# Untargeted metabolomics to identify potential metabolite alterations after exposing to DHP (Dihexyl phthalate)

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**Abstract**: Dihexyl phthalate (DHP) is one of the most commonly used phthalate esters in various plastic and consumer products. Although several animal experiments have revealed that DHP can cause multiple toxicities, information about DHP toxicity on human cells is very limited. In the present study, we performed liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) based metabolomics to detect metabolic profiles of $U_2OS$ cells after exposing to DHP. Significant differences in metabolic profiles were observed in cells exposed to DHP compared with controls. By mass spectrometry we detected 58 putative metabolites in electrospray ionization source (ESI)+ mode and 32 putative metabolites in ESI- mode, the majority of the differential metabolites being lipids and lipid-like molecules. In addition, DHP exposure decreased gene expression levels of enzymes related to fatty acids synthesis and oxidation. In summary, our results suggested that combination of metabolomics analysis and other molecular biology methods could be an efficient way to detect potential biomarkers of exposure to and toxicity of DHP.

**Keywords:** Dihexyl phthalate (DHP); LC-Q-TOF-MS; multivariate analysis; metabolic profiles

**Key Contribution:** This study is the first to analyze metabolic profiles of DHP exposure by

LC-Q-TOF-MS based metabolomics technique and in combination with other molecular biological methods to investigate the potential toxicity mechanism of DHP.

## 1. Introduction

Phthalic acid esters (PAEs) are used broadly as industrial plasticizers in the making of polyvinyl chloride plastics and consumer products, such as plastic food packaging, toys, and paints. They are important additives to make polyvinylchloride (PVC) products more flexible and durable [1-4]. There are several typical phthalates including dimethyl phthalate (DMP), diethyl phthalate (DEP), di-n-butyl phthalate (DBP), di-iso-butyl phthalate (DIBP), benzyl butyl phthalate (BzBP), bis(2-ethylhexyl) phthalate (DEHP), di-n-hexyl phthalate (DnHP), dicyclohexyl phthalate (DCHP), and di-n-octyl phthalate (DnOP) [5].

Dihexyl phthalate (DHP), one of the phthalate esters produced in a great quantity, is widely used in various plastic materials [6]. It is frequently found in indoor and outdoor environments, has been classified as an environmental endocrine disruptor and possesses estrogenic activity [7,8]. Under certain conditions, DHP can migrate from plastic packaging materials to foods and drinking water. Dietary intake is the main source of human exposure to DHP [9,10]. Phthalates and phthalate metabolites have been detected in human blood, urine and semen [11-13]. There is a growing concern about the possibility of phthalates harming human health because multiple toxicities were observed in several rat models after exposure. A number of studies have pointed out that exposure to DHP resulted in reproductive, developmental, and genetic toxicity in most rat animal model. Saillenfait et al. has reported that exposure to DHP induced reproductive abnormalities syndrome in the male rat [14]. DHP-induced DNA damage and increase of apoptotic cells in testicular cells of male rats were

50 considered to be a signal of reproductive risk [8,15]. Another study by Saillenfait demonstrated that a
51 significant decrease of fetal weight and increase of embryo mortality were caused by DHP in rats [16].
52 Other investigators reported that exposure to DHP caused a decrease in proliferation of cell nuclear
53 antigen (PCNA) of placenta in rats, meaning that DHP inhibited cell proliferation [17]. Yet, despite the
54 evidence that toxicity of DHP exposure in rat animal models has been established, information about
55 DHP toxicity on human health is very limited.

56 Metabolomics analysis is a process of untargeted identification and quantitative analysis of
57 metabolites in sample. It is widely applied in many fields, and considered to be an excellent tool for
58 diagnosing diseases, identifying disturbed pathway, discovering potential biomarkers and drug
59 development [18,19]. There are three main analytical technologies, including nuclear magnetic
60 resonance (NMR), gas-chromatography-mass spectrometry (GC-MS), and liquid chromatography mass
61 spectrometry (LC-MS) commonly used for investigations of metabolites [20]. Among them, NMR is
62 less sensitivity than MS, and usually only the most abundant substances can be detected [21]. GC-MS
63 can be used to isolate and identify low molecular weight and volatile metabolites, it is very sensitive to
64 the polarity of compounds. For molecules with high polarity or thermal instability detection, it needs
65 many chemical derivatization steps to improve the volatility [22].However, this method is
66 time-consuming and analysis at high temperature may lead to isomerization and/or decomposition of
67 analytes [23]. LC-MS shows higher sensitivity compared to GC-MS [24]. It is extensively used in
68 identifying and quantifying metabolites, and maay reveal previously unidentified or annotated
69 metabolites. LC-Q-TOF-MS possess the advantages of high sensitivity, acquisition speed, accurate
70 mass measurement, and high repeatability [25,26]. It can identify hundreds of changed endogenous
71 metabolites and related metabolic pathways,, and according to the changes in endogenous metabolites

which could contribute to explain the state of different organisms and elucidate potential molecular toxicity mechanisms [27,28]. To our knowledge, no studies have previously assessed the effects of DHP exposure by LC-MS analysis on human cells.

In order to acquire more information about the effects of DHP on human cells, we used a LC-Q-TOF-MS based metabolomic approach to carry out a comprehensive analysis of the metabolic profiles of DHP treated and control cells. The purpose of this study was to reveal putative biomarkers that caused metabolic changes by DHP treatment, which may contribute to understanding the possible toxicity mechanisms of DHP exposure.

## 2. Results

### *2.1 Multivariate Statistical Analysis*

In order to compare classification variations and trends between the DHP treated group and the control group, partial least squares-discriminate analysis (PLS-DA) score plot, a supervised analysis method, was performed to process the data in two different groups, which can reflect sample clustering in the data. PLS-DA showed an obvious separation, there was non-overlap between the two groups as shown in Fig. 1 A, B. (The PLS-DA model parameters were $R^2$=0.9347, $Q^2$=0.5604 in ESI+ mode and $R^2$=0.9402, $Q^2$=0.207 in ESI- mode). This result suggested that the metabolic profiling of two groups were significantly different both in ESI+ and ESI- modes.

**Figure 1.** PLS-DA score plot of DHP treated and control groups on $U_2OS$ cells. (A) Score plot in ESI+ mode ($R^2$=0.9347, $Q^2$=0.5604). (B) Score plot in ESI- mode ($R^2$=0.9402, $Q^2$=0.207).

*2.2 Univariate Statistical Analysis*

In this study, we used univariate analysis method (T-test ($p<0.05$) and fold change ($\geq 1.2$ or $\leq 0.83$)) to screen for metabolites which were present in different amounts with and without DHP treatment. All of the detected metabolites were presented in the volcano plot as shown in Fig. 2 A, B, where the red dots represent significantly changed metabolites after DHP treatment. 316 and 199 differential ions were detected in ESI+ mode and ESI- mode, respectively.

**Figure 2.** Volcano plot of DHP treated and control groups on $U_2OS$ cells in ESI+ mode (A) and in ESI- mode (B). Each dot represents a detected metabolite. Red dots represent metabolites influenced by DHP treatment with upregulated metabolites to the right and downregulated metabolites to the left. Blue dots are unaffected by the DHP treatment.

*2.3 Identification of differential metabolites*

Variable importance in the projection (VIP) is used to assist in screening metabolic markers. According to the standard metabolite identification established by the Chemical Analysis Working Group Metabolomics Standards Initiative[29], we identified 58 putatively characterized metabolites which were influenced by the DHP treatment in ESI+ mode and 32 putatively characterized metabolites in ESI- mode based on three criteria: VIP value $\geq 1$, fold-change $\geq 1.2$ or $\leq 0.8333$, and p-value $< 0.05$ in the PLS-DA model, as shown in Table 1 and 2. These differential metabolites could be grouped into several categories: lipids and lipid-like molecules, organoheterocyclic compounds, phenylpropanoids and polyketides, organic acids and derivatives and others. Among these metabolites, 50 lipids and

lipid-like molecules were identified in both ESI modes, and 17 of them were increased, while 33 of them were decreased after DHP treatment. In addition, 5 and 6 organoheterocyclic compounds, 1 and 6 phenylpropanoids and polyketid compounds, 6 and 1 organic acids and derivatives were identified in ESI+ and ESI- modes, respectively. Briefly, terpenoids, amino acids, peptides, and analogues, and bile acids, alcohols and derivatives appeared significantly reduced after DHP exposure, whereas fatty aldehydes and glycerophospholipids showed the opposite trend. In addition, fatty acids and conjugates demonstrated the different changes (Fig. 3).

**Table1.** List of putatively characterized metabolites between DHP and control groups in ESI+ mode

| Compound name | Formula | m/z | RT (min) | VIP | FC | p-value |
|---|---|---|---|---|---|---|
| Putatively annotated compounds or compound class (level 2) | | | | | | |
| Purines and purine derivatives | C5H4N4O | 136.0383 | 0.83 | 1.71 | 0.77 | 0.0341 |
| Purine nucleosides | C10H13N5O4 | 268.1037 | 0.83 | 2.46 | 1.59 | 0.0142 |
| Putatively characterized compounds or compound class (level 3) | | | | | | |
| Fatty acids and conjugates | C23H40O3 | 365.3028 | 10.01 | 2.29 | 1.54 | 0.0203 |
| Fatty acids and conjugates | C26H52O2 | 414.4305 | 6.96 | 2.46 | 1.61 | 0.0288 |
| Fatty alcohols | C37H68O5 | 592.5079 | 12.58 | 2.20 | 1.39 | 0.0068 |
| Fatty aldehydes | C26H52O | 398.4369 | 7.83 | 2.74 | 1.67 | 0.0041 |
| Eicosanoids | C22H38O5 | 405.2581 | 8.89 | 2.25 | 0.69 | 0.0015 |
| Glycerophosphoinositols | C27H51O12P | 621.3036 | 9.49 | 2.85 | 0.59 | 0.0017 |
| Glycerophosphocholines | C34H68NO7P | 651.5020 | 12.33 | 1.78 | 1.27 | 0.0233 |
| Glycerophosphoserines | C44H80NO10P | 813.5493 | 12.58 | 1.63 | 1.24 | 0.0106 |
| Glycerophosphoserines | C46H82NO10P | 839.5665 | 12.81 | 1.63 | 1.24 | 0.0446 |
| Glycerolipids | C15H32O6 | 331.2079 | 6.24 | 1.67 | 0.72 | 0.0389 |
| Monoterpenoids | C12H22O | 200.2003 | 9.37 | 1.95 | 1.45 | 0.0386 |
| Monoterpenoids | C35H56O5 | 595.3787 | 9.01 | 1.49 | 0.81 | 0.0216 |
| Triterpenoids | C27H36O6 | 439.2467 | 9.76 | 2.63 | 0.65 | 0.0006 |
| Triterpenoids | C27H42O6 | 463.3019 | 8.45 | 3.34 | 0.50 | 0.0002 |
| Triterpenoids | C29H42O4 | 455.3133 | 10.11 | 1.04 | 0.80 | 0.0454 |
| Triterpenoids | C40H56O5 | 616.4132 | 9.01 | 1.48 | 0.83 | 0.0156 |
| Terpene glycosides | C22H40O11 | 463.2584 | 8.65 | 1.66 | 0.80 | 0.0020 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sesquiterpenoids | C27H42O5 | 447.3091 | 8.94 | 3.05 | 0.51 | 0.0032 |
| Sesterterpenoids | C27H36O2 | 410.3082 | 10.00 | 1.73 | 1.29 | 0.0022 |
| Retinoids | C28H28O3 | 413.2086 | 9.64 | 1.46 | 1.22 | 0.0203 |
| Bile acids, alcohols and derivatives | C27H42O4 | 413.3047 | 8.94 | 3.99 | 0.38 | 0.0006 |
| Bile acids, alcohols and derivatives | C27H42O4 | 413.3050 | 9.25 | 4.40 | 0.33 | 0.0002 |
| Bile acids, alcohols and derivatives | C27H44O4 | 415.3203 | 10.03 | 4.80 | 0.26 | 0.0002 |
| Cholestane steroids | C27H42O | 382.3231 | 9.74 | 4.81 | 0.24 | 0.0007 |
| Cholestane steroids | C27H42O | 382.3234 | 10.31 | 3.96 | 0.38 | 0.0009 |
| Steroidal glycosides | C27H42O3 | 414.3113 | 9.81 | 3.82 | 0.41 | 0.0008 |
| Steroidal glycosides | C42H72O15 | 834.5204 | 12.84 | 1.40 | 1.21 | 0.0359 |
| Vitamin D and derivatives | C23H34O4 | 357.2394 | 9.72 | 3.07 | 0.58 | 0.0003 |
| Steroid esters | C45H74O2 | 647.5736 | 9.52 | 2.13 | 1.51 | 0.0268 |
| Steroid lactones | C23H36O5 | 393.2667 | 8.80 | 1.73 | 0.80 | 0.0015 |
| Androstane steroids | C21H34O2 | 341.2432 | 12.65 | 2.33 | 1.45 | 0.0049 |
| Stigmastanes and derivatives | C29H50O4 | 462.3711 | 10.79 | 2.04 | 1.39 | 0.0255 |
| Ergostane steroids | C28H40O2 | 447.2700 | 8.51 | 1.38 | 0.81 | 0.0174 |
| Amino acids, peptides, and analogues | C12H21NO8S | 339.0958 | 0.83 | 2.35 | 1.45 | 0.0197 |
| Amino acids, peptides, and analogues | C26H39NO3 | 414.3043 | 8.66 | 1.11 | 0.75 | 0.0318 |
| Amino acids, peptides, and analogues | C27H43NO3 | 452.3105 | 9.49 | 1.46 | 0.83 | 0.0179 |
| Amino acids, peptides, and analogues | C27H45NO3 | 454.3260 | 8.90 | 1.78 | 0.74 | 0.0240 |
| Depsipeptides | C23H36N2O11 | 539.2177 | 8.55 | 2.43 | 0.50 | 0.0487 |
| Phosphate esters | C16H34NO5P | 369.2536 | 9.32 | 1.67 | 0.82 | 0.0115 |
| Carbohydrates and carbohydrate conjugates | C23H27N2O11 | 525.1921 | 8.92 | 1.87 | 0.74 | 0.0230 |
| Carbonyl compounds | C15H26O | 205.1952 | 9.21 | 2.16 | 1.46 | 0.0361 |
| Carbonyl compounds | C9H16O2 | 139.1119 | 9.19 | 1.40 | 1.22 | 0.0260 |
| Amines | C22H37NO4 | 397.3069 | 9.39 | 3.75 | 0.43 | 0.0008 |
| Quaternary ammonium salts | C10H28N2OP2 | 233.1320 | 8.63 | 2.02 | 0.75 | 0.0033 |
| Azaspirodecane derivatives | C46H69NO13 | 844.4798 | 12.84 | 1.42 | 1.23 | 0.0445 |
| Pyrazoles | C17H12F3N3O4S | 429.0877 | 12.32 | 1.49 | 1.27 | 0.0445 |
| Heteroaromatic compounds | C14H24O | 191.1788 | 9.19 | 2.72 | 1.73 | 0.0030 |
| Benzoic acids and derivatives | C15H22O5 | 305.1352 | 6.91 | 1.08 | 0.78 | 0.0206 |
| Naphthalenes | C14H16O | 223.1108 | 7.61 | 1.63 | 0.80 | 0.0423 |
| Quinone and hydroquinone lipids | C27H40O2 | 379.2957 | 8.77 | 3.76 | 0.42 | 0.0003 |
| Gamma butyrolactones | C12H20O2 | 214.1806 | 8.92 | 4.27 | 0.33 | 0.0013 |
| Ketones | C25H36O4 | 400.2594 | 9.32 | 1.85 | 1.27 | 0.0019 |
| Sulfoxides | C7H14OS3 | 211.0268 | 9.64 | 1.47 | 1.21 | 0.0401 |
| Olefins | C8H12 | 109.1012 | 10.45 | 2.19 | 1.48 | 0.0127 |
| Hydroxycinnamic acids and derivatives | C27H44O3 | 439.3194 | 9.81 | 2.81 | 0.56 | 0.0070 |
| Cytochalasans | C28H37NO4 | 305.1352 | 9.22 | 3.16 | 0.52 | 0.0008 |

m/z: mass-to-charge ratio; RT: retention time; VIP: variable importance in the projection; FC: fold change; p-value: based on analysis of T-tests.

129 **Table2.** List of putatively characterized metabolites between DHP and control groups in ESI- mode

| Compound name | Formula | m/z | RT (min) | VIP | FC | p-value |
|---|---|---|---|---|---|---|
| Putatively annotated compounds or compound class (level 2) | | | | | | |
| Amino acids, peptides, and analogues | C9H11NO3 | 180.0663 | 0.83 | 1.45 | 1.21 | 0.0358 |
| Putatively characterized compounds or compound class (level 3) | | | | | | |
| Fatty acids and conjugates | C20H36O3 | 323.2584 | 8.98 | 3.72 | 0.35 | 0.0015 |
| Fatty acids and conjugates | C20H34O3 | 321.2424 | 8.79 | 3.37 | 0.34 | 0.0123 |
| Fatty alcohol esters | C22H36O3 | 347.2582 | 9.05 | 3.69 | 0.36 | 0.0294 |
| Fatty alcohols | C14H28O3 | 243.1961 | 8.79 | 1.30 | 0.78 | 0.0035 |
| Eicosanoids | C20H38O5 | 393.2427 | 8.35 | 1.88 | 0.73 | 0.0459 |
| Glycerophosphocholines | C40H76NO8P | 728.5225 | 11.43 | 1.28 | 1.33 | 0.0182 |
| Glycerophosphocholines | C48H86NO10P | 866.5901 | 11.92 | 1.21 | 1.24 | 0.0341 |
| Glycerophosphocholines | C50H88NO10P | 892.6151 | 11.92 | 1.48 | 1.37 | 0.0114 |
| Glycerophosphoglycerols | C24H49O9P | 511.3034 | 9.87 | 1.75 | 0.79 | 0.0056 |
| Diterpenoids | C20H34O3 | 321.2427 | 8.96 | 2.71 | 0.49 | 0.0223 |
| Diterpenoids | C22H34O2 | 329.2476 | 9.01 | 1.93 | 0.69 | 0.0289 |
| Diterpenoids | C20H34O4 | 337.2373 | 8.47 | 4.56 | 0.25 | 0.0006 |
| Monoterpenoids | C21H32O5 | 363.2147 | 8.76 | 1.84 | 0.67 | 0.0301 |
| Terpene glycosides | C36H60O8 | 619.4271 | 11.67 | 1.91 | 0.68 | 0.0061 |
| Physalins and derivatives | C28H31ClO10 | 597.1324 | 12.78 | 1.49 | 0.78 | 0.0046 |
| Steroidal glycosides | C30H48O11 | 619.2882 | 11.48 | 3.89 | 0.37 | 0.0021 |
| Carbohydrates and carbohydrate conjugates | C8H14O8 | 273.0374 | 0.62 | 1.87 | 1.40 | 0.0128 |
| Carbohydrates and carbohydrate conjugates | C16H23NO6S | 356.1195 | 0.81 | 3.96 | 2.65 | 0.0089 |
| Delta valerolactones | C5H8O5 | 147.0290 | 0.81 | 2.71 | 0.43 | 0.0370 |
| Naphthyridines | C20H15F3N4O3 | 451.0767 | 8.47 | 1.49 | 0.79 | 0.0095 |
| Thienopyridines | C14H14ClNOS | 278.0413 | 0.61 | 2.97 | 1.96 | 0.0298 |
| Piperazines | C21H31N5O3 | 400.2333 | 11.92 | 2.54 | 1.82 | 0.0136 |
| Porphyrins | C34H38N4O4 | 601.2595 | 11.92 | 1.34 | 1.34 | 0.0173 |
| Cyclic diarylheptanoids | C21H24O5 | 391.1334 | 11.92 | 1.02 | 1.21 | 0.0169 |
| Flavonoid glycosides | C22H22O10 | 445.1129 | 9.40 | 1.21 | 1.20 | 0.0378 |
| Flavonoid glycosides | C34H30O14 | 661.1510 | 9.66 | 1.45 | 0.80 | 0.0494 |
| Furanocoumarins | C30H34O14 | 618.1909 | 10.48 | 2.19 | 0.69 | 0.0031 |
| Hydroxycinnamic acids and derivatives | C26H42O4 | 417.2996 | 9.28 | 5.44 | 0.15 | 0.0002 |
| 2-arylbenzofuran flavonoids | C39H36O8 | 631.2324 | 8.98 | 1.72 | 0.68 | 0.0304 |
| Enynes | C13H8 | 199.0332 | 0.59 | 1.52 | 1.26 | 0.0170 |
| 2-benzopyrans | C22H34O3 | 345.2428 | 8.76 | 3.52 | 0.38 | 0.0027 |

130 m/z: mass-to-charge ratio; RT: retention time; VIP: variable importance in the projection; FC: fold change; p-value:

131 based on analysis of T-tests.

**Figure. 3** Content comparison of putatively characterized metabolites in DHP treated and control groups. Data are shown as Mean±SEM, Students' t-test. *P<0.05, ** P<0.01, *** P<0.001.

In addition, in order to better visualize differences between the two groups, heat maps were constructed using identified metabolites influenced by the DHP treatment according to the the PLS-DA analysis, which showed a distinct hierarchical clustering both in ESI+ and ESI- modes (Fig. 4 A, B).

**Figure 4.** Hierarchical clustering analysis of DHP treated and control groups on $U_2OS$ cells. (A) Heat map of identified differential metabolites in ESI+ mode. (B) Heat map of identified differential metabolites in ESI- mode. Each row and column represents putatively differential metabolites and samples, respectively. The change in color from green to red indicates the signal intensity from low to high. Fold change is indicated.

*2.4 Exposure to DHP decreases expression levels of enzymes involved in fatty acid synthesis and oxidation*

Fatty acid synthesis and oxidation has been shown to produce a marked role in the cell survival and growth [30]. In our study, we investigated gene expression levels of enzymes involved in fatty acids synthesis and oxidation by quantitative real-time PCR (qPCR) (Fig. 4). The mRNA levels of fatty acid synthase (FASN), fatty acid desaturases (FADS), elongation of very-long-chain fatty acids (ELOVLs), acyl-COA synthetase long-chain family member 1 (ACSL1), carnitine palmitoyltransferase 1B (CPT1B) , acyl-CoA dehydrogenase, C-4 to C-12 straight chain (ACADM), and acyl-CoA dehydrogenase, long chain (ACADL) were markedly decreased on $U_2OS$ cells under DHP treatment.

**Figure 5.** Expression levels of FASN, FADS, ELOVL5, ACSL1 and CPT1B genes in different groups. (A) mRNA expression levels of FASN, FADS1, FADS2, and ELOVL5 were determined using qPCR. (B) mRNA expression levels of ACSL1 and CPT1B were determined using qPCR. Data are presented as mean±standard error of mean (SEM) of three experiments, analyzed by Student's t-test,* P<0.05, ** P<0.01, *** P<0.001.

## 3. Discussion

Several different "omics" techniques have been used to study the interaction between small molecules and hosts, such as genomics, transcriptomics, proteomics, and metabolomics. The application of these omics methods has revealed important information about many biological systems and has contributed greatly to the development of systems biology [31]. Metabolomics is an established omics method based on the analysis of low molecular weight metabolites that provides a snapshot of the host's overall physiology and its response to the environment. In our study, we conducted a LC-Q-TOF-MS based untargeted metabolomics approach to investigate whether the metabolic profile can differentiate DHP exposure from controls. Our results suggested that DHP exposure caused significant dynamic changes in metabolite balance, as there was distinct separation between DHP and control groups. A total of 90 putatively annotated/characterized compound or compound class were found, of which 58 were detected in the ESI+ mode and 32 in the ESI- mode. These putatively annotated/characterized metabolites could be classified into several categories: lipids and lipids-like molecules, organoheterocyclic compounds, phenylpropanoids and polyketides, and other metabolites. Terpenoids are natural components of animals and plants, mainly in the form of monoterpenoids, diterpenoids, triterpenoids, sesquiterpenoids, and tetraterpenoid carotenoids [32]. Hundreds of terpenoids have been reported to have chemopreventive activity [33], and animals can utilize these

compounds to achieve hormone growth regulation. The presence of terpenoids can prevent certain diseases, especially those associated with chronic damage and growth disorders. Significant differences in terpenoids, including monoterpenoids, diterpenoids, triterpenoids, and sesquiterpenoids have been detected between DHP group and control group in our experiments.. We found that DHP exposure significantly reduced the levels of most detected terpenoids. Studies have shown that monoterpenoids can be used in the prevention and treatment of a variety of cancers, including prostate cancer [34-36]. A previous study reported that phthalates induced prostate cancer cells proliferation [37]. Thus, the reduced levels of monoterpenoids by DHP may be associated with the occurrence of prostate cancer. In addition, triterpenoids, as natural substances including steroids and sterols [38], have been proved to possess the properties of anti-inflammatory and anti-cancer [39,40]. The reduction of metabolites may imply a reduced resistance to inflammation after exposing to DHP. It is worth noting that, a kind of retinoid level was significantly elevated, which is one of the important diterpenoids in animals, possesses anti-inflammatory and anti-cancer activity [41]. It may act as compensatory effect to counteract the inflammatory response caused by DHP.

Fatty acids are one of the body's main sources of energy. It has been reported that fatty acid synthesis and oxidation can affect cell survival and growth [30]. In our results, we observed that some fatty acids and conjugates were exhibited different changes, implying that DHP exposure may affect fatty acids metabolism. Moreover, Shen et al. have demonstrated that DEHP affected fatty acid synthesis by decreasing the enzyme expression of fatty acid synthesis in mouse testes and inhibited fatty acids oxidation degradation, thereby reducing the energy supply and causing testicular cells atrophy [42]. Both DHP and DEHP are substances with large molecular weights, have similar structures and long alcohol chain residues. Despite the similarities with DEHP, whether DHP affects

198 the expression of fatty acid synthesis and oxidation-related enzyme genes are still poorly known. In the
199 present study, we detected that key fatty acid synthesis-related enzymes expression levels were
200 significantly reduced, which explains the reduction in fatty acids level. Similarly, the expression of
201 fatty acid oxidation-related enzyme genes were also significantly decreased when exposed to DHP, this
202 may be responsible for the accumulation of certain fatty acids. Taken together, these results indicated
203 that exposure to DHP could induce abnormal fatty acid metabolism, it may retard fatty acids oxidation
204 and reduce energy supply.

205 In addition to changes in fatty acids and conjugates, we found that there was a significant increase
206 in fatty aldehyde levels, Fatty aldehydes can be oxidized and converted into plmitoyl-CoA under the
207 synergistic effect of various enzymes, and then enter the glycerophspholipid biosynthetic pathway [43].
208 Significant up-regulated in glycerophospholipids were observed upon DHP treatment. As a major
209 membrane lipid, glycerophospholipids participate in many physiological and pathological processes,
210 and play a vital role in cell signal transduction and material changes in life processes[44]. Early studies
211 have reported that increased levels of glycerophospholipids was related to liver injury [45], dementia
212 [46,47]. A previous study reported that exposure to phthalate mixtures or metabolites of phthalates
213 induced abnormal cognitive ability in adult rats [48,49]. So, the increase of glycerophospholipids may
214 be responsible for the decreased cognitive flexibility.

215 Moreover, significant differences in several amino acids, purines and purine derivatives were
216 found between the two groups. Some were significantly increased and others were decreased. Amino
217 acids are important components of proteins, they are involved in various metabolism pathways as
218 intermediates. Abnormal amino acid metabolism can lead to serious diseases in energy metabolism,
219 immunity, and nervous systems [48,50-52]. Purines are important components of DNA and RNA, they

also play a critical role in the process of metabolic regulation and energy supply [53]. Some amino acids and purines have been identified as potential biomarkers of type 2 diabetes and cancers [54,55]. Previous studies have shown that exposure to phthalates caused reduced cognitive flexibility in the brain of adult rats, and increase DNA damage [56], this may be related to altered amino acids and purines levels.

Furthermore, changes in bile acids, alcohols and derivatives were observed, they were significantly decreased by DHP treatment. Bile acids as common signal molecules are involved in regulating the bile acid themselves, and also regulate glucose and lipid metabolism in a variety of ways. Their main function is to dissolve dietary lipids, sterols and fat-soluble vitamins in the gut, and active pancreatic lipase to promote fat absorption [57]. Therefore, changes in bile acids and derivatives levels and composition may affect other systems, which in turn affect the homeostasis. Bile acids are produced by the metabolism of cholesterol in the liver [58]. We found that several cholestane steroids levels were significantly decreased under the treatment of DHP. That implied that DHP may decrease bile acids levels by affecting cholesterol metabolism. In addition, previous evidence has suggested that cholesterol metabolism is involved in Alzheimer's disease (AD) development [59,60], speculating that abnormal cholesterol metabolism caused by DHP may cause cognitive impairment.

## 4. Conclusions

This is the first report of a metabolomics approach to analyze endogenous differential metabolites under DHP treatment. In the present study, we used LC-Q-TOF-MS based metabolomics to capture differential metabolites in different groups. Our PLS-DA score plots analysis showed a clear separation,

indicating that the metabolic profiles of two groups were significantly different. A total of 90 putatively annotated/characterized metabolites were detected, they represent interference in a variety of metabolism pathways. In addition, exposure to DHP reduced the expression of crucial enzyme genes involved in fatty acids synthesis and oxidation. These findings will be helpful in exploring the possible toxic mechanisms of DHP exposure in certain chronic diseases.

## 5. Materials and method

### *5.1 Chemicals*

DHP (≥99.0%) and methanol (chromatographic grade, ≥99.9%) were purchased from Sigma-Aldrich Co. (St. Louis, USA). Analytical grade of chloroform, ethanol and isopropyl alcohol were obtained from the office of laboratory and equipment management of Nanjing Agricultural University. RNA extraction regent TRIzol was purchased from TransGen Biotech Co., Ltd. (Beijing, China). PrimeScript™ II 1st Strand cDNA Synthesis Kit was from TaKaRa Biotechnology Co., Ltd. (Dalian, China). SYBR Green Supermix was from Chun Lei Co., Ltd. (Beijing, China). DHP was dissolved in DMSO to obtain a stock solution of 100 mM, stored at 4°C, and then diluted to the desired concentration.

### *5.2 Cell culture and treatment*

The $U_2OS$ cells were cultured in a Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, USA) containing 10% fetal bovine serum (FBS, Cegrogen, South America), 1% Penicillin-Streptomycin solution (Hyclone, Logan, USA) and maintained at 37°C in 5% $CO_2$ under a humidified atmosphere. The $U_2OS$ cells were treated with DHP at a dose of 10 μM for 6 h in growth

medium before harvesting and analysis of metabolites and mRNA analysis.

*5.3 RNA extraction and Quantitative real-time PCR*

Quantitative real-time PCR was conducted on a CFX96 Touch real-time PCR detection system using a SYBR Green reporter as described previously [68]. The gene for glyceraldehyde -3- phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences used for qPCR were listed in Supplementary Materials, Table S1. Experiments were repeated 3 times on different days.

*5.4 Hydrophobic metabolite extraction*

$U_2OS$ cells were washed three times with sterile cold DPBS buffer before extraction with 1 mL pre-chilled 60% (v/v) methanol extraction solvent. Then cells were transferred to a new 2 mL frozen storage tube and quickly frozen with liquid nitrogen for 15 min. The samples were stored at -80°C and transported on dry ice. For detection, each sample was added with 800 µL 50% methanol extraction solvent and two small steel beads. The frequency of TissueLyser was adjusted to 50 HZ for 5 min, and the samples ground. After grinding, the steel beads were removed and the samples precipitated at -20 °C for 2 hours, then centrifuged at 10, 000 × g at 4 °C for 15 min. Subsequently, the supernatant (650 µL) was collected into a new tube and centrifuged again as described above. Finally, 50 µL of the supernatant was used for LC-MS analysis.

*5.5 LC-Q-TOF-MS analysis*

All chromatographic separations were conducted on an ACQUITY UPLC HSS T3 column (100mm*2.1mm, 1.8µm, Waters, UK). The column temperature was kept at 50 °C and the flow rate

was maintained at 0.4 mL/min. A binary gradient solvent mode including mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid) was run as follows for eluting the samples: 0~2 min, 100% phase A; 2~11 min, 0% to 100% B; 11~13 min, 100% B; 13~15 min, 0% to 100%. The injection volume of each sample was 10 μL. Mass spectrometric detection was performed on a high-resolution tandem mass spectrometer Xevo G2 XS QTOF (Waters, UK) equipped with an electrospray ionization source (ESI) operating in ESI+ and ESI- modes. The detailed parameters were set as below: capillary voltages, 3kV (ESI+) or 2 kV (ESI-); cone voltage, 40V; TOF mass range, 50–1200 Da; scan time: 0.2 s; trap CE ramp 20–40 eV, and the scan time was 0.2 s. In addition, take 50 μL of each sample and mix into one quality control (QC) sample, the detailed sample list is in Table S1. quality control (QC) samples were used to evaluate the reliability of the LC-MS system. Moreover, we used blank samples for pre-machine instrument quality control, mainly to ensure that there was no residual impuritie in the chromatographic column, and the blank sample data was not used for subsequent analysis.

*5.6 Data processing and statistical analysis*

The original data were processed by Progenesis QI v2.2 Software (Waters, Newcastle, UK), its detailed parameter information is in Table S2. This metabolomics analysis software package is widely used to process high-resolution LC-MS data. We obtained a data matrix containing mass-to-charge (m/z) ratio, retention time (RT), and peak intensities. The missing values are processed as follows: 1) Delete the ions whose missing value exceeds 50% in the QC sample; 2) Delete the ions whose missing value exceeds 80% in the sample; 3) Use k-nearest neighbors (KNN) method to perform the remaining missing values filling. The acquired data were imported into metaX software [69], an R language

analysis package for subsequent statistical analysis. PLS-DA is a supervised discriminant analysis statistical method that can best reflect the differences between classification groups [70]. The PLS-DA model quality was assessed by model parameters $R^2$ and $Q^2$ ($R^2$ represents the cumulative interpretation of model differences, $Q^2$ can reflect the predictive ability of the model), and 200 times permutation tests were conducted to verify the reliability of PLS-DA model. At the same time, the variable importance of the Projection (VIP) value summarizes the intensity and explanatory power of each metabolite to the classification and discrimination of each group of groups, and a VIP value >1 reflects important variables affecting the model [71]. Differential metabolites were screened in combination with T-test and fold change analysis (FC analysis) of univariate analysis ($p<0.05$ and $FC\geq1.2$ or $\leq0.8333$). FC can be used to measure the relative changes in specific metabolite concentrations under different conditions in the study. FC values are calculated on the average original signal strength relative to the given reference sample [72]. Cluster analysis was carried out on the selected differential metabolites and presented in the form of heat map, which was generated by MetaboloAnalyst 3.0. The putative metabolites were first identified by searching for exact molecular mass data from redundant m/z peaks based on the online HMDB and KEGG databases. When the match between the observed mass and the theoretical mass is less than 10 ppm, the specific metabolite will be screened out. The metabolite formula was then further confirmed by isotope distribution measurements.

Other data were showed as mean $\pm$ SEM and statistical analysis were performed using GraphPadPrism, version6.0 (GraphPad Software, SanDiego, CA, USA). Data were evaluated by two-tailed Student's *t*-test to detect the differences among different group. $P< 0.05$ was considered statistically significant. Experiments were repeated at least 3 times on different days.

## Supplemental Materials

**Table S1** Primers for qPCR analysis

| Genes | Primer sequence (5'-3') |
|---|---|
| GAPDH | F: GTGGTCTCCTCTGACTTCAACAG |
| | R: CTGTAGCCAAATTCGTTGTCATAC |
| FASN | F: ACAGCGGGGAATGGGTACT |
| | R: GACTGGTACAACGAGCGGAT |
| FADS1 | F: GTTATCCAGCGAAAGAAGTGGG |
| | R: CCAATAGTGGCACATAAGTGAGG |
| FADS2 | F: GACCACGGCAAGAACTCAAAG |
| | R: GAGGGTAGGAATCCAGCCATT |
| ELOVL5 | F: ATGGTTTGTCGTCAGTCCCTT |
| | R: CCACCAGAGGTATGGACGC |
| ACSL1 | F: CGACGAGCCCTTGGTGTATTT |
| | R: GGTTTCCGAGAGCCTAAACAA |
| CPT1B | F: CCTGCTACATGGCAACTGCTA |
| | R: AGAGGTGCCCAATGATGGGA |
| CPT2 | F: CATACAAGCTACATTTCGGGACC |
| | R: AGCCCGGAGTGTCTTCAGAA |
| ACADM | F: ACAGGGGTTCAGACTGCTATT |
| | R: TCCTCCGTTGGTTATCCACAT |
| ACADL | F: GATTAAAAGCCCAGGATACCGC |
| | R: AGGTGAGCAACTGTTTTGCCA |


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## Conflicts of Interest

The authors declare that there are no conflicts of interest

## Graphical abstract

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