Reproductive Toxicology 46 (2014) 98–105

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Dialkyl phosphates in amniotic fluid as a biomarker of fetal exposure to organophosphates in Crete, Greece; association with fetal growth

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ARTICLE INFO

Article history:
Received 14 April 2013
Received in revised form 21 January 2014
Accepted 20 March 2014
Available online 29 March 2014

Keywords:
Amniotic fluid
Birth weight
Dialkyl phosphates
Fetal exposure
Fetal growth
Gas chromatography–mass spectrometry
Organophosphate pesticides
Pregnancy

ABSTRACT

The aim of this study was to evaluate fetal exposure to organophosphate pesticides (OPs) by measuring their non-specific dialkyl-phosphate metabolites (DAPs) in amniotic fluid (AF), and to examine the potential association between prenatal exposure and fetal growth. AF samples were collected from 415 women during the second gestational trimester. The determined OPs metabolites were DMP, DMTP, DEP, DETP, and DEDTP. DAPs were extracted by liquid–solid extraction, derivatized and analyzed by gas chromatography–mass spectrometry. 97.8% of AF samples were positive for at least one DAP. DAPs levels did not differ between urban and rural areas. Macrosomic neonates have significantly higher sum levels of DMPs ($p = 0.043$), which exerted a linear positive association with birth-weight centile ($b = 4.43$, $p = 0.016$). Conclusively, as DAPs are detectable in AF they may be used as a potential biomarker of fetal exposure to OPs. Sum levels of DMPs appear to be associated with birth weight independently of other covariates.

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1. Introduction

The residents of the island of Crete, Greece are a mixture of rural and urban population whose most important source of income is agriculture, mainly based on the production of olive oil, vegetables and fruits. In these types of crops, organophosphate pesticides (OPs) consist of a class of widely used neurotoxic insecticides and it is well established that environmental and/or dietary exposure to OPs results in the bioaccumulation of these chemicals in the human body, especially in adipose tissue, urine and breast milk [1–6]. During pregnancy, OPs which are lipophilic chemicals stored in maternal adipose tissue can be mobilized to the blood stream reaching the fetus through the placenta [7]. The next step is their rapid metabolism in the human body by hydrolysis or oxidative desulfuration, giving the non-specific metabolites dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP), referred as dialkyl phosphate metabolites (DAPs) [7–9]. These metabolites are polar water-soluble compounds and are used as biomarkers of OPs exposure on human population in various biological samples such as blood, urine, post-mortem tissue, hair, AF and meconium [2–4,10–12].

Maternal exposure to pesticides during pregnancy is a topic of major public concern as there are human and animal studies that associate the prenatal exposure to these compounds with aberrations in neuronal proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis. Prenatal exposure to organochlorine pesticides has been associated with adverse effects in neurodevelopment and growth in infancy and childhood [13–15]. Similarly, significant associations have been reported after prenatal exposure to OPs with structural changes in the developing human brain in terms of abnormal reflexes [16], reduced cognitive abilities [17–19], and attention problems [20,21]. Recent studies associate the prenatal exposure to OPs with pregnancy-associated complications such as shorter length of gestation and lower birth-weight [22]. In addition, there is increasing evidence in support of a

Abbreviations: AF, amniotic fluid; CV, coefficient of variability; DAPs, dialkyl phosphate metabolites; DEDTP, diethyl dithiophosphate; DEP, diethyl phosphate; DETP, diethyl thiophosphate; DMP, dimethyl phosphate; DMTP, dimethyl thiophosphate; OPs, organophosphate pesticides; GC–MS, gas chromatography–mass spectrometry; sumDMPs, sum levels of DMP and DMTP; sumDEPs, sum levels of DEP, DETP and DEDTP; sumDAPs, sum levels of DMP, DMTP, DEP, DETP and DEDTP.

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http://dx.doi.org/10.1016/j.reprotox.2014.03.010
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potential link between increased risk for childhood leukemia, brain cancer, neuroblastoma, non-Hodgkin's lymphoma, Wilms' tumor, and Ewing's sarcoma with pesticide exposure [23,24]. Although there is an increasing number of studies that determine pesticide metabolites levels in maternal urine or serum samples, there is no extending literature for measuring pesticides or metabolites in AF samples [25–27], and there is only one study that evaluates OP metabolite levels in AF [28]. The concentration of OP metabolites in AF collected during amniocentesis from pregnant women who are exposed to pesticides through a number of sources, including residential and agricultural applications, is likely to be a useful biomarker of direct fetal exposure to these chemicals between 16th and 20th weeks of gestation which is a critical period for the developing embryo.

The aim of this study was first, to determine the presence of OP non-specific metabolites (DAPs) in AF as an index for in utero exposure to these contaminants among a cohort of pregnant women in the island of Crete and second, to evaluate any potential link between prenatal exposure to OPs and birth weight. This is in continuation and advancement of the authors' studies hitherto focused on the determination of the parent compounds in fetal and maternal compartments [1–6,9].

2. Materials and methods

2.1. Study's design

The study was carried out between August 2006 and May 2008. 415 women with singleton pregnancies, who were permanent residents of Crete for at least two years, were enrolled. Women were recruited at the time of referral for amniocentesis to the Fetal-Maternal Unit, Department of Obstetrics and Gynecology, University Hospital of Heraklion, Crete, Greece. The indications for referral included increased risk for chromosomal abnormalities (based on advanced maternal age or the results of the first trimester combined test for fetal aneuploidies, or sonographically detected markers or abnormalities between 18 and 22 weeks of gestation) suspicion of genetic syndromes or increased risk for single gene disorders (based on history or sonographic findings), abnormal fetal growth, and suspicion of congenital infection. Written informed consent was obtained from each woman that agreed to participate. The Ethics Committee of the University Hospital of Crete approved the study's protocol.

2.2. Data collection

82.5% of the amniocenteses were carried out between 16th and 20th weeks of gestation and an additional volume of 8–10 ml of AF was obtained during each procedure. Each AF sample was frozen at −20 °C immediately after collection. The samples were analyzed to the laboratory of Toxicology, Medical School, University of Crete. The participants were asked to complete a detailed questionnaire about their medical history, demographic data, socioeconomic status, occupational and residential status and exposure to other potentially embryotoxic factors.

2.3. Materials and assays

Diethyl ether (95.5%), toluene (99.5%), hydrochloric acid (37%), natriumdisulfit (98%) and potassium carbonate were obtained from Merck (Darmstadt, Germany). Dimethyl phosphate (DMP, 98%) and dimethyl chlorothiophosphate (DMClTP, 97%) were purchased from Acros Organics (Geel, Belgium). Diethyl phosphate (DEP, 98.9%) was obtained from Chem Service (West Chester, USA), *O*,*O*-diethyl thiophosphate potassium salt (DETP, 98%) and diethyl dithiophosphate salt (DEDTP, 95%) from Sigma–Aldrich (Steinheim, Germany). Methanol and acetonitrile, both HPLC-grade, were purchased from Roth (Karlsruhe, Germany). Sodium chloride (NaCl) was from Riedel-de Haen (Seelze, Germany). The derivatization agent 2,3,4,5,6-pentafluoro benzylbromide (PFBBr, 99%) was purchased from Sigma–Aldrich (Steinheim, Germany) and water (LC–MS grade) from Sigma–Aldrich (Buchs, Switzerland). The synthesis of dimethyl thiophosphate (DMTP) was achieved by hydrolysis of 5 g of DMClTP in a solution of 40 ml of HPLC grade water–acetonitrile (10:30, v/v) and triethylamine (3 ml). After hydrolysis, acetonitrile was added in order to achieve a final volume of 200 ml [9,29].

2.4. Stock solutions

Stock solutions (1 mg/ml) of each individual DAP were prepared in methanol and stored at −20 °C. Mixed working solutions of DMP, DEP, DMTP, DETP and DEDTP were prepared monthly and stored at 0 °C, in the dark, covering concentration range from 0 to 500 ng/ml.

2.5. Sample treatment

2.5.1. Liquid–liquid extraction

AF samples were treated according to previously reported procedure by Ueyama et al. [30] with slight modifications. Briefly, 5 ml of AF were transferred to a clean 15 ml screw-top glass vial. Four grams of NaCl, 1 mL of HCl (6 M), 50 mg of $Na_2S_2O_5$ were added. Liquid–liquid extraction was performed by adding 4 ml of diethyl ether–acetonitrile (1:1, v/v) followed by mechanical shaking for 5 min. After the extraction, the samples were centrifuged at 2000 × *g* (5 min) at 4 °C. The supernatant was collected in another vial containing 15 mg of K_2CO_3 and the liquid–liquid extraction step was repeated. The two extracts were combined and evaporated to dryness under a stream of nitrogen at 30 °C.

2.5.2. Derivatization procedure

Fifteen milligrams of K_2CO_3 was added to the residue, which was reconstituted in 1 ml of acetonitrile and 0.1 ml of PFBBr in acetonitrile (1:3, v/v) and incubated in a water bath at 80 °C for 30 min with occasional shaking [30]. After incubation, the mixture was brought to room temperature and acetonitrile was evaporated to dryness under a stream of nitrogen at 35 °C. The residue was dissolved in 50 μl of toluene and 2 μl was injected to GC–MS.

2.6. Chromatography and mass spectrometry conditions

Electron ionization mass spectrometric analysis was performed on a GC-2010 Shimadzu system equipped with a BPX5 (30 m × 0.25 mm × 0.25 μm) capillary column (SGE, Argent Place, Ringwood, Victoria, Australia). Pure helium (99.999%) was used as a carrier gas. The column temperature was initially held at 60 °C for 1 min, raised to 180 °C at 20 °C/min, held for 1 min, raised to 250 °C at 4 °C/min, held for 1 min and finally raised to 300 °C, at 25 °C/min. The injector, interface and ion source temperatures were 270 °C, 310 °C and 230 °C, respectively.

Quantitative analysis was performed in selected ion monitoring (SIM) mode with a total run time 32.2 min per sample, using one target ion for quantification and one fragment ion for confirmation for each DAP. Specifically, m/z = 110, 306 for DMP; 258, 334 for DEP; 322, 211 for DMTP; 350, 274 for DETP; 366, 185 for DEDTP; and 335 for DBP (IS) [4,9].

2.7. Data management and statistical analysis

Exploratory data analysis was carried out to investigate the distributions of the examined continuous variables. Normality of

the studied parameters was assessed by Shapiro Wilk test. Comparisons among groups for variables that demonstrated Gaussian distribution was done by independent samples *t*-test and one-way analysis of variance (ANOVA), whereas for skewed parameters, non-parametric Mann–Whitney *U* test, and Kruskal–Wallis test were applied. Dialkylphosphate metabolites were $\log_{10}$ transformed to approximate Gaussian distribution for the purpose of the subsequent regression analysis. Dichotomous variables were compared by chi-square test.

We plotted biometric parameters namely head circumference, body length, weight and body mass index, to published reference ranges, to obtain the corresponding centiles [31,32]. We constructed multiple linear regression models to investigate the association between the levels of sum of dialkylphosphate metabolites (dimethyl metabolites – sumDMPs, diethyl metabolites – sumDEPs, dialkyl metabolites – sumDAPs) and neonatal biometric indices after adjustment for the potential confounding effect of maternal age (in years), gestational age at delivery (weeks), neonate gender (male or female) and exposure to pesticides (sum of DAPs). A limit of a 0.200 was set for independent variables to be included in the linear models. An IBM SPSS Statistics 21.0 was used for statistical analysis of data and a level of significance of $a = 0.05$ was set.

3. Results

3.1. Linearity, limits of determination and extraction recoveries

Mix DAPs solutions with concentrations 0, 25, 50, 100, 250 and 500 ng/ml were used to prepare the standard curves. Pooled AF samples, with DAP levels below LOQ values, were used as blank for the preparation of spiked solutions in concentrations 0, 0.125, 2.5, 0.5, 1, 2.5 and 5 ng/ml. Peak area ratios (DAP response/internal standard response) were used for quantification. The spiked sample curves were linear in the above concentration range for all DAPs examined, with $r^2 = 0.9841$ for DMP; 0.9923 for DEP; 0.9761 for DMTP; 0.9972 for DETP; and 0.9934 for DEDTP. The limit of quantification (LOQ) of the method was calculated based on the signal to noise ratio being 10 and defined as 0.13, 0.07, 0.05, 0.05, 0.04 ng/ml for DMP, DEP, DMTP, DETP and DEDTP, respectively. The recovery of the extraction method was estimated on spiked AF samples at six concentrations levels (0.125, 0.25, 0.5, 1, 2.5 and 5 ng/ml), in triplicate. The mean recovery was 59.2% for DMP, 62.0% for DEP, 113.3% for DMTP, 114.4% for DETP and 67.8% for DEDTP.

3.2. Method precision and accuracy

The precision of the method was evaluated by preparing and extracting blank spiked AF samples at concentrations of 2.5 and 5 ng/ml for each DAP and then injecting them into the GC–MS system during one working day (within-day precision) or during consecutive working days (between-day precision). For DMP, DEP, DMTP, DETP and DEDTP the within-day precision (% RSD) was 18.4%, 4.9%, 4.6%, 0.1% and 0.5%, respectively, at the concentration of 2.5 ng/ml, and 2.5%, 2.8%, 16.4%, 4.5% and 4.6%, respectively, at the concentration of 5 ng/ml. The values (% RSD) of between-day precision was 27.1%, 11.5%, 12.9%, 0.4%, 1.1%, respectively, at the concentration 2.5 ng/ml and 0.5%, 3.5%, 11.6%, 1.0% and 3.2% at the concentration of 5 ng/ml respectively for DMP, DEP, DMTP, DETP and DEDTP.

For the estimation of accuracy four spiked AF samples at concentrations of 0.25, 0.5, 1 and 2.5 ng/ml were prepared and extracted in five consecutive working days and injecting into the GC–MS system. The mean accuracy of the method at the above spiked levels was estimated at 81.2%, 83.3%, 99.5%, 114.9% and 104.5% for DMP, DEP, DMTP, DETP and DEDTP, respectively.

Table 1
Demographic characteristics and medical history of the study's sample.

Variable	Subgroups	*N*	%
Age groups	≤25	54	13.0
	26–30	77	18.6
	31–35	133	32.0
	36–40	128	30.8
	41+	23	5.5
Region of living	Urban	271	65.3
	Rural	144	34.7
Place of birth	Greece	361	87.0
	Other	54	13.0
Agriculture work	No	390	94.0
	Yes	25	6.0
Agricultural activities	No	288	69.4
	Yes	127	30.6
Insecticides use at home	No	278	67.0
	Yes	137	33.0
Education	Primary	40	9.7
	Secondary (Gymnasium)	66	16.0
	Secondary (Lyceum)	228	55.3
	Post-Lyceum	42	10.2
	University	36	8.7
Parity	0	138	33.3
	1	151	36.5
	2	86	20.8
	≥3	39	9.4
Abortion	0	334	80.7
	>1	80	19.3
Admission in pregnancy	No	365	88.4
	Yes	48	11.6
Smoking before pregnancy	No	202	51.8
	Yes	188	48.2
Smoking during pregnancy	No	209	68.1
	Yes	98	31.9

3.3. Maternal characteristics

The study population consisted of 415 women carrying a singleton pregnancy. The demographic characteristics are presented in Table 1. Twenty two pregnancies resulted in either fetal demise or termination of pregnancy due to chromosomal abnormalities or major fetal defects. Mean maternal age was 32.7 (SD = 5.7). The mean gestational age at amniocentesis was 18.5 weeks (SD = 2.5).

3.4. Biological monitoring

Univariate comparisons did not reveal any statistically significant difference between rural and urban population, for the subgroup of women with positive samples (>LOQ) for sumDAPs, sumDMPs and sumDEPs (Table 2).

Exposure to OPs, expressed as sums of DMPs, DEPs, DAPs in AF samples ranged from 0.07 to 222.9 ng/ml, 0.19 to 254.3 ng/ml, and 0.05 to 252.6 ng/ml, respectively. DEP was the metabolite with the higher frequency of detection (90.5%), followed by DETP (74.0%), DMP (60.0%) and DEDTP (39.5%) (data not shown in tables for simplicity reasons).

The sample was stratified by maternal age (≤25, 26–30, 31–35, 36–40, >41). Analysis of the variance demonstrated that the levels of sumDMPs, sumDEPs and sumDAPS in AF samples did not differ significantly amongst the examined age subgroups (Table 2). Log_{10}-transformed levels of sumDMPs and sumDEPs were significantly correlated (Pearson correlation coefficient, $r = 0.453$, $p < 0.001$). The abovementioned association is graphically depicted in Fig. 1. Moreover, comparisons between the levels of the individual DAPs in pairs, showed the existence of positive correlations ($p < 0.002$).

Table 2
Levels of dialkyl phosphate metabolites (DAPs) in amniotic fluid for different regions of living and age groups.

	Area	N[a]	%[b]	Mean[c]	±SD	25th	50th	75th	p
sumDMPs	Urban	198	73.1	7.1	22.5	1.1	2.4	4.9	0.885
	Rural	100	69.4	7.5	19.2	1.4	3.3	7.3	
sumDEPs	Urban	263	97.0	5.8	17.6	0.6	1.7	4.9	0.571
	Rural	138	95.8	6.8	15.7	0.8	1.7	5.8	
sumDAPs	Urban	265	97.8	11.0	26.5	1.7	4.4	10.7	0.730
	Rural	141	97.9	11.9	23.1	1.9	4.9	13.0	
	Age groups	N	%	Mean	±SD	25th	50th	75th	p
sumDMPs	≤25	39	72.2	5.3	9.5	1.5	2.9	5.2	0.864
	26–30	51	66.2	8.9	31.1	1.0	2.1	5.6	
	31–35	99	74.4	8.4	22.7	1.1	2.9	6.4	
	36–40	94	73.4	6.0	18.9	1.3	2.4	4.6	
	41+	15	65.2	5.9	7.6	1.9	3.6	4.9	
sumDEPs	≤25	53	98.1	6.6	13.9	0.9	2.0	6.2	0.648
	26–30	73	94.8	6.2	12.7	0.8	2.5	6.1	
	31–35	129	97.0	4.6	7.8	0.5	1.3	4.9	
	36–40	124	96.9	7.8	26.2	0.7	1.5	5.2	
	≥41	22	95.7	4.3	5.8	0.9	2.5	4.7	
sumDAPs	≤25	53	98.1	10.5	15.8	2.0	5.9	12.7	0.951
	26–30	74	96.1	12.2	28.9	1.6	5.4	13.2	
	31–35	130	97.7	11.0	22.5	1.6	4.4	12.4	
	36–40	126	98.4	12.1	30.8	1.9	4.3	10.4	
	≥41	23	100.0	7.9	8.1	2.6	4.4	8.9	

[a] *N*: number of AF samples.
[b] %: percentage of positive samples.
[c] Mean: Mean values (ng/ml).

The strength of the associations ranged from 0.194 to 0.941 for DEP/DEDTP and DETP/DEDTP, respectively (data not shown).

We subdivided the neonates according to their birth weight and head circumference centile. Subsequently we stratified neonates into 5 distinct subgroups by using the calculated centiles (<3th centile, 3–10th centile, 10–90th centile, 90–97th centile, >97th centile). The respective comparisons between groups, categorized by the biometric indices, are presented in Table 3. Overall the magnitude of biometric parameters was not associated with the levels of DAPs, except for the birth weight. In particular, ANOVA has shown that for the levels of sumDMPs statistically significant differences exist for the different subgroups. The group of macrosomic neonates (>97%) had the highest levels of sumDMPs (median = 4.6 ng/ml). Furthermore we found that there is no effect of neonatal gender in the levels of DAPs (Table 3).

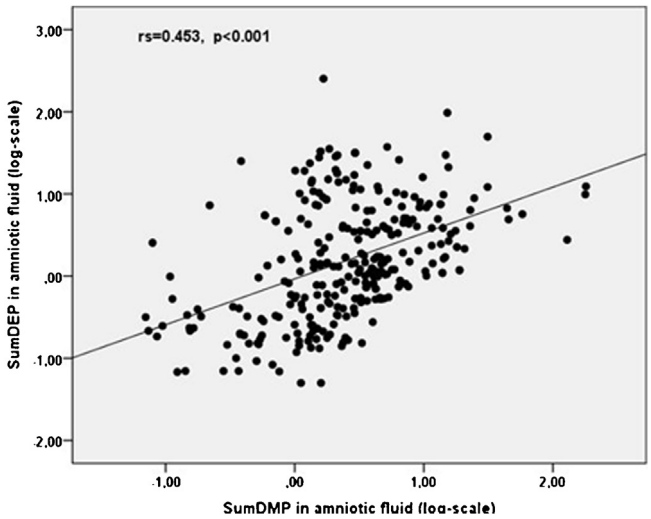

Fig. 1. Correlation of logarithmically transformed levels of sumDMP and sumDEP measured in amniotic fluid.

We constructed multiple linear regression models to describe the potential association between sumDMPs, sumDEPs and sumDAPs treated as dependent variables whereas birth weight and head circumference were the predictors. The aforementioned associations were adjusted for the confounding effect of neonatal gender, maternal age, agricultural activities and gestational age at amniocentesis. SumDMPs was positively related with birth weight centile (Table 4). The remaining DAPs were not correlated with biometric indices. Similar results were found after missing data imputation with LOD/2 values. However, the relation between sumDMPs and biometric parameters was attenuated being at the same level of statistical significance.

4. Discussion

Our study describes a sensitive analytical method for the measurement of OP non-specific metabolites in AF. We investigated the potential value of DAPs as biomarkers of the fetal exposure to OPs. DAPs were correlated with maternal characteristics and fetal biometric parameters. Macrosomic neonates were found to have higher levels of sumDMPs. Interestingly, birth weight centile was positively associated with sumDMPs in a linear regression model after adjustment for the confounding effect of demographic characteristics. This novel finding suggests a continuous linear relation for the whole range of the birth weight distribution. Specifically, the deviation of birth weight from the expected median, as expressed by the computed centile, appears to be correlated with the levels of sumDMPs, rather than the absolute birth weight. The presented data are in agreement with our previous study that demonstrated that significant levels of OPs metabolites exist in meconium [9]. Thus, we provide evidence for pervasive fetal exposure to OPs in the second trimester of pregnancy. To the best of our knowledge this is the second study that analyzes AF samples. Our study was carried out in an area with increased exposure to the examined chemical compounds due to extensive occupational and residential use.

A significant literature has linked intra-uterine pesticide exposure with adverse neurodevelopmental and fetal growth outcomes [33–37]. Consequently research has focused in the evaluation of

Table 3
Median (2nd) and quartiles (1st and 3rd) of sum DMPs, DEPs and DAPs of positive samples.

	sumDMPs			sumDEPs			sumDAPs		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Gender									
Male (214)	1.4	2.8	5.6	0.7	1.5	4.9	1.7	4.5	11.4
Female (185)	1.1	2.7	5.2	0.5	2.0	5.6	1.7	4.7	11.9
p^{*}		0.431 (0.547)			0.849 (0.858)			0.686 (0.924)	
BW (centile)									
≤3% (24)	0.9	1.6	4.1	0.6	1.1	1.9	1.6	4.4	11.5
3–10% (23)	1.0	2.1	3.6	0.3	.8	3.0	1.6	3.6	15.7
10–90% (305)	1.3	2.9	5.7	0.4	1.5	5.1	2.4	5.9	12.3
90–97% (17)	1.0	2.2	2.9	0.2	6.7	16.5	1.3	17.1	30.9
>97% (19)	2.2	4.3	6.0	0.8	1.1	3.2	3.2	5.9	9.2
p^{**}		0.043 (0.186)			0.314 (0.387)			0.422 (0.605)	
HC (centile)									
≤3% (23)	1.1	2.7	3.3	0.4	0.9	2.3	1.9	3.7	6.2
3–10% (40)	1.3	3.0	6.5	0.6	1.1	12.4	3.7	8.8	16.0
10–90% (289)	1.3	2.6	5.3	0.4	1.5	5.0	2.3	5.7	13.1
90–97% (12)	1.5	5.7	8.0	0.7	1.2	8.3	2.4	8.3	15.7
>97% (20)	0.4	2.7	3.9	0.4	0.5	1.7	0.7	3.1	5.6
p^{**}		0.817 (0.655)			0.714 (0.585)			0.698 (0.549)	

BW: birth weight; HC: head circumference.
* *p*: values as resulted from independent samples *t*-test and Mann–Whitney (inside brackets) tests.
** *p*: values as resulted from one-way ANOVA and Kruskal–Wallis (inside brackets) tests.

markers for fetal exposure to these chemicals. The majority of studies that examined the relation of prenatal OP exposure with perinatal outcomes, measured DAP metabolite concentrations in maternal urinary samples or maternal serum. However, the main drawback of these studies is that the exposure to OPs suggested by measurements in the above mentioned biological materials is rather a temporary than a long-term indicator; only after the examination of samples such as meconium or amniotic fluid can estimates be made of pesticide exposures at earlier gestational weeks in pregnancy. The results of previous studies are consistent and reproducible and DAP metabolites were detected in the vast majority of the examined populations with percentages varying from 92.7% to 100% [18,21,22,38,39]. However, the lack of established physiologically based pharmacokinetic models which will serve as valuable tools for estimating fetal exposure based only on maternal biologic samples explains the research interest in other sources such as meconium and AF to evaluate direct fetal exposure. Meconium is likely to represent exposures from the second trimester through delivery whereas AF collected during amniocentesis is the only biologic medium available that can be used to characterize fetal exposure between 16th and 20th weeks of gestation when fetuses are particularly vulnerable to the adverse health effects induced by environmental contaminants due to rapid growth and development and limited ability to detoxify harmful substances [28,33].

Amniotic fluid surrounds and protects the developing embryo and fetus. At the beginning of the 11th week of gestation, the fetus begins to produce urine that enters the AF which in turn is swallowed by the fetus and absorbed by the gastrointestinal and respiratory tracts [40]. Toxic substances can be excreted in the AF via the placenta or can be transferred directly from maternal blood through the amniotic sac [41]. In the studies that measure DAPs in amniotic fluid samples [28, present study] there is no a clear explanation about the mechanism of transport of these water-soluble metabolites across the placenta. In our study, almost all AF samples (97.8%) provided at least one positive pesticide metabolite (DMP, DEP, DMTP, DETP, DEDTP), which are common metabolites of about 75% of the OPs. The presence of DAPs metabolites indicates exposure to one or more OP pesticides but does not indicate exposure to any particular pesticide. The ability to identify specific OP exposures would improve etiological studies because OPs differ widely in their use and relative toxicity. The fact that there was no difference between urban and rural population (97.8% in urban areas vs. 97.9% in rural areas) makes difficult to pinpoint if

Table 4
Results of multiple linear regressions for the prediction of sumDMPs, sumDEPs and sumDAPs levels by biometric indices after adjustment for maternal age, agricultural activities, gestational week at sampling and neonatal gender.

Somatometric data of neonates	sumDMPs	sumDEPs	sumDAPs
Weight (percentiles)			
Beta (95%CI)	4.43 (0.83–8.03)	0.88 (−1.55 to 3.31)	3.43 (−0.13 to 6.99)
p-Value	0.016	0.478	0.059
Head circumference (percentiles)			
Beta (95%CI)	2.11 (−1.43 to 5.65)	−0.20 (−2.57 to 2.17)	0.83 (−2.66 to 4.32)
p-Value	0.242	0.867	0.641

	sumDMPs*	sumDEPs*	sumDAPs*
Weight (percentiles)			
Beta (95%CI)	2.65 (0.20–5.01)	0.76 (−1.57 to 3.10)	3.51 (−0.04 to 7.03)
p value	0.034	0.521	0.050
Head circumference (percentiles)			
Beta (95%CI)	0.98 (−1.43 to 3.40)	−0.34 (−2.63 to 1.95)	0.78 (−2.68 to 4.24)
p-Value	0.425	0.770	0.657

* Values of pesticides with an asterisk are referred to missing values which imputed with LOD/2.

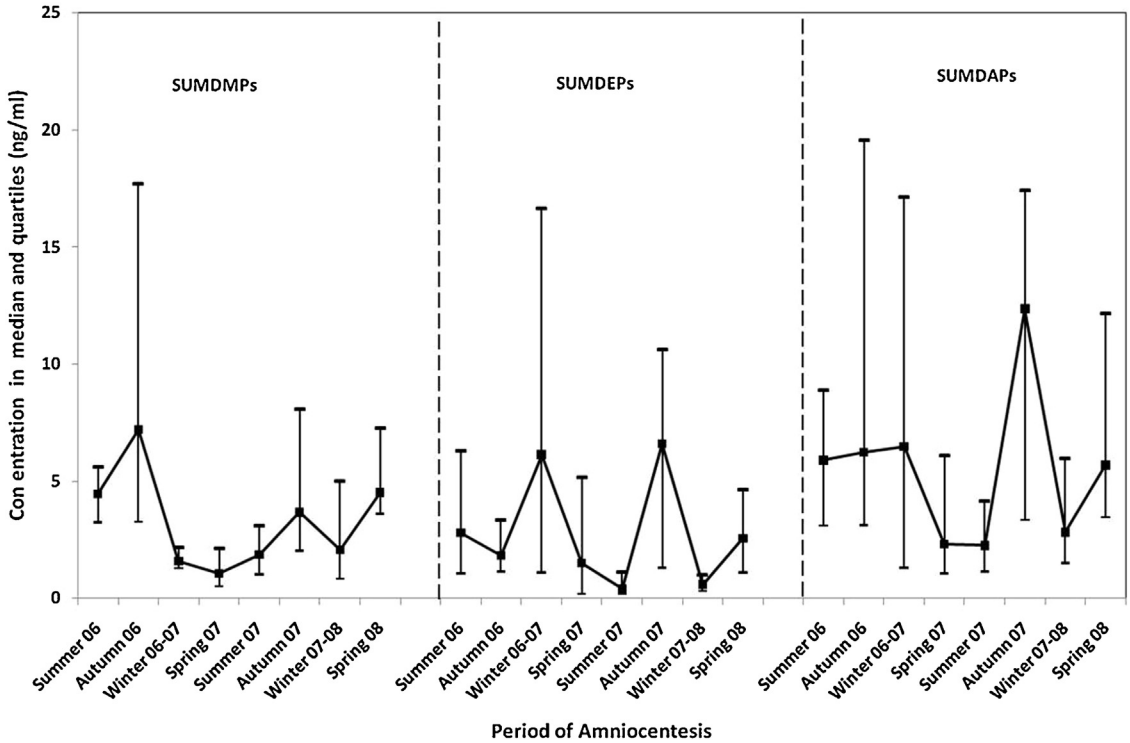

Fig. 2. Time dependence of sum of DAPs metabolites measured in amniotic fluid.

the dominant source of exposure is the diet and home pesticide use or agriculture. There is also a seasonal variation in the levels of DAPs which can be explained by the use of specific OPs in certain types of cultivations (Fig. 2). The sumDMPs showed elevated levels in autumn of 2006 and 2007 and spring 2008. DMPs derived of *O*,*O*-dimethyl-substituted OPs such as malathion and dimethoate which are used for spraying apple, pear, apricot, cherry, peach, grapevine, figs, olives, vegetables, and other common agricultural products in Crete and oxydemeton methyl is used as insecticide. The most probable cause of increased dimethyl phosphate metabolite levels in the autumns of 2006 and 2007 is the systematic spraying of olive oil trees for dacus oleae using fenthion and dimethoate during the period between August and September which was approved by the Ministry of Rural Development & Food in Greece. The sumDEPs showed elevated levels in winter 2006–2007 and autumn 2007. DEPs derived from *O*,*O*-diethyl-substituted OPs such as chlorpyrifos and diazinon and their elevated levels may be due to the widespread use of diazinon in vineyards and fruit. In addition, our results showed that maternal age does not affect the levels of sumDAPs ($p > 0.400$). Based on Spearman's rho coefficients, all measured DAPs in AF seem to positively associate with each other ($p < 0.002$) and this could possibly be attributed to the concomitant exposure of the participants in methyl and ethyl OPs.

To the best of our knowledge, there is only one previous pilot study conducted by Bradman et al. [28], in an agricultural area in California and evaluated the presence of synthetic pyrethroids, chemical-specific and class-specific metabolites of OP pesticides, carbamates, herbicides, and pesticides with chlorinated phenol metabolites, such as disinfectants in AF. This research group applied GC/MS analysis in 20 AF samples collected during amniocentesis and found that five of 20 samples (25%) in the nonspecific OP pesticide metabolite assay had detectable levels of DEP (2 samples, 10%), DMP (2 samples, 10%), or DMTP (1 samples, 5%), with concentrations ranging from 0.26 to 0.43 μg/L [28]. Possible explanations for the rather low frequency of detection levels of the chemicals detected in this study compared with our findings may include technical improvements in the laboratory methods applied resulting in high sensitivity and lower detection limits, our large sample size, differences in the target population and the wide use of pesticides in the region of Crete. Similar inconsistency is observed among studies that evaluate the level of contamination of AF by other classes of pesticides such as organochlorines for which one research group recruited 100 pregnant women from Tenerife island and showed some detectable OC-residue in the majority of the AF samples (67%) though a longitudinal study in 323 pregnant women from Western Canada concluded that there were no organochlorines present in the AF at their applied level of quantification [26,27].

Early intrauterine exposure to OPs is an area of intense research due to the potential long-term effects of this chemical class in the fetus and newborn. OP pesticides share a common mechanism of toxicity that is the inhibition of acetylcholinesterase activity [42]. Animal studies have shown that low-level exposure to various OP pesticides could affect neurodevelopment and growth [43,44]. In human studies, most of published reports showed no associations for any of the OP exposure measures and the birth weight, suggesting that OP may have no effect on fetal growth [45,46]. In particular, Wang et al. found no association even though the maternal urine pesticide levels in their study population were much higher than those reported in developed countries [45]. In addition, Eskenazi et al. failed to demonstrate an adverse relationship between fetal growth and in utero organophosphate exposure as assessed by multiple measures of exposure including levels of blood cholinesterase, urinary dialkyl phosphate metabolites, and some pesticide-specific metabolites of organophosphates [38]. However, the authors note that higher urinary sumDMAP concentrations were associated with shortened gestation and small increases in gestational age-adjusted birth weight in a primary Latin cohort. Other epidemiological studies have shown that maternal–fetal transfer of OP could be related to reduced fetal growth and this association appears to be stronger in black newborns than white newborns [22,47,48]. This inconsistency is also evident in a recently published meta-analysis about

the potential effects of chlorpyrifos (one of the most widely used organophosphate insecticides that is also used in our region) on fetal growth outcome both in human and animal studies [49]. Other research groups have also reported effect modification by child sex but a more recent study found no evidence for this interaction [21,22]. This could be due to geographical and regulatory variations in the use and restriction of OPs around the world and second, methodological (extraction and purification of the sample) and/or analytical differences between laboratories. Another possible explanation for the lack of consistency between studies could be that single nucleotide polymorphisms in paroxanase (PON1) enzyme, a high-density lipoprotein-associated enzyme involved in the metabolism and detoxification of OP pesticides, may modify the relationships between OP exposure and perinatal outcomes [22,50]. Specifically, increased risk for premature birth and fetal growth restriction has been observed among infants who might be more susceptible to the effects of exposure to OPs either because they had lower PON1 activity or they were of a susceptible genotype ($PON1_{108TT}$ and PON_{192QR}) [22,50]. There is also evidence that PON1 distributions differ across racial groups [51]. Unfortunately we do not have data about the PON1 enzyme activity in the fetuses from our study population. In our study, we focused on the associations between increased levels of DAPs and birth weight as well as with head circumference. Interestingly, birth weight centile was positively associated with sumDMPs in a linear regression model after adjustment for the confounding effect of demographic characteristics. Overall, our results are in disagreement with previous studies, who found a negative association [22,47,48] or no effect [38,45,46]. Nevertheless, there can be no direct comparison between the results of the previous reports and our conclusions due to various differences in the studies. Firstly, we analyzed AF samples, which were collected during amniocentesis, while in other studies only urine samples were used. Moreover, we must take into account the different genetic backgrounds and the homogeneity of the populations studied in each case. Finally, our population is primarily of Greek decent and have been living on the island of Crete for the last 2 years, while in the other studies not only the ethnicity differed between the participants, but also the race. A limitation of our study however, is that the study population is consisted of women undergo an amniocentesis at 18.5 weeks of gestation for a variety of indications. The mean maternal age of the study group was 32.7 (SD = 5.7) showing that it is not a population of advanced maternal age (one common indication for amniocentesis). Unfortunately, a study with a random sampling of pregnant women at the second trimester of pregnancy is not possible to be conducted.

Another consideration in our findings would be to examine if the association between macrosomia and high levels of organophosphates in the amniotic fluid may act through disrupted maternal glucose metabolism. Human and animal studies have shown that long-term exposure to organophosphate insecticides may be associated with increased risk of developing diabetes [52–54]. In particular, Slotkin has demonstrated that when neonatal rats were exposed to organophosphate insecticides in early life, the animals developed obesity and metabolic dysfunction resembling prediabetes [54]. Moreover, there is increasing evidence of a strong and continuous association of maternal glucose levels below those diagnostic of diabetes with increased birth weight [55]. Based on all these observations from previous studies, it could be suggested that the association between macrosomia and organophosphates in our study is mediated by a disrupted maternal glucose metabolism in women exposed to organophosphates. In our study population, however, there were no women with preexisting diabetes. Furthermore, the diagnosis of gestational diabetes is made later in pregnancy between 24 and 28 weeks of gestation after a glucose tolerance test; unfortunately, we do not have such data from our cohort, and we are not able to support further this hypothesis. Another mechanism that could be proposed is an organophosphate-altered glucose intolerance risk through prior generational exposure. There is increasing evidence supporting the epigenetic transgenerational inheritance of adult-onset disease and phenotypic variation mediated in part by epigenetic mechanisms, as pesticides seem to modify gene promoter DNA methylation levels [56–58]. This association, however, cannot be supported by the conduction of the present study, and requires further investigation.

5. Conclusion

Overall, the results of this study demonstrate that DAPs in AF may be considered as a potential biomarker for the assessment of fetal exposure to OPs and this is an important objective as there is a link between the in utero exposure to this chemical class and the incidence and/or prevalence of short and long term health effects linked to OPs. Our findings add to the growing body of literature regarding the sensitivity of developing fetus to OP pesticide exposure in a direct way rather than from maternal biologic monitoring data. The next step is to compare the levels of DAPs in AF and in maternal blood in the same cohort of pregnant women and evaluate if there is any relation between DAP levels in AF and neurodevelopment in these fetuses. Further studies are required to determine the persistence of these associations and their generalizability to other populations and to address concerns about the potential health effects of pre- and postnatal exposure to pesticides and develop suitable regulatory reference doses.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

The authors thank the staff of the Maternal-Fetal union at Department of Obstetrics & Gynecology, University Hospital of Heraklion, and the Laboratory of Toxicology, Medical School, University of Crete.

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