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Seminal plasma metabolites mediate the associations of multiple environmental pollutants with semen quality in Chinese men

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ABSTRACT

Environmental exposure to arsenic, phthalate esters (PAEs) and perfluorinated compounds (PFCs) has been associated with human semen quality. However, the epidemiological "black-box" of these associations remains poorly uncovered. In this study, based on the association analysis between arsenic, PAE and PFC exposure and semen quality parameters (i.e., semen volume, sperm concentration, sperm count, progressive motility, total motility and normal morphology) in a Chinese male population, we explored the seminal plasma metabolic signatures that may mediate the exposure-outcome relations by using the meet-in-metabolite-analysis (MIMA) approach. As a result, a negative association was found between DMA and sperm concentration, whereas MEHP and PFHxS were positively associated with sperm count and concentration, respectively. Metabolomics analysis revealed that sixteen and twenty-two seminal plasma metabolites were related to sperm concentration and count, respectively, and they are mainly involved in fatty acid, lipid and amino acid metabolism. Moreover, it was further indicated that eicosatetraenoate, carnitines and DHA may impact the inverse association between DMA and sperm concentration, while eicosatetraenoate, carnitines, DHA, PGB2 and tocotrienol are possible mediators of the positive association between PFHxS and sperm concentration. As these metabolic biomarkers are relevant to antioxidation and fatty acid β-oxidation, we suggest that redox balance and energy generation shifts in seminal plasma are involved in the association of human semen quality with environmental DMA and PFHxS exposure.

1. Introduction

Arsenic, phthalate esters (PAEs) and perfluorinated compounds (PFCs) are three types of pollutants ubiquitously present in the environment, and human exposure to them has been associated with a variety of adverse health outcomes including male reproductive dysfunction. To date, the associations between environmental chemical exposure and human semen quality have been widely studied. It was revealed that arsenic exposure is inversely associated with sperm concentration (Xu et al., 2012; Li et al., 2012) and progressive and total sperm motility but is positively associated with the percentage of sperm tail DNA in human populations (Wang et al., 2017a). Significant correlations of phthalates exposure with reduced semen quality have been reported by numerous epidemiological studies (Cai et al., 2015; Pan et al., 2015; Wang et al., 2016; Thurston et al., 2016); however, MEHP exposure was found to be associated with higher sperm motility (Bloom et al., 2015; Wang et al., 2018). In addition, environmental exposure to PFCs was negatively related to sperm concentration, count, motility and morphology (Vested et al., 2013; Louis et al., 2015; Song et al., 2018), while a positive association between PFOA and sperm motility was observed (Toft et al., 2012). Although these associations have been observed, the molecular linkages between these pollutant (arsenic, PAEs and PFCs) exposures and human semen quality still need to be elucidated due to the "black-box" character of traditional epidemiological research.

Metabolites in organisms are the endpoints of enzyme/protein actions, which could reflect the final consequences of functional changes induced by environmental and disease stresses. Metabolic biomarkers

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can therefore provide biological information to map the potential pathway indicating the physiological/pathological changes involved. Metabolomics, a global chemical phenotyping approach that quantitatively measures the metabolome alteration, is a powerful tool to screen the biomarkers related to health outcomes (Huang et al., 2018). There have been studies addressing the metabolic differences between fertile and infertile men by metabolomics. As the microenvironment of sperm before ejaculation, seminal plasma has been considered as a better sample for metabolomic analysis in the evaluation of male fertility than other biofluids (e.g., serum and urine) (Courant et al., 2013; Zhang et al., 2014a, 2014b; Chen et al., 2015; Qiao et al., 2017; Wang et al., 2019).

Since semen quality is an important indicator of male fertility, a few studies have investigated the metabolic alterations in seminal plasma of patients with low semen quality. Metabolomics analysis of seminal plasma samples comparing healthy and asthenozoospermic men revealed that elevation of fatty acids and oxysterols indicated oxidative stress to the spermatozoa, and that a lack of valine may contribute to poor sperm motility (Tang et al., 2017; Zhang et al., 2015). Gupta et al. (2013) reported that the contents of lactate, citrate, GPC and several amino acids (alanine, histidine and phenylalanine) in seminal plasma were perturbed in oligozoospermic and asthenozoospermic patients. Furthermore, different levels of a series of metabolites were also found when comparing idiopathic infertility (normozoospermia) and other forms of male infertility, and lysine has been suggested as a potential biomarker for the detection and diagnosis of idiopathic infertility (Jayaraman et al., 2014).

Although the associations of arsenic, PAE and PFC exposure with semen quality have been fully characterized, their underlying mechanism of action remains largely unclear in humans. In view of the metabolic disturbance involved in poor semen quality, we hypothesized that the human metabolome can serve as a hub in which semen quality-related metabolic biomarkers may link pollutant exposure to male fecundity. Therefore, by using the meet-in-metabolite-analysis (MIMA) strategy (Huang et al., 2018; Wu et al., 2018), we investigated the associations of arsenic, PAE and PFC exposure with human semen quality and the metabolome differences in seminal plasma between male participants with low and high semen quality, aiming to identify the metabolic biomarkers linking arsenic, PAE and PFC exposure to semen quality. We hope that these results will aid in better understanding the mode of action of environmental pollutants in affecting male reproduction.

2. Materials and methods

2.1. Participant recruitment and sample collection

One hundred and eighteen men were enrolled by the Reproduction Department of the Chongqing Institute of Science and Technology for Population and Family Planning (CISTPFP), China between July 2009 and August 2010. They were male partners in couples who came for fertility assessment. The local ethics committee approved the study. The purposes of this study were explained to the participants, and written informed consent was obtained before the study began. The individual information including age, height, weight, abstinence time, smoking and alcohol drinking status were collected by questionnaire. Participants with diagnosed metabolic or urogenital diseases were excluded from this study.

Urine, blood and semen samples were collected on the same day when the participants had their clinic visits. However, only 86 and 99 urine samples were used for arsenic and phthalates analysis, respectively, and 57 blood samples for PFC analysis due to limited sample amounts. The participants were requested to abstain from sexual activity for at least two days before contributing a semen sample. The semen samples were generated by masturbation and the semen parameters including semen volume, sperm concentration, count, progressive motility, total motility and morphology were analyzed according to World Health Organization (WHO) guidelines as described previously (Shen et al., 2013). Among the 118 subjects, there were 98 semen samples with sufficient seminal plasma for metabolomics analysis (Table 1). Finally, a total of 67, 67 and 57 subjects who provided urine and blood samples for arsenic, PAE and PFC measurement, respectively, also provided sufficient seminal plasma for metabolomics analysis. Details of the subject selection are shown in Fig. S1.

Table 1
Demographics and semen quality parameters of the study participants (n = 98).

Characteristic	Mean ± standard deviation (SD)	Median	n (%)
Age (years)	30.9 ± 5.2	30	
BMI (kg/m^2)	24.1 ± 4.0	23.7	
Smoking			
Yes			53(54.1%)
No			45(45.9%)
Alcohol drinking			
Yes			53(54.1%)
No			45(45.9%)
Abstinence time (day)	4.3 ± 1.6	4.0	
Semen parameters			
Semen volume (mL)	2.7 ± 0.9	2.7	
< 1.5 mL			6 (6.1%)
Sperm concentration (×10^6/mL)	77.4 ± 59.0	70.7	
< 15 × 10^6/mL			6 (6.1%)
Sperm count (×10^6)	204.5 ± 163.3	175.7	
< 39 × 10^6			7 (7.1%)
Progressive motility (%)	48.6 ± 18.3	50.1	
< 32%			17 (17.3%)
Total motility (%)	58.5 ± 20.4	60.4	
< 40%			17 (17.3%)
Normal sperm morphology (%)	15.6 ± 7.8	14.9	
< 4%			3 (3.1%)

2.2. Urinary arsenic species, phthalate metabolites and blood PFCs analysis

The arsenic species of As_i^{III}, As_i^{V}, methylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenobetaine (AsB), and six major phthalate metabolites, including monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP) and mono-2-ethyl-5-oxohexyl phthalate (MEOHP) in urine samples were measured as reported previously (Xu et al., 2012; Liu et al., 2017). Eleven perfluorinated compounds in blood samples, including perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorobutanoic acid (PFBA), perfluorobutane sulfonate (PFBS), perfluorodecanoic acid (PFDA), perfluoroheptanoic acid (PFHpA), perfluorohexanoic acid (PFHxA), perfluorohexane sulfonate (PFHxS), perfluorononanoic acid (PFNA), perfluoroundecanoic acid (PFUnA) and perfluorododecanoic acid (PFDoA) were determined according to our previously reported method (Liu et al., 2015). The six PAEs and eleven PFCs are widespread and have a relatively higher concentration in the environment, which are usually measured to be representative of environmental PAEs and PFCs exposure in humans (Liu et al., 2017; Wang et al., 2017b). The details of chemical analysis are provided in the Supporting Information.

2.3. Seminal plasma metabolome analysis

The details of sample preparation, metabolome profiling acquisition, data processing and quality control procedures are described in the Supporting Information. The processed mass feature tables were Pareto-scaled and introduced to SIMCA-P software (v13.0, Umetrics, Uppsala, Sweden) for multivariate statistical analysis. The semen parameters were classified into quartiles from the highest to the lowest

levels, and the data set was then categorized into four groups (1st, 2nd, 3rd and 4th quartile) accordingly. Quartile-based partial least squares-discriminant analysis (PLS-DA) models were established by taking the semen parameters (i.e., semen volume, sperm concentration, sperm count, progressive motility, total motility and normal sperm morphology) as classifiers. The 999-time permutation tests were performed to validate the developed PLS-DA models. The metabolite biomarkers were screened based on the following criteria: 1) variable importance in projection (VIP) value > 2; 2) jack-knifing confidence interval > 0; 3) intensity difference in variables between the 1st and 4th quartile groups was significant ($p < 0.05$); and 4) the features significantly correlated with semen parameters after adjustment for age, body mass index (BMI), abstinence time, smoking and alcohol drinking status. Metabolite identification was carried out by searching the Human Metabolome Database (HMDB, http://www.hmdb.ca) based on accurate mass measurement. The accepted mass difference was set as 20 mDa during the search. Furthermore, the UPLC/MS/MS product ion spectrum of a metabolite was matched with the MS spectra available in HMDB to confirm the identification. Metabolic pathways were analyzed by using the web-based MetaboAnalyst software (http://www.metaboanalyst.ca/) and KEGG database (https://www.kegg.jp/).

2.4. Data analysis

The Mann-Whitney nonparametric test was used to evaluate the significance of intergroup differences for each potential biomarker. Partial correlation analysis was performed to investigate the associations of biomarkers with semen parameters in which the variables of age, BMI, abstinence time, smoking and alcohol drinking status were adjusted. Furthermore, the associations of pollutants (arsenic, PAEs and PFCs) with semen parameters and biomarkers were analyzed with multiple linear regression model, and the false discovery rate (FDR) correction was applied to correct for multiple hypothesis tests (Wang et al., 2019). The effects of pollutant-metabolite combinations on semen quality were expressed by the adjusted odds ratios (AORs), and their dose-related trends were analyzed by using binary logistic regression in which the defined outcomes of low and high semen quality were counted based on the median cutoffs of the pollutants and metabolites. Pollutant concentrations below LOD were replaced by half of their corresponding LOD. These statistical analyses were performed by using SPSS 19 (SPSS Inc.).

Receiver operating characteristic (ROC) analysis was applied to assess the specificity and sensitivity of the biomarkers. Classical univariate ROC analysis was performed by using SPSS 19 (SPSS Inc.); multivariate analyses of combinational biomarker patterns were performed using online ROCCET (ROC Curve Explorer & Tester) software (http://www.metaboanalyst.ca/).

The average causal mediation effect was performed by using the R Package for Causal Mediation Analysis according to the quasi-Bayesian Monte Carlo method with 1000 simulations (Tingley et al., 2014).

3. Results

3.1. Participant characteristics and semen parameters

The demographic characteristics of our 98 participants who provided seminal plasma for metabolomics analysis are listed in Table 1. The participants were from 21 to 42 years old (median 30 years old) and their median BMI was 23.7 kg/m^2. The consumption of tobacco and alcohol both accounted for 54.1% of the subjects, and the abstinence time ranged from 2 to 8 days (median 4 days). The ranges of the major semen parameters, including semen volume, sperm concentration, count, progressive motility, total motility and normal sperm morphology were 0.6–5.2 mL (median 2.7 mL), 0–402.7 × 10^6/mL (median 70.7 × 10^6/mL), 0–1167.7 × 10^6 (median 175.7 × 10^6), 0–82.0% (median 50.1%), 0–97.8% (median 60.4%) and 0–42.6% (median 14.9%), respectively (Table 1). Only 6 (6.1%), 6 (6.1%), 7 (7.1%), 17 (17.3%), 17 (17.3%) and 3 (3.1%) subjects had the parameters below WHO references for semen volume (1.5 mL), sperm concentration (15 × 10^6/mL), count (39 × 10^6), progressive motility (32%), total motility (40%) and normal sperm morphology (4%), respectively (WHO, 2010). In addition, there are not any statistically significant differences ($p > 0.05$) in the demographics and semen parameters between this population ($n = 98$) and the three subpopulations, which provided urine and blood samples for arsenic ($n = 67$), PAE (n = 67) and PFC ($n = 57$) measurement, respectively (Table S1).

Table 2
Urinary arsenic, PAEs and blood PFCs concentration of the participants.

Pollutant	Mean ± SD[a]	Median	Range	Detection rate
Arsenic ($n = 86$), μg/g creatinine				
AsB	16.1 ± 32.0	7.8	0.4–212.0	100%
MMA	3.4 ± 3.0	2.5	0.1–16.8	100%
DMA	23.9 ± 16.3	19.5	4.0–102.8	100%
As_i^{III}	4.1 ± 2.9	3.4	0.1–16.2	100%
As_i^{V}	0.6 ± 1.7	0.1	0.03–10.5	100%
As_i	4.7 ± 3.4	3.6	0.4–16.7	
As_t	48.0 ± 40.4	37.3	6.4–222.0	
PAEs ($n = 99$), μg/g creatinine				
MMP	42.6 ± 91.3	19.6	0.0–745.4	99%
MEP	52.7 ± 121.6	15.2	0.5–964.3	100%
MBP	35.4 ± 63.2	14.2	0.0–382.3	99%
MBzP	0.3 ± 1.1	0.0	0.0–10.4	46.5%
MEHP	2.8 ± 6.5	0.8	0.0–48.8	98%
MEOHP	3.3 ± 4.2	2.2	0.3–29.4	100%
ΣDEHP	6.1 ± 10.2	3.5	0.5–75.8	
PFCs (n = 57), μg/L				
PFOA	2.5 ± 2.6	1.7	0.5–16.6	100%
PFOS	6.5 ± 4.6	5.3	1.4–29.4	100%
PFHxS	0.7 ± 0.9	0.6	0.0–6.3	70.2%
PFUnA	0.5 ± 0.5	0.5	0.0–2.1	70.2%
PFDA	0.3 ± 0.3	0.0	0.0–1.2	47.4%
PFNA	1.0 ± 0.5	0.9	0.0–2.7	96.5%
ΣPFCs	11.5 ± 6.8	10.2	2.3–40.4	

[a] SD: standard deviation.

3.2. Urinary arsenic, PAEs and blood PFCs levels and their associations with semen parameters

Urinary arsenic, phthalates and blood PFCs levels of the participants are listed in Table 2. The five arsenic species were detected in all of the samples, and DMA had a much higher concentration (median 19.5 μg/g) than the other four species. The median creatinine-adjusted concentrations of inorganic arsenic (As_i) and total arsenic (As_t) were 3.6 and 37.3 μg/g, respectively. For urinary phthalates, MMP, MEP, MBP, MEHP and MEOHP were detected in most of the samples, while the detection rate of MBzP was only 46.5%. We detected much higher concentrations of MMP, MEP and MBP (median 19.6, 15.2 and 14.2 μg/g) than the other phthalate metabolites, and the median concentration of ΣDEHP was 3.5 μg/g. In addition, PFOA and PFOS were detected in all of the blood samples while PFHxS, PFUnA, PFDA and PFNA were detected in 70.2%, 70.2%, 47.4% and 96.5% of the subjects, respectively. However, PFHxA, PFHpA, PFDoA, PFBA and PFBS were not detected in any blood samples. The median blood concentrations of PFOA, PFOS and total PFCs were 1.7, 5.3 and 10.2 μg/L, respectively.

By using multiple linear regression, we analyzed the associations of arsenic, phthalate and PFC exposure levels with semen quality parameters (i.e., semen volume, sperm concentration, count, progressive motility, total motility and morphology) in the male subjects, respectively (Table 3). After adjustment for age, BMI, abstinence time, smoking and alcohol drinking status, it was found that among the arsenic species, only DMA was negatively associated with sperm concentration (−0.688, 95% CI: −1.32, −0.056), while PFHxS in PFCs

Table 3
Associations of arsenic, PAE and PFC concentrations with semen quality parameters.

Pollutant	Semen volume β (95% CI)	Sperm concentration β (95% CI)	Sperm count β (95% CI)	Progressive motility β (95% CI)	Total motility β (95% CI)	Sperm morphology β (95% CI)
Arsenic (n = 86)						
AsB	−0.001 (−0.007, 0.005)	−0.03 (−0.352, 0.292)	−0.238 (−1.156, 0.679)	0.007 (−0.109, 0.123)	0.026 (−0.098, 0.15)	0 (0, 0.001)
MMA	0.006 (−0.059, 0.07)	−1.434 (−5.017, 2.15)	−3.605 (−13.83, 6.62)	−0.325 (−1.615, 0.966)	−0.408 (−1.793, 0.977)	−0.001 (−0.006, 0.005)
DMA	0.002 (−0.009, 0.014)	**−0.688 (−1.32, −0.056)***	−1.723 (−3.537, 0.09)	2.44E-05 (−0.234, −0.234)	−0.062 (−0.313, 0.188)	−3.66E-05 (−0.001, 0.001)
As_i^{III}	0.012 (−0.053, 0.078)	−1.19 (−4.833, 2.453)	−2.157 (−12.56, 8.245)	−0.458 (−1.767, 0.85)	−0.525 (−1.929, 0.879)	0 (−0.005, 0.006)
As_i^{V}	0.011 (−0.104, 0.125)	−4.687 (−10.966, 1.623)	−13.106 (−31.103, 4.148)	0.027 (−2.271, 2.326)	−0.153 (−2.62, 2.315)	−0.001 (−0.011, 0.009)
As_i	0.011 (−0.044, 0.066)	−1.934 (−4.984, 1.116)	−4.575 (−13.298, 4.148)	−0.32 (−1.424, 0.785)	−0.409 (−1.594, 0.776)	−4.60E-05 (−0.005, 0.005)
As_t	0 (−0.005, 0.004)	−0.149 (−0.405, 0.108)	−0.475 (−1.204, 0.253)	0 (−0.092, 0.093)	0.002 (−0.098, 0.101)	0 (0, 0.001)
PAEs (n = 99)						
MMP	0.002 (0, 0.004)	−0.026 (−0.138, 0.086)	0.077 (−0.241, 0.395)	0.033 (−0.011, 0.077)	0.033 (−0.015, 0.08)	−1.08E-05 (0, 0)
MEP	5.00E-05 (0, 0)	−0.001 (−0.006, 0.003)	0.001 (−0.012, 0.013)	0.001 (−0.001, 0.003)	0.001 (−0.001, 0.002)	5.55E-06 (0, 0)
MBP	0.001 (−0.001, 0.004)	−0.073 (−0.232, 0.087)	0.014 (−0.441, 0.47)	0.048 (−0.015, 0.111)	0.044 (−0.024, 0.112)	0 (0, 0)
MBzP	0.16 (0.005, 0.315)	−6.107 (−15.178, 2.963)	−8.383 (−34.346, 17.58)	1.441 (−2.169, 5.051)	0.584 (−3.324, 4.492)	0 (−0.017, 0.018)
MEHP	0.023 (−0.002, 0.049)	1.028 (−0.46, 2.515)	**5.204 (1.077, 9.331)***	0.448 (−0.139, 1.035)	0.537 (−0.095, 1.168)	0 (−0.003, 0.003)
MEOHP	0.023 (−0.018, 0.064)	0.916 (−1.465, 3.297)	5.128 (−1.571, 11.828)	0.698 (−0.235, 1.631)	0.768 (−0.239, 1.774)	0 (−0.005, 0.004)
ΣDEHP	0.013 (−0.003, 0.029)	0.561 (−0.386, 1.508)	2.918 (0.277, 5.56)	0.292 (−0.08, 0.665)	0.339 (−0.062, 0.74)	−8.93E-05 (−0.002, 0.002)
ΣPAEs	5.05E-05 (0, 0)	−0.001 (−0.006, 0.003)	0.001 (−0.011, 0.013)	0.001 (−0.001, 0.003)	0.001 (−0.001, 0.002)	5.31E-06 (0, 0)
PFCs (n = 57)						
PFOA	−0.004 (−0.09, 0.082)	−0.069 (−6.368, 6.23)	−0.686 (−16.981, 15.61)	−0.239 (−2.197, 1.719)	−0.285 (−2.495, 1.926)	−0.002 (−0.011, 0.007)
PFOS	0.009 (−0.04, 0.058)	0.145 (−3.452, 3.742)	−0.548 (−9.854, 8.758)	0.078 (−1.041, 1.197)	0 (−1.264, 1.263)	0.003 (−0.002, 0.008)
PFHxS	−0.082 (−0.33, 0.166)	**23.733 (6.81, 40.656)***	43.722 (−1.778, 89.221)	1.148 (−4.517, 6.812)	3.026 (−3.322, 9.374)	0.009 (−0.018, 0.036)
PFUnA	−0.097 (−0.613, 0.42)	−18.744 (−56.279, 18.791)	−50.17 (−147.211, 46.871)	−2.602 (−14.372, 9.169)	−4.754 (−18.001, 8.494)	0.003 (−0.053, 0.058)
PFDA	−0.123 (−0.895, 0.649)	−21.591 (−77.907, 34.725)	−60.869 (−206.407, 84.669)	5.956 (−11.583, 23.495)	3.427 (−16.448, 23.301)	−0.015 (−0.098, 0.069)
PFNA	0.167 (−0.306, 0.639)	0.374 (−34.424, 35.172)	15.706 (−74.212, 105.624)	0.66 (−10.162, 11.482)	0.588 (−11.633, 12.808)	−0.011 (−0.062, 0.04)
ΣPFCs	0.002 (−0.031, 0.036)	0.376 (−2.078, 2.83)	0.19 (−6.164, 6.545)	0.024 (−0.74, 0.788)	0 (−0.862, 0.863)	0.001 (−0.002, 0.005)

CI: confidence intervals.
Arsenic species, PAEs and PFCs were all ln-transformed.
* $p < 0.05$ (FDR corrected).

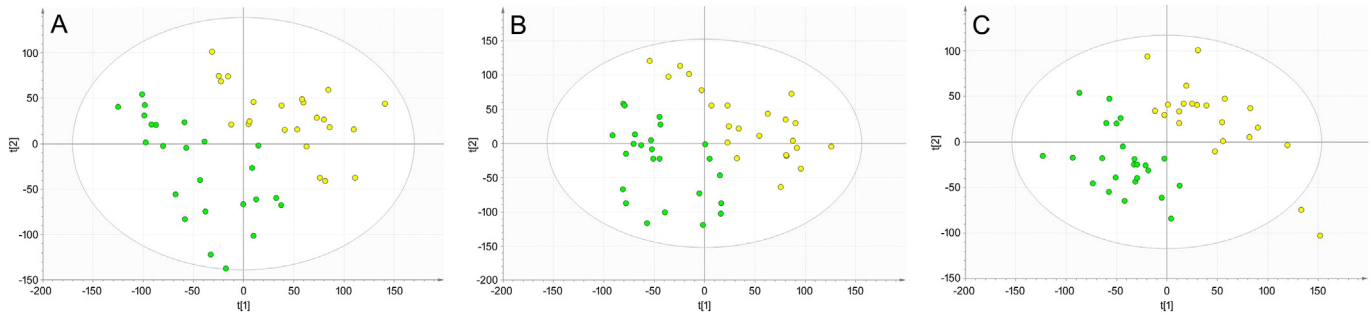

Fig. 1. Scoring plots of the developed PLS-DA models with sperm concentration (A) and sperm count (B, C) as the classifiers. (A) positive mode, ● 1st quartile group, ● 4th quartile group; (B) positive mode, ● 1st quartile group, ● 4th quartile group; (C) negative mode, ● 1st quartile group, ● 4th quartile group.

had a positive correlation with sperm concentration (23.733, 95% CI: 6.81, 40.656). For phthalates, only MEHP was positively related to sperm count (5.204, 95% CI: 1.077, 9.331). However, all the other pollutants were not significantly associated with any semen parameters. Since our participants may contain some subfertile men, we conducted a sensitivity analysis on the subjects with normal semen parameters (at/above WHO references), which showed the same significant associations between pollutants and semen parameters (Table S2), indicating the representativeness of this study population.

3.3. Semen parameter-oriented seminal plasma metabolome

In the seminal plasma metabolome analysis, a total of 5232 and 3496 metabolic features were obtained under positive and negative ion mode, respectively (Supporting Information). To identify the sperm quality-related metabolic biomarkers, PLS-DA models were developed by using various semen parameters as classifiers. Finally, PLS-DA models were successfully established only for sperm concentration and sperm count. As can be seen in Fig. 1, good separations of metabolic profiles were observed between the 1st (highest) and 4th quartile (lowest) groups in sperm concentration model for only positive mode (Fig. 1A), and in sperm count model for both positive and negative modes (Fig. 1B, C). These PLS-DA models were further validated by a strict permutation test (999 random permutations), and no overfitting of the data was observed (Fig. S2), indicating that the models were robust. However, no satisfactory PLS-DA models can be generated for semen volume, progressive motility, total motility or morphology (data not shown), which suggested that the alterations of seminal plasma metabolome in this population may not relate to these semen parameters.

Based on the screening criteria, 16 and 22 biomarkers were selected from sperm concentration and count models, respectively. Ten of these biomarkers were the same and showed similar change trends in both models (Table 4). After adjustment for age, BMI, abstinence time, smoking and alcohol drinking status, it was found that 10 biomarkers were positively associated with sperm concentration and 6 were negatively associated, while 11 biomarkers were negatively related to sperm count and another 11 were positively related. Moreover, most of the biomarkers were monotonically changed (increase or decrease) when the sperm concentration (or count) decreased (Fig. S3), showing that these metabolic alterations were semen quality dependent. These biomarkers indicated that the disturbed metabolic pathways, including fatty acid biosynthesis and metabolism, lipid metabolism, amino acid metabolism, oxidative phosphorylation and steroid hormone biosynthesis are involved in the changes of sperm concentration and count (Table 4).

3.4. ROC analysis

ROC curve is extensively used to evaluate biomarker diagnostic performance. The closer the AUC value approaches to 1, the better diagnostic performance the biomarker provides. Our results showed that eleven concentration-related biomarkers and fifteen sperm count-related biomarkers had AUC values between 0.7 and 0.9 (Table 4), indicating moderate to high discriminating abilities of the biomarkers for sperm concentration and count levels. A multiple biomarker model may provide a better discriminating capability than a single biomarker model (Huang et al., 2018). It was shown that the combination of the top five concentration-related biomarkers (with AUC = 0.852) and the combination of ten count-related biomarkers (with AUC = 0.908) turned out to be the best indicators for sperm concentration and count level, respectively (Fig. 2). A confusion matrix can show the predictive accuracy as the percentage of correctly classified samples in a given class. For the concentration model, the predicative accuracies were calculated as 80.8% and 86.4% for the lowest (1st quartile) and highest (4th quartile) sperm concentration groups, respectively (Fig. 2A); for the count model, the accuracies were 84.6% and 90.9% for the lowest and highest sperm count groups, respectively (Fig. 2B).

3.5. Associations of metabolic biomarkers with arsenic, PAEs and PFCs

The associations of arsenic, PAEs and PFCs with seminal plasma metabolic biomarkers were further investigated by using multiple linear regression analysis adjusting by age, BMI, abstinence time, smoking and alcohol drinking status (Table S3-S5), and the observed significant results were shown in Fig. S4. Among the sperm concentration-related metabolites, a 1-unit increase of DMA was positively associated with a 40.64%, 50.98% and 77.00% increase in L-carnitine, pivaloylcarnitine and docosahexaenoic acid (DHA), respectively (Table S3). In addition, a 22.39% and 69.05% increase in pivaloylcarnitine and glycerophosphocholine (GPC) were associated with a 1-unit increase of PFHxS, respectively (Table S5). However, no sperm count-related metabolites were significantly related to MEHP (Table S4).

3.6. Metabolic biomarkers link pollutant exposure to semen quality

To study the impacts of pollutants on semen quality via metabolic biomarkers, the concentrations of pollutants (i.e., risk factor of DMA and preventive factors of MEHP and PFHxS) and metabolites, as well as sperm concentration and count were dichotomized with a cut-off of the median and then the participants were categorized into low and high groups. Each observation with the binary outcome of low or high semen quality in the pollutant-metabolite two-dimension space was coded in three status, i.e., low risk, medium risk and high risk (Fig. 3A). Binary logistic regression analysis showed that the combinational patterns of DMA with 5 metabolites (1 risk biomarker and 4 preventive biomarkers) and PFHxS with 7 metabolites (2 risk biomarkers and 5 preventive biomarkers) indicated an increased risk of low sperm concentration. For these pollutant-metabolite combinations, the AORs of medium risk combinations significantly increased when compared to their corresponding references but were lower than the utmost risk combinations (Fig. 3B, C; Table S6), suggesting that DMA and PFHxS

Table 4
Seminal plasma metabolites associated with sperm concentration and count.

Biomarkers	VIP score		Fold change[a]		AUC (95% CI)[b]		Correlation with semen quality[c]		Pathway
	Sperm concentration	Sperm count	Sperm concentration	Sperm count	Sperm concentration	Sperm count	Sperm concentration	Sperm count	
N-Acryloylglycine[d]	10.97	13.70	1.20*	1.25*	0.707 (0.560–0.853)*	0.667 (0.513–0.820)*	−0.251*	−0.226*	Fatty acid metabolism
Aminoadipic acid[d]	3.99	3.91	0.14*	0.20*	0.682 (0.528–0.837)*	0.677 (0.523–0.831)*	0.421**	0.331**	Lysine biosynthesis and degradation
L-Carnitine[d]	5.90	5.22	0.50**	0.61**	0.863 (0.749–0.977)**	0.769 (0.632–0.906)**	0.351**	0.295**	Fatty acid metabolism
Pivaloylcarnitine[d]	8.27	5.01	0.50**	0.48**	0.861 (0.740–0.982)**	0.851 (0.731–0.971)**	0.361**	0.344**	Fatty acid metabolism
Glycerophosphocholine[d]	4.93	3.66	0.41**	0.50*	0.748 (0.604–0.892)**	0.700 (0.549–0.851)*	0.430**	0.342**	Glycerophospholipid metabolism
Hydroxyhexanoycarnitine[d]	2.41	2.25	0.66**	0.68*	0.760 (0.619–0.901)**	0.708 (0.559–0.858)*	0.463**	0.455**	Fatty acid metabolism
Docosahexaenoic acid[d]	2.78	2.87	0.47**	0.52**	0.847 (0.724–0.970)**	0.806 (0.673–0.938)**	0.278**	0.300**	Unsaturated fatty acid biosynthesis
Prostaglandin B2[d]	6.71	8.77	1.43**	1.49**	0.752 (0.613–0.890)**	0.797 (0.671–0.923)**	−0.338**	−0.300**	Arachidonic acid metabolism
Eicosatetraenoate[d]	3.12	4.08	1.41**	1.49**	0.748 (0.609–0.888)**	0.793 (0.666–0.920)**	−0.335**	−0.299**	Arachidonic acid metabolism
Tocotrienol[d]	2.16	2.39	0.26**	0.28**	0.839 (0.718–0.960)**	0.828 (0.705–0.952)**	0.362**	0.373**	Ubiquinone biosynthesis
Imidazole-4-acetaldehyde[e]	2.90		0.78*		0.694 (0.544–0.845)*		0.306**	0.156	Histidine metabolism
L-Lysine[e]	2.11		0.09**		0.757 (0.620–0.893)**		0.254*	0.087	Lysine biosynthesis and degradation
Malonylcarnitine[e]	2.59		2.63*		0.684 (0.530–0.838)*		−0.247*	−0.257*	Fatty acid metabolism
5-Aminoimidazole ribonucleotide[e]	2.61		0.34*		0.712 (0.559–0.865)*		0.431**	0.385**	Purine nucleotide biosynthesis
Tryptophyl-Aspartate[e]	2.53		2.08*		0.684 (0.534–0.834)*		−0.294**	−0.204	
11b-Hydroxyprogesterone[e]	2.44		1.32*		0.670 (0.513–0.827)*		−0.270**	−0.189	Steroid hormone biosynthesis
Uracil[f]		7.62		2.13**		0.759 (0.611–0.907)**	−0.160	−0.214*	β-Alanine metabolism
Capryloylglycine[f]		3.04		0.34*		0.696 (0.545–0.847)*	0.295**	0.244*	Mitochondrial fatty acid beta-oxidation
Isobutyryl-L-carnitine[f]		10.19		0.57*		0.681 (0.529–0.832)*	0.301**	0.308**	Fatty acid metabolism
Imidazoleacetic acid riboside[f]		6.74		1.33*		0.708 (0.561–0.856)*	−0.274**	−0.296**	Histidine metabolism
Glucosamine 6-phosphate[f]		2.26		1.33*		0.708 (0.561–0.856)*	−0.218*	−0.257*	Aminosugar metabolism
Tyramine glucuronide[f]		2.19		2.38*		0.677 (0.525–0.829)*	−0.216*	−0.210*	Tyramine metabolism
Ubiquinone-2[f]		2.27		3.57**		0.840 (0.725–0.956)**	−0.151	−0.226*	Oxidative phosphorylation
Prostaglandin E2[f]		2.31		1.47**		0.766 (0.628–0.903)**	−0.330**	−0.288**	Arachidonic acid metabolism
Glutaconic acid[f]		5.15		0.56*		0.684 (0.533–0.835)*	0.383**	0.409**	
3-Oxohexanoic acid[f]		3.26		1.23*		0.677 (0.525–0.830)*	−0.209*	−0.212*	Fatty acid biosynthesis
LysoSM(d18:1)[f]		2.01		0.24**		0.842 (0.726–0.958)**	0.522**	0.552**	Sphingolipid metabolism
PS[f]		2.32		1.72**		0.738 (0.595–0.881)**	−0.323**	−0.286**	Glycerophospholipid metabolism

[a] Fold change of metabolite abundance is expressed as the average ratio of low semen quality group/high semen quality group.
[b] Area under curve (AUC) derived from ROC analysis. CI = confidence intervals.
[c] Partial correlation analysis was performed to investigate the associations between the biomarkers and semen quality after adjustment by age, BMI, abstinence time, smoking and alcohol drinking status.
[d] Screened from both sperm concentration and count models.
[e] Screened from sperm concentration model.
[f] Screened from sperm count model.
* $p < 0.05$.
** $p < 0.01$.

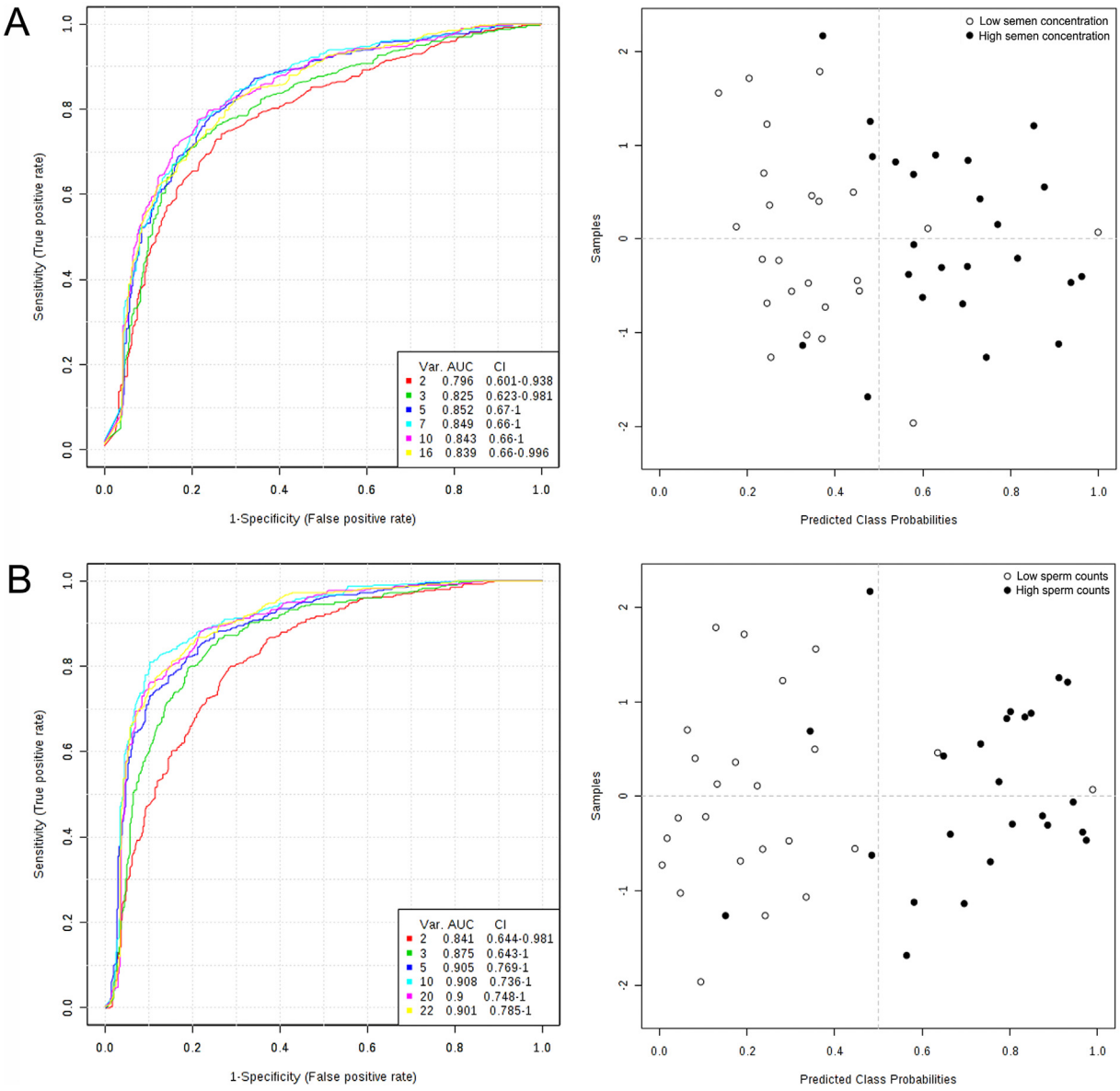

Fig. 2. ROC curves and probability views of the combined biomarker patterns for sperm concentration (A) and sperm count (B). ROC curves were generated by Monte Carlo cross validation using balanced subsampling. The predicted class probabilities were calculated for each sample using the developed ROC models.

(but not MEHP) and metabolites performed their actions on sperm concentration dependently.

The mediation effects of the metabolic biomarkers (Fig. 3) between the pollutants (i.e., DMA and PFHxS) and semen quality were further calculated by causal mediation analysis. As shown in Fig. 4, among the 7 metabolites, 25.1%, 17.0% and 18.7% of the positive association between PFHxS and sperm concentration was mediated by pivaloylcarnitine, eicosatetraenoate and tocotrienol, respectively. However, no significant mediation effects of the 5 metabolites were found between DMA and sperm concentration (data not shown).

4. Discussion

Although the statistical associations have been widely studied, the molecular linkages between general arsenic, PAE and PFC exposure and human semen quality are still rarely elucidated. In the current study, by conducting an untargeted metabolomics analysis of human seminal plasma, we identified a set of metabolites related to sperm concentration and count. In addition, the observed mediation effects of metabolic biomarkers on the associations between pollutants and semen quality may enable us to partly understand the modes of action, via which arsenic and PFC exposure affects sperm concentration in men.

4.1. General arsenic, PAE and PFC exposure associated with human semen quality

A myriad of epidemiological studies have reported the associations between various environmental pollutants and human semen quality but have shown inconsistent findings. In our study, a negative association was found between DMA and sperm concentration, while MEHP and PFHxS were positively associated with sperm count and concentration, respectively. In support of our findings, two previous studies of Chinese men also found the associations between DMA/total arsenic and reduced sperm concentration (Xu et al., 2012; Li et al., 2012). However, other reports showed different results in which no significant associations were found between arsenic exposure and semen quality (Zeng et al., 2015; Oguri et al., 2016). The mechanisms underlying the link between arsenic exposure and reduced semen quality has been primarily derived from animal studies, and the diminished androgen production caused by arsenic might be one of the reasons for semen

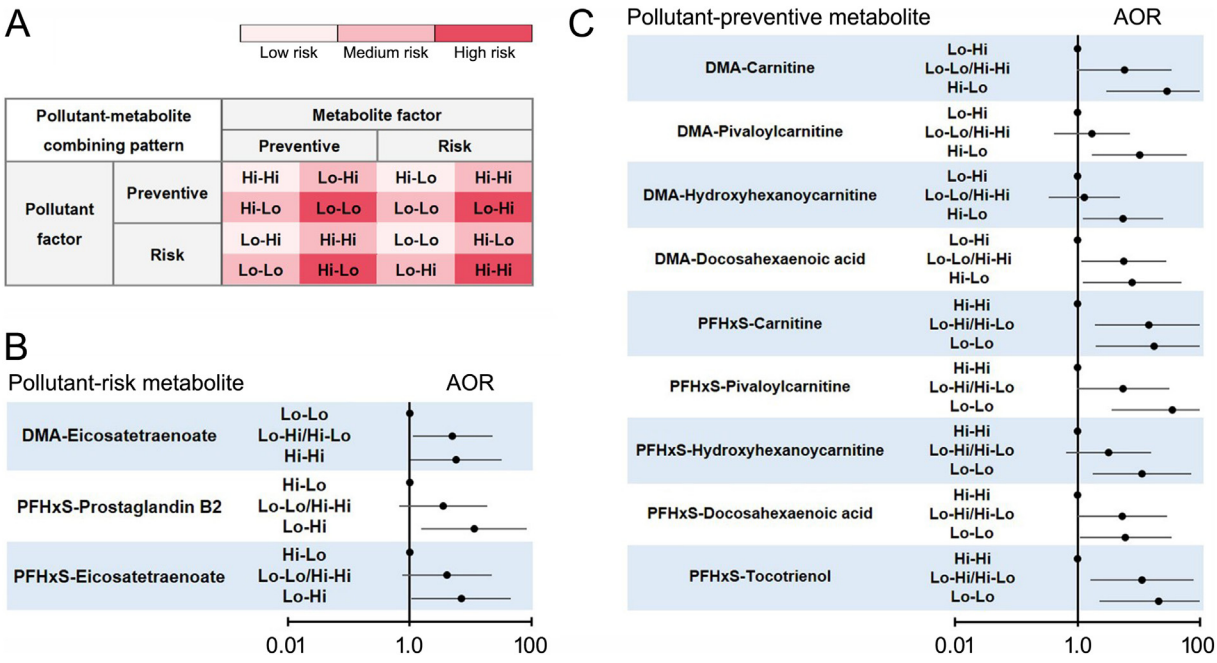

Fig. 3. Pollutant-metabolite interactions on low-sperm-concentration risk. (A) pollutant-metabolite combination patterns. Factors of pollutants and metabolites are categorized into the high level (Hi) and low level (Lo) subsets by their concentration medians. The risk factor assigned with Hi or Lo indicated a higher risk or lower risk, while the preventive factor assigned with Hi or Lo indicated a lower risk or higher risk, respectively. Each observation with the binary outcome of low or high sperm concentration in the pollutant-metabolite two-dimension space was coded into three status, i.e., low risk, medium risk and high risk. The adjusted odds ratio (AOR) was used to express the relative risk of low-sperm-concentration. The AOR was analyzed by using a binary logistic regression model adjusting for age, BMI, abstinence time, smoking and alcohol drinking status. The low risk was defined as AOR = 1. (B) pollutant-risk metabolite patterns; (C) pollutant-preventive metabolite patterns.

quality reduction (Huang et al., 2016).

Extensive reports have also demonstrated inverse correlations between phthalate exposure and semen quality. Although it was revealed that MBP and MEHHP were significantly associated with a lower total sperm count (Wang et al., 2015a; Bloom et al., 2015), MEHP was more frequently found to be negatively associated with sperm motility (Wang et al., 2016; Cai et al., 2015; Jurewicz et al., 2013). Thus, our observation regarding the positive correlation between MEHP and sperm count was not in line with most of these findings but was consistent with a positive relationship between DEHP and sperm motility found by Bloom et al. (2015). Similarly, in very few studies of the associations between PFCs and semen quality, only PFOA was found to be inversely related to sperm concentration (Vested et al., 2013), and no significant associations of PFHxS with semen quality were reported hitherto. However, our result may be supported by Toft et al. (2012) who found that PFOA was associated with higher sperm motility in an arctic population.

One plausible explanation for the observed positive associations between MEHP and PFHxS concentrations and semen quality may lie in different pollutant exposure levels and sample sizes between various studied populations. The preventive effects of pollutants on semen quality may be ascribed to low-level exposure, while the effects could

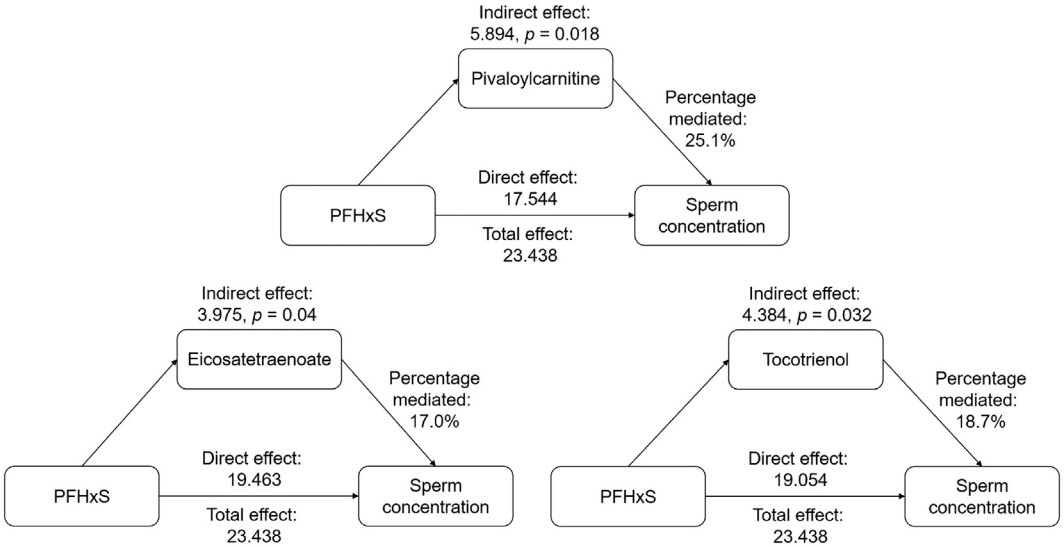

Fig. 4. Mediation effects of metabolic biomarkers (pivaloylcarnitine, eicosatetraenoate and tocotrienol) on the associations between PFHxS exposure and sperm concentration. Mediation effect (indirect effect) with $p < 0.05$ was considered as statistically significant.

be reversed when higher-level doses are applied due to hormesis (Calabrese, 2008). Actually, the median urinary concentrations of MEHP in our participants (0.5 μg/L, unadjusted by creatinine) were much lower than those of previous studies (ranged from 6.1 to 10.1 μg/L), where the inverse associations were observed (Duty et al., 2004; Jurewicz et al., 2013; Wirth et al., 2008). This can be supported by our previous findings that low-level phthalate exposure elevated androgen levels in a male population (Tian et al., 2018) and that DEHP metabolites were positively associated with preventive metabolic markers of male fertility, which may contribute to a decreased risk of male infertility (Liu et al., 2017). Moreover, our recent study also found that low-dose PFOA stimulated reproductive hormone production while a higher dose reduced hormone levels in Leydig cells (Tian et al., 2019), which may provide evidence in support of a positive association between PFHxS and semen quality.

4.2. Predictabilities of seminal plasma metabolites for semen quality

In the present study, 16 and 22 metabolites in human seminal plasma were identified to be related to sperm concentration and count, respectively (Table 4). ROC curve analysis is widely accepted as the most objective and statistically valid method in defining the clinical utility of a biomarker. A biomarker with AUC > 0.7 is usually acceptable for most clinical applications (Zhang et al., 2014a, 2014b). Here, eleven of the sixteen sperm concentration-related biomarkers had AUC values > 0.7, indicating their moderate to high diagnostic power for sperm concentration. The combination of five biomarkers (AUC = 0.852) showed a great capability in the evaluation of sperm concentration. In addition, there were fifteen sperm count-related biomarkers with AUC > 0.7, and the combination of the top ten metabolites gave an AUC of 0.908, which also suggested the great power of these metabolites in sperm count assessment. Taken together, a series of metabolic biomarkers identified in this study are proposed to have great potentials in evaluating human semen quality (sperm concentration and count).

4.3. Metabolic biomarkers link DMA and PFHxS to sperm concentration

In this study, the identified sperm concentration and count-related metabolites were mainly associated with lipid metabolism, fatty acid biosynthesis and metabolism as well as amino acid metabolism, and they were found to be closely involved in a metabolic pathway network (Fig. S5). In view of the significant associations found between DMA and sperm concentration, between MEHP and sperm count, and between PFHxS and sperm concentration, we further investigated the potential roles of metabolic biomarkers in these associations. However, only the mediation effects of biomarkers between DMA, PFHxS and sperm concentration were observed (Figs. 3 and 4), and most of these biomarkers were involved in fatty acid metabolism. We found that the reduced L-carnitine, pivaloylcarnitine, hydroxyhexanoycarnitine and DHA, as well as the elevated eicosatetraenoate, were associated with an increased risk of low-sperm-concentration for DMA exposure. Conversely, the higher levels of carnitines, DHA and tocotrienol, together with the decreased eicosatetraenoate and prostaglandin B2 (PGB2), would contribute to the positive effect of PFHxS on sperm concentration. The casual mediation analysis further confirmed that pivaloylcarnitine, eicosatetraenoate and tocotrienol mediate the association between PFHxS and sperm concentration.

Carnitine is an essential factor for fatty acid metabolism by transporting long-chain acyl-CoA into mitochondria. Infertile patients were reported to have lower levels of carnitine in seminal plasma and urine, and a positive correlation between carnitine levels and sperm concentration has been found (Agarwal and Said, 2004; Ruiz-Pesini et al., 2000; Shen et al., 2013). Sperm cells have high levels of omega-3 and -6 polyunsaturated fatty acids (PUFAs) crucial for sperm membrane stability, which confer sperm motility and fertilizing capacity (Amaral et al., 2016). Prostaglandins are a subclass of the lipid mediator group known as eicosanoids derived from C-20 PUFAs like arachidonic acid (AA), and eicosatetraenoate is a long-chain fatty acid related to AA metabolism. Docosahexaenoic acid (DHA) is also a major PUFA in sperm phospholipids. It was reported that prostaglandins are present in semen with high concentrations and that prostaglandin E2 (PGE2) affects sperm motility (Colon et al., 1986). In addition, DHA supplementation fully restores the sperm count and spermatogenesis in male delta-6 desaturase-null mice, while insufficient DHA causes asthenozoospermia with hypomotility and infertility (Roqueta-Rivera et al., 2010; Lenzi et al., 2000). It was shown that increased PUFAs and their metabolites promote sperm fertility (Murase et al., 2016). A recent study also reported that human semen quality is related to PUFA and acylcarnitine metabolism in seminal plasma (Wang et al., 2019). Here, the elevation of PGB2, PGE2 and eicosatetraenoate, as well as the depletion of L-carnitine, pivaloylcarnitine, hydroxyhexanoycarnitine, isobutyrylcarnitine and DHA indicate the disruption of fatty acid metabolism in seminal plasma, which may reduce human semen quality.

Tocotrienols are members of the vitamin E family. As an antioxidant, vitamin E has a preeminent role in protecting sperm membranes against reactive oxygen species (ROS) and lipid peroxidation, which is important for spermatogenesis and sperm maturation (Amaral et al., 2016). It has been demonstrated that vitamin E supplementation improves semen quality, including sperm count (Yue et al., 2010; Suleiman et al., 1996). In addition, carnitine is highly concentrated in the epididymis, and has important roles in sperm maturation and maintenance of sperm quality due to its antioxidant properties. Therefore, the decreased tocotrienol and carnitine imply that oxidative stress occurred in men with a lower sperm concentration.

Our previous studies have pointed out that arsenic exposure alters the levels of carnitines in rat serum and testis (Wang et al., 2015b; Huang et al., 2016). It was also found that carnitines are regulated by PFOA and that AA is negatively associated with PFC exposure in humans (Peng et al., 2013; Wang et al., 2017b). Moreover, there were reports suggesting that arsenic and PFOA exposure disrupted fatty acid metabolism (Jia et al., 2019; Shao et al., 2018). Carnitine and tocotrienol are antioxidants, and carnitine, acyl-carnitines, PGB2, eicosatetraenoate and DHA are all involved in fatty acid β-oxidation (providing a large amount of energy for human body). Since most of these metabolites were decreased in the subjects with lower semen quality, we propose that DMA exposure may reduce sperm concentration via lowering the participants' antioxidative capacity and decreasing sperm energy generation. Consistently, it has been found that urinary metabolic biomarkers (e.g., carnitines) link arsenic-induced oxidative stress to male infertility (Shen et al., 2013). Nevertheless, through enhancing sperm antioxidative capacity and energy production, low-level PFHxS exposure could contribute to the promotion of sperm concentration.

4.4. Limitations

To our best knowledge, this work is the first data-driven epidemiological study to investigate the potential roles of seminal plasma metabolites in mediating the associations between multiple pollutants exposure (i.e., arsenic, PAEs and PFCs) and human semen quality. However, our study still has some limitations. Firstly, the participants may contain some subfertile men, thus limiting the possible generalization of our results to broader populations. In addition, although we have observed some significant associations, the sample size is relatively small, which could weaken the statistical correlations. To avoid decreasing the sample size and lowering the statistical power, we used different sample sizes to explore the associations of pollutant exposures with semen quality, the associations of pollutant exposures with seminal plasma metabolites, and the mediation effects of metabolites, respectively. Finally, the urine, blood and seminal plasma samples were only collected once for pollutant measurements and metabolomics, which could lead to exposure misclassification and biased estimation

for certain associations (Wang et al., 2018). Therefore, our findings should be verified in larger and diverse populations with more than one time point of sample collection.

In conclusion, we found that a higher level of DMA was associated with a decreased human sperm concentration, whereas MEHP and PFHxS exposure was positively correlated with sperm count and concentration, respectively. Through metabolomics analysis of seminal plasma, a series of metabolites related to sperm concentration and count were identified. We further revealed that several seminal plasma metabolites mediate the negative association between DMA and sperm concentration, and the positive association between PFHxS and sperm concentration, which suggests that general DMA and PFHxS exposure may affect human semen quality by shifting oxidation status and energy supply in seminal plasma. Our study could shed new light on the potential mechanisms linking environmental pollutant exposure to health outcomes based on the general framework of molecular epidemiology (Schulte et al., 2011).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105066.

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