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Yi Xin Wang
Tongji Medical College

Yan Wu
Chinese Academy of Sciences

Heng Gui Chen
Tongji Medical College

Peng Duan
Hubei University of Medicine

Liang Wang
Harvard T.H. Chan School of Public Health, wangl2@etsu.edu

See next page for additional authors

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Creator(s)

Yi Xin Wang, Yan Wu, Heng Gui Chen, Peng Duan, Liang Wang, He Qing Shen, Wen Qing Lu, Bin Sun, Qi Wang, Bo Zhang, Jorge E. Chavarro, Jie Zhang, and An Pan

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Yi-Xin Wang[a,b,1], Yan Wu[c,1], Heng-Gui Chen[a], Peng Duan[d], Liang Wang[b,e], He-Qing Shen[c], Wen-Qing Lu[a], Bin Sun[a], Qi Wang[f], Bo Zhang[b,g], Jorge E. Chavarro[b], Jie Zhang[c,h,**], An Pan[a,*]

[a] Department of Epidemiology and Biostatistics, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Departments of Nutrition, Harvard TH Chan School of Public Health, Boston, MA, USA
[c] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China
[d] Center for Reproductive Medicine, Xiangyang No. 1 People's Hospital, Hubei University of Medicine, Xiangyang, China
[e] Department of Biostatistics and Epidemiology, College of Public Health, East Tennessee State University, Johnson City, TN, USA
[f] Department of Pathology, Bengbu Medical College, Anhui, China
[g] Department of Biochemistry, Fukuoka University School of Medicine, Fukuoka, Japan
[h] School of Public Health, Xiamen University, Xiamen, China

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ABSTRACT

Background: A growing body of evidence has found links between endocrine disruptor phthalates and male reproductive disorders, but the mechanisms underlying these relationships are poorly known. Seminal plasma metabolomes may mediate associations of phthalate exposure with impaired semen quality.

Objective: To identify seminal plasma metabolomes associated with poor semen quality and evaluate their associations with urinary phthalate metabolites among 660 Chinese adult men.

Method: The seminal plasma metabolic profiles were acquired using an untargeted approach based on liquid chromatography-high resolution mass spectrometry. We explored the differences in seminal plasma metabolites between participants with poor and good semen quality and evaluated cross-sectional associations between discriminatory metabolic biomarkers and urinary phthalate metabolites.

Results: Differences between poor and good semen quality groups were observed in relation to 25 seminal plasma metabolites, mostly related to the metabolism of polyunsaturated fatty acids (PUFA) and acylcarnitine (all $p < 0.05$). After adjusting for various confounders and multiple tests, metabolites were all significantly associated with one or more individual sperm quality parameters (motility, concentration, total count, and morphology) (all $p < 0.05$). Among identified metabolic biomarkers, seminal plasma L-palmitoylcarnitine, linoelaidyl carnitine, and oleic acid were inversely associated with urinary mono-(2-ethylhexyl) phthalate (MEHP), and seminal plasma L-acetylcarnitine was inversely associated with the proportion of di-(2-ethylhexyl)-phthalate metabolites (DEHP) excreted as MEHP in urine (%MEHP) (all $p < 0.05$). Mediation analysis revealed that oleic acid and L-acetylcarnitine mediated significant proportions (6.7% and 17%, respectively) of the positive associations between urinary DEHP metabolites and the percentage of spermatozoa with an abnormal head.

Conclusions: Elevated urinary phthalate metabolites may impact semen quality by causing metabolic disorders of seminal plasma PUFAs and acylcarnitine. These pathways warrant further investigation.

1. Introduction

A recent systematic review reported a significant decline in sperm count and concentration among men from North America, Europe, and Australia between 1973 and 2011 (Levine et al., 2017); these trends were consistent with the findings from many retrospective studies in various countries (Almagor et al., 2003; Feki et al., 2009; Huang et al., 2017; Jorgensen et al., 2011; Minguez-Alarcon et al., 2018; Rolland

* Correspondence to: An Pan, Department of Epidemiology and Biostatistics, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.
** Correspondence to: Jie Zhang, School of Public Health, Xiamen University, Xiamen, China.
E-mail addresses: jie.zhang@xmu.edu.cn (J. Zhang), panan@hust.edu.cn (A. Pan).
[1] Authors equally contributed.

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et al., 2013; Shine et al., 2008; Adiga et al., 2008). Although the causes of global declines in semen quality are debated (Bonde and Te Velde, 2017), growing evidence indicates that environmental endocrine disrupting chemicals may partly contribute to these trends, as well as similar changes observed in other male reproductive disorders (e.g., low testosterone, testicular cancer, cryptorchidism, and hypospadias) (Skakkebaek et al., 2016).

Phthalates are a class of chemicals that possess endocrine-disrupting properties and are widely used as plasticizers. In 2009, the estimated production of phthalates was 6.2 million tons worldwide, of which > 1.0 million were manufactured in China (Qian, 2010). As a result, these compounds are widespread in environmental media and human biospecimens (Guo et al., 2011). Toxicological studies have found that certain phthalates, including di(2-ethylhexyl) phthalate (DEHP), butyl benzyl phthalate (BBzP), di-*n*-butyl phthalate (DBP), and diisononyl phthalate (DiNP), act as endocrine disruptors, leading to testicular toxicity in different experimental animal models (Foster et al., 2001; Gray et al., 2000). Recently, we conducted a large cross-sectional study among 1040 nonoccupational Chinese men whose exposure levels were assessed using phthalate metabolites in repeated urine specimens (Wang et al., 2015). We found that higher monobutyl phthalate (MBP) was associated with decreasing sperm concentration and total count and that higher levels of di-(2-ethylhexyl)-phthalate (DEHP) metabolites were associated with increasing percentage of abnormal head; these findings support prior toxicological evidence (Bao et al., 2011; Erkekoglu et al., 2011; Foster et al., 2001), and epidemiological results (Duty et al., 2003; Hauser et al., 2006; Liu et al., 2012; Pant et al., 2008; Zhang et al., 2006). However, the mechanisms underlying these associations are poorly understood.

Metabolic profiles (or metabolomes) reflect the distribution of small molecules in biological specimens that are intermediates or endpoints of metabolism (Nicholson et al., 2002; Nicholson and Wilson, 2003). An individual's metabolome can be used to identify biomarkers of toxicity and pathways of adverse outcomes resulting from transcriptional and translational changes. Emerging human and animal evidence suggests that exposure to phthalates at relatively low levels can adversely affect urinary and blood metabolic profiles (Houten et al., 2016; Liu et al., 2017; Manteiga and Lee, 2017; Shen et al., 2017; Zhang et al., 2016). Seminal metabolic profiles play important roles in energy production, protection, motility, and regulation of metabolic activity for spermatozoa (Bieniek et al., 2016) and are increasingly used for the evaluation of human infertility (Gilany et al., 2014; Gilany et al., 2017; Jafarzadeh et al., 2015; Jayaraman et al., 2014). However, these studies were based on small sample size that may have been insufficient to generate precise estimations. Additionally, associations between phthalate exposure and seminal plasma metabolites have never been investigated.

In this study, we sought to identify seminal plasma metabolites related to poor semen quality using an untargeted approach based on liquid chromatography (LC)-high resolution mass spectrometry (HRMS) among 660 Chinese adults with well-characterized phthalate exposure. We further evaluated associations between discriminatory metabolic biomarkers and urinary phthalate metabolites and assessed the mediating role of seminal plasma metabolome in the associations between urinary phthalate metabolites and semen quality.

2. Materials and methods

2.1. Study volunteers

The protocol of this study was authorized by the Ethics Committee of Tongji Medical College. This cross-sectional study was conducted between March and June 2013, as described previously (Wang et al., 2015). Eligible volunteers of this study were male partners in couples who came to the Reproductive Medicine Centre of Wuhan Tongji Hospital for semen quality inspection without knowledge of their fertility status. Anyone who met this criterion was invited to participate. A total of 1490 men were invited during the study period, of which 1247 ultimately agreed (participation rate: 83.7%). Refusal to participate in the study was mostly due to time constraints. After providing written informed consent, the volunteers were required to have a blood sample drawn, provide two spot-urine specimens (at least 2 h apart) and a semen sample, and complete a questionnaire under the guidance of trained research staff. The information collected included demographic characteristics [e.g., age, body mass index (BMI) and abstinence time], lifestyle factors (e.g., smoking status and drinking habits), medical history, and occupational exposure. Of the 1247 men enrolled, 149 men were excluded due to missing urine samples (n = 22), self-reported diseases (e.g., testis injury, epididymitis, vasectomy, orchiditis, diabetes and adrenal disorder) that may cause male reproductive dysfunction (n = 121) and occupational exposure to synthetic materials that contain phthalates (n = 6). We further excluded 58 azoospermic men because this condition might be related to either a Y chromosome deletion or obstruction. Out of the 1040 remaining participants, 660 had adequate semen volumes (usually > 1.5 mL) for determination of seminal plasma metabolome. We explored potential selection bias in a subanalysis by comparing the demographic characteristics between the entire population and the men included in our present analysis.

2.2. Urine collection and measurement

Because urinary phthalate metabolite concentrations exhibited a high intraday but limited interday variation, repeated urine specimens (separated by a mean interval: 4.4 ± 3.7 h; range: 2.0–11 h) were thereby gathered from each volunteer on a given day. Concentrations of monomethyl phthalate (MMP), monoethyl phthalate (MEP), MBP, monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-n-octyl phthalate (MOP) were detected using LC-MS with preceding enzymatic deconjugation and solid phase extraction, which has been reported in our prior study (Wang et al., 2015). Urinary creatinine in repeated specimens was measured to correct for urine dilution using a clinical chemistry analyzer (Wang et al., 2015).

2.3. Semen collection and analysis

The participants ejaculated semen specimens into a sterile polyethylene cup by masturbation in a private chamber next to an andrology laboratory. After liquefaction, semen quality parameters, including sperm concentration, progressive motility, total motility, total count, percentage of normal morphology and the percentage of spermatozoa with an abnormal head, were analyzed according to a WHO (2010) laboratory manual, which has been described in our prior study (Wang et al., 2016a).

2.4. Determination of seminal plasma metabolites

After determination of semen quality, the remaining semen samples were centrifuged at 15,000 rpm/min for 5 min. The resulting seminal plasma were subpacked and stored at −80 °C until determination of seminal plasma metabolites. A 200 μL of seminal plasma was added to precooled methanol (−20 °C) and shaken vigorously for 1 min. Next, the mixture was sonicated in cold water (4 °C) for 10 min, and then centrifuged at 12,000*g* for 15 min at 4 °C. The resulting supernatant was collected and dried, and then redissolved with a 100 μL solution of 50% (*v*/v) methanol. Seminal plasma metabolomes were detected using a LC coupled with Q Exactive™ high-resolution mass spectrometry (HRMS) system (Thermo Scientific, Waltham, MA, USA). Chromatographic separations were performed on an ACQUITY UPLC HSS T3 column (1.8 μm, 100 mm × 2.1 mm ID) (Waters, Milford, MA, USA), using the following gradient: 0.1% B at 0 min, 6% B at 2 min, 20% B at 11 min, 60% B at 24 min, 99.9% B at 30 min, 99.9% B at 32 min, 0.1% B at

32.2 min and 0.1% B at 35 min (mobile phase A: ultrapure water with 0.1% formic acid; phase B: methanol with 0.1% formic acid). The flow rate was 0.4 mL/min. The mass spectrometer was operated on a positive-negative ion switching mode with a scan range of 100 to 1000 *m/z*. To identify potential biomarkers, the MS analysis was conducted with 1×10^5 automatic gain control (AGC) target, the collision gas was nitrogen, and the collision energy ranged from 25 to 45 eV to obtain comprehensive MS information. To test the stability and reproducibility of retention time and mass measurement for the sequence, a blank specimen (pure acetonitrile) and quality control (QC) samples (mixed aliquots of each sample) was prepared and determined each 25 samples during the whole sequence, as previously described (Zhang et al., 2014; Zhang et al., 2016). The blank sample was used to monitor sample carryover. A tight QC clustering was shown in the scores plot on principal component analysis (see Supplemental Material Fig. S1).

2.5. Metabolome data processing

Metabolome data were processed with Compound Discovery (version 2.1; Thermo Scientific, Waltham, MA, USA) for retention time alignment, compound area correction and metabolite identification, according to the manufacturer protocols. A metabolic feature table was exported and processed with a quality control-based robust loess signal correction (Dunn et al., 2011). To reduce the variations resulting from sample injection and enrichment factor (related to semen volume), we further normalized the intensity of extracted variables (metabolome concentrations) using a probabilistic quotient normalization method, in which the total spectral area of sample was assumed to be constant and the intensity of extracted variables was normalized according to the total spectral area (Dieterle et al., 2006).

Multivariable analyses were conducted using SIMCA-P (version 14; Umetrics, Umea, Sweden). The participants were classified as good semen quality groups (total motility > 50%, sperm concentration > 40 million/mL and morphology > 9%) or poor semen quality groups (total motility < 32%, and/or concentration < 15 million/mL, and/or normal morphology < 4%); all other volunteers were categorized as intermediate semen quality group (Damsgaard et al., 2016). The orthogonal projections to latent structures discriminant analysis (OPLS-DA) model was used to screen the differences in seminal metabolic profiles between the poor and good semen quality groups. We randomly selected 80% of the samples (n = 528) as a training set; the other 20% of samples (n = 132) were used as a test set to validate the predictive ability of the model (Cuadros-Inostroza et al., 2010). The metabolic features with variable influence on projections (VIP) > 1.0 and jackknifing confidence intervals > 0 were considered to be potential biomarkers, which were further identified by mzCLOUND results from Compound Discovery, or by comparing the Human Metabolome Database (http://www.hmdb.ca).

2.6. Data analyses

%MEHP, calculated as the proportion of total molar concentrations of DEHP metabolites (MEHP, MEHHP and MEOHP) excreted as MEHP, is a marker of DEHP metabolism for “less toxic” metabolites (Joensen et al., 2012). Urinary phthalate metabolite concentrations were log-transformed and then averaged to represent volunteers' exposure levels. We calculated the intraclass correlation coefficients (ICCs) to characterize the variability of repeated phthalate metabolite measures using multilevel mixed models. Differences in demographic characteristics between the subjects of our current analysis and the entire population were analyzed using Chi-square tests or independent sample *t*-test where appropriate. The Mann-Whitney test and multivariable logistic models were used to evaluate differences in metabolic features between the poor and good semen quality groups. We also explored the association between identified seminal plasma metabolites and each individual sperm-quality parameter using multivariable logistic models, where subjects were dichotomized as either below or at/above WHO reference levels for progressive sperm motility (32%), total motility (40%), concentration (15 million/mL) and total count (39 million) (WHO, 2010). Subjects who had values equal to or greater than the WHO reference levels for all 4 parameters were treated as the comparison group (WHO, 2010). Linear regression models were applied to explore the associations between identified seminal plasma metabolomes and continuous measures of semen quality parameters. The false discovery rate (FDR) correction was applied to correct for multiple-hypothesis tests (Pike, 2011). For seminal plasma metabolites meeting the p value threshold after accounting for multiple tests, we further constructed multivariable linear regressions to explore their associations with average urinary phthalate metabolites.

Age, BMI, liquefaction time, smoking status, daily cigarette consumption, drinking status, education background, and having ever fathered a pregnancy were retained as potential confounders if they caused a > 10% change in effect estimations of the associations of seminal plasma metabolomes with sperm-quality parameters or urinary phthalate metabolites in multivariable models (Greenland, 1989). To keep consistency, all models were finally adjusted for a same set of covariates. Average urinary creatinine concentrations were included as a covariate to account for urinary dilution where appropriate.

To determine if seminal plasma metabolome mediated the associations of urinary phthalate metabolites and semen quality parameters, we performed a mediation analysis (Tingley et al., 2014). We decomposed the total effect of urinary phthalate metabolites on semen quality into two components: the natural direct effect (the effect of phthalate metabolites on semen quality that was not mediated through changing seminal plasma metabolome) and the natural indirect effect (the effect that was mediated through changing seminal plasma metabolome). The mediated percentage was calculated by dividing the natural indirect effect by the total effect. All data analyses were performed using the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC, USA) or the R software (R Foundation for Statistical Computing, Austria). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the selected subjects

The demographic characteristics of the 660 men whose seminal plasma metabolomes have been analyzed are shown in Table 1. Volunteers were mostly of Han ethnic background, with a mean (± SD) age of 32 (± 5.5) years and BMI of 23 (± 3.1) kg/m^2. Most men (99%) were married, and 263 (40%) had fathered a child. Half of the participants currently smoked, and 394 (60%) had a weekly intake of alcohol. There was no significant difference in demographic characteristics between the men of our current analysis and the entire study sample. Semen quality parameters of the 660 men are shown in Table 2. The number of men who had poor, intermediate, and high good semen quality was 140, 300, and 220, respectively.

3.2. Urinary phthalate metabolites

The distribution and reproducibility of urinary phthalate metabolites in repeated specimens are presented in Table 3. MMP, MEP, MBP, MBzP, MEHP, MEHHP, and MEOHP were detected in > 90% of the specimens. The detection rate for MOP was relatively low (< 15%); thus, data for MOP were not analyzed subsequently. Concentrations of MBP and MEHHP in repeated urine samples showed excellent reproducibility (ICCs = 0.75 and 0.72, respectively), while MMP showed poor reproducibility (ICC = 0.26). The reproducibility of urinary MEP, MBP, MBzP, MEHP, MEOHP, and %MEHP were fair-to-good (ICCs = 0.52–0.63).

Table 1
Comparison of the characteristics of the study population [*n* (%) or mean ± SD].

Characteristic	The men of present study[a] (*n* = 660)	Total population[b] (*n* = 1040)
Age, years	32 ± 5.5	32 ± 5.4
BMI, kg/m^2	23 ± 3.1	23 ± 3.2
Daily cigarette consumption	7.4 ± 8.3	7.3 ± 8.4
Abstinence time, days		
< 3	64 (10%)	119 (12%)
3	153 (23%)	255 (25%)
4	156 (24%)	231 (22%)
5	123 (19%)	183 (17%)
> 5	164 (24%)	251 (24%)
Race		
Han	643 (97%)	1013 (97%)
Other	17 (3%)	27 (3%)
Marital status		
No	6 (1%)	15 (1%)
Yes	654 (99%)	1025 (99%)
Ever fathered a child		
No	394 (60%)	615 (60%)
Yes	263 (40%)	419 (40%)
Education level		
Less than high school	261 (40%)	390 (38%)
High school and above	391 (60%)	641 (62%)
Smoking status		
Never	249 (38%)	406 (39%)
Former	81 (12%)	113 (11%)
Current	330 (50%)	521 (50%)
Alcohol use		
No	266 (40%)	405 (39%)
Yes	394 (60%)	635 (61%)
Income, RMB yuan/month		
< 3000	297 (45%)	456 (44%)
3000–6000	251 (38%)	397 (38%)
> 6000	111 (17%)	185 (18%)

[a] A total of 2 subjects had missing information on age; 3, on the history of a successful child; 8, on the education level; and 1, on income.
[b] A total of 2 subjects had missing information on age; 6, on the history of a successful child; 1, on abstinence time; 9, on the education level; and 2, on income.

3.3. Seminal plasma metabolic biomarkers

A total of 2770 and 2642 metabolic features were extracted with electrospray ionization (ESI) positive and negative modes, respectively. For the training set, the poor and good semen quality groups showed good separation in OPLS-DA scoring plots (Fig. 1A and B). The proportion of men in the test set who had poor and good semen quality in the positive modes and who were correctly predicted as such using the OPLS-DA model based on the training set were 69% and 70%, respectively (Fig. 1C). In the negative nodes, the proportions were 71% and 60%, respectively (Fig. 1D).

Table 2
Semen quality parameters of the study population (*n* = 660)[a].

Semen quality parameters	Arithmetic mean ± SD	Median (25th, 75th)	Range
Volume (mL)	3.4 ± 1.6	3.0 (2.0, 4.0)	0.5–12
Concentration (10^6/mL)	49 ± 32	42 (25, 64)	3.4–211
Progressive motility (%)	43 ± 18	42 (31, 57)	0–94
Total motility (%)	50 ± 20	49 (37, 67)	0–96
Total count (10^6/ejaculate)	153 ± 111	119 (68, 188)	9.7–828
Normal morphology (%)	20 ± 8.4	21 (15, 24)	0–53
Percent of abnormal head	66 ± 10	65 (59, 76)	0–95

[a] The number of participants who had poor (total motility < 32%, and/or concentration < 15 million/mL, and/or normal morphology < 4%), intermediate and good semen quality (total motility > 50%, sperm concentration > 40 million/mL and morphology > 9%) were 140, 300, and 220, respectively.

A total of 35 seminal plasma metabolites were identified as potential biomarkers of low semen quality (Table 4). Among them, higher seminal plasma levels of hexanoylcarnitine, propionylcarnitine, carnitine, glycerophosphocholine, L-palmitoylcarnitine, L-acetylcarnitine, adenine, arginine, Linoelaidyl carnitine, docosahexaenoic acid, epitestosterone, docosapentaenoic acid, 8-iso-15-keto-PGF2α, prostaglandin E2, L-3-phenyllactic acid, hydroxyphenyllactic acid, L-histidine, 11b-hydroxyprogesterone, (9*S*,10*S*)-9,10-dihydroxyoctadecanoate xanthine, 12,13-DHOME, ent-7alpha,12beta-dihydroxy-16-kauren-19,6beta-olide, 6-keto-prostaglandin F1a, and oleic acid were significantly correlated with a lower risk of low semen quality after adjusting for various covariates and multiple testing. Higher seminal plasma oleamide, however, was correlated with a higher risk of low sperm quality (all FDR-corrected $p < 0.05$). Further analyses showed that all these 25 biomarkers were significantly associated with one or more sperm parameters (i.e., progressive motility, total motility, concentration, and total count, and morphology) based on multivariable logistic or linear models (all FDR-corrected $p < 0.05$; Supplemental Material Tables S1 and S2). Using online database of metabolic pathways (KEGG PATHWAY Database, www.genome.jp/kegg), we established a molecular network of the potential biomarkers related to male semen quality, mostly involving in the metabolism of polyunsaturated fatty acids (PUFA) and acylcarnitine (see Supplemental Material Fig. S2).

3.4. Urinary phthalate metabolites and seminal plasma metabolome

The associations of average urinary phthalate metabolites with seminal plasma metabolic biomarkers are shown in Table 5. Significantly inverse associations were found between urinary MEHP and L-palmitoylcarnitine, linoelaidyl carnitine, and oleic acid, and between urinary %MEHP and L-acetylcarnitine (all FDR-corrected $p < 0.05$). A 2-fold increase in urinary MEHP was associated with a 5.1% (95% CI: −7.9, −2.3%), 8.3% (95% CI: −14, −2.7%) and 3.6% (95% CI: −6.0, −1.1%) decline in L-palmitoylcarnitine, linoelaidyl carnitine, and oleic acid, respectively. A 2-fold increase in urinary %MEHP was associated with a 1.8% (95% CI: −2.9, −0.74%) decline in L-acetylcarnitine.

3.5. Mediation analysis

Mediation analyses were only conducted for the intermediates (i.e., L-palmitoylcarnitine, oleic acid and L-acetylcarnitine) that satisfied the following assumptions: *a*) a significant association between an exposure and health indicator, *b*) a significant association between a mediator and exposure, and *c*) a significant association between a mediator and health indicator. In this population, higher urinary di-(2-ethylhexyl)-phthalate (DEHP) metabolites were significantly associated with increased percentage of spermatozoa with an abnormal head (Wang et al., 2015). Therefore, we assessed the mediating effect of L-palmitoylcarnitine, oleic acid and L-acetylcarnitine in the associations between DEHP metabolites and the percentage of spermatozoa with an abnormal head. We found that seminal plasma oleic acid significantly mediated the association between MEHP and percentage of abnormal head (mediated proportion = 6.7%); seminal plasma L-acetylcarnitine significantly mediated the association between %MEHP and percentage of abnormal head (mediated proportion = 17%) (Table 6).

4. Discussion

Seminal plasma not only provides nutritional supplement for spermatozoa but also modulates the process of sperm maturation (Bieniek et al., 2016). Among 660 Chinese adults, 25 seminal plasma metabolites, mostly related to metabolism of PUFA and acylcarnitine, were significantly different between poor and good semen quality groups. These discriminatory biomarkers were all significantly associated with

Table 3
Distribution and intraclass correlation coefficients (ICCs) of phthalate metabolite concentrations in repeated urine samples (n = 660).

Metabolites	First urine sample		Second urine sample		Variance component		
	% > LOD	Geometric mean	% > LOD	Geometric mean	Interindividual (%)	Intraindividual (%)	ICCs
MMP	93	13	91	11	1.3 (26%)	3.7 (74%)	0.26
MEP	97	17	97	15	1.3 (52%)	1.2 (48%)	0.52
MBP	100	65	100	59	0.49 (75%)	0.16 (25%)	0.75
MBzP	99	2.4	98	2.3	0.77 (63%)	0.45 (37%)	0.63
MEHP	98	5.4	97	5.0	0.93 (61%)	0.59 (39%)	0.61
MEHHP	100	14	100	13	0.34 (72%)	0.13 (28%)	0.72
MEOHP	100	8.1	99	7.1	0.29 (57%)	0.22 (43%)	0.57
MOP	14	0.02	13	0.02	–	–	–
%MEHP[a]	–	19	–	20	0.41 (51%)	0.38 (49%)	0.51

Urinary quartile levels of creatinine (g/L): 0.93, 1.4, and 2.1 (the first urine sample); and 0.72, 1.3, and 2.1 (the second urine sample).
[a] %MEHP (%) = [MEHP (nmol/L)/the sum of MEOHP (nmol/L), MEHHP (nmol/L) and MEHP (nmol/L)] × 100.

one or more individual sperm quality parameters (i.e., progressive motility, total motility, concentration, or total count), controlling for various confounders and multiple comparisons. Further analysis showed that L-palmitoylcarnitine, linoelaidyl carnitine and oleic acid were inversely associated with urinary MEHP, and L-acetylcarnitine was inversely associated with urinary %MEHP. Assuming that the underlying causal assumption of the mediation analysis was valid, seminal plasma oleic acid and L-acetylcarnitine mediated significant proportions of the positive association between urinary DEHP metabolites and the percentage of spermatozoa with an abnormal head.

Seminal plasma, as a proximal fluid for metabolite biomarker discovery, provides more abundant molecular composition than blood and urine. Additionally, high-throughput mass spectrometry platform and newly developed techniques for metabolome analysis make it possible

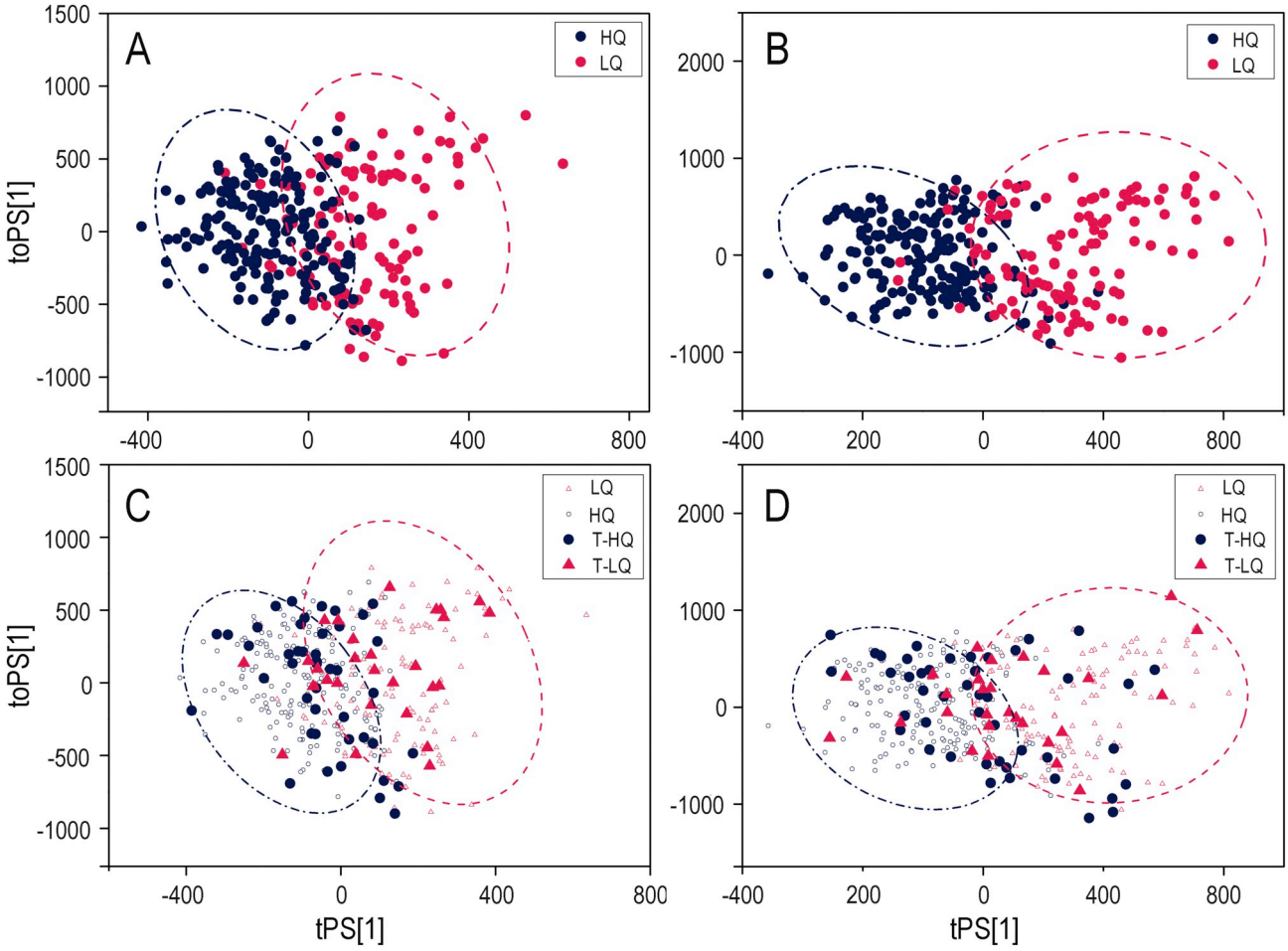

Fig. 1. Seminal plasma metabolites of men with low semen quality and high semen quality. (A) Scores plot [first predictive component (tPS[1]) vs. first orthogonal component (toPS[1])] of a training set of men based on positive electrospray ionization; (B) scores plot of a training set of men based on negative electrospray ionization; (C) scores plot of a test set of men based on positive electrospray ionization; and (D) scores plot of a test set of men based on negative electrospray ionization. LQ: low semen quality based on the training set; HQ: high semen quality based on the training set; T-LQ: low semen quality based on the testing set; H-HQ: high semen quality based on the testing set.

Table 4
Potential seminal plasma biomarkers of poor semen quality.

Compounds	Formula	Models	Fold change	VIP score	P[a]	Logistic models[b]	
						OR (95% CI)	P
Hexanoylcarnitine	C13H25NO4	Positive	1.3	1.4	< 0.001	0.59 (0.43, 0.81)	0.001*
Leukotriene E4	C23H37NO5S	Positive	−1.5	1.4	0.006	1.10 (0.96, 1.25)	0.17
Propionylcarnitine	C10H19NO4	Positive	1.3	1.6	< 0.001	0.53 (0.37, 0.75)	< 0.001*
Carnitine	C7H15NO3	Positive	1.6	2.2	< 0.001	0.57 (0.45, 0.72)	< 0.001*
Glycerophosphocholine	C8H20NO6P	Positive	1.3	1.1	< 0.001	0.72 (0.56, 0.92)	0.009*
Prostaglandin J2	C20H30O4	Positive	1.3	1.2	0.001	0.78 (0.60, 1.02)	0.07
5′-Methylthioadenosine	C11H15N5O3S	Positive	1.3	1.2	0.001	0.80 (0.63, 1.02)	0.08
Cortolone	C21H34O5	Positive	1.3	1.2	0.002	0.82 (0.65, 1.03)	0.09
L-palmitoylcarnitine	C23H45NO4	Positive	1.4	1.3	< 0.001	0.51 (0.37, 0.71)	< 0.001*
L-acetylcarnitine	C9H17NO4	Positive	1.6	2.2	< 0.001	0.51 (0.39, 0.66)	< 0.001*
Adenine	C5H5N5	Positive	1.9	1.6	< 0.001	0.65 (0.51, 0.82)	< 0.001*
Arginine	C6H14N4O2	Positive	1.6	1.6	0.003	0.81 (0.69, 0.96)	0.01*
17alpha,21-dihydroxypregnenolone	C21H32O4	Positive	1.6	2.2	0.003	0.87 (0.73, 1.02)	0.09
17a-ethynylestradiol	C20H24O2	Positive	−1.5	2.8	0.043	1.10 (0.92, 1.32)	0.32
Dehydrophytosphingosine	C18H37NO3	Positive	1.2	1.1	< 0.001	0.94 (0.74, 1.20)	0.63
Linoelaidyl carnitine	C25H45NO4	Positive	2.7	3.0	< 0.001	0.61 (0.52, 0.72)	< 0.001*
Docosahexaenoic acid	C22H32O2	Positive	1.3	1.0	< 0.001	0.53 (0.38, 0.72)	< 0.001*
Epitestosterone	C19H28O2	Positive	1.2	1.1	0.003	0.74 (0.56, 0.98)	0.03*
Dihydrocortisol	C21H32O5	Positive	1.3	1.1	0.012	0.86 (0.70, 1.06)	0.15
Oleamide	C18H35NO	Positive	−1.8	1.8	< 0.001	1.32 (1.12, 1.55)	0.001*
Docosapentaenoic acid	C22H34O2	Positive	1.2	1.0	< 0.001	0.36 (0.21, 0.59)	< 0.001*
8-Iso-15-keto-PGF2α	C20H32O5	Negative	1.1	1.2	0.009	0.58 (0.43, 0.79)	0.01*
Prostaglandin E2	C20H32O5	Negative	1.2	1.3	0.001	0.64 (0.49, 0.83)	< 0.001*
L-3-phenyllactic acid	C9H10O3	Negative	2.8	3.0	< 0.001	0.49 (0.40, 0.59)	< 0.001*
Hydroxyphenyllactic acid	C9H10O4	Negative	1.3	1.3	0.002	0.82 (0.69, 0.97)	< 0.001*
L-histidine	C6H9N3O2	Negative	1.2	1.3	0.005	0.74 (0.62, 0.88)	< 0.001*
11b-hydroxyprogesterone	C20H28O4	Negative	1.4	1.5	0.048	0.78 (0.64, 0.95)	0.02*
(9*S*,10*S*)-9,10-dihydroxyoctadecanoate	C18H36O4	Negative	1.3	1.3	0.004	0.77 (0.62, 0.96)	0.02*
Indolelactic acid	C11H11NO3	Negative	1.2	1.1	0.009	0.87 (0.73, 1.03)	0.11
Xanthine	C5H4N4O2	Negative	1.3	1.3	0.009	0.74 (0.59, 0.92)	0.007*
12,13-DHOME	C18H34O4	Negative	1.3	1.4	0.002	0.71 (0.60, 0.84)	< 0.001*
ent-7alpha,12beta-Dihydroxy-16-kauren-19,6beta-olide	C20H28O4	Negative	1.4	1.8	0.001	0.78 (0.66, 0.92)	0.001*
Arachidonic acid	C20 H32 O2	Positive	1.1	NA	0.025	0.85 (0.68, 1.06)	0.15
6-Keto-prostaglandin F1a	C20H34O6	Positive	1.2	NA	0.014	0.75 (0.59, 0.94)	0.01*
Oleic acid	C18H34O2	Negative	1.2	NA	0.004	0.55 (0.39, 0.77)	0.001*

* Statistically significant after FDR correction. VIP: Variable influence on projection.
[a] Calculated from nonparametric Wilcoxon-Mann-Whitney test.
[b] Adjusted ORs (95% CIs) for low semen quality associated with seminal plasma metabolites (ln-transformed), adjusted for age, BMI, creatinine level, abstinence duration, smoking status, daily cigarette consumption and having ever fathered a pregnancy.

to seek novel biomarkers relevant to male infertility. Several prior epidemiologic studies have investigated potential seminal plasma metabolome related to male infertility, using nuclear magnetic resonance spectroscopy (Jayaraman et al., 2014; Zhang et al., 2015), gas chromatography (GC)-MS (Gilany et al., 2017; Qiao et al., 2017; Tang et al., 2017), and Raman spectroscopy (Gilany et al., 2014; Jafarzadeh et al., 2015). The identified metabolites were related to amino acid metabolism, nucleoside metabolism, lipids metabolism, carbohydrate/energy metabolism, and oxidative stress. However, very little overlap was observed among the metabolites identified in prior studies and those found in our present study, probably because of differences in metabolomics platforms, population characteristics, and forms of infertility (e.g., oligozoospermia, asthenozoospermia, azoospermia, and unexplained infertility). Additionally, previous studies have been performed on a limited number of subjects, ranging from 10 to 260, which may be insufficient to generate precise estimation.

In this study, elevated seminal plasma PUFA [i.e., docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), oleic acid, and 9,10-dihydroxystearic acid] and carnitine or its derivatives (i.e., carnitine, hexanoylcarnitine, propionylcarnitine, L-palmitoylcarnitine, L-acetylcarnitine, and linoelaidyl carnitine) were all significantly associated with a reduced OR of poor semen quality. PUFAs are present in mammalian sperm at very high levels and are crucial for sperm motility, membrane fluidity, and susceptibility to lipid peroxidation (Safarinejad et al., 2010). However, few epidemiological investigations have assessed associations between PUFAs and semen quality. In support of our results, Safarinejad et al. (2010) reported a positive association between serum and spermatozoa levels of DPA and DHA and sperm motility, total count, concentration, and normal forms among 156 Iranian males from an infertility clinic. Additionally, we found that prostaglandin E2 and 6-Keto-prostaglandin, the downstream metabolites of arachidonic acid (AA), were associated with a reduced risk of being below WHO reference values for sperm quality. Emerging evidence shows that PUFAs affect the biosynthetic pathway of prostaglandin (Lands, 1992; Watanabe, 2002; Wathes et al., 2007), which is crucial for maintaining male reproductive function (Dolatpanah et al., 2008; Titiroongruang et al., 2012). The carnitine system plays an important role in cell energy metabolism as a reservoir pool of acyl-coenzyme A or as a carrier of long-chain fatty acids for β-oxidation (Jones et al., 2010). It is also reported that carnitine has antioxidant properties, protecting spermatozoa membrane and DNA from oxidative damage (Agarwal and Said, 2004; Ng et al., 2004). Both experimental and epidemiological studies have revealed that carnitine is beneficial for semen quality (Lenzi et al., 2003; Lenzi et al., 2004; Moore, 1998). In contrast, we found that higher seminal plasma oleamide was correlated with an increased risk of poor sperm quality. Oleamide is a messenger of prototype long chain primary fatty acid amide lipid and has been found to inhibit gap junctional communication in male seminiferous tubule cells (Decrouy et al., 2004).

We found that urinary DEHP metabolites were inversely associated

Table 5
Percentage differences (95% CI) in seminal plasma metabolic biomarkers associated with a 2-fold increase in average concentrations of urine phthalate metabolites[a].

Metabolomics	lnMMP	lnMEP	lnMBP	lnMBzP
Hexanoylcarnitine	0.38% (−1.4, 2.2%)	−0.05% (−2.3, 2.2%)	1.1% (−3.0, 5.4%)	−0.80% (−3.9, 2.4%)
Propionylcarnitine	0.43% (−1.2, 2.1%)	0.23% (−1.8, 2.3%)	1.1% (−2.6, 4.9%)	−0.39% (−3.1, 2.4%)
Carnitine	0.40% (−2.1, 3.0%)	0.97% (−2.2, 4.3%)	4.6% (−1.3%, 11%)	−0.66% (−4.9, 3.8%)
Glycerophosphocholine	2.4% (0.13, 4.7%)	1.4% (−1.5, 4.3%)	1.9% (−3.2, 7.4%)	−2.8% (−6.6, 1.0%)
L-palmitoyl carnitine	0.48% (−1.4, 2.4%)	0.25% (−2.1, 2.7%)	0.04% (−4.2, 4.5%)	−2.7% (−5.9, 0.47%)
L-acetylcarnitine	0.47% (−1.7, 2.7%)	1.3% (−1.4, 4.1%)	2.8% (−2.2, 8.1%)	1.8% (−2.0, 5.7%)
Adenine	3.8% (0.86, 6.8%)	−0.48% (−4.1, 3.2%)	1.4% (−5.1, 8.3%)	0.60% (−4.3, 5.7%)
Arginine	0.32% (−7.5, 8.6%)	1.3% (−3.1, 5.9%)	0.72% (−7.1, 9.2%)	−0.20% (−6.1, 6.0%)
Linoelaidyl carnitine	2.2% (−1.6, 6.1%)	−2.8% (−7.3, 2.0%)	−6.6% (−14, 1.9%)	−2.3% (−8.5, 4.4%)
Docosahexaenoic acid	1.3% (−0.47, 3.2%)	0.54% (−1.8, 2.9%)	1.6% (−2.6, 5.9%)	−0.80% (−3.9, 2.4%)
Epitestosterone	0.63% (−1.3, 2.5%)	−1.2% (−3.6, 1.2%)	6.5% (2.0, 11%)	−0.65% (−3.9, 2.7%)
Oleamide	−3.7% (−7.0, −0.29%)	−2.3% (−6.6, 2.2%)	−5.8% (−13, 2.1%)	−3.0% (−8.7, 3.1%)
Docosapentaenoic acid	0.034% (−1.8, 1.9%)	1.4% (−0.98, 3.9%)	2.1% (−2.2, 6.6%)	−0.54% (−3.8, 2.8%)
8-iso-15-keto-PGF2α	0.16% (−1.6, 1.9%)	0.018% (−2.2, 2.3%)	5.3% (1.1, 9.7%)	−0.50% (−3.5, 2.6%)
Prostaglandin E2	0.43% (−1.2, 2.1%)	−0.51% (−3.2, 2.2%)	3.9% (−1.0, 9.1%)	−0.82% (−4.4, 2.9%)
L-3-phenyllactic acid	0.42% (−2.7, 3.6%)	2.1% (−1.9, 6.3%)	−1.2% (−8.1, 6.3%)	−1.5% (−6.7, 4.1%)
Hydroxyphenyllactic acid	−0.57% (−3.7, 2.6%)	1.1% (−2.9, 5.3%)	3.2% (−4.0, 11%)	2.2% (−3.3, 8.0%)
L-histidine	0.40% (−2.8, 3.7%)	−2.9% (−6.9, 1.3%)	−6.4% (−13, 1.0%)	−4.6% (−9.9, 1.0%)
11b-hydroxyprogesterone	−0.56% (−3.7, 2.6%)	1.1% (−2.9, 5.2%)	3.2% (−4.0, 11%)	2.2% (−3.2, 8.0%)
(9*S*,10*S*)-9,10-dihydroxyoctadecanoate	0.36% (−2.1, 2.8%)	0.51% (−2.6, 3.7%)	8.1% (2.2, 14%)	−2.2% (−6.3, 2.1%)
Xanthine	0.86% (−1.8, 3.6%)	0.91% (−2.5, 4.4%)	1.3% (−4.7, 7.7%)	4.0% (−0.74, 8.9%)
12,13-DHOME	−0.29% (−3.5, 3.1%)	−0.23% (−4.4, 4.1%)	−0.72% (−8.0, 7.2%)	−0.10% (−5.7, 5.9%)
ent-7alpha,12beta-dihydroxy-16-kauren-19,6beta-olide	−0.93% (−4.2, 2.4%)	−0.84% (−5.0, 3.5%)	−8.2% (−15, −0.78%)	−4.5% (−9.9, 1.3%)
Oleic acid	0.19% (−1.4, 1.8%)	−1.0% (−3.1, 1.1%)	−0.73% (−4.4, 3.1%)	1.2% (−1.6, 4.2%)
6-Keto-prostaglandin F1a	0.46% (−1.9, 2.9%)	−0.69% (−3.7, 2.4%)	3.4% (−2.2, 9.3%)	0.46% (−3.7, 4.8%)

Metabolomics	lnMEHP	lnMEHHP	lnMEOHP	%MEHP
Hexanoylcarnitine	−0.96% (−3.7, 1.9%)	−0.46% (−5.3, 4.6%)	−0.34% (−5.2, 4.8%)	−0.74% (−1.6, 0.15%)
Propionylcarnitine	−0.64% (−3.1, 1.9%)	2.0% (−2.5, 6.6%)	0.96% (−3.4, 5.6%)	−1.0% (−1.7, −0.13%)
Carnitine	−2.3% (−6.1, 1.7%)	3.2% (−3.8, 11%)	4.2% (−2.8, 12%)	−1.5% (−2.7, −0.23%)
Glycerophosphocholine	−2.4% (−5.8, 1.1%)	0.91% (−5.2, 7.4%)	−0.3% (−6.3%, 6.1%)	−1.2% (−2.3, −0.12%)
L-palmitoyl carnitine	−5.1% (−7.9, −2.3%)*	−5.0% (−9.8, 0.06%)	−5.7% (−10, −0.74%)	−1.3% (−2.2, −0.34%)
L-acetylcarnitine	−2.1% (−5.4, 1.3%)	4.6% (−1.5, 11%)	1.9% (−4.0, 8.2%)	−1.8% (−2.9, −0.74%)*
Adenine	−0.53% (−4.9, 4.1%)	5.7% (−2.4, 14%)	5.4% (−2.6, 14%)	−0.80% (−2.2, 0.62%)
Arginine	−4.5% (−9.6, 0.88%)	−3.4% (−12, 6.4%)	−4.2% (−13, 5.4%)	−1.4% (−3.1, 0.36%)
Linoelaidyl carnitine	−8.3% (−14, −2.7%)*	−6.2% (−16, 4.1%)	−6.7% (−16, 3.5%)	−1.9% (−3.7, −0.01%)
Docosahexaenoic acid	−0.34% (−3.1, 2.6%)	−0.64% (−5.5, 4.5%)	−1.0% (−5.8, 4.1%)	−0.33% (−1.2, 0.57%)
Epitestosterone	1.9% (−1.1, 5.0%)	1.5% (−3.7, 7.0%)	−0.66% (−5.7, 4.7%)	0.63% (−0.32, 1.6%)
Oleamide	−2.0% (−7.2, 3.6%)	5.0% (−4.7, 16%)	−0.43% (−9.6, 9.7%)	−1.0% (−2.7, 0.74%)
Docosapentaenoic acid	−0.87% (−3.8, 2.1%)	1.8% (−3.4, 7.2%)	−0.08% (−5.2, 5.3%)	−0.98% (−1.9, −0.04%)
8-iso-15-keto-PGF2α	1.9% (−0.87, 4.8%)	2.8% (−2.1, 7.9%)	0.88% (−3.9, 5.9%)	0.48% (−0.41, 1.4%)
Prostaglandin E2	2.8% (−0.60, 6.3%)	2.7% (−3.1, 9.0%)	2.4% (−3.5, 8.5%)	0.97% (−0.09, 2.1%)
L-3-phenyllactic acid	−0.73% (−5.6, 4.4%)	−0.14% (−8.5, 9.0%)	0.65% (−7.8, 9.8%)	−0.37% (−1.9, 1.2%)
Hydroxyphenyllactic acid	3.6% (−1.4, 8.9%)	3.6% (−5.0, 13%)	4.1% (−4.6, 14%)	0.63% (−0.95, 2.2%)
L-histidine	−5.9% (−11, −0.89%)	−3.7% (−12, 5.5%)	−5.4% (−14, 3.6%)	−1.3% (−2.9, 0.35%)
11b-hydroxyprogesterone	3.6% (−1.4, 8.9%)	3.6% (−5.1, 13%)	4.1% (−4.6, 14%)	0.63% (−0.95, 2.2%)
(9*S*,10*S*)-9,10-dihydroxyoctadecanoate	1.7% (−2.1, 5.8%)	8.1% (0.98, 16%)	1.8% (−4.9, 8.9%)	0.19% (−1.0, 1.4%)
Xanthine	5.0% (0.67, 9.4%)	4.2% (−3.2, 12%)	6.5% (−1.0, 15%)	1.4% (0.06, 2.7%)
12,13-DHOME	1.4% (−3.8, 6.8%)	−6.0% (−14, 3.0%)	−0.51% (−9.3, 9.1%)	1.1% (−0.59, 2.8%)
ent-7alpha,12beta-dihydroxy-16-kauren-19,6beta-olide	−6.6% (−11, −1.5%)	−1.3% (−10, 8.4%)	−5.1% (−14, 4.1%)	−1.9% (−3.5, −0.25%)
Oleic acid	−3.6% (−6.0, −1.1%)*	−3.2% (−7.5, 1.3%)	−3.8% (−8.1, 0.66%)	−1.1% (−1.9, −0.24%)
6-Keto-prostaglandin F1a	1.8% (−1.9, 5.8%)	1.3% (−5.2, 8.3%)	1.2% (−5.3, 8.1%)	0.36% (−0.83, 1.6%)

* Statistically significant after FDR correction. Abbreviations: CI, confidence interval.

[a] Adjusted for age, BMI, creatinine level, abstinence duration, smoking status and daily cigarette consumption. Seminal plasma metabolomics were transformed by the natural logarithm and then back-transformed {100 × [exp(beta) − 1]} to obtain the percent changes.

Table 6
The estimated proportions of associations between urinary phthalate metabolites and the percentage of spermatozoa with an abnormal head mediated through seminal plasma metabolomics.

Exposure and mediators	Estimated effect			Percent mediated[a]
	Natural direct effect (95% CI)	Natural indirect effect (95% CI)	Total effect (95% CI)	
lnMEHP				
L-palmitoylcarnitine	1.1 (0.44, 1.8)*	0.077 (−0.015, 0.20)	1.2 (0.52, 1.9)*	6.0%
Oleic acid	1.1 (0.44, 1.8)*	0.086 (0.0058, 0.20)*	1.2 (0.52, 1.9)*	6.7%*
ln%MEHP				
L-acetylcarnitine	0.09 (0.001, 0.17)*	0.02 (0.006, 0.040)*	0.11 (0.02, 0.19)*	17%*

* Statistically significant. Abbreviations: CI, confidence interval.
[a] Percent mediated = natural indirect effect/(natural direct effect + natural indirect effect) × 100.

with seminal plasma oleic acid, as well as L-palmitoylcarnitine, L-acetylcarnitine, and linoelaidyl carnitine, indicating inhibited fatty acid and carnitine metabolism. The hypolipidemic effect of DEHP has been consistently demonstrated in different animal models (Dirven et al., 1990; Hayashi et al., 2011; Nakashima et al., 2013), probably by affecting peroxisome proliferation (Reddy et al., 1976), fatty acid catabolism (Feige et al., 2010), and expression of hepatic microsomal triglyceride transfer protein (Hayashi et al., 2011). Carnitine and its derivatives were found to modulate fatty acid oxidation, energy metabolism, and oxidative stress (Marcovina et al., 2013). Treatment of DEHP or its metabolites was found to disrupt carnitine acetyltransferase activities both in vitro and in vivo (Gray et al., 1982; Grolier and Elcombe, 1993; Meng et al., 2018). In support of our results, Jia et al. (2015) found an inverse association between blood levels of MEHP and oleic acid among 318 Japanese pregnant mothers. In our recent study of 364 Chinese adult men, urinary palmitoleyl carnitine was significant lower in high exposure group of ΣDEHP compared to the low exposure group (Zhang et al., 2016).

Among these subgroup subjects, the direct effect of urinary MEHP and %MEHP on percentage of spermatozoa with an abnormal head were significant, supporting the findings observed in our overall population (Wang et al., 2015), and that from other studies (Pant et al., 2008; Zhang et al., 2006). Our mediation analysis showed that seminal plasma oleic acid and L-acetylcarnitine mediated significant proportions of the positive association between urinary DEHP metabolites and percentage of abnormal head, suggesting that PUFA and acylcarnitine metabolism play an intermediating role. However, mediation analysis was conducted within the counterfactual framework, which makes a strong assumption that there is no unmeasured confounding between exposures, mediators, and outcomes. Although we have accounted for a number of potential confounders in the models, the possibility of unmeasured confounders cannot be fully excluded.

Strengths of our present study include a sufficient sample size to analyze metabolomics, and collection of repeated urine samples to determine phthalate metabolites that would improve within-individual exposure estimation. We also used seminal plasma as a proximal fluid that is directly reverent to male infertility. Our study also has several limitations. Firstly, there is a possibility of measurement error in seminal plasma metabolome due to the collection of single specimen from each individual. However, we expect measurement error to be nondifferential, and therefore to attenuate observed significant associations rather than induce them. Secondly, although we repeatedly measured phthalate metabolite concentrations in urine, they might not represent the exposure status of reproductive organs because some phthalate metabolites were differently distributed in seminal plasma and urine (Wang et al., 2016b). Thirdly, our study volunteers were enrolled from an infertility clinic. This study design helped us enroll participants but resulted in the inclusion of more subfertile men, which restricts the generalization of our findings. Finally, this study design was cross-sectional (i.e., we measured biomarkers of phthalate exposures, seminal plasma metabolome, and semen quality at a similar time point), making it impossible to establish a causal relationship.

5. Conclusions

Using an untargeted approach based on LC-MS platform, we identified 25 seminal plasma metabolomes that were significantly associated with semen quality, mostly related to PUFA and acylcarnitine metabolism. Of these potential biomarkers, L-palmitoylcarnitine, L-acetylcarnitine, linoelaidyl carnitine, and oleic acid were inversely associated urinary DEHP metabolites. Mediation analysis showed that oleic acid and L-acetylcarnitine mediated significant proportions of the positive association between urinary DEHP metabolites and the percentage of spermatozoa with an abnormal head. Our finding provides preliminary evidence that seminal plasma metabolome may serve as intermediating biomarkers linking phthalate exposures and impaired semen quality, which is worthy of further investigations.

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Competing financial interests

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.05.043.

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