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Oxidative stress of glyphosate, AMPA and metabolites of pyrethroids and chlorpyrifos pesticides among primary school children in Cyprus

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ABSTRACT

Background: Exposure to various pesticides, such as pyrethroids and chlorpyrifos, has been previously associated with adverse effects on children's health. Scientific evidence on the human toxicity of glyphosate (GLY) and its primary metabolite, aminomethylphosphonic acid (AMPA) is limited, particularly for children. This study aimed to i) assess the exposure determinants of the studied pesticides measured in children in Cyprus, and ii) determine the association between the urinary pesticides and the biomarkers of DNA and lipid oxidative damage.

Methods: A children's health study was set up in Cyprus (ORGANIKO study) by aligning it with the methodology and tools used in the European Human Biomonitoring Initiative (HBM4EU). Urinary GLY and AMPA, pyrethroid metabolites and the chlorpyrifos metabolite TCPy were measured in 177 children aged 10–11 years old, using mass spectrometry. Oxidative stress was assessed with 8-iso-prostaglandin F2a (8-iso-PGF2α) as a marker of lipid damage and 8-hydroxy-2′-deoxyguanosine (8-OHdG) as a DNA oxidative damage marker, both measured with immunoassays. Questionnaires about demographic characteristics, pesticide usage, and dietary habits were filled out by the parents. Multivariable regression models examined associations between pesticides and biomarkers of effect using two creatinine adjustments (cr1: adding it as covariate and cr2: biomarkers of exposure and effect were creatinine-adjusted).

Results: Parental educational level was a significant predictor of urinary pyrethroids but not for GLY/AMPA. Median [interquartile range, IQR] values for GLY and AMPA were <LOQ [<LOQ, 0.19] μg/L and 0.18 [0.10, 0.29] μg/L, respectively, while a moderate correlation was shown between GLY and AMPA ($r = 0.45$). 8-OHdG was positively associated with AMPA (beta = 0.17; 95% CI: 0.02, 0.31, $p = 0.03$ cr2, and beta = 0.12; 95% CI: 0.0,0.24, $p = 0.06$, cr1), albeit not with GLY ($p > 0.05$). Similar significant associations with 8-OHdG were shown for a pyrethroid metabolite (3-PBA) and the chlorpyrifos metabolite (TCPy). No associations were observed between the aforementioned pesticides and 8-iso-PGF2α ($p > 0.05$).

Conclusions: This is the first children's health dataset demonstrating the association between AMPA and DNA oxidative damage, globally. More data is needed to replicate the observed trends in other children's populations around the globe.

1. Introduction

Numerous studies suggest that pesticides are potentially hazardous to humans (Sabarwal et al., 2018). Due to differences in physiology, children are more susceptible to pesticides than adults. Earlier research suggests multifactorial etiology for higher levels of environmental chemical exposures in children, including, but not limited to, smaller body mass, and higher likelihood of ingestion via hand to mouth activities (Gillezeau et al., 2020).

Glyphosate (GLY) is a broad-spectrum herbicide and the active ingredient of Roundup®, the most heavily used herbicide in the world (IARC Working Group on the Evaluation of Carcinogenic Risks to

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Humans, 2017). GLY may be degraded in the environment by a suite of microorganisms in the course of a few days (range of 5–20 days) (PPDB, 2013a) giving rise to aminomethylphosphonic acid (AMPA), which is highly persistent in the environment ($t_{1/2}$ of 151 days) (PPDB, 2013b). Pyrethroids are also commonly used worldwide, being among the pesticides with high volume usage in the EU (Eurostat, 2021). Some of the pyrethroids have been shown to exert endocrine disrupting health effects (Mnif et al., 2011). Chlorpyrifos, a historic organophosphoric pesticide was banned in 2019 by the European Food Safety Authority, being classified as toxic for reproduction (category 1B).(European Food Safety Authority, 2019).

Several human biomonitoring studies measured urinary pyrethroids and chlorpyrifos metabolites in children populations over the world and showed that their levels vary (Wang et al., 2016; van Wendel de Joode et al., 2016; CDC, 2019; Bravo et al., 2019; Health Canada, 2019; Panuwet et al., 2009; Muñoz-Quezada et al., 2020; Fernández S et al., 2020). Indicatively, the median urinary levels of the common metabolite (3-PBA) for many pyrethroids was <LOD (8 ng/L) in 406 Chinese children aged 3–6 years in 2014 (Wang et al., 2016), 0.38 μg/L in 534 children aged 6–11 years in Canada in 2016–2017 (Health Canada, 2019) and 1.63 μg/L in 568 Spanish children aged 5–12 years in 2016 (Fernández S et al., 2020). Similarly, the median urinary levels of the chlorpyrifos metabolite TCPy were 0.36 μg/L in 199 Italian children aged 7 years in 2014–2015 (Bravo et al., 2019), 1.4 μg/L in 140 children aged 6–9 years in Costa Rica in 2007 (van Wendel de Joode et al., 2016), 1.4 μg/L in 489 children aged 6–11 years in Canada in 2014–2015 (Health Canada, 2019) and 2.64 μg/L in 207 children aged 12–13 years in Thailand in 2003 (Panuwet et al., 2009).

GLY data is available for only about 500 children worldwide, with urinary levels of GLY being higher in children than in adults, in studies where data from both exist (Gillezeau et al., 2020). A 2015 review of nonfarmer U.S. and European adults reported mean urinary GLY levels of 1.35 μg/L and 0.215 μg/L, respectively (Niemann et al., 2015). Only a few studies have measured urinary AMPA when compared to the number of studies on urinary GLY (Connolly et al., 2020). Of the 12 peer-reviewed, non-occupational human biomonitoring studies published between 2007 and 2020 that investigated urinary GLY exposures, only half of them co-analyzed AMPA, of which only two studies had median levels above the limit of quantification (LOQ = 0.1 μg/L) (Connolly et al., 2020). More recent studies showed that the geometric means of urinary GLY and AMPA levels of nonfarmer children in Germany were 0.107 μg/L and 0.10 μg/L, respectively (Lemke et al., 2021), while in Slovenia, both analytes were <LOQ (0.1 μg/L) (Stajnko et al., 2020).

The latest IARC monograph on the carcinogenicity of GLY and its formulations stated that there is strong evidence that GLY causes genotoxicity and oxidative stress (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017). The evidence base for GLY includes studies with largely positive results in human cells *in vitro*, in mammalian model systems *in vivo and in vitro*, and studies in other non-mammalian organisms (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017). The evidence for AMPA genotoxicity is moderate, since the number of studies is limited and no human genotoxicity studies on AMPA exist (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017). Further, little is known, globally, about the relationship between AMPA and oxidative stress in sensitive subpopulation groups, as in children and pregnant women (Silver et al., 2021; Eaton et al., 2022). To date, a single human study in pregnant women (n=227) explored the association between AMPA and measures of oxidative stress; results from the prospective birth cohort study in Puerto Rico showed a positive association between urinary AMPA and a metabolite of 8-iso-PGF2α, a biomarker of oxidative stress (Eaton et al., 2022).

Towards this, a cross-sectional study was set up using the baseline dataset of the ORGANIKO trial to measure a suite of pesticides in a non-occupational sample of children. The main objectives of this study were to: i) assess the exposure determinants of the studied pesticides' metabolites measured in children in Cyprus, and ii) determine the association between the urinary pesticides and the biomarkers of oxidative stress among primary school children.

2. Methods

2.1. Oversight

This was a cross-sectional study of the baseline dataset of the ORGANIKO LIFE + trial (ClinicalTrials.gov number: NCT02998203) (Makris et al., 2019). Out of 191 children recruited through their primary schools located in the Limassol urban area of Cyprus, a total of 179 children, aged 10–11 years were included in this analysis. The original study protocol was approved by the Cyprus National Bioethics Committee (ΕΕΒΚ/ΕΠ/2016/25) and the Cyprus Ministry of Education and Culture (7.15.06.15/2). The study was performed in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from the school principal, written informed consent was provided by the children's parents or legal guardians, and verbal assent was obtained from the children. In 2020, ORGANIKO was approved for alignment in the frame of the European Human Biomonitoring Initiative, HBM4EU (Gilles et al., 2021) with the aim to collect harmonized HBM data of children's exposure to prioritized pesticides with EU wide coverage. This new use of ORGANIKO urine samples and data from Cypriot children was approved by the Cyprus National Bioethics Committee (6/7/2020, ΕΕΒΚ/ΕΠ/2016/25).

2.2. Population

Six primary schools, located in the urban Limassol, Cyprus, were randomly selected and agreed to participate in the study between October–December 2016, with the trial taking place during January–April 2017 (Makris et al., 2019). The following eligibility criteria were set for the clusters (schools): i) being a public primary school, and ii) located in the urban areas of Limassol, Cyprus. Eligible participants were healthy 10-12 year-old primary school children (5th - 6th grade) who had been living in Cyprus for at least the previous five years and were systematically consuming conventional (non-organic) food (>80% of a week's meals) prior to the study recruitment. In the original ORGANIKO trial, children with any self-reported chronic disease conditions (e.g., asthma, type I diabetes, or other chronic diseases) or food allergies (e.g., gluten or lactose tolerance) were excluded (Makris et al., 2019). For this study, participants with urinary creatinine levels outside the WHO guideline range (i.e., 0.3–3 g/L) (Wessels et al., 2003; World Health Organization, 1996) were excluded (n = 2).

2.3. Procedures

Recruitment began by randomly contacting 12 primary schools in the urban area of Limassol using a list publicly available by the Cyprus Ministry of Education, Culture, Youth and Sports. Upon each school's headmaster approval, we organized meetings with parents and children to inform them about the study. With the expression of interest to participate in the study, eligibility criteria were checked by the research team. After signing of informed consent forms by the children's parents or legal guardians, participants received the study materials (i.e., collection instructions for first-morning urine void samples, coded vials for urine collection, and food diaries). Weight, height, and waist circumference were measured using standardized methods) (NHANES) and age-and-sex-standardized BMI was calculated (Nihiser et al., 2007). Besides the baseline questionnaire, a food frequency questionnaire was also administered to the parents through a telephone interview to collect information about the children's food habits. The study questionnaires can be found in the supporting information of the original ORGANIKO + article (Makris et al., 2019).

2.4. Urine sample collection

First-morning urine voids were collected at home in polypropylene, sterilized urine vials and collected at school by the research team. Urine vials were temporarily (≤1 h) stored in a school/home fridge (4 °C) until transferred to laboratory facilities for long-term storage (−80 °C).

2.5. Biomarkers of exposure and effect

Urinary biomarkers of exposure to a suite of pesticides were measured in two HBM4EU-accredited labs using mass spectrometry: i) the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine (IPASUM) in Germany and ii) the Wageningen Food Safety Research Laboratory in the Netherlands. The following pesticides' metabolites were measured: glyphosate (GLY); aminomethylphosphonic acid (AMPA); 3,5,6-trichloro-2-pyridinol (TCPy), the main chlorpyrifos pesticide metabolite; cis-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylic acid (cis-DBCA); cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis-DCCA); trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (trans-DCCA); 3-phenoxybenzoic acid (3-PBA); 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA); and cis-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylic acid (CIF3CA or CFMP). The pyrethroid metabolites and the TCPy in urine samples were measured by the Wageningen team with a Sciex Q-Trap 6500 plus, ESI negative (LC-MS/MS (TQ-ESI)), while the GLY and AMPA were measured by the IPASUM German laboratory. Urinary creatinine was measured using the colorimetric Jaffé method (Angerer and Hartwig, 2010).

Limits of quantification (LOQ) for all urinary pesticides were equal to 0.1 μg/L, except for DBCA (0.2 μg/L). Strict QA/QC protocols were followed, including randomly measured negative and positive controls, including field blanks, method blanks, and reference material from the 2020 ICI HBM4EU study 2020.

Competitive ELISA kits were used to determine urinary concentrations of 8-iso-PGF2α (catalog no: STA-337; Cell Biolabs, Inc, California, USA) and 8-OHdG (catalog no: ABIN2964843; antibodies-online, Aachen, Germany). Analyses were performed according to the manufacturer's instructions. Detection limits for 8-iso-PGF2α and 8-OHdG were 49 pg/mL and 590 pg/mL, respectively.

2.6. Statistical analysis

Continuous variables were checked for normality with QQ plots and histograms. Categorical variables were described with frequencies and percentages, normally distributed continuous variables with means and standard deviations, and non-normal continuous variables with medians and interquartile ranges (IQR, 25th-75th percentiles). Study population demographic and other characteristics' data were tested for differences between sexes by chi-squared test for categorical variables, *t*-test for normally distributed continuous variables, and Wilcoxon test for non-normally distributed continuous variables.

Outcomes were the urinary biomarkers of exposure to pesticides and the biomarkers of oxidative stress (8-iso-PGF2α and 8-OHdG). They were used both as raw (μg/L) and adjusted with creatinine levels (μg/g cr) to account for urine dilution. If outcomes were not normally distributed and the analyses required the normality assumption, then outcomes were log-transformed (natural logarithm). We imputed the missing values of continuous variables in the questionnaire with the median and of categorical variables with the mode (the most frequent categorical level). For the biomarker data below LOQ, imputation was performed using kernel density estimation (Sabarwal et al., 2018). Pesticide metabolites with more than 70% of values below LOQ were dropped from the analysis.

One set of multivariate linear regression models examined the influence of standard demographic variables (sex, age, BMI z scores and parents' educational level) on urinary pesticide levels. No other variables (e.g. food consumption frequency, use of pesticides frequency) were included as covariates in the models as the p-value of their univariate associations with pesticides was higher than 0.08. Another set of multivariate linear regression models was used to examine the association between pesticide biomarkers (used as independent variables in separate models) and biomarkers of oxidative stress (outcomes). The second set of models were adjusted for the standard demographic variables used in the first set of models plus the covariate of physical activity (hours/week), due to its association with oxidative stress and possibly with pesticide use.

Both sets of models were adjusted for urinary creatinine via two approaches (Barr et al., 2005): i) by inclusion in the regression models as covariate (cr1), and ii) by dividing the levels of the urinary biomarkers of exposure and effect by creatinine levels (μg/g cr) (cr2). Forest plots were constructed to better visualise beta coefficients and 95% CI values of the associations between pesticides and the biomarkers of oxidative stress.

A sensitivity analysis, using the same models and including participants with urinary creatinine levels falling outside the WHO guideline range (i.e., 0.3–3 g/L) (Wessels et al., 2003; World Health Organization, 1996) was performed.

All analyses were performed in R (4.12) with RStudio Server (2021.09.1, Build 372) (R Core Team, 2017; RStudio Team, 2015). The statistical analysis plan and the input data can be found in the supporting information.

3. Results

3.1. Participant characteristics and pesticide use

A total of 177 children were included in this cross-sectional study. Overall, the sex distribution of the children was balanced (53% males) with a mean age of 11 years, while the majority of them had a normal weight (66%) (Table 1). A higher percentage of obese boys (18%) than that of girls (8%) was observed. The majority of participating children's parents held at least a university/college degree (82% for mothers and 69% for fathers).

Overall, fruits and vegetables weekly consumption of participating children was 4.5 [2.8,8] and 7.8 [4,14] portions, with boys consuming about half the weekly portions of vegetables that girls consumed; boys also consumed fewer fruits and spent more time on sedentary activities than girls (Table 1).

The frequency of using various pesticides inside and outside the house of participating children varied among participants and across pesticide types (Table 2). For example, rodenticide, herbicide and fungicide use inside and outside the house during the last 90 days was not practiced for more than 90% of the participants. The highest frequencies of use inside the house were reported for insecticides (29%) and insect repellents (20%), while their use outside the house was minimal (<10% of the participants) for all pesticide types. The last usage of insecticides and insect repellents inside the house among those who used them took place 11 or more days before the questionnaire completion, and >31 days, respectively (Table 2).

3.2. Biomarkers of exposure to pesticides

The proportion of samples with values below LOQ were 0% for 3-PBA, 94% for 4F-3-PBA, 70% for ClF3CA, 3% for DBCA, 1% for DCCA-cis, 0% for DCCA-trans, 54% for GLY, 25% for AMPA, and 0% for TCPy (LOQ = 0.1 μg/L for all metabolites except for DBCA, which was 0.2 μg/L). The metabolite 4F-3-PBA was excluded from the analysis due to very high percentage of values <LOQ (94%).

The summarized concentrations of the biomarkers of exposure to GLY/AMPA, pyrethroids and chlorpyrifos are presented in Table 3. The median [IQR] values for GLY and AMPA were <LOQ [<LOQ, 0.19] μg/L and 0.18 [0.10, 0.29] μg/L, respectively. The urinary pesticides were

Table 1
Basic characteristics of the study population (overall and by sex).

	Overall	Male	Female				
	median [IQR]	n (%)	median [IQR]	n (%)	median [IQR]	n (%)	p-value^
N		177		93		84	
Age (years)	10.97 [10.53,11.52]		10.97 [10.49,11.48]		10.97 [10.53,11.55]		0.879
Mother educational level							0.929
Primary/Secondary		32 (18)		16 (17)		16 (19)	
University/college		100 (57)		54 (58)		46 (55)	
Master/PhD		44 (25)		23 (25)		21 (25)	
Father educational level							0.496
Primary/Secondary		53 (31)		29 (32)		24 (30)	
University/college		72 (42)		41 (45)		31 (39)	
Master/PhD		46 (27)		21 (23)		25 (31)	
Weight status*							0.129
Underweight		4 (2)		1 (1)		3 (4)	
Normal Weight		111 (66)		54 (61)		57 (71)	
Overweight		32 (19)		18 (20)		14 (18)	
Obese		22 (13)		16 (18)		6 (8)	
Waist (cm)	69 [63,76]		72 [65,81]		66.5 [61,73.25]		<0.001
Hand in mouth activities							0.731
Yes		46 (26)		23 (25)		23 (27)	
No		131 (74)		70 (75)		61 (73)	
Physical activities (hours/week)**	3.5 [2,6]		3 [0,5.5]		4 [2,6]		0.117
Sedentary activities (hours/week)***	20 [13,28.5]		21 [14,29]		16 [11.75,27.25]		0.055
Time spent outside (hours/week)****	2 [0,4.5]		2 [0,5]		1 [0,4]		0.092
Milk products (portions/week)*****	14.62 [10.53,19.5]		15 [11.5,19.5]		14 [8.5,19]		0.174
Meat-Fish-Eggs-Nuts-Legumes (portions/week)*****	13.75 [10,18]		13.75 [10.31,17.25]		14 [9.13,18.25]		0.929
Vegetables (portions/week)*****	4.5 [2.81,8]		3.5 [2,7]		6 [3.5,8]		0.001
Fruits (portions/week)*****	7.78 [4,14]		7.38 [5,12]		9 [4,17]		0.133
Cereals (portions/week)*****	20.25 [15.38,27.78]		20.75 [16,26]		20.25 [13.75,29]		0.883

^the above variables were tested for differences between the two groups by $\chi 2$ tests for categorical variables, t-tests for normally distributed continuous variables and Wilcoxon tests for non-normally distributed continuous variables.
*Based on CDC cut-off points for BMI-for-age taking in account age and sex, the BMI-for-age weight status categories were created (Underweight: Less than the 5th percentile; Healthy Weight: 5th percentile to less than the 85th percentile; Overweight: 85th to less than the 95th percentile; Obesity: 95th percentile or greater).
**Summary of time spent in physical activities including hours per week spent on running, cycling, basketball, football, volleyball, swimming, dancing and other physical activities.
***Summary of time spent in sedentary activities including hours per week spent on TV, computer, tablet, mobile phones, or other sedentary activities.
****Food categories summarized based on consumption per week of food items belonging in each category as reported in the food frequency questionnaire for the conventional period of the study (food portion sizes were denoted in the questionnaire) – Food categories based on Children's Diet Pyramid for children aged 6–12 years (Ministry of Health, Cyprus).

Table 2
Frequency of pesticide usage during the last 90 days in the children's households (Table 2A) and the number of days passed since last usage of pesticides by the participating families (Table 2B).

	Days	Insect repellents (n, %)	Insecticides (n, %)	Fungicides (n, %)	Herbicides (n, %)	Rodenticides (n, %)
Inside the house	Never	143 (81)	125 (71)	177 (100)	177 (100)	175 (99)
	1–10	19 (11)	39 (22)	0 (0)	0 (0)	2 (1)
	11–30	8 (5)	5 (3)	0 (0)	0 (0)	0 (0)
	31+	7 (4)	8 (5)	0 (0)	0 (0)	0 (0)
Outside the house	Never	173 (98)	161 (91)	162 (92)	168 (95)	173 (98)
	1–10	2 (1)	14 (8)	15 (8)	9 (5)	4 (2)
	11–30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	31+	2 (1)	2 (1)	0 (0)	0 (0)	0 (0)

	Days	Insect repellents (n, %)	Insecticides (n, %)	Fungicides (n, %)	Herbicides (n, %)	Rodenticides (n, %)
Inside the house	Never	152 (86)	129 (73)	177 (100)	177 (100)	177 (100)
	1–10	2 (1)	5 (3)	0 (0)	0 (0)	0 (0)
	11–30	5 (3)	18 (10)	0 (0)	0 (0)	0 (0)
	31+	18 (10)	25 (14)	0 (0)	0 (0)	0 (0)
Outside the house	Never	173 (98)	162 (92)	164 (93)	171 (97)	175 (99)
	1–10	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)
	11–30	3 (2)	6 (3)	1 (1)	1 (1)	0 (0)
	31+	1 (1)	9 (5)	11 (6)	5 (3)	1 (1)

more correlated within each class of pesticides rather than between classes (e.g., GLY and AMPA were moderately correlated with each other, r = 0.45) (Fig. 1, Fig. S1).

3.3. Determinants of urinary pesticides

Pyrethroid and chlorpyrifos metabolites were negatively associated with mother's educational level (Master/PhD) when compared with the reference group of high school level or lower (cr1 3-PBA: beta = −0.55, 95% CI: −0.95, −0.14, p = 0.01), but this was not the case for GLY/

Table 3
Percentiles for the biomarkers of exposure to pesticides and the biomarkers of effect.

Biomarker [μg/L]	Mean	sd	min	q25	q50	q75	q90	q95	max	count	Biomarker [μg/g]	mean	sd	min	q25	q50	q75	q90	q95	max
3-PBA	2.72	2.54	0.34	1.28	1.93	3.29	5.40	6.59	20.02	177	3-PBA	2.48	2.14	0.29	1.24	1.92	3.05	4.20	5.90	14.35
ClF3CA	0.11	0.14	0.06	0.06	0.07	0.10	0.21	0.27	1.66	177	ClF3CA	0.11	0.13	0.03	0.06	0.08	0.10	0.18	0.23	1.23
DBCA	1.02	1.18	0.11	0.32	0.60	1.10	2.49	3.85	6.06	177	DBCA	0.91	0.96	0.08	0.31	0.63	1.09	1.84	2.73	6.35
DCCA-cis	0.89	1.06	0.06	0.33	0.61	1.03	1.81	2.22	9.63	177	DCCA-cis	0.80	0.91	0.07	0.32	0.57	0.97	1.48	2.12	8.31
DCCA-trans	1.45	1.56	0.10	0.50	0.93	1.83	3.01	4.28	10.84	177	DCCA-trans	1.34	1.43	0.11	0.46	0.88	1.76	2.70	3.64	10.49
Glyphosate	0.23	0.45	0.06	0.06	0.06	0.19	0.37	1.01	3.18	177	Glyphosate	0.20	0.40	0.03	0.06	0.08	0.16	0.42	0.72	3.20
TCPy	7.02	3.43	1.58	4.23	6.72	9.22	11.41	13.71	17.65	177	TCPy	6.47	2.86	1.66	4.52	6.20	8.34	9.74	11.01	20.76
AMPA	0.24	0.23	0.06	0.10	0.18	0.29	0.52	0.65	1.44	177	AMPA	0.23	0.25	0.04	0.10	0.16	0.23	0.46	0.58	1.83
8-iso-PGF2α	3.35	1.71	0.53	2.28	3.04	4.09	5.30	5.95	13.13	158	8-iso-PGF2α	3.45	2.60	0.46	1.77	2.99	4.11	5.88	7.31	19.25
8-OHdG	394.53	246.90	26.09	251.87	327.99	494.70	665.17	764.61	2039.20	139	8-OHdG	395.16	285.78	18.14	210.57	328.16	466.84	737.51	953.39	1663.85
Creatinine [g/L]	1.13	0.43	0.22	0.82	1.05	1.38	1.66	1.82	3.07	177										

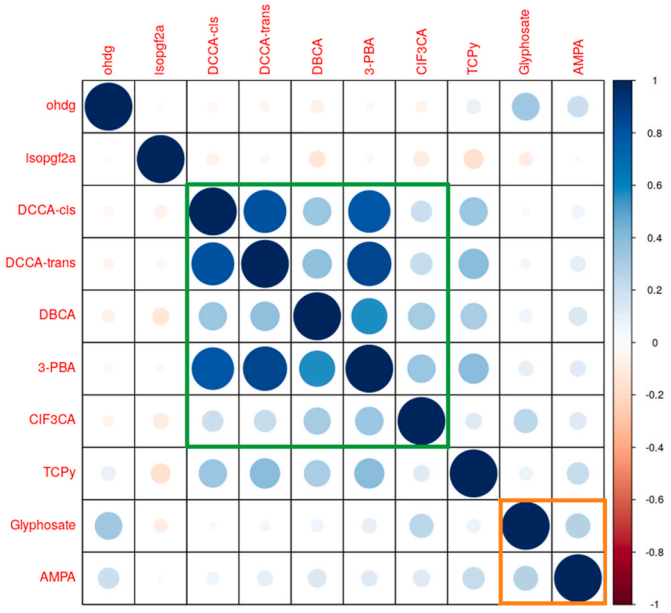

Fig. 1. Correlation matrix of the biomakers of exposure to pesticides and biomarkers of oxidative stress (μg/L). The green colour denotes the correlations between pyrethroid pesticides and the orange colour denotes the corellations between GLY and AMPA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

AMPA ($p > 0.05$) (Table 4). The pyrethroid metabolite DBCA was marginally significantly associated with BMI (cr1 beta = 0.01, 95% CI: 0.00, 0.01, p = 0.05) and father's educational level (Master/PhD) when compared with the reference group of high school level or lower (cr1 beta = 0.48, 95% CI: 0.02, 0.95, p = 0.04). No other exposure determinant was significantly associated with most of the measured pesticides.

3.4. Association between pesticides and oxidative stress biomarkers

The DNA oxidative damage marker (8-OHdG) was positively associated with AMPA after adjusting for age, sex, BMI, parental education level, physical activity and creatinine (beta = 0.17; 95% CI: 0.02, 0.31, p = 0.03, cr2, and beta = 0.12; 95% CI: 0.00, 0.24, p = 0.06, cr1) (Fig. 2, Fig. S2; Table S1); however, no significant association was observed between GLY and 8-OHdG ($p > 0.05$, Fig. S3). Similar significant associations with 8-OHdG were observed for the pyrethroid metabolite 3-PBA (beta = 0.19, 95% CI: 0.02, 0.37; p = 0.03, cr2 and beta = 0.14, 95% CI: 0.00, 0.28; p = 0.05, cr1) and the chlorpyrifos metabolite (TCPy) (beta = 0.42, 95% CI: 0.16, 0.68; $p < 0.001$, cr2 and beta = 0.20, 95% CI: -0.01, 0.41; p = 0.06, cr1) (Fig. 2, Table S1). In the case of the lipid oxidation models, only the pyrethroid metabolite ClF3CA showed significant association with 8-iso-PGF2α (beta = 0.31, 95% CI: 0.12, 0.50; $p < 0.001$, cr2). In a sensitivity analysis, we reanalyzed the data after including the two subjects that were excluded from the primary analysis, because their urinary creatinine levels were outside the acceptable range. In the sensitivity analysis, the coefficients and relationships remained essentially unchanged (Table S2).

4. Discussion

To the best of our knowledge, this is the first non-occupational children's health dataset that presents evidence of AMPA's oxidative stress toxicity, albeit this was not the case for GLY. While we cannot rule out that these significant associations may be due to chance or non-causal, these findings are of concern, because they refer to a vulnerable subpopulation group (10–12 years old) with widespread pesticide

Table 4
Linear multivariate regression models of each biomarker of exposure to pesticides (log transformed) -creatinine added as covariate in the model) (beta, 95% CI).

Predictors	DCCA-cis			DCCA-trans			DBCA			3-PBA	
	Estimates	CI	p	Estimates	CI	p	Estimates	CI	P	Estimates	CI
Creatinine	0.06	−0.29–0.41	0.74	0.09	−0.26–0.44	0.61	0.06	−0.31–0.44	0.73	0.07	−0.21–0.35
Male	0.10	−0.19–0.38	0.51	−0.02	−0.30–0.25	0.86	−0.27	−0.57–0.03	0.08	−0.10	−0.33–0.12
Age	0.04	−0.20–0.28	0.74	0.08	−0.15–0.32	0.48	0.03	−0.22–0.28	0.82	−0.02	−0.21–0.17
BMI	0.00	−0.00–0.01	0.64	0.00	−0.00–0.01	0.13	0.01	0.00–0.01	**0.05**	0.00	−0.00–0.01
Mother educational level - University/ college	−0.35	−0.74–0.04	0.07	−0.42	−0.80–−0.03	**0.03**	−0.30	−0.71–0.12	0.16	−0.23	−0.54–0.08
Mother educational level - Master/PhD	−0.65	−1.15–−0.15	**0.01**	−0.76	−1.26–−0.27	**<0.001**	−0.40	−0.93–0.14	0.15	−0.55	−0.95–−0.14
Father educational level - University/college	0.14	−0.20–0.47	0.43	0.15	−0.18–0.49	0.37	0.21	−0.15–0.57	0.26	0.03	−0.24–0.30
Father educational level - Master/PhD	0.23	−0.20–0.67	0.29	0.36	−0.07–0.79	0.10	0.48	0.02–0.95	**0.04**	0.17	−0.18–0.52
Observations	175			175			175			175	

exposures. It was speculated that the lack of evidence of GLY toxicity in this study would be partially attributed to its faster environmental degradation than that of AMPA (Eaton et al., 2022). GLY degradation in the environment by a suite of microorganisms is relatively fast (range of 5–20 days) (PPDB, 2013a) giving rise to AMPA, which is highly persistent in the environment ($t_{1/2}$ of 151 days) (PPDB, 2013b). The varying environmental half-lives of GLY and AMPA may not be directly responsible for their association with oxidative damage, but they are responsible for the relative abundance of GLY and AMPA in the environmental medium that humans come in contact with. The higher probability of detecting AMPA in the environment due to its longer persistence may be responsible for the higher children's body burden of AMPA (urine) than that of GLY. In our study, a higher proportion of urine samples had GLY levels <LOQ (54% for GLY), while this was the case for only 25% of samples for AMPA.

Earlier evidence of oxidative stress caused by GLY, glyphosate-based formulations and AMPA was strong, based on human and animal studies on various end-points (e.g. lipid peroxidation markers, oxidative DNA adducts, dysregulation of antioxidant enzymes) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2017; Wang et al., 2022). A recent study in pregnant women of Puerto Rico showed that an interquartile range (IQR) increase in AMPA and GLY was associated with 2.3% (95% CI: 2.9, 7.8) and 4.7% (95% CI: -0.9, 10.7) higher 8-iso--PGF2α concentrations (Eaton et al., 2022). A newly published study showed that urinary AMPA levels in an adult population were associated with leukocyte telomere length, which is a biomarker of biological aging (Cosemans et al., 2022). However, GLY and AMPA studies in children populations examining associations with oxidative stress and genotoxicity are currently lacking. The inherent difficulty discriminating the portion of the AMPA that originates from GLY from the portion originating from other formulations/products (e.g., detergents) still remains. The literature suggests a difficulty in distinguishing between the scenario of observing a small transformation rate of GLY to AMPA by the liver enzymes and the scenario of no internal metabolism of GLY to AMPA, suggesting external AMPA exposure sources (Zoller et al., 2020). In our study, GLY and AMPA were moderately correlated ($\rho = 0.43$), indicating that at least some of the AMPA source was likely ascribed to GLY, with similar results obtained elsewhere (Silver et al., 2021).

Both AMPA and GLY are co-occurring external environmental contaminants that could cause harm to humans (Connolly et al., 2020). There are only a few original, peer-reviewed children's studies that measured biomarkers of exposure to GLY and/or AMPA using mass spectrometry, instead of immunoassays (Connolly et al., 2020). In adults, average GLY and AMPA (μg/L) concentrations ranged between <LOQ – 3.4 and <LOQ – 0.3, respectively (Connolly et al., 2020). Children studies showed that medians of both AMPA and GLY were below or at the limit of quantification (≤0.1 μg/L) for 149 children aged 7–10 years from rural areas of Northeastern Slovenia (Stajnko et al., 2020). Measurements of GLY in children (5–15 yrs old) from two agricultural communities of Mexico showed a geometric mean of 0.27 μg/L (Sierra-Diaz et al., 2019), while a U.S. children and neonates (0–8 years old) population had mean (sd) GLY levels of 0.28 (0.23) μg/L, that was detected in only 11% of the study population (Trasande et al., 2020). Urinary GLY and AMPA geometric mean levels in children in Germany were 0.107 μg/L and 0.100 μg/L, respectively (Lemke et al., 2021), while in Slovenia, both were below LOQ (Stajnko et al., 2020). However, it becomes evident that additional children's health studies on the toxicity of AMPA and GLY are greatly needed.

It has been suggested that both AMPA and GLY present with a similar toxicological profile (EFSA (European Food Safety Authority), 2015). The combined exposure to both GLY and AMPA should be considered, as suggested by the JMPR, proposing a GLY/AMPA group-based acceptable daily intake value (ADI) (Glyphosate and Metabolites, 2011). This suggests a GLY-independent source of AMPA in the environment, such as environmental metabolites of amino-polyphosphonates (Grandcoin et al., 2017). The WHO also reported that both GLY and AMPA along with some other degradation products should be regarded as residues of toxicological concern (JMPR, 2017). In some studies, GLY levels were higher than those of AMPA, while in other studies the opposite trend was shown.

Parental education level was the sole significant exposure determinant for most of the tested pesticides in this study with children of parents holding a Master or PhD, having lower urinary pesticides levels. This was similarly reported in a U.S. children population (Trasande et al., 2020), while no sociodemographic factors were identified as exposure sources in a German children population (Lemke et al., 2021). However, it should be noted that our study participants' parents had high educational attainment (82% of mothers and 65% of fathers held at least a university/college degree), hence a small number of children came from families with low educational background.

This study was the first examining the association between AMPA and oxidative stress in a children's subpopulation group. This study also measured classical (e.g., TCPy) and emerging (CFMP) pesticide metabolites abiding by a strict human biomonitoring-based pan-EU laboratory inter-comparison scheme within the HBM4EU project. The pesticide metabolites 3-PBA and TCPy were significantly associated with the oxidative DNA adduct marker in this study. Compared to studies in other children populations (for example USA, Canada, Costa Rica, Spain, Italy, Thailand) (Wang et al., 2016; van Wendel de Joode et al., 2016; CDC, 2019; Bravo et al., 2019; Health Canada, 2019; Panuwet et al., 2009; Muñoz-Quezada et al., 2020; Fernández S et al., 2020), the median levels of 3-PBA and TCPy in this study population of Cyprus were higher (Table S3).

The collective results from the HBM4EU aligned studies for

3-PBA	TCPy			ClF3CA			Glyphosate			AMPA		
p	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p
0.62	0.11	−0.09–0.31	0.26	0.04	−0.17–0.25	0.72	−0.11	−0.49–0.27	0.55	−0.03	−0.34–0.28	0.85
0.37	0.01	−0.14–0.17	0.86	−0.12	−0.28–0.05	0.17	0.14	−0.16–0.45	0.35	0.18	−0.07–0.43	0.15
0.84	0.07	−0.07–0.20	0.32	−0.04	−0.18–0.11	0.62	−0.04	−0.30–0.21	0.75	−0.05	−0.26–0.16	0.65
0.11	−0.00	−0.00–0.00	0.90	−0.00	−0.00–0.00	0.75	−0.00	−0.01–0.00	0.85	−0.00	−0.01–0.00	0.77
0.15	−0.16	−0.38–0.06	0.15	−0.14	−0.37–0.09	0.23	−0.11	−0.53–0.31	0.60	0.01	−0.33–0.35	0.95
0.01	−0.30	−0.58–−0.01	**0.04**	−0.16	−0.45–0.14	0.30	−0.23	−0.77–0.32	0.41	0.21	−0.23–0.65	0.35
0.82	0.01	−0.18–0.20	0.90	0.00	−0.20–0.20	0.99	0.02	−0.35–0.39	0.91	0.12	−0.18–0.42	0.43
0.34	0.03	−0.22–0.28	0.82	−0.04	−0.30–0.22	0.74	0.01	−0.46–0.48	0.97	0.04	−0.34–0.43	0.82
175	175			175			175			175		

pesticides exposures of European children will be presented elsewhere. A strength of the study was the choice of first morning urine void to control for possible diurnal variation and metabolism of xenobiotics instead of spot urine sampling (Makris, 2021). We also controlled for urine output variation employing two approaches of creatinine adjustment in the regressions. A potential limitation of the study would be the single urine sample measurement for both the pesticides and the biomarkers of effect and the cross-sectional study design with a relatively small sample size. Another limitation would be that often common pesticide metabolites or specific metabolites may form as a result of environmental degradation processes. It should be mentioned here that CIF3CA analysis was accompanied by bioanalytical challenges and difficulties in accurately and reliably measuring it, by being detected in the low-level ppt range (<0.1 μg/L). CIF3CA/CFMP is an emerging metabolite for bifenthrin, cyhalothrin, tefluthrin (Khemiri et al., 2017).

In summary, this was a pesticide-focused children's biomonitoring study in Cyprus aligned with the methodology and tools used in the HBM4EU project. Although a significant association was observed between AMPA and the DNA oxidative stress marker in this children's population, these results need to be replicated in a larger study. The lack of significant associations between AMPA/GLY and lipid damage may suggest a biological mechanism of DNA damage for AMPA. This is the first epidemiological study exploring the association between AMPA/GLY and oxidative stress in children. Our results indicate that Cypriot children are co-exposed to a mixture of pesticides likely originating from both dietary and non-dietary sources. On average, these pesticide exposures appear at higher levels than those typically measured in other EU populations. The population health risk associated with such mixture exposures needs to be further investigated.

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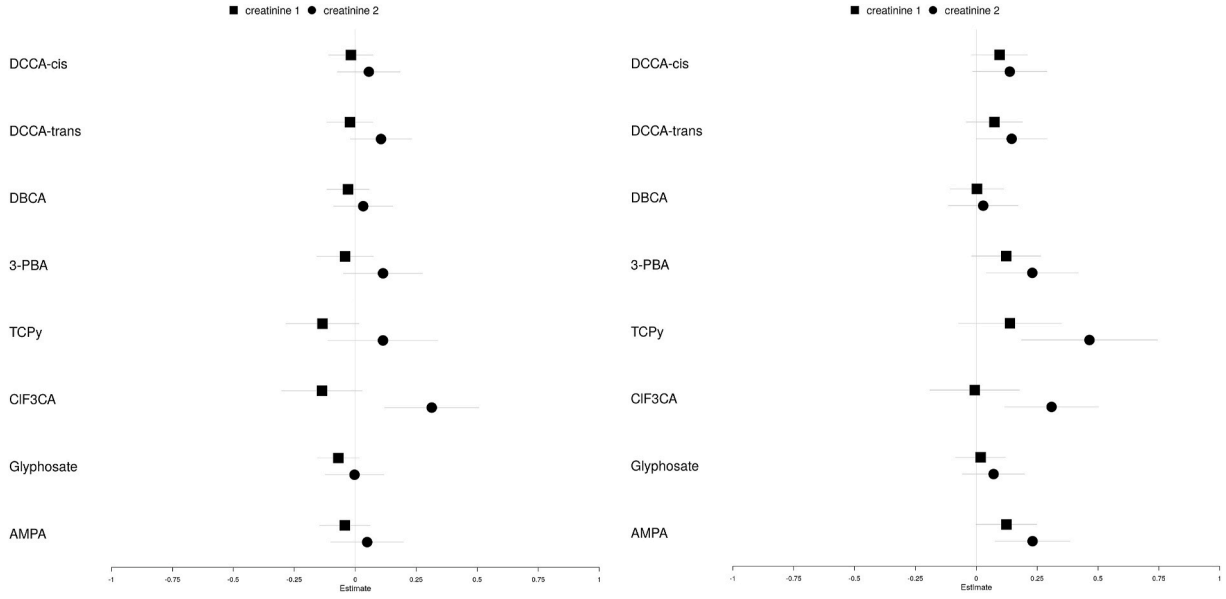

Fig. 2. Forest plots of the beta coefficients from the multivariate linear regression models of DNA oxidation (8-OHdG, right graph) and lipid damage (8-iso-PGF2α, left graph) regressed on the biomarkers of exposure to pesticides. Adjusted for age, sex, BMI, parental education level, physical activity and urinary creatinine (creatinine 1 models treated creatinine as a covariate in the regression model; creatinine 2 models had biomarker data dividing their unadjusted levels by their urinary creatinine, μg/g cr).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113316. Data is available upon request to the corresponding authors.

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