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# The interactions among organophosphate pesticide exposure, oxidative stress, and genetic polymorphisms of dopamine receptor D4 increase the risk of attention deficit/hyperactivity disorder in children

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ABSTRACT

*Objective:* The aim of this study was to clarify the association between organophosphate pesticides (OPs) and attention-deficit/hyperactivity disorder (ADHD) related to oxidative stress and genetic polymorphisms.
*Methods:* This case-control study enrolled 93 children with ADHD and 112 control children in north Taiwan. Six dialkyl phosphate (DAP) metabolites of OPs and oxidative stress biomarkers were analyzed. Polymorphisms of the dopamine receptor D4 gene (*DRD4*) were identified.
*Results:* Children with ADHD had significantly higher dimethylphosphate (DMP, 236.69 nmol/g cre. vs. 186.84 nmol/g cre., p value = 0.01) and 4-hydroxy-2-nonenal-mercapturic acid (HNE-MA, 28.95 μg/g cre. vs. 16.55 μg/g cre., p value < 0.01) concentrations than control children. Children who carried *DRD4* GA/AA genotypes (rs752306) were less likely than those who carried the *DRD4* GG genotype to have ADHD (odds ratio [OR]: 0.45, 95% CI: 0.24–0.84). The estimated value of the AP (attributable proportion due to interaction) was 0.59 (95% CI: 0.13–1.05), indicating that 59% of ADHD cases in DMP-exposed children with the *DRD4* GG genotype were due to the gene-environment interaction. After adjustment for other covariates, children who carried the *DRD4* GG genotype, had been exposed to high DMP levels (more than the median), and had high HNE-MA levels had a significantly increased risk for developing ADHD (OR = 11.74, 95% CI: 2.12–65.04).
*Conclusion:* This study indicated a gene-environment interaction in the risk of ADHD in children. The association between DMP and ADHD in children might relate to the mechanism of lipid peroxidation. Dose-response relationships and the combined effects of OPs, oxidative stress, and genetic polymorphism on ADHD should not be neglected.

## 1. Introduction

Organophosphate pesticides (OPs) are synthetic chemicals that are widely used in agricultural applications and household pest control. It has been confirmed that OPs cause both acute and chronic toxic effects by inhibiting acetyl cholinesterase (AChE) or by affecting target organs directly (Karami-Mohajeri and Abdollahi, 2011; Pakravan et al., 2016; Surajudeen et al., 2014). Both in vitro and in vivo, OPs affect the peripheral and central nervous system by disturbing redox processes, inducing oxidative stress, and disrupting neuronal development (Karami-Mohajeri and Abdollahi, 2011; Ojha and Srivastava, 2012; Ojha et al., 2013; Singh et al., 2004, 2006). Pregnancy and early childhood are critical stages during which the development of the human brain and neural structures is sensitive. However, fetuses and young children are vulnerable to OPs and have low detoxifying enzyme activity (Furlong et al., 2006; Holland et al., 2006).

Attention deficit/hyperactivity disorder (ADHD) is the most frequently diagnosed neurobehavioral disorder in children. The estimated

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**Nomenclature**

| | |
|---|---|
| 8-iso-$PGF_{2\alpha}$ | 8-iso-prostaglandin $F_{2\alpha}$ |
| 8-$NO_2$Gua | 8-nitroguanine |
| 8-OHdG | 8-hydroxy-2-deoxyguanosine |
| ADHD | Attention-deficit/hyperactivity disorder |
| DAP | Dialkyl phosphate |
| DEP | Diethyl phosphate |
| DETP | Diethylthio phosphate |
| DEDTP | Diethyldithio phosphate |
| DMP | Dimethyl phosphate |
| DMTP | Dimethylthio phosphate |
| DMDTP | Dimethyldithio phosphate |
| DRD4 | Dopamine receptor D4 gene |
| HNE-MA | N-acetyl-S-(tetrahydro-5-hydroxy-2-pentyl-3-furanyl)-L-cysteine |
| OPs | Organophosphate pesticides |

prevalence of ADHD in children ranged from 6.7% to 7.8% in a meta-analytic study (Thomas et al., 2015). The etiology of ADHD is still unclear and is mainly associated with disturbances in dopaminergic activity (Takeda et al., 2014; Wohleb et al., 2014). Recently, the associations between gestational exposure to OPs and neurobehavioral outcomes of neonates were indicated, but the detrimental effects of low-level exposure were still controversial (Yolton et al., 2013; Zhang et al., 2014). Furthermore, several studies suggested low-level OP exposure correlated with neurobehavioral effects in children, affecting domains such as motor skills, short-term memory, and ADHD (Bouchard et al., 2010; Eskenazi et al., 2007; Ruckart et al., 2004).

An increasing body of evidence indicates that the developing brain is especially vulnerable to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Excessive production of ROS and RNS causes progressive oxidative damage (Ikonomidou and Kaindl, 2011). In animal studies, OPs caused marked DNA damage in the rat brain and disturbed redox homeostasis (Ojha and Srivastava, 2012; Ojha et al., 2013). Treatment with OPs also produced a dose- and time-dependent increase in lipid peroxidation in peripheral blood lymphocytes of rats (Ojha and Gupta, 2016). However, the associations among OPs, oxidative stress, and ADHD in children are still not well explored.

ADHD has a high degree of heritability, estimated at approximately 76% (Faraone et al., 2005). Single-nucleotide polymorphisms (SNPs) contribute to individual genetic susceptibility to ADHD. Polymorphism of the dopamine D4 receptor gene (*DRD4*) is significantly associated with ADHD risk (Brookes and others, 2006; Gehricke et al., 2015; Gizer et al., 2009). Even though the etiology of ADHD remains unclear, it is believed that individual genetic and environmental factors as well as gene-environment interaction play important roles (Banerjee et al., 2007; Lee and Humphreys, 2014). Our previous study showed that children with ADHD had higher OP levels than control children, and the relationship was dose dependent (Yu et al., 2016a). This association between OPs and child ADHD related to oxidative stress and genetic polymorphisms needs to be clarified. The objective of this study is to explore the association among OPs exposure, oxidative stress, and genetic polymorphisms in child ADHD and to further characterize the gene-environment interaction.

## 2. Materials and methods

### 2.1. Study design and subject recruitment

The study subjects in this case-control study in North Taiwan were the same subjects used in the previous study (Yu et al., 2016b, 2016c), and the protocol was approved by the Taipei City Hospital institutional review board. Children aged 4–15 years who had been diagnosed with ADHD and clinically assessed at least three times were enrolled. ADHD symptoms were identified in accordance with the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th edition, text revision (DSM-IV-TR), by board-certified pediatricians or psychiatrists. The control subjects were age-matched children who visited Taipei City Hospital for non-ADHD-related purposes. The Chinese version of the Swanson, Nolan and Pelham, Fourth Revision (SNAP-IV) questionnaire was used to assess control children's behavior at home and in the classroom by the reports of their parent(s) and teachers, respectively (Bussing et al., 2008; Gau et al., 2009, 2008). Children with suspected ADHD symptoms were further evaluated by pediatricians or psychiatrists. The exclusion criterion was a diagnosis of neurological deficits (e.g. cerebral palsy, spinal bifida, epilepsy) or intellectual disability. In addition, children with medical disease or neurologic condition (e.g. seizure disorder, bipolar disorder) which might interfere with the assessment of ADHD were also excluded.

After written consent forms were signed by each children's parents or guardians, a structured questionnaire was distributed to request information about socio-demographic characteristics, lifestyle, family history of nervous system diseases (e.g. Parkinson's disease, Alzheimer's disease, ADHD, intellectual disability, cerebral palsy, autism, epilepsy, developmental delay, multiple sclerosis, and peripheral neuromuscular disease in grandparents, parents, or siblings of the child), and maternal pregnancy conditions (e.g. smoking, drinking, medication use, and environmental tobacco smoke exposure during pregnancy). A spot urine and oral swab specimen were collected and stored at − 20 °C until analysis.

### 2.2. Measurement of organophosphate pesticides

The analytical method was identical to that of the previous study (Kupfermann et al., 2004; Yu et al., 2016a). A volume of 750 μL of isopropanol was added to a 250 μL urine sample and then dried under nitrogen at 65 °C. Subsequently, 500 μL of acetonitrile and 25 μL of benzyl bromide were added to the residue and incubated in a water bath at 65 °C for 15 min. The mixtures were extracted twice using 2 mL hexane per extraction. The upper-phase solvent was dried under nitrogen at 40 °C, dissolved with 250 μL of hexane, and placed on a 100-mg silica cartridge. The lower-phase residue was added to a solution of methanol, Amberlite (IR) 120 (Sigma-Aldrich, USA), and diazotoluene; evaporated under 40 °C; dissolved with 250 μL of hexane; and placed on a 100-mg silica cartridge. Each SPE (solid-phase extraction) cartridge (Waters, Ireland) was preconditioned with 5 mL of hexane. After sample application, the analytes were eluted with 1 mL of methanol/dichloromethane (50:50, v/v) and evaporated at 40 °C. Finally, each sample was reconstituted in 100 μL of toluene, and an aliquot of 1 μL was injected into a gas chromatography-mass spectrometry (GC-MS) system. Urinary dialkyl phosphate (DAP) metabolites of OPs were analyzed. The six DAPs of interest were dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP). Each DAP was adjusted by the corresponding molecular weight and normalized to creatinine.

### 2.3. Measurement of oxidative stress

The analytical method was identical to that of the previous study (Wang et al., 2015a, 2015b; Wu et al., 2016). Urinary 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-nitroguanine (8-$NO_2$Gua), 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), and N-acetyl-S-(tetrahydro-5-hydroxy-2-pentyl-3-furanyl)-L-cysteine (HNE-MA) were simultaneously analyzed using high-performance liquid chromatography-electrospray ionization

tandem mass spectrometry (HPLC-ESI-MS/MS). In brief, each urine sample was centrifuged at 10,000 rpm for 10 min, and 100 μL of supernatant was diluted 10-fold with deionized water containing 1 mM ammonium acetate. Each SPE cartridge (Oasis HLB cartridge, Waters, USA) was preconditioned with 2 mL of methanol and 2 mL of water. After sample application, each cartridge was washed with 2 mL of water. The analytes were eluted with 1 mL of methanol, evaporated to dryness with a vacuum centrifuge, and redissolved in 200 μL of 5% methanol containing 1 mM ammonium acetate. Finally, an aliquot of 25 μL was injected into an HPLC-ESI-MS/MS system. Each oxidative stress biomarker was normalized to creatinine.

### 2.4. Measurement of creatinine

Concentrations of urinary creatinine were determined using a commercial kit (Eagle Diagnostics, Desoto, Texas, USA). Briefly, 0.1 mL of each urine sample was added to 3 mL picric acid (3.3 mM) mixed with sodium hydroxide (0.17 M) and sodium tetraborate (26 mM). After incubation at 37 °C for 15 min in a shaker bath, the creatinine concentration was quantified with a spectrophotometer at a wavelength of 510 nm.

### 2.5. DRD4 genetic polymorphism determination

The method was identical to that of the previous study (Yu et al., 2016a, 2016b, 2016c). Genomic DNA was extracted from mouth swabs using a QIAamp DNA Mini Kit in accordance with the manufacturer's instructions (QIAGEN, Hilden, Germany). SNP (single-nucleotide polymorphism) genotyping was performed using Sequenom MassARRAY technology with iPLEX gold chemistry (Sequenom, San Diego, CA). In total, 4 *DRD4* SNPs, namely, rs7395429, rs3758653, rs11246228, and rs752306, were identified. Briefly, 10 ng of the DNA sample was subjected to PCR in a reaction volume of 5 μL containing 0.2 units of Taq polymerase, 2.5 pmol of each PCR primer and 25 mM of each dNTP. Thermocycling was set at 94 °C for 2 min followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final extension was done at 72 °C for 1 min. The amplified DNA was then subjected to primer extension using an iPLEX Gold Reagent Kit. Primer extension was performed with a cycling program of 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 5 cycles of 52 °C for 5 s, and 80 °C for 5 s within 40 cycles. The final extension was performed at 72 °C for 3 min. The extended reaction products were purified by cation exchange resins and then spotted onto a 384-format SpectroCHIP II array using a MassARRAY Nanodispenser RS1000. The genotypes were tested for deviation from Hardy-Weinberg equilibrium, and associations were analyzed using standard chi-squared goodness-of-fit tests.

### 2.6. Statistical analysis

SAS version 9.3 (SAS Institute, Cary, NC, USA) was used for the statistical analysis. The level of significance was set to $p < 0.05$ for all statistical tests. The differences between children with ADHD and control children were initially explored in terms of demographic characteristics, maternal pregnancy conditions, family history of nervous system diseases, and polymorphisms of *DRD4*. DAPs and oxidative stress levels were compared between these two groups using the Wilcoxon rank-sum test. To explore the association of genetic polymorphisms with OPs exposure in children with ADHD, the odds ratio (OR) with 95% confidence interval (CI) and additive interactions including relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP), and synergy index (S) were calculated. Potential confounder associated with ADHD and OPs was mainly considered based on gender. Though the etiologies of various nervous system diseases were still unclear, studies showed that there might be familial clustering across generations and deficits in the neuroprotection function against assorted risk factors (Biederman et al., 1990; Millichap, 2008). Family history of nervous system disorders, such as Parkinson's disease, Alzheimer's disease, epilepsy, autism, etc. were also selected as confounders for the high degree of heritability in ADHD (Bertelsen et al., 2016; Ghirardi et al., 2017; Walitza et al., 2007; Zhang et al., 2015). The age and BMI of the children were viewed as modifiers. Other covariates associated with ADHD at $p < 0.1$, including maternal education level, alcohol use and environmental tobacco smoke exposure during pregnancy were adjusted in the analysis. To clarify the combined effects of OPs, oxidative stress levels, and genetic variants on ADHD, children were further categorized into several subgroups by the median of OPs or oxidative stress levels, and adjusted ORs were estimated using multiple logistic regression.

**Table 1**
Demographic characteristics and *DRD4* polymorphisms among all children.

| | ADHD, N (%) | Control, N (%) | OR (95% CI) |
|---|---|---|---|
| Total | 93 | 112 | |
| Age (years, mean ± SD) | 8.8 ± 2.7 | 8.9 ± 2.0 | |
| Gender | | | |
| Male | 77 (82.8) | 67 (59.8) | 1 |
| Female | 16 (17.2) | 45 (40.2) | 0.31 (0.16–0.60)* |
| Maternal educational levels | | | |
| High school and below | 40 (43.0) | 30 (26.8) | 1 |
| College or above | 53 (57.0) | 82 (73.2) | 0.48 (0.27–0.87)* |
| Maternal drinking during pregnancy | | | |
| No | 78 (83.9) | 107 (95.5) | 1 |
| Yes | 15 (16.1) | 5 (4.5) | 4.12 (1.44–11.80)* |
| Family history of nervous system diseases | | | |
| No | 62 (66.7) | 91 (81.3) | 1 |
| Yes | 31 (33.3) | 21 (18.7) | 2.17 (1.14–4.11)* |
| Environmental tobacco smoke exposure | | | |
| No | 50 (53.8) | 82 (73.9) | 1 |
| Yes | 43 (46.2) | 29 (26.1) | 2.43 (1.35–4.38)* |
| Polymorphisms of dopamine receptor D4 | | | |
| rs7395429 | | | |
| TT | 58 (62.4) | 54 (48.2) | 1 |
| TC | 28 (30.1) | 47 (42.0) | 0.56 (0.31–1.01) |
| CC | 7 (7.5) | 11 (9.8) | 0.59 (0.21–1.64) |
| rs3758653 | | | |
| TT | 45 (48.4) | 44 (39.3) | 1 |
| TC | 43 (46.2) | 56 (50.0) | 0.75 (0.42–1.33) |
| CC | 5 (5.4) | 12 (10.7) | 0.41 (0.13–1.25) |
| rs11246228 | | | |
| TT | 19 (20.4) | 36 (32.1) | 1 |
| TC | 47 (50.5) | 52 (46.4) | 1.71 (0.87–3.39) |
| CC | 27 (29.1) | 24 (21.4) | 2.13 (0.98–4.66) |
| rs752306 | | | |
| GG | 72 (77.4) | 68 (60.7) | 1 |
| GA/AA | 21 (22.6) | 44 (39.3) | 0.45 (0.24–0.84)* |

One child with ADHD and five control subjects carried the *DRD4* AA genotype (rs752306).
* p value < 0.05.

## 3. Results

Among the 205 children, comprising 93 children with ADHD and 112 control children, the average age was 8 years and the majority of children with ADHD were male (82.8% vs. 59.8%) (Table 1). The children's gender, maternal education level, alcohol consumption and environmental tobacco smoke exposure during pregnancy, and family history of nervous system diseases carried significant ORs for ADHD risk. There were no significant differences in the *DRD4* genotype frequency of rs7395429, rs3758653, or rs11246228 between these two groups. Children who carried *DRD4* GA/AA genotypes (rs752306) were less likely than those with the *DRD4* GG genotype to have ADHD (OR: 0.45, 95% CI: 0.24–0.84).

Table 2 summarizes the concentrations of six DAPs for all children

**Table 2**
The distribution of urinary organophosphate metabolites among children.

| | Group | Detection rate (%) | Mean (SE) | Range | 25th %ile | 50th %ile | 75th %ile | p value |
|---|---|---|---|---|---|---|---|---|
| DMP | Total | 99.0 | 271.59 (17.39) | 3.25–2306.18 | 124.77 | 208.73 | 351.19 | 0.01 |
| | ADHD | 100 | 316.77 (31.50) | 22.98–2306.18 | 148.56 | 236.69 | 366.33 | |
| | Control | 98.2 | 234.09 (17.49) | 3.25–1279.90 | 112.22 | 186.84 | 310.58 | |
| DMTP | Total | 97.6 | 189.46 (19.19) | 1.66–1885.63 | 39.06 | 96.29 | 197.37 | 0.65 |
| | ADHD | 97.8 | 220.38 (35.05) | 1.73–1885.63 | 40.06 | 96.72 | 262.54 | |
| | Control | 97.3 | 163.79 (19.48) | 1.66–1111.52 | 38.51 | 89.93 | 183.14 | |
| DMDTP | Total | 83.9 | 24.93 (2.18) | 0.65–201.29 | 3.87 | 16.21 | 32.75 | 0.52 |
| | ADHD | 89.2 | 25.46 (3.42) | 0.71–201.29 | 7.17 | 16.35 | 29.31 | |
| | Control | 79.5 | 24.49 (2.83) | 0.65–164.42 | 2.19 | 15.65 | 33.58 | |
| DEP | Total | 85.8 | 72.11 (13.57) | 1.86–2316.89 | 16.15 | 39.18 | 68.81 | 0.79 |
| | ADHD | 84.9 | 92.38 (28.72) | 1.86–2316.89 | 19.41 | 38.44 | 70.38 | |
| | Control | 86.6 | 55.27 (6.77) | 3.71–629.14 | 14.55 | 40.10 | 65.28 | |
| DETP | Total | 94.6 | 102.95 (11.64) | 0.81–1163.61 | 25.48 | 52.75 | 103.10 | 0.85 |
| | ADHD | 100 | 87.23 (14.54) | 5.54–1157.27 | 28.40 | 44.38 | 90.80 | |
| | Control | 90.2 | 116.01 (17.51) | 0.81–1163.61 | 23.51 | 59.04 | 110.68 | |
| DEDTP | Total | 59.8 | 16.66 (3.69) | 0.75–516.10 | 1.59 | 4.56 | 13.38 | 0.28 |
| | ADHD | 61.3 | 10.67 (2.58) | 0.75–228.34 | 1.59 | 4.03 | 11.14 | |
| | Control | 58.0 | 21.63 (6.38) | 0.75–516.10 | 1.58 | 5.29 | 14.68 | |

Concentrations less than LOD were replaced with values equal to LOD/2.
Unit of concentration: nmol/g cre.

(Table 2). The detection rates were 59.8% for DEDTP, 83.9% for DMDTP, 85.8% for DEP, 94.6% for DETP, 97.6% for DMTP, and 99.0% for DMP. Concentrations less than the limit of detection (LOD) were replaced with values corresponding to LOD/2 (Hornung and Reed, 1990). The medians for DMP, DMTP, DMDTP, DEP, DETP, and DEDTP were 208.73, 96.29, 16.21, 39.18, 52.75, and 4.56 nmol/g cre. (creatinine), respectively. Children with ADHD had significantly higher DMP concentrations than control children had (236.69 nmol/g cre. vs. 186.84 nmol/g cre., p value = 0.01).

Table 3 summarizes the concentrations of four urinary oxidative stress biomarkers for all children (Table 3). The detection rates were 76.1% for 8-$NO_2$Gua, 90.7% for 8-iso-$PGF_{2\alpha}$, 97.6% for 8-OHdG, and 100% for HNE-MA. The LODs were 0.40 ng/mL for 8-$NO_2$Gua, 0.20 ng/mL for HNE-MA, 0.20 ng/mL for 8-iso-$PGF_{2\alpha}$, and 0.10 ng/mL for 8-OHdG. Concentrations less than the LOD were replaced with values equal to LOD/2. The medians for 8-$NO_2$Gua, HNE-MA, 8-iso-$PGF_{2\alpha}$, and 8-OHdG were 0.50, 20.42, 4.67, and 5.97 μg/g cre. respectively. Children with ADHD had significantly higher HNE-MA concentrations than control children had (28.95 μg/g cre. vs. 16.55 μg/g cre., p value < 0.01).

To further explore gene-environment interaction in ADHD, children were stratified by DMP quartile and *DRD4* SNP genotype (rs752306).

**Table 4**
The additive interaction between *DRD4* polymorphism and urinary DMP levels on children with ADHD.

| | rs752306 (GA/AA) | | rs752306 (GG) | |
|---|---|---|---|---|
| | Control/ ADHD | OR (95% CI) | Control/ ADHD | OR (95% CI) |
| DMP | | | | |
| < 25th percentile | 13/5 | 1 | 23/10 | 1.13 (0.32–4.03) |
| 25th–75th percentile | 22/10 | 1.18 (0.33–4.22) | 31/39 | 3.27 (1.05–10.17)* |
| > 75th percentile | 9/6 | 1.73 (0.40–7.46) | 14/23 | 4.27 (1.25–14.57)* |
| RERI (95% CI) | 2.11 (− 0.03 to 4.25) | | | |
| AP (95% CI) | 0.59 (0.13–1.05)** | | | |
| S index (95% CI) | 5.47 (0.05–602.96) | | | |

* p value < 0.05.
** A significant additive interaction between rs752306 and DMP levels greater than Q1, as indicated by a 95% CI that does not include the null value (0 for AP).

**Table 3**
The distribution of urinary oxidative stress biomarkers among children.

| Oxidative stress | Group | Detection rate (%) | Mean (SE) | Range | 25th %ile | 50th %ile | 75th %ile | p value |
|---|---|---|---|---|---|---|---|---|
| 8-$NO_2$Gua | Total | 76.1 | 1.21 (0.38) | 0.08–76.79 | 0.35 | 0.50 | 0.96 | 0.59 |
| | ADHD | 76.3 | 1.72 (0.82) | 0.08–76.79 | 0.36 | 0.53 | 0.89 | |
| | Control | 75.9 | 0.79 (0.08) | 0.08–4.03 | 0.34 | 0.48 | 1.03 | |
| HNE-MA | Total | 100 | 48.42 (5.32) | 2.21–478.89 | 10.30 | 20.42 | 43.80 | < 0.01 |
| | ADHD | 100 | 62.72 (8.72) | 2.62–432.56 | 12.84 | 28.95 | 73.72 | |
| | Control | 100 | 36.55 (6.33) | 2.21–478.89 | 9.31 | 16.55 | 27.99 | |
| 8-iso-$PGF_{2\alpha}$ | Total | 90.7 | 8.50 (1.00) | 0.05–153.83 | 1.65 | 4.67 | 10.79 | 0.66 |
| | ADHD | 90.3 | 7.68 (1.08) | 0.05–81.40 | 1.79 | 4.23 | 10.54 | |
| | Control | 91.1 | 9.19 (1.61) | 0.07–153.83 | 1.64 | 5.05 | 11.02 | |
| 8-OHdG | Total | 97.6 | 10.83 (3.88) | 0.02–796.88 | 3.77 | 5.97 | 8.64 | 0.54 |
| | ADHD | 96.8 | 15.80 (8.54) | 0.05–796.88 | 3.68 | 5.23 | 8.75 | |
| | Control | 98.2 | 6.70 (0.36) | 0.02–21.01 | 4.06 | 6.23 | 8.38 | |

Concentrations less than LOD were replaced with values equal to LOD/2.
Unit of concentration: μg/g cre.

Children who carried the *DRD4* GG genotype and had DMP concentrations above the first quartile had a significantly higher risk of ADHD than those who carried the *DRD4* GA/AA genotypes and had DMP concentration below the first quartile (Table 4). A dose-response relationship also existed. There was a significant additive interaction between *DRD4* polymorphism and DMP levels for ADHD. The estimated AP was 0.59 (95% CI: 0.13–1.05), indicating that 59% of ADHD cases in children who carried the *DRD4* GG genotype and had DMP concentrations above 124.77 nmol/g cre. were attributable to the gene-environment interaction.

The combined effects of *DRD4* polymorphism (rs752306), DMP concentrations, and internal HNE-MA level on the risk of developing ADHD were estimated by multivariable logistic regression (Table 5). After adjustment for other covariates, no significant associations between ADHD and genetic polymorphism or oxidative stress existed in children with DMP concentrations below the median level. Children who carried the *DRD4* GG genotype, had been exposed to high DMP levels (above the median), and had low HNE-MA levels (below the median) had a significantly increased risk for developing ADHD (OR = 6.41, 95% CI: 1.15–35.82) compared with a reference group. The risk increased to 11-fold in children who carried the *DRD4* GG genotype and simultaneously had high DMP and HNE-MA levels (OR = 11.74, 95% CI: 2.12–65.04).

## 4. Discussion

The neurobehavioral effects of low-level OP exposure on children have been reported to include motor inhibition; learning impairments; behavioral problems; and deficits in social functioning, working memory, and verbal comprehension (Furlong et al., 2014; Kofman et al., 2006; Oulhote and Bouchard, 2013; Wang et al., 2016). In the National Health and Nutrition Examination Survey (NHANES) in the United States, children with higher urinary concentrations of dialkyl phosphates (DAPs) were more likely to be diagnosed with ADHD (Bouchard et al., 2010). This case-control study also demonstrated dose-response effects of DMP and oxidative stress (HNE-MA) levels on child ADHD.

Some studies indicated that OPs induced oxidative stress that could cause DNA damage and lipid peroxidation. In vitro, OPs increased DNA strand breaks, ROS superoxide anion concentration, and hydrogen peroxide in rat peripheral blood lymphocytes (Ojha and Srivastava, 2014). In rat erythrocytes (red blood cells, RBCs) treated with OPs, oxidative stress increased with decreasing glutathione (GSH) content, and lipid peroxidation occurred through interaction with free radicals (Singh et al., 2004, 2006). A biomonitoring study showed that occupational pesticide applicators had high serum myeloperoxidase (MPO) and malondialdehyde (MDA) levels (Surajudeen et al., 2014). In this study, 8-$NO_2$Gua was significantly associated with DETP (r = 0.18, p value = 0.01) as well as DEDTP (r = 0.16, p value = 0.03), while HNE-MA was significantly associated with DMP, DMDTP, and DETP (r = 0.14–0.15, p value = 0.04).

There is increasing evidence of the role of oxidative stress in the function and dopaminergic activity of the brain (Avshalumov et al., 2005; Kiss et al., 2004; Sezen et al., 2016). The susceptibility of the developing brain is influenced by its high oxygen utilization and lipid concentration and low antioxidant concentrations, which make the brain vulnerable to ROS and RNS (Ikonomidou and Kaindl, 2011). Intracellular hydrogen peroxide ($H_2O_2$) was found to modulate the excitability of midbrain dopamine neurons (Avshalumov et al., 2005). Nitric oxide (NO) showed an inhibitory effect on dopamine transporters (Kiss et al., 2004). In epidemiological studies, ADHD patients had a higher total oxidant status (TOS) and oxidative stress index than the control group (Guney et al., 2015; Kul et al., 2015; Sezen et al., 2016). In a meta-analysis, a significant association was found between ADHD and oxidative stress but not antioxidant status (Joseph et al., 2015). In this study, HNE-MA, a biomarker of lipid peroxidation, was nearly twice as high in children with ADHD as in control children. It is worth noting that there is an increased odds of ADHD among children who simultaneously have high DMP and HNE-MA concentrations.

Polymorphisms of *DRD4* have been extensively studied, and polymorphic variants could alter the activity of the gene and modulate individual susceptibility to ADHD occurrence and development (Gehricke et al., 2015; Nikolas and Momany, 2017; Pappa et al., 2014; Schweren et al., 2016). The high heritability of ADHD suggests that *DRD4* variants moderate the impact of parental ADHD and neurocognitive functioning on children's ADHD symptoms (Nikolas and Momany, 2017). *DRD4* may be involved in brain activity in response to negative emotional stimuli, and it may thereby be associated with increased ADHD symptoms (Gehricke et al., 2015; Pappa et al., 2014). Our results demonstrated not only an effect of *DRD4* genotype but an interaction between DMP levels and *DRD4* genotype on the risk of ADHD. To further clarify whether the ADHD risk associated with *DRD4* (rs752306) is attributable to high levels of DMP or HNE-MA, this study compared these biomarkers among children with different genotypes, and no significant difference existed (DMP, genotype GA/AA vs. GG = 36.20 vs. 54.10 nmol/g cre. p value = 0.12; HNE-MA, genotype GA/AA vs. GG = 234.42 vs. 288.86 μg/g cre., p value = 0.29). In addition, it has been shown that lipid peroxidation modulates the function of dopamine receptors and that 4-hydroxynonenal (4-HNE) can influence their binding activity (Shin et al., 2003). In this study, the mean HNE-MA levels increased as the DMP levels increased among children with the genotype GA/AA (DMP levels were classified by 1st quartile, interquartile range, and 3rd quartile, and the HNE-MA levels were 19.98, 37.82, and 47.91 μg/g cre., respectively) or GG (the HNE-MA levels were 32.71, 52.23, and 63.63 μg/g cre., respectively) of *DRD4*. This study suggests that the association between DMP and child ADHD might relate to the mechanism of lipid peroxidation. The combined effect of genetic polymorphism, OPs exposure, and oxidative stress on child ADHD should be a concern.

Our previous study indicated that a number of risk factors can contribute to child ADHD, such as alcohol consumption during pregnancy, parental education level, dietary habits, family history of nervous system diseases, gender, and exposure to environmental toxicants (Yu et al., 2016a, 2016b, 2016c). The levels of co-exposure to

**Table 5**
The association among *DRD4* polymorphism, HNE-MA, and ADHD, stratified by median DMP levels.

| | | DMP < Median | | | DMP ≥ Median | | |
|---|---|---|---|---|---|---|---|
| | | Control/ADHD | OR (95% CI) | AOR (95% CI) | Control/ADHD | OR (95% CI) | AOR (95% CI) |
| HNE-MA | rs752306 | | | | | | |
| < Median | GA/AA | 14/4 | 1 | 1 | 13/3 | 1 | 1 |
| ≥ Median | GA/AA | 10/5 | 1.75 (0.37–8.20) | 2.28 (0.39–13.30) | 7/9 | 5.57 (1.13–27.52)* | 5.66 (0.88–36.45) |
| < Median | GG | 25/13 | 1.82 (0.50–6.66) | 1.99 (0.46–8.61) | 15/16 | 4.62 (1.10–19.50)* | 6.41 (1.15–35.82)* |
| ≥ Median | GG | 15/17 | 3.97 (1.07–14.71)* | 4.14 (0.93–18.49) | 13/26 | 8.67 (2.09–35.89)* | 11.74 (2.12–65.04)* |

AOR (95% CI): Adjusted for age, sex, BMI, maternal education, pregnancy alcohol consumption, environmental tobacco smoke exposure and family history of nervous system diseases.
* p value < 0.05.

nonylphenol (NP) and lead among these children were also determined, but their effect on child ADHD was minor. In this study, although the ADHD patients were predominantly male (82.8%), the small number of females with ADHD presented similar results in the gender-stratified analysis, indicating that males and females with high levels of both DMP and HNE-MA had the highest risk of ADHD compared to the reference group (the ORs for males and females were 3.00 (95% CI: 1.20–7.51) and 6.00 (95% CI: 1.05–34.21), respectively). Organophosphorus pesticide interfered with hypothalamic neuroendocrine pathways regulating steroidogenesis in the sex-dependent pattern, which affected neurobehavioral differences between males and females (Venerosi et al., 2012). The sex-specific effects of pesticides exposure on neurobehavioral deficits were reported that girls tended to have impaired neuropsychological function, developmental delay in language function, and low motor speed functions (Andersen et al., 2015). In this study, the DMP (221.85 vs. 180.73 nmol/g cre., p value = 0.03) and HNE-MA (22.09 vs. 16.63 μg/g cre., p value = 0.06) in male were higher than those in female. Even though the high ORs of ADHD in female, differences in sex-specific susceptibility to OPs should be taken into consideration. OPs have been used for household and agricultural applications for decades, and the major pathways through which children are exposed to OPs are ingestion of food containing OPs residues and OPs-laden dust (Berman et al., 2016; Harnpicharnchai et al., 2013; Jaipieam et al., 2009; Julien et al., 2008). A variety of foods, such as vegetables, fruits, and juices, showed positive correlation with urinary dimethyl metabolites (Holme et al., 2016). In urban public housing, pesticides were detected in kitchen floor wipes and living room vacuum dust (Julien et al., 2008). Urinary DMP levels in this study were high compared to values reported from other countries, suggesting possible exposure to phenthoate, phosmet, dichlorvos, naled, trichlorfon, and malathion (Aprea et al., 2000; Barr et al., 2004; Becker et al., 2006; Bravo et al., 2004).

The half-life of OPs metabolites is short (24–27 h), and complete excretion from the body takes approximately 3–6 days (Bradway et al., 1977; Huen et al., 2012). Despite the rapid metabolism of OPs, the high detection rate of DAPs indicated a persistent exposure profile among children. The ubiquitous and relative high DAP level might derive from daily OPs intake. One study replaced of children's conventional diets with organic food and found that urinary DAP decreased to undetectable levels until the conventional diets were resumed (Lu et al., 2006). Even though DAP variation depends exclusively on dietary patterns, most children are already established in their eating behavior and food choices. Given this stable dietary intake, the spot urine samples could be representative of chronic internal OPs concentrations among these children.

The SNAP-IV questionnaire is a widespread instrument proven to have excellent validity and reliability in screening ADHD. The Chinese version of the SNAP-IV has been applied to determine ADHD symptoms and types (Gau et al., 2009, 2008). Once the SNAP-IV scores reached the criteria for inattention or hyperactivity/impulsivity, children were referred to pediatricians or psychiatrists for ADHD diagnosis in accordance with the DSM-IV-TR. Therefore, misclassification of ADHD should be avoided. The relatively small sample size, which was due to the challenge of recruiting school-age children, could be a limitation of this study. This study also cannot neglect the potentially significant results of recall bias regarding pregnancy conditions and family history of nervous diseases. Other factors, such as epigenetic modification, immunological activity, and other environmental toxicants, need to be explored further in relation to this complex and multifactorial neurobiological disorder.

## 5. Conclusion

This study indicates a dose-response relationship between the risk of child ADHD and OPs exposure as well as oxidative stress. There is a significant additive interaction between *DRD4* polymorphism and DMP levels for ADHD. The increased HNE-MA levels accompanying DMP suggest that child ADHD might relate to the mechanism of lipid peroxidation. The combined effects of OPs, oxidative stress, and genetic polymorphism on ADHD should not be neglected.

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## Conflicts of interest

The authors have no conflicts of interest to declare.

## Author contributions

Yu CJ, Du JC, Yang W, Chung MY, Hwang B, and Chen ML conceived and designed this study. Yu CJ, Du JC, Chiou HC, and Chen YS performed the experiments. Chang CH and Yu CJ analyzed the data. Du JC, Chen HC, and Mao IF contributed reagents/materials/analysis tools. Chang CH and Chen ML wrote the draft of the manuscript. Each author listed on the manuscript has seen and approved the submission of this version of the manuscript and takes full responsibility for the manuscript.

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