		36%		36%
Carnitine shuttle	35	34%				43%
Beta-Alanine metabolism	13	46%				
Bile acid biosynthesis	33	33%	27%		18%	27%
Ascorbate (Vitamin C) and Aldarate Metabolism	11		36%			
Prostaglandin formation from arachidonate	37		22%			
Phosphatidylinositol phosphate metabolism	25		24%			
Hyaluronan Metabolism	4		50%			
Biopterin metabolism	16			44%		
De novo fatty acid biosynthesis	20			35%		
Vitamin E metabolism	26			31%		
Vitamin B2 (riboflavin) metabolism	3			67%		
C5-Branched dibasic acid metabolism	2				100%	
Prostaglandin formation from dihomo gama-linoleic acid	6				50%	
Arachidonic acid metabolism	30				23%	
Tyrosine metabolism	110					24%
Propanoate metabolism	3					67%
Squalene and cholesterol biosynthesis	15					33%

*Percentages in the cells represent the proportion of overlapping metabolites

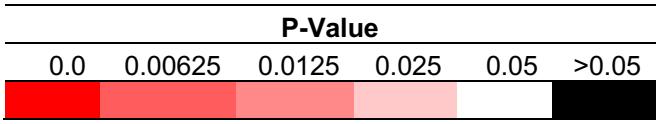

Table 2. Chemical identity of the annotated metabolites in the follicular fluid significantly associated with at least one air pollutant and number of mature oocytes.

m/z	RT	Validated Metabolite	Adduct Form	Associated Pollutant	% Change (95% CI) per SD Increase in Pollutant	% Change (95% CI) per Mature Oocyte Retrieved	Column
130.0873	23	L-ISOLEUCINE LEUCINE NORLEUCINE	M-H	Black Carbon	-14 (-24, -3)	-3 (-6, -1)	C18 neg
131.0825	24	D-ORNITHINE L-ORNITHINE	M-H	Black Carbon	-13 (-23, -2)	-3 (-5, -1)	C18 neg
				PM2.5	-13 (-22, -2)		
135.0302	23	HYPOXANTHINE	M-H	NO2	11 (1, 22)	-3 (-5, -1)	C18 neg
377.0863	22.2	D-LACTOSE SUCROSE MELIBIOSE MALTOSE D-(+)-CELLOBIOSE PALATINOSE	M+Cl	NO2	51 (14, 102)	-7 (-13, -2)	C18 neg
391.287	170.9	DEOXYCHOLATE	M-H	PM2.5	-18 (-30, -4)	-5 (-8, -2)	C18 neg
118.0863	60.7	L-VALINE L-NORVALINE 5-AMINOPENTANOATE	M+H	PM2.5	-10 (-17, -2)	-3 (-4, -1)	HILIC pos
176.103	93.5	CITRULLINE	M+H	PM2.5	-14 (-22, -5)	-3 (-5, -1)	HILIC pos
195.0875	30.4	CAFFEINE	M+H	NO2	47 (15, 88)	5 (0, 11)	HILIC pos

Supplemental Table 1. Descriptive characteristics of the EARTH Study women with a follicular fluid sample from their primary follicle (n=125) who were included in this analysis.

	Mean ± SD or N (%)
Demographics	
Age, years	34.7 ± 3.7
BMI, kg/m^2	24.0 ± 4.7
Race, n (%)	
White	108 (86%)
African-American	2 (2%)
Asian	10 (8%)
Other	5 (4%)
Smoking Status, n (%)	
Never	96 (77%)
Ever	29 (23%)
Education Level, n (%)	
<College	9 (7%)
College Graduate	37 (30%)
Graduate Degree	79 (63%)
Census-Tract Median Income, $	106,282 ± 42,954*
Distance to A1/A2 Roadway, m	1,345 ± 1,898
ART Cycle Characteristics	
Year of Oocyte Retrieval, n (%)	
2005-2008	11 (9%)
2009-2012	77 (62%)
2013-2015	37 (30%)
Season of Oocyte Retrieval, n (%)	
Jan-Mar	34 (27%)
Apr-Jun	40 (32%)
Jul-Sep	31 (25%)
Oct-Dec	20 (16%)
Initial Infertility Diagnosis, n (%)	
Male	37 (30%)
Female	34 (27%)
Unexplained	54 (43%)
Treatment protocol, n (%)	
Luteal phase agonist	90 (72%)
Flare or antagonist	35 (28%)
Outcomes of Oocyte Retrieval	
Total Number of Oocytes	11.8 ± 5.9
Total Number of Mature Oocytes	9.7 ± 4.8

* There were 3 women with missing information

Supplemental Table 2. Distribution of ambient air pollution exposure concentrations in the 2 weeks prior to oocyte retrieval.

	Minimum	Q1	Median	Q3	Maximum
NO_2, ppb	2.7	15.3	22.3	36.0	187.8
O_3, ppb	0.0	26.1	32.2	42.0	200.0
$PM_{2.5}$, μg/m³	4.8	7.3	8.4	10.1	16.7
BC, μg/m³	0.3	0.4	0.5	0.7	1.1

Supplemental Table 3. Spearman correlation coefficients between average air pollutant concentrations in the 2 weeks prior to oocyte retrieval.

		2 weeks			
		NO_2	**O_3**	**$PM_{2.5}$**	**BC**
2 weeks	**NO_2**	1	0.25	0.07	0.39
	O_3		1	0.11	0.27
	$PM_{2.5}$			1	0.34
	BC				1

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