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Exposure to non-persistent pesticides, BDNF, and behavioral function in adolescent males: Exploring a novel effect biomarker approach

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ABSTRACT

Background: Numerous contemporary non-persistent pesticides may elicit neurodevelopmental impairments. Brain-derived neurotrophic factor (BDNF) has been proposed as a novel effect biomarker of neurological function that could help to understand the biological responses of some environmental exposures.
Objectives: To investigate the relationship between exposure to various non-persistent pesticides, BDNF, and behavioral functioning among adolescents.
Methods: The concentrations of organophosphate (OP) insecticide metabolites 3,5,6-trichloro-2-pyridinol (TCPy), 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPy), malathion diacid (MDA), and diethyl thiophosphate (DETP); metabolites of pyrethroids 3-phenoxybenzoic acid (3-PBA) and dimethylcyclopropane carboxylic acid (DCCA), the metabolite of insecticide carbaryl 1-naphthol (1-N), and the metabolite of ethylene-bis-dithiocarbamate fungicides ethylene thiourea (ETU) were measured in spot urine samples, as well as serum BDNF protein levels and blood DNA methylation of Exon IV of BDNF gene in 15–17-year-old boys from the INMA-Granada cohort in Spain. Adolescents' behavior was reported by parents using the Child Behavior Check List (CBCL/6–18). This study included 140 adolescents of whom 118 had data on BDNF gene DNA methylation. Multivariable linear regression, weighted quantile sum (WQS) for mixture effects, and mediation models were fit.
Results: IMPy, MDA, DCCA, and ETU were detected in more than 70% of urine samples, DETP in 53%, and TCPy, 3-PBA, and 1-N in less than 50% of samples. Higher levels of IMPy, TCPy, and ETU were significantly associated with more behavioral problems as social, thought problems, and rule-breaking symptoms. IMPy, MDA, DETP, and 1-N were significantly associated with decreased serum BDNF levels, while MDA, 3-PBA, and ETU were associated with higher DNA methylation percentages at several CpGs. WQS models suggest a mixture effect on more behavioral problems and BDNF DNA methylation at several CpGs. A mediated effect of serum BDNF within IMPy-thought and IMPy-rule breaking associations was suggested.
Conclusion: BDNF biomarkers measured at different levels of biological complexity provided novel information regarding the potential disruption of behavioral function due to contemporary pesticides, highlighting exposure to diazinon (IMPy) and the combined effect of IMPy, MDA, DCCA, and ETU. However, further research is warranted.

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1. Introduction

The adolescence dramatically reshapes teenagers' perception of themselves, as well as their social context through complex neural mechanisms (Gore et al., 2018). They must exhibit social communicative skills, reproductive behaviors, adequate anxiety responses, and affective states according to their age and sex, among other factors (Spear, 2000). Thus, dendritic spine turnover, enhancing synaptic plasticity and consequently experience-dependent learning, and inhibitory neurotransmission, enhancing an adequate social behavior, are two of the potential mechanisms of action orchestrating adolescence brain development (Pfeifer and Allen, 2021). Exposure to environmental neurotoxic chemicals could be involved in the increasing incidence observed for mental health disorders such as social anxiety, depression, or eating disorders, among adolescents through alterations on these mechanisms of action (Bjørling-Poulsen et al., 2008; Bouchard et al., 2010; Pfeifer and Allen, 2021; Shoaff et al., 2020; Supke et al., 2021).

A growing body of epidemiological research suggests that prenatal exposures to contemporary non-persistent pesticides, mainly organophosphate (OP) and pyrethroid insecticides, are associated with neurodevelopmental impairments (González-Alzaga et al., 2014; Hernández et al., 2016; Oulhote and Bouchard, 2013; Wagner-Schuman et al., 2015). However, limited evidence is available regarding the potential impact of pesticide exposures during childhood or adolescence (Bouchard et al., 2010; Dalsager et al., 2019; Damgaard et al., 2006) with inconclusive results (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016; Wagner-Schuman et al., 2015).

The general population is widely exposed to many non-persistent pesticides. Although some of them are banned in the European Union (EFSA, 2020) (Supplementary Material, Table S1), and the use of others (e.g. some OPs) is decreasing, the employment of pyrethroids is increasing as agriculture and indoor biocides, being frequently detected in urinary samples of mothers, children, and neonates (Andersen et al., 2021; Dalsager et al., 2019). Parent compounds of some specific and non-specific pesticide metabolites are known developmental neurotoxicants (Andersen et al., 2021; Bjørling-Poulsen et al., 2008).

Under the framework of the Human Biomonitoring for Europe Initiative (HBM4EU), brain-derived neurotrophic factor (BDNF) has been proposed as a promising effect biomarker to improve the causal inference between exposure to environmental chemicals and altered neurodevelopment (Mustieles et al., 2020, 2022; Rodríguez-Carrillo et al., 2022). BDNF plays an important role in synaptic plasticity and is expressed throughout the brain, especially in the hippocampus, cortex, and other basal forebrain areas, which are susceptible to both exogenous and endogenous stressors. Thus, alterations in hippocampal BDNF expression could contribute to the development of mood disorders (Polyakova et al., 2015; Zaletel et al., 2017).

In this regard, epigenetic mechanisms, such as DNA methylation that regulate BDNF expression patterns (e.g. higher DNA methylation implies lower protein expression) could be related to the development of neurodevelopmental diseases (Ikegame et al., 2013). In previous epidemiological studies, BDNF disruption has been assessed by measuring its protein levels in serum or plasma, or its degree of blood DNA methylation at six CpGs from Exon IV of the BDNF gene concerning behavioral outcomes, including depression, bipolar disorder, attention-deficit hyperactivity disorder (ADHD), and even suicidal behavior at different stages of life (Bilgiç et al., 2020; Heinrich et al., 2017; Kim et al., 2007; Kundakovic et al., 2015; Polyakova et al., 2015). Recent results from our research group highlighted the advantages of including BDNF as an effect biomarker to address the relationship between exposure to bisphenol A and toxic metals, and altered/inadequate neurodevelopment (Mustieles et al., 2022; Rodríguez-Carrillo et al., 2022).

This study aimed to explore the potential role of BDNF on the relationship between exposure to non-persistent pesticides and behavior in adolescents and to assess the mixture effect of pesticides exposure on behavioral symptoms and BDNF levels. To do so, we assessed the cross-sectional relationship of urinary pesticide metabolites, both individually and combined, with: i) the behavioral function of adolescents and ii) the effect biomarker BDNF measured at two levels of biological complexity (DNA methylation and serum protein levels), and; iii) the association of BDNF with adolescents' behavior. In addition, and if relevant, we aimed to test the potential mediation role of BDNF in exposure-effect relationships.

2. Material and methods

2.1. Study population

The Environment and Childhood (INMA) Project is a multicenter population-based birth cohort study that aims to investigate the impact of environmental exposures and diet on health and development during key windows of susceptibility, *i.e.* pregnancy, childhood, and adolescence, in different geographical areas of Spain (Guxens et al., 2012). During the last follow-up of the INMA-Granada cohort of boys (2017–2019), 151 adolescents aged 15–17 years agreed to participate in a clinical visit at the San Cecilio University Hospital of Granada, which included physical examination and behavioral assessments (Castiello et al., 2020). All participants provided a first-morning urine sample, and most of them (n = 135, 89%) also provided a blood sample. The present study included participants with available data on i) urinary pesticide metabolite concentrations and behavioral outcomes (n = 140); ii) urinary pesticide metabolite concentrations, behavioral outcomes, and serum total BDNF protein levels (n = 130); and iii) urinary pesticide metabolites, behavioral outcomes, and blood DNA methylation of the Exon IV of BDNF gene at six CpGs (n = 118). Further details on the INMA-Granada cohort recruitment and follow-ups have been previously described (Castiello et al., 2020; Fernandez et al., 2007; Freire et al., 2018). The informed consent was signed by adolescents' parents and the study protocol was approved by the Biomedical Research Ethics Committee of Granada (reference number 0509-N17, date of approval March 28, 2017).

2.2. Analysis of urinary pesticides metabolites concentrations

A first morning spot urine sample was collected from each participant on the day of the clinical visit and kept at −80 °C until analysis. Urine samples were analyzed for 3,5,6-trichloro-2-pyridinol (TCPy), 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), and malathion diacid (MDA), specific metabolites of the OP insecticides chlorpyrifos/chlorpyrifos-methyl, diazinon, and malathion, respectively; dialkyl phosphates diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP), non-specific metabolites of OP insecticides; 3-phenoxybenzoic acid (3-PBA) and dimethylcyclopropane carboxylic acid (DCCA) (sum of *cis* and *trans* isomers), metabolites of pyrethroids; 1-naphthol (1-N), the primary metabolite of the carbamate insecticide carbaryl; and ethylene thiourea (ETU), the major metabolite of ethylene-bis-dithiocarbamate (EBDC) fungicides such as mancozeb (Supplementary Material, Table S1). It was not possible to measure other DAPs such as diethyl phosphate (DEP) or dimethyl phosphate (DMP) metabolites because their reference standards were not available.

The urinary pesticide metabolites TCPy, IMPy, DETP, DEDTP, 3-PBA, 1-N, and ETU were measured by ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS), using a UHPLC Ultimate 3000 (Thermo Fischer) and a Q Exactive Focus mass spectrometer (Thermo Fischer) at the "UNETE Research Unit'' of the Biomedical Research Center (CIBM), University of Granada (Spain). The urinary acid metabolites MDA and DCCA were measured by liquid chromatography coupled to mass spectrometry (LC-MS/MS), using an Agilent 1290 liquid chromatography (Agilent) and API 4000 mass spectrometry (AB Sciex Instruments) at the facilities of the MEDINA Foundation, Granada (Spain). All metabolites, including MDA and DCCA, were first extracted and calibrated according to Suárez et al.

(2021). No deconjugation procedure was performed. Standards solutions and Internal Standards (IS) were added to 1 mL of urine samples. Successively, 2 mL of aqueous ammonium hydroxide solution at 1% was added and kept at room temperature for 20 min. Cleanup and preconcentration were performed with solid-phase extraction (SPE). Briefly, activation was done using 3 mL of methanol, 3 of deionized water, and 2 mL of aqueous ammonium hydroxide solution at 1% (v/v). Cartridge was rinsed with 2 mL of formic acid at 1% with deionized water and dried under vacuum. Further information regarding the analytical method and quality control procedures has been fully described previously (Freire et al., 2021; Suárez et al., 2021).

Data regarding retention times, analytical parameters, calibration curves, mean accuracy, selected reaction monitoring (SRM), and relative standard deviation (RSD) values are reported in Supplementary Material (Table S2). Limits of detection (LOD) were 0.039 μg/L for TCPy, 0.117 μg/L for IMPy, 0.052 μg/L for MDA, 0.116 μg/L for DETP, 0.142 μg/L for DEDTP, 0.117 μg/L for 3-PBA, 0.055 μg/L for DCCA, 0.156 μg/L for 1-N, and 0.072 μg/L for ETU (Table S2). To account for urine dilution, concentrations of creatinine in urine were assessed using a commercial kit (CREJ2) through the Jaffe method in a Roche Cobas C-311 system (mg/dL).

2.3. Serum BDNF and whole blood DNA methylation of BDNF gene assessment

On the same day of urine sample collection, peripheral venous blood samples were collected from participants under non-fasting conditions between 5 and 7 p.m. and were immediately processed to obtain serum and whole blood aliquots and stored at −80 °C. Whole blood was sent on dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Center. DNA extraction was performed using Maxwell® RSC equipment and quantified by PicoGreen assay, sowing an average concentration of 50 ng/μL.

Total serum BDNF levels, which contain mature and immature isoforms of BDNF, were measured with an enzyme-linked immunosorbent assay using the commercial Quantikine® ELISA kit (R&D Systems, Minneapolis, MN, USA) at the CIBM, University of Granada (Spain), following manufacturer's instructions. The full procedure was described elsewhere (Rodríguez-Carrillo et al., 2022). Serum total BDNF protein showed an intra- and inter-assay coefficient of variation (%) of <3% and 15%, respectively.

DNA methylation of the BDNF gene was performed by bisulfite pyrosequencing analysis at IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085), Rennes, France. Exon IV of BDNF gene was the targeted region (genomic coordinates: chr11:27, 723, 070–27,723,280 retrieved from UCSC Genome Browser Human (GRCh37/hg19), previously validated in rodents and humans (Kundakovic et al., 2015) which contains 6 CpGs including a CREB-binding site (cAMP response element-binding site). Further information regarding the measurement of BDNF DNA methylation was published according to Mustieles et al. (2022). Biotinylated primers used for BDNF amplification (0.4 μM final concentrations) are provided in Table S2. The degree of methylation at each CpG was expressed as percentage of DNA methylation.

2.4. Behavioral functioning assessment

The behavioral function of adolescents was evaluated using the Spanish version of the validated Child Behavior Checklist (CBCL/6–18) (Achenbach and Rescorla, 2013; Sardinero García et al., 1997). In this questionnaire, parents report on their sons' behavior during the previous 6 months. It is based on 118 items rated on a three-point scale (not true, somewhat true, or very/often true) and contains a total of eight syndrome scales: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. These scales are then summarized in three composite scales obtained from the sum of the mentioned syndrome scales, as follows: internal problems (sum of scores from anxious/depressed, withdrawn/depressed, and somatic complaints scales), externalizing problems (the sum of scores from the rule-breaking behavior and aggressive behavior scales) and total problems. All scales were normalized by age and sex and finally reported as T-scores. For all the above-mentioned scales, higher scoring means more behavioral problems (Achenbach and Rescorla, 2013).

2.5. Statistical analysis

Absolute and relative frequencies (categorical variables) and central tendency measures and dispersion profiles (continuous variables) were calculated to describe sociodemographic and lifestyle characteristics of the study participants. For descriptive purposes, detection frequencies were calculated for urinary pesticides concentrations, while percentiles (25th, 50th, and 75th) were calculated for both pesticide metabolites and BDNF effect biomarker levels. DEDTP was detected in only 1 urine sample and was thus excluded from further analyses. For pesticide metabolites detected above 70% of samples, concentrations below the LOD were imputed with $LOD/\sqrt{2}$ (Schisterman et al., 2006). The total concentration of OP (ΣOPs) and pyrethroid (ΣPYR) urinary metabolites was further calculated by summing concentrations of their respective metabolites (i.e., ΣOPs = TCPy + IMPy + MDA; ΣPYR = DCCA + 3-PBA). A small number of DNA methylation measurements (<4% of all CpG measurements performed) could not be quantified, and thus were multiple imputed (20 imputations) using the regression method. Spearman's correlation test was used to assess the relationship among concentrations of non-persistent pesticides metabolites.

Multivariable linear regression models were performed to assess the association of i) urinary pesticide metabolite concentrations with adolescents' behavioral function; ii) urinary pesticide metabolites with biomarkers of effect (serum BDNF and methylation profile of the BDNF gene), and iii) BDNF biomarkers with behavioral outcomes. Before regression analysis, pesticide metabolites detected in more than 70% of urine samples (IMPy, MDA, DCCA, and ETU) were natural log-transformed to reduce distribution skewness (including ΣOPs and ΣPYR), while those detected in less than 70% of samples were dichotomized into detected vs. non-detected values. BDNF biomarkers (normally distributed) and behavioral outcomes were modeled as continuous variables. Metabolites detected in >70% of samples as well as ΣOPs, ΣPYR, and all BDNF effect biomarkers were further categorized into tertiles to assess possible non-linear associations. DETP (detected in 54% of samples) was categorized as follows: from below the LOD to the 50th percentile of urinary concentration, from the 50th to 75th percentile, and above the 75th percentile.

All models were adjusted for adolescent's age (continuous), body mass index (BMI, continuous kg/m^2), alcohol consumption (less than one beverage/month or at least 1 beverage/month), and maternal education (primary education/secondary education/university education), which were selected based on their known influence on neurodevelopment and previous studies exploring pesticide exposure and behavioral function (Patra et al., 2016; Pyman et al., 2021; Wirt et al., 2015). Maternal education, a proxy for socioeconomic status, had a 6% of missing values (n = 10). To maintain the initial sample size, this covariable was imputed using multiple imputation through regression method (20 imputations). Season of the urinary collection (spring/-summer/autumn/winter) was added to models with pesticide metabolites, since it may influence pesticides exposure (Fortenberry et al., 2014). To control for urine dilution variability and reduce potential error bias, urinary creatinine concentration (ng/mL) was included in models as a separate covariate as recommended in previous studies (Barr et al., 2005; O'Brien et al., 2016). Regression estimates reflect the mean change in behavioral outcome score/serum BDNF level/methylation percentage for each log unit increase in urinary pesticide metabolite concentration in linear models. For models with the independent

variable categorized into tertiles, regression estimates reflect the mean change in the dependent variable taking the first tertile (T1) as the reference; in models with dichotomized pesticide exposure, regression estimates reflect the mean change in the dependent variable for detected *versus* undetected concentrations.

Weighted Quantile Sum (WQS) analysis was performed to assess the combined effect of IMPy, MDA, DCCA, and ETU (>70% detection) on behavioral functioning and BDNF biomarkers. The WQS index is regressed from multivariable linear models, which constructs the unidirectional weighted index from quantiled chemical exposure variables, thus reducing potential multicollinearity and dimensionality while providing an overall mixture effect estimate (Tanner et al., 2020). Weights expressed as percentages, show the relative strength of each mixture component. Pesticide metabolites were binned as quartiles. Since we hypothesized that the mixture effect would be associated with more behavioral problems and BDNF DNA methylation, but with lower serum BDNF protein levels (Mustieles et al., 2020), the index was constructed using weights in *positive mode* when addressing CBCL subscales and BDNF DNA methylation, and as *negative mode* when addressing serum BDNF. Mean weights of chemicals exceeding 25% (100%/4 chemicals) were considered as chemicals of concern, meaning that their contribution to the outcome would be larger than expected by chance. Bootstrapping was set to 100. Finally, to evaluate the stability and generalizability of our results, repeated holdout validation was performed. This method combines cross-validation and bootstrap resampling by splitting data into 40–60% training test sets and repeating WQS regression 100 times as previously performed in other epidemiological studies (Galbán-Velázquez et al., 2021; Tanner et al., 2020).

To assess the role of BDNF as a potential mediator of significant/suggestive exposure-effect associations, total, direct, and indirect effects were analyzed using mediation analysis in the PROCESS macro v3.5 (http://processmacro.org/index.html). According to Hayes (2009), the magnitude of the effect and confidence intervals (95% CI) was estimated by conducting 10,000 bootstrapped replicates. The indirect or mediated-effect represents the proportion of the statistical relationship between the exposure (*i.e.,* pesticides) and outcome (*i.e.,* CBCL) that is driven by the mediator (*i.e.,* BDNF). The percentage of mediation was calculated as indirect effect/(direct + indirect effect) x 100, where the *direct effect* represents the proportion of the statistical relationship between the exposure and the outcome not attributable to the mediator.

The significance level was set at $p < 0.05$; however, results were also interpreted based on patterns of associations, internal validity, and coherence with previous toxicological and epidemiological evidence (Amrhein et al., 2019). SPSS v26.0 (IBM, Chicago, IL) and R statistical software version 3.4.3, package WQS (https://cran.r-project.org/web/packages/gWQS/index.html), were used for data analyses.

3. Results

3.1. Characteristics of study participants

Sociodemographic characteristics of study participants are described in Table 1. Adolescents had a mean (standard deviation, SD) age and BMI of 16.2 (0.4) years and 23.2 (4.9) kg/m^2, respectively. Less than a half of participants consumed more than one alcoholic beverage per month (39.7%) and had mothers with university education (25.9%). Most urine samples were collected in autumn (42.6%) (Table 1).

Urinary DCCA was detected in all urine samples (median = 1.06 ng/mL). MDA, IMPy, and ETU were detected in 83.0%, 74.8% and, 74.2% of samples, respectively (median = 0.30, 0.25, and 0.26 ng/mL, respectively). DETP was detected in 54.3% (median = 0.25 ng/mL) and TCPy, 3-PBA, and 1-N in <40% of samples (Table 2). Urinary concentrations of IMPy and TCPy were positively and significantly correlated with 3-PBA (Spearman's rho, $\rho = 0.20$ and 0.17, respectively), DETP with DCCA and 1-N ($\rho = 0.20$ and 0.21, respectively); no significant correlation was found for MDA and ETU (Table S3).

Table 1
General characteristics of study participants and their mothers (n = 140).

Adolescents	Mean ± SD/n (%)
Age (years)	16.2 ± 0.4
BMI (kg/m^2)	23.2 ± 4.9
Area of residence	
Urban	97 69.5)
Suburban/rural	43 (30.5)
Passive smoking	
Yes	59 (43.2)
No	81 (56.8)
Alcohol consumption	
Never or <1 beverage per month	85 (60.3)
≥1 beverage per month	55 (39.7)
Season of urine collection	
Spring	33 (24.1)
Summer	16 (12.1)
Autumn	62 (42.6)
Winter	29 (21.3)
Annual family income (euros)	
<25,000	54 (38.3)
25,000–35,000	54 (38.3)
>35,000	32 (23.4)
Mothers	
Age (years)	39.6 ± 4.7
Maternal education	
Primary	58 (41.0)
Secondary	46 (33.1)
University	36 (25.9)

SD: standard deviation; BMI: Body mass index.

Median total serum BDNF level was 32.59 ng/mL, median blood BDNF gene DNA methylation percentages at CpGs #1 to #6 were: 4.45%, 3.18%, 3.21%, 5.69%, 3.17%, and 2.42%, respectively; and the total median CpGs DNA methylation was 3.70% (Table 2). Finally, adolescents' externalizing problems (16%) were less prevalent than internalizing problems (32%) (Table S2).

3.2. Pesticide's exposure and adolescents' behavioral functioning

Multivariable linear regression models based on tertiles of urinary concentrations of pesticide metabolites showed that adolescents with IMPy concentrations at the third tertile (T3), *versus* first (T1), were associated with more social problems, rule-breaking, aggressive behavior, externalizing, and total problems [$\beta = 3.34$ (95%CI = 0.65,6.02), $\beta = 3.76$ (95%CI = 1.06,6.45), $\beta = 3.77$ (95%CI = 1.07,4.46), $\beta = 5.50$ (95%CI = 1.58,9.42), $\beta = 4.60$ (95%CI = 0.68,8.52), respectively] (Table 3). An apparent dose-dependent association with more thought problems was also found [$\beta_{T2} = 2.33$ (95%CI = −0.24,4.90) $\beta_{T3} = 2.56$ (95%CI = −0.04,5.16)] (Table 3). Detected *versus* undetected urinary TCPy was significantly associated with more social and thought problems (Table 3). ΣOPs at T3 was associated with more social problems and rule-breaking behavior [$\beta = 3.53$ (95%CI = 0.77,6.29), $\beta = 3.11$ (95%CI = 0.30,5.92), respectively]. DETP at T3 was associated with less withdrawn symptoms ($\beta = -3.54$, 95%CI = −6.85,0.23).

The pyrethroid metabolite DCCA at T2 and T3 was associated with more attention problems, rule-breaking, and aggressive behaviors, although without reaching statistical significance. Detected *versus* undetected urinary 3-BPA was associated with less somatic, attention, and internalizing problems. No relevant associations were observed for ΣPYR (Table 3). Finally, urinary ETU at T2 and T3 was associated with more social problems and less anxiety problems [($\beta = 3.18$, (95%CI = 0.64,5.71), $\beta = -2.70$, 95%CI = −5.25–0.14), respectively] (Table 3). No associations were observed for MDA and 1-N. Statistical analyses using pesticide metabolites as continuous showed similar results to those reported from categorized exposures (Table S4).

The WQS model revealed some associations regarding the mixture effect of IMPy, MDA, DCCA, and ETU on increasing withdrawn

Table 2
Distribution of urinary pesticide metabolites concentrations (ng/mL) (n = 140), serum BDNF protein levels (n = 130), and methylation levels at CpGs (n = 118).

Pesticide metabolites		IMPy	MDA	TCPy	DETP	ΣOPs	DCCA	3-PBA	ΣPYR	1-N	ETU
% Detection	74.8	83.0	32.5	54.3	–	100	19.9	–	38.0	74.2	
Percentiles	25	0.08	0.14	<LOD	<LOD	0.67	0.12	<LOD	0.21	<LOD	0.05
	50	0.25	0.30	<LOD	0.25	1.29	1.06	<LOD	1.17	<LOD	0.26
	75	0.81	0.50	0.08	0.74	2.27	3.45	0.083	3.53	0.34	0.70
Effect biomarkers		**Serum BDNF (ng/mL)**			**CpG1 (%)**	**CpG2 (%)**	**CpG3 (%)**	**CpG4 (%)**	**CpG5 (%)**	**CpG6 (%)**	**∑CpG (%)**
Percentiles	25	25.41			3.89	2.90	2.85	5.18	2.70	2.04	3.45
	50	32.59			4.45	3.18	3.21	5.69	3.17	2.42	3.70
	75	39.40			4.87	3.50	3.64	6.30	3.68	3.13	4.04

BDNF = Brain-derived neurotrophic factor; LOD = Limit of detection; IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA = malathion dicarboxylic acid; TCPy = 3,5,6-trichloro-2-pyridinol; DETP = diethyl thiophosphate; ΣOPs = sum of organophosphate metabolites (IMPy + MDA + TCPy); DCCA = 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3-PBA = 3-phenoxybenzoic acid; ΣPYR = sum of pyrethroid metabolites (DCCA+3-PBA); 1-N = 1-naphthol; ETU = ethylene thiourea.

problems, with MDA and IMPy presenting the greatest influence on this effect (34% and 50%, respectively). In the case of social problems, IMPy, DCCA, and MDA had the greatest weights (33%, 29%, and 28% of the association, respectively) (Fig. 1). The mixture effect model also revealed associations with more thought problems, with IMPy and MDA presenting the greatest influence (41% and 35%, respectively); however, statistical significance was not reached (Fig. 1). The remaining associations are available in Table S5.

3.3. Urinary pesticide metabolites and BDNF levels

Adolescents with urinary IMPy, MDA, DETP, ETU, and 1-N concentrations at T3 were associated with lower serum BDNF levels [β = −4.29 (95%CI = −8.33,-0.25) β = −6.74 (95%CI = −11.38,-2.10), β = −3.82 (95%CI = −8.25,0.61), β = −3.27 (95%CI = −7.36,0.82), respectively] (Table 4). Interestingly, exposure to ΣOPs was associated significantly and dose-dependently with lower serum BDNF levels [βT2 = −4.34 (95%CI = −8.58,-0.09), βT3 = −5.89 (95%CI = −10.19,-1.58)].

Adolescents with urinary MDA at T2 were associated with increased DNA methylation at CpG 2 (β = 0.26, 95%CI = 0.04,0.46). T3 urinary MDA levels were associated with higher DNA methylation at CpGs 1, 3, and total CpGs, although without reaching statistical significance. ΣOPs levels at T3 also showed a suggestive association with higher DNA methylation at CpG2 (β2 = 0.21, 95%CI = −0.02,0.45) (Table 4). Detected *versus*undetected concentrations of urinary 3-PBA were associated with higher BDNF DNA methylation at CpGs #4, 5, 6 and total CpGs methylation [β = 0.65 (95%CI = 0.03,1.26), β = 0.38 (95%CI = −0.01,0.76), β = 0.57 (95%CI = 0.02,1.12), β = 0.30 (95%CI = 0.00,0.60), respectively] (Table 4). Finally, ETU concentrations at T3 and T2 were associated with more DNA methylation percentages at CpGs: # 2, 3, 4, 5, 6 and total CpGs [CpG2: βT3 = 0.27 (95%CI = 0.05,0.49); CpG3: βT3 = 0.41 (95%CI = 0.15,0.67); total CpGs βT3 = 0.32 (95%CI = 0.04,0.60)] (Table 4). In the linear regression analysis, overall patterns evidenced with the logistic regression were preserved although the associations tended to be attenuated (Table S6).

The WQS model revealed associations between the mixture effect and higher BDNF DNA methylation at CpG3 and total CpGs DNA methylation, with ETU and MDA showing the greatest influence on the mixture effect (37% and 42%, respectively, for methylation at CpG3, and 58% and 25%, respectively, for total CpGs methylation) (Fig. 1). Finally, the mixture effect was also associated with higher DNA methylation at CpG6 (MDA and IMPy showing the greatest influence, 51% and 32%, respectively), although without reaching statistical significance (Fig. 1). The remaining associations are shown in Table S5.

3.4. BDNF and adolescents' behavior

Adolescents with BDNF protein levels at T2 were associated with lower thought (β = −2.93, 95%CI = −5.47,-0.38), rule-breaking (β = −2.71, 95%CI = −5.39,-0.02), and total problems (β = −3.57, 95%CI = −7.72,0.58) (Fig. 2, red and green diamonds), and those with BDNF at T3 were associated with lower thought (β = −3.03, 95%CI = −5.65; −0.40) and rule-breaking behavior problems (β = −2.35, 95%CI = −5.11,0.42) (Fig. 2). However, continuous BDNF levels were not associated with any CBCL subscales, suggesting non-dose response associations (Table S7).

Adolescents with CpG6 DNA methylation at T3 were associated with higher thought problems (β = 2.86, 95%CI = 0.14,5.57). Meanwhile, adolescents showing CpG 2 DNA methylation at T3 were associated with lower anxiety (β = −3.17, 95%CI = −6.00,-0.35), somatic (β = −3.81, 95%CI = −7.30,-0.31), attention (β = −4.60, 95%CI = −8.04,-1.16) and internalizing problems (β = −5.33, 95%CI = −9.88,-0.79) (Fig. S1).

3.5. Mediation analysis

Because urinary IMPy levels were dose-dependently associated with higher thought and rule-breaking problems, and with lower serum BDNF levels (Tables 3 and 4), and additionally serum BDNF levels non-linearly associated with lower thought and rule-breaking problems (Fig. 2); we investigated the potential mediation role of BDNF in exposure-effect relationships. The potential mediation role of categorized serum BDNF protein within IMPy (categorized)-thought problems (continuous) and IMPy (categorized)-rule breaking behavior (continuous) relationships was explored. The covariate-adjusted mediation model revealed a non-significant indirect effect between serum BDNF and IMPy-thought problems (β = 0.17, 95%CI: −0.07, 0.57), which accounted for 21.5% of the association. Direct and total effects were not statistically significant. Additionally, serum BDNF showed a potential small indirect effect within IMPy-rule-breaking problems that accounted for 7.6% (β = 0.11, 95%CI: −0.08, 0.45) of the association, although both total and direct effects were significant (Fig. 3).

4. Discussion

Our results suggest a possible association between IMPy, ΣOPs, and ETU levels with behavioral problems among Spanish adolescent males, which could be partly explained by BDNF protein levels. A possible combined effect for some pesticides with more withdrawn, social, and thought problems, and higher BDNF DNA methylation percentage at CpG3, and total CpGs methylation was observed. At the same time, serum BDNF levels were associated with more thought problems and rule-breaking behavior, and BDNF DNA methylation percentage at CpG6 with more thought problems.

4.1. OP insecticides

Urinary TCPy and MDA concentrations were generally lower than previously reported for children in Spain, the USA, and Costa Rica

Table 3
Regression estimates (β, 95% CI) for the associations between urinary pesticide metabolites concentrations and CBCL behavioral scoring (n = 140).

		Syndrome scores								Composite scores		
		Anxious depressed	Withdrawn	Somatic complaints	Social problems	Thought problems	Attention problems	Rule-breaking behavior	Aggressive behavior	Internalizing problems	Externalizing problems	Total problems
IMPy	T2	1.85 (−0.79,4.49)	1.82 (−1.30,4.94)	−0.59 (−3.75,2.56)	1.47 (−1.19,4.13)	**2.33 (-0.24,4.90)†**	1.10 (−1.87,4.07)	0.76 (−1.90,3.43)	**2.47 (-0.20,5.13)†**	2.19 (−1.83,6.21)	2.46 (−1.43,6.34)	2.54 (−1.34,6.42)
	T3	1.70 (−0.96,4.37)	2.04 (−1.11,5.19)	−0.99 (−4.17,2.20)	**3.34 (0.65,6.02)***	**2.56 (-0.04,5.16)†**	2.28 (−0.72,5.28)	**3.76 (1.06,6.45)****	**3.77 (1.07,6.46)****	1.13 (−2.93,5.20)	**5.50 (1.58,9.42)****	**4.60 (0.68,8.52)***
	p-trend	0.76	0.57	0.24	**0.04**	0.20	0.18	**< 0.01**	**< 0.01**	0.59	**< 0.01**	**0.07**
MDA	T2	1.47 (−1.21,4.07)	−0.06 (−3.20,3.07)	0.71 (−2.36,3.78)	−0.28 (−2.94,2.38)	−0.16 (−2.72,2.41)	0.67 (−2.25,3.59)	1.61 (−1.07,4.29)	0.17 (−2.52,2.86)	1.82 (−2.11,5.75)	1.22 (−2.69,5.12)	2.19 (−1.66,6.04)
	T3	0.47 (−2.50,3.44)	0.35 (−3.05,3.75)	−1.42 (−4.94,2.09)	0.37 (−2.67,3.41)	0.08 (−2.86,3.01)	−0.67 (−4.02,2.67)	0.73 (−2.34,3.79)	−0.17 (−3.25,2.91)	−0.36 (−4.86,4.14)	−0.37 (−4.84,4.09)	−0.31 (−4.71,4.09)
	p-trend	0.70	0.82	0.46	0.83	0.97	0.73	0.59	0.92	0.93	0.91	0.96
TCPy	D vs ND	0.88 (−1.39,3.14)	−0.98 (−3.66,1.69)	−1.05 (−3.73,1.64)	**2.13 (-0.16,4.42)†**	**2.48 (0.29,4.67)***	1.05 (−1.49,3.60)	−0.61 (−2.95,1.74)	0.21 (−2.13,2.56)	−0.09 (−3.53,3.36)	−0.74 (−4.14,2.67)	0.58 (−2.80,3.95)
DETP	T2	0.33 (−2.36,3.03)	−1.44 (−4.57,1.68)	−2.04 (−5.21,1.13)	−1.62 (−4.36,1.11)	−1.98 (−4.60,0.65)	−0.54 (−3.56,2.49)	−0.84 (−3.59,1.92)	−1.91 (−4.66,0.85)	−1.79 (−5.86,2.27)	−2.44 (−6.46,1.57)	−2.75 (−6.72,1.23)
	T3	0.08 (−2.77,2.93)	**−3.54 (-6.85,-0.23)***	0.00 (−3.36,3.36)	−1.01 (−3.91,1.88)	−0.42 (−3.20,2.36)	0.36 (−2.85,3.56)	1.67 (−1.25,4.59)	−0.15 (−3.07,2.77)	−1.67 (−5.98,2.64)	−0.17 (−4.42,4.09)	−1.01 (−5.22,3.21)
	p-trend	0.88	**0.05**	0.94	0.49	0.61	0.79	0.32	0.86	0.47	0.92	0.64
DCCA	T2	0.58 (−2.04,3.21)	−1.39 (−4.48,1.70)	0.13 (−2.99,3.24)	1.72 (−0.95,4.38)	1.10 (−1.47,3.68)	**2.09 (-0.84,5.03)**	0.29 (−2.41,2.98)	0.91 (−1.80,3.61)	−0.06 (−4.05,3.92)	0.73 (−3.19,4.65)	1.28 (−2.61,5.17)
	T3	−0.33 (−3.01,2.35)	−1.00 (−4.16,2.15)	0.74 (−2.44,3.92)	1.54 (−1.18,4.26)	0.82 (−1.81,3.45)	1.17 (−1.82,4.17)	**1.88 (-0.87,4.63)**	**1.27 (-1.49,4.03)**	0.47 (−3.59,4.54)	**2.43 (-1.58,6.43)**	1.93 (−2.05,5.90)
	p-trend	0.81	0.53	0.64	0.26	0.53	0.44	0.18	0.36	0.82	0.23	0.34
3-PBA	D vs ND	−1.52 (−4.20,1.16)	−0.77 (−3.94,2.40)	**−5.18 (-8.25,-2.12)****	−2.16 (−4.88,0.57)	−0.70 (−3.34,1.95)	**−2.79 (-5.78,0.20)†**	−2.01 (−4.78,0.75)	−0.34 (−3.12,2.44)	**−3.73 (-7.76,0.30)†**	−0.60 (−4.64,3.44)	−2.99 (−6.96,0.98)
1-N	D vs ND	−1.29 (−3.47,0.89)	1.48 (−1.08,4.05)	0.02 (−2.58,2.61)	0.39 (−1.84,2.62)	0.25 (−1.90,2.39)	−0.27 (−2.73,2.18)	−0.65 (−2.91,1.60)	−0.40 (−2.66,1.85)	0.34 (−2.97,3.65)	0.47 (−2.81,3.75)	0.46 (−2.79,3.71)
ETU	T2	0.01 (−2.49,2.50)	1.52 (−1.47,4.52)	−1.73 (−4.34,1.27)	**3.18 (0.64,5.71)***	1.59 (−1.25,4.44)	0.41 (−2.09,2.90)	−0.56 (−3.18,2.07)	1.15 (−1.46,3.76)	−0.87 (−4.69,2.96)	0.10 (−3.69,3.89)	0.28 (−3.47,4.02)
	T3	**−2.70 (-5.25,-0.14)***	0.80 (−2.26,3.86)	−1.15 (−4.23,1.92)	0.48 (−2.12,3.07)	−0.15 (−3.06,2.77)	−0.89 (−3.44,1.66)	−1.16 (−3.85,1.53)	−0.78 (−3.45,1.89)	−3.00 (−6.91,0.92)	−2.60 (−6.48,1.27)	−2.75 (−6.58,1.09)
	p-trend	**0.04**	0.60	0.45	0.69	0.94	0.50	0.39	0.58	0.13	0.19	0.16
ΣOPs	T2	−0.65 (−3.34,2.05)	−1.35 (−4.50,1.80)	−1.07 (−4.27,2.13)	1.16 (−1.53,3.86)	1.79 (0.85,4.42)	−0.07 (−3.09,2.96)	1.17 (−1.57,3.92)	1.12 (−1.63,3.88)	−1.46 (−5.55,2.64)	0.33 (−3.68,4.34)	0.70 (−3.28,4.68)
	T3	0.96 (−1.79,3.72)	1.47 (−1.76,4.69)	−1.61 (−4.88,1.66)	**3.53 (0.77,6.29)***	2.22 (−0.48,4.91)	1.62 (−1.47,4.72)	**3.11 (0.30,5.92)***	**2.71 (-0.10,5.53)†**	0.24 (−3.95,4.42)	**3.52 (-0.58,7.61)†**	3.29 (−0.78,7.36)
	p-trend	0.46	0.33	0.34	0.01	0.11	0.28	**0.03**	**0.06**	0.87	**0.08**	**0.10**
ΣPYR	T2	0.22 (−2.36,2.80)	−1.03 (−4.07,2.01)	−1.86 (−4.91,1.18)	0.89 (−1.73,3.52)	0.36 (−2.18,2.90)	2.26 (−0.62,5.13)	−2.07 (−4.67,0.53)	0.38 (−2.27,3.03)	−0.22 (−4.15,3.71)	−0.74 (−4.57,3.09)	0.50 (−3.33,4.32)
	T3	−0.58 (−3.46,1.76)	−0.74 (−3.81,2.33)	−0.00 (−3.08,3.07)	1.23 (−1.42,3.88)	1.03 (−1.54,3.60)	0.87 (−2.04,3.78)	1.46 (−1.16,4.09)	1.27 (−1.41,3.95)	0.13 (−3.84,4.10)	2.52 (−1.35,6.39)	1.66 (−2.20,5.53)
	p-trend	0.53	0.63	0.97	0.36	0.43	0.53	0.31	0.35	0.95	0.21	0.40

D = Detected; ND= Non detected; IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA = malathion dicarboxylic acid; TCPy = 3,5,6-trichloro-2-pyridinol; DETP = diethyl thiophosphate; ΣOPs = sum of organophosphate metabolites (IMPy + MDA + TCPy); DCCA = 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3-PBA = 3-phenoxybenzoic acid; ΣPYR = sum of pyrethroid metabolites (DCCA+3-PBA); 1-N = 1-naphthol; ETU = ethylene thiourea. Models were adjusted for adolescents' age (continuous), BMI (continuous), alcohol consumption (>1 beverage/month; ≤1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university). *p < 0.05; **p ≤ 0.01; †p < 0.10.

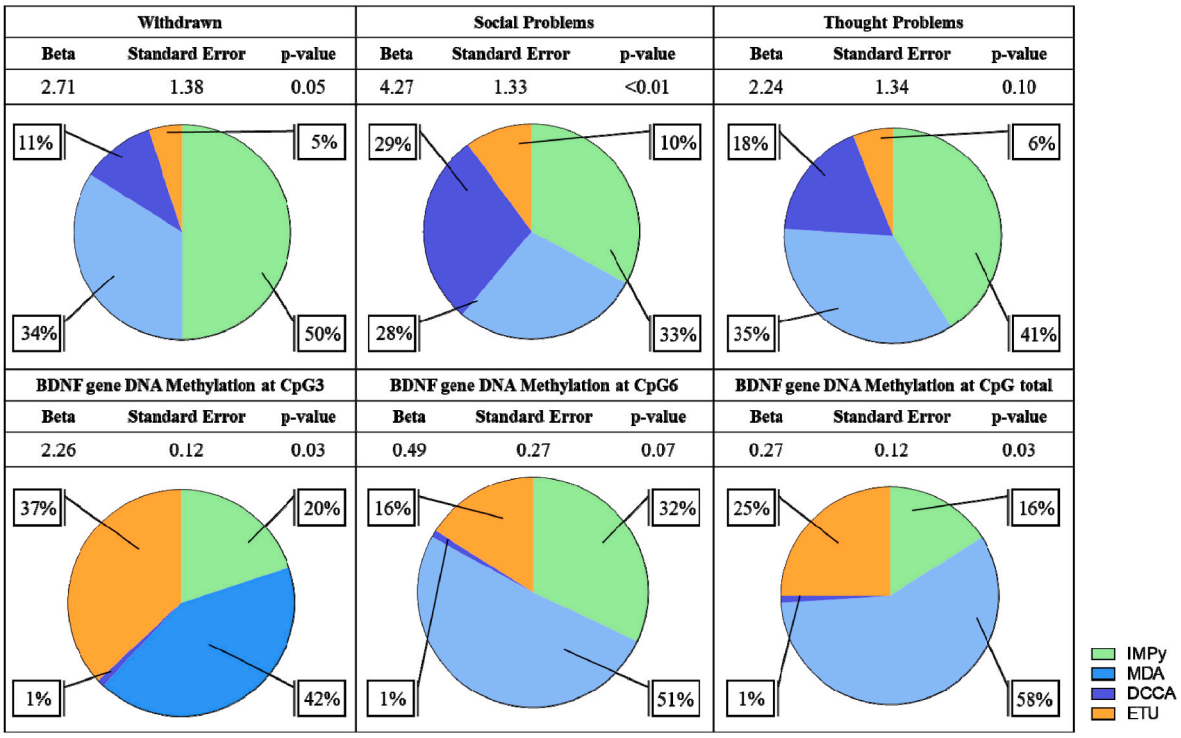

Fig. 1. Mixture effect of urinary IMPy, MDA, DCCA, and ETU (IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA = malathion dicarboxylic acid; DCCA = 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; ETU = ethylene thiourea.) on behavioral functioning (n = 140) and BDNF gene DNA methylation at several CpGs (n = 118) (BDNF= Brain-derived neurotrophic factor). Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; ≤1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university).

(Hyland et al., 2019; Roca et al., 2014; van Wendel de Joode et al., 2016), while IMPy and DETP were higher than reported for Spanish and US children (Hernández et al., 2019; Hyland et al., 2019; Roca et al., 2014). In our study, exposure to diazinon (IMPy) and chlorpyrifos (TCPy) metabolites was associated with more social-related problems among male adolescents aged 15–17 years. Results regarding TCPy are partially consistent with the results of three epidemiological studies among Egyptians and Latino American subjects (12–21 years of age) working or living near plantations, which found that urinary TCPy was associated with deficits in cumulative neurobehavioral performance and a higher prevalence of depression, ADHD, irritability, and superficial sensation of abnormality (Ismail et al., 2017; Rohlman et al., 2016; van Wendel de Joode et al., 2016). To our knowledge, no previous study assessed exposure to diazinon concerning child neurodevelopment; however, some *in-vivo* studies found behavioral functioning alterations after diazinon exposure, supporting our data (Hawkey et al., 2020; Shin et al., 2001; Velki et al., 2017).

Interestingly, IMPy and DETP were associated with lower serum BDNF protein levels, MDA with lower serum BDNF, and higher BDNF DNA methylation percentage, while no association was observed for TCPy. ΣOPs was also strongly and dose-dependently associated with lower serum BDNF levels. Although OP pesticides are known to exert adverse effects on the nervous system through acetylcholinesterase (AChE) inhibition (Bjørling-Poulsen et al., 2008; Richendrfer and Creton, 2015), growing evidence also suggest non-cholinergic mechanisms, such as alterations of synaptic formation and neuronal cell development (Rauh et al., 2011). Thus, OP pesticides are known to alter the dopaminergic system, which plays a key role in the regulation of BDNF through the inhibition of tyrosine hydroxilase (TH) activity (Küppers and Beyer, 2001) (Fig. 4, key event #3). This enzyme anabolizes L-tyrosine, the precursor of the dihydroxyphenylalanine (DOPA), leading to decreasing dopamine levels (Shin et al., 2001) (Fig. 4, #1). *In vivo* studies observed TH inhibition after diazinon and malathion exposure, leading to behavioral alterations and increased anxiety behavior in Japanese medaka and rats, respectively (Ahmed et al., 2017; Shin et al., 2001) (Fig. 4, #1 and #2). Additionally, exposure to chlorpyrifos decreased the cholinergic system and down-regulated BDNF expression in zebrafish, resulting in increased impulsive rates (Perez-Fernandez et al., 2020) (Fig. 4, #5). Finally, another *in vivo* study observed that malathion-exposed rats showed increased reactive oxidative stress species (ROS) and reduced hippocampal BDNF expression (Ardebili Dorri et al., 2015) (Fig. 4, #4). This evidence could support, from a mechanistic perspective, our findings regarding OP pesticides exposure and decreased BDNF.

4.2. *Pyrethroids*

3-PBA urinary concentrations among the Spanish adolescents were lower, and concentrations of DCCA were higher than reported for North American children aged 6–15 years (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014). Urinary 3-PBA levels were associated with fewer somatic problems, whereas DCCA tended to be associated with more behavioral problems. Previous studies in North America found an increased risk of ADHD in 8- to 15-year-old boys with high urinary 3-PBA concentrations (Wagner-Schuman et al., 2015), and more behavioral difficulties in children aged 6–11 years with high urinary DCCA concentrations (Oulhote and Bouchard, 2013). In contrast, in other studies conducted among 6- to 15-year-old children from North and Latin America, 3-PBA and DCCA were not associated with behavioral functioning (Oulhote and Bouchard, 2013; Quirós-Alcalá et al., 2014; van Wendel de Joode et al., 2016). Discrepancies among studies could be due to, at least in part, differences in design, age of the children, pesticide exposure levels, and inter-individual variability of urinary pesticide metabolites. Moreover, studies use different tests to assess behavioral outcomes, which may be another source of heterogeneity.

The observed association between 3-PBA and increased BDNF gene

Table 4
Regression estimates (β, 95%CI) for the association between tertiles of urinary pesticide metabolites concentrations and serum BDNF protein levels (n = 130) and BDNF gene DNA methylation percentage (n = 118).

		BDNF protein	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpGt
IMPy	T2	−1.77 (−6.03,2.50)	0.00 (−0.37,0.37)	−0.11 (−0.34,0.13)	0.04 (−0.24,0.32)	0.10 (−0.51,0.71)	0.01 (−0.37,0.39)	0.18 (−0.36,0.73)	0.04 (−0.26,0.33)
	T3	**−4.29 (-8.33,-0.25)***	0.12 (−0.24,0.49)	0.02 (−0.21,0.26)	−0.01 (−0.28,0.27)	−0.16 (−0.76,0.44)	0.01 (−0.37,0.39)	0.09 (−0.45,0.63)	0.01 (−0.28,0.31)
	p-trend	**0.04**	0.39	0.57	0.88	0.46	0.81	0.99	0.94
MDA	T2	−2.71 (−6.88,1.46)	0.21 (−0.15,0.57)	**0.26 (0.04,0.46)***	0.12 (−0.16,0.39)	0.04 (−0.57,0.65)	0.18 (−0.21,0.56)	−0.07 (−0.62,0.48)	0.12 (−0.17,0.42)
	T3	**−6.74 (-11.38,-2.10)****	0.31 (−0.08,0.71)	**0.21 (-0.04,0.46)†**	0.24 (−0.06,0.54)	0.25 (−0.41,0.91)	0.23 (−0.18,0.64)	0.05 (−0.54,0.65)	0.22 (−0.10,0.53)
	p-trend	< **0.01**	0.11	**0.08**	0.12	0.46	0.26	0.87	0.18
TCPy	D vs ND	0.09 (−3.59,3.76)	−0.10 (−0.41,0.21)	−0.04 (−0.23,0.16)	−0.14 (−0.37,0.10)	−0.04 (−0.56,0.47)	0.07 (−0.25,0.39)	0.08 (−0.38,0.54)	−0.03 (−0.28,0.22)
DETP	T2	**−0.68 (-7.87,0.52)†**	−0.09 (−0.45,0.26)	−0.00 (−0.23,0.23)	0.04 (−0.23,0.32)	0.09 (−0.51,0.68)	−0.03 (−0.40,0.34)	0.13 (−0.40,0.65)	0.02 (−0.27,0.31)
	T3	**−3.82 (-8.25,0.61)†**	−0.19 (−0.57,0.19)	0.09 (−0.15,0.33)	0.13 (−0.16,0.42)	0.19 (−0.44,0.82)	−0.02 (−0.42,0.38)	0.31 (−0.25,0.87)	0.08 (−0.22,0.39)
	p-trend	**0.09**	0.31	0.49	0.34	0.57	0.97	0.25	0.57
DCCA	T2	2.93 (−1.21,7.07)	0.07 (−0.29,0.42)	−0.18 (−0.41,0.05)	−0.01 (−0.28,0.27)	−0.30 (−0.88,0.30)	−0.18 (−0.55,0.19)	−0.07 (−0.60,0.46)	−0.11 (−0.40,0.18)
	T3	0.03 (−4.34,4.40)	0.14 (−0.23,0.51)	−0.07 (−0.31,0.16)	−0.10 (−0.38,0.18)	−0.35 (−0.97,0.27)	−0.17 (−0.56,0.21)	−0.21 (−0.76,0.34)	−0.13 (−0.42,0.17)
	p-trend	0.93	0.44	0.56	0.48	0.25	0.38	0.46	0.40
3-PBA	D vs ND	−2.60 (−6.90,1.64)	0.01 (−0.37,0.39)	−0.00 (−0.24,0.24)	0.21 (−0.08,0.50)	**0.65 (0.03,1.26)***	**0.38 (-0.01,0.76)†**	**0.57 (0.02,1.12)***	**0.30 (0.00,0.60)***
1-N	D vs ND	**−3.91 (-7.35,-0.46)***	−0.20 (−0.51,0.10)	0.13 (−0.07,0.32)	0.01 (−0.22,0.24)	0.37 (−0.14,0.86)	0.25 (−0.06,0.56)	0.30 (−0.15,0.75)	0.14 (−0.10,0.39)
ETU	T2	−1.23 (-5.43,2.97)	0.20 (−0.16,0.57)	**0.23 (0.01,0.46)***	**0.27 (0.01,0.54)***	**0.68 (0.09,1.27)***	**0.36 (-0.02,0.73)†**	0.40 (−0.14,0.93)	**0.36 (0.07,0.64)***
	T3	−3.27 (−7.36,0.82)	0.18 (−0.17,0.54)	**0.27 (0.05,0.49)***	**0.41 (0.15,0.67)****	**0.53 (-0.05,1.11)†**	0.22 (−0.15,0.58)	0.32 (−0.21,0.84)	**0.32 (0.04,0.60)***
	p-trend	0.16	0.46	**0.07**	**0.01**	**0.08**	0.35	0.29	**0.05**
ΣOPs	T2	**−4.34 (-8.58,-0.09)***	0.13 (−0.24,0.50)	0.18 (−0.05,0.42)	0.08 (−0.20,0.36)	0.12 (−0.50,0.73)	0.20 (−0.18,0.59)	0.02 (−0.54,0.57)	0.12 (−0.18,0.42)
	T3	**−5.89 (-10.19,-1.58)***	**0.30 (-0.06,0.67)†**	0.17 (−0.06,0.41)	0.02 (−0.26,0.30)	−0.15 (−0.77,0.46)	0.06 (−0.32,0.45)	−0.04 (−0.60,0.51)	0.06 (−0.23,0.36)
	p-trend	< **0.01**	**0.10**	0.17	0.94	0.58	0.81	0.86	0.73
ΣPYR	T2	−1.96 (−6.14,2.23)	−0.02 (−0.38,0.34)	−0.15 (−0.38,0.08)	0.06 (−0.21,0.34)	0.09 (−0.51,0.69)	0.07 (−0.31,0.44)	1.00 (−0.44,0.64)	0.02 (−0.27,0.32)
	T3	−0.74 (−4.92,3.44)	0.24 (−0.12,0.59)	−0.04 (−0.26,0.19)	−0.05 (−0.32,0.22)	−0.17 (−0.76,0.41)	−0.10 (−0.47,0.26)	−0.06 (−0.58,0.47)	−0.03 (−0.32,0.25)
	p-trend	0.71	0.18	0.77	0.69	0.55	0.57	0.82	0.82

D = Detected; ND= Non detected; BDNF= Brain-derived neurotrophic factor; IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA = malathion dicarboxylic acid; TCPy = 3,5,6-trichloro-2-pyridinol; DETP = diethyl thiophosphate; ΣOPs = sum of organophosphate metabolites (IMPy + MDA + TCPy); DCCA = 2,2-dichlorovinyl-2,2-dimethylcyclopropane-1-carboxiclic acid; 3-PBA = 3-phenoxybenzoic acid; ΣPYR = sum of pyrethroid metabolites (DCCA+3-PBA); 1-N = 1-naphthol; ETU = ethylene thiourea.
Models were adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; ≤1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university). *p < 0.05; **p ≤ 0.01; †p < 0.10.

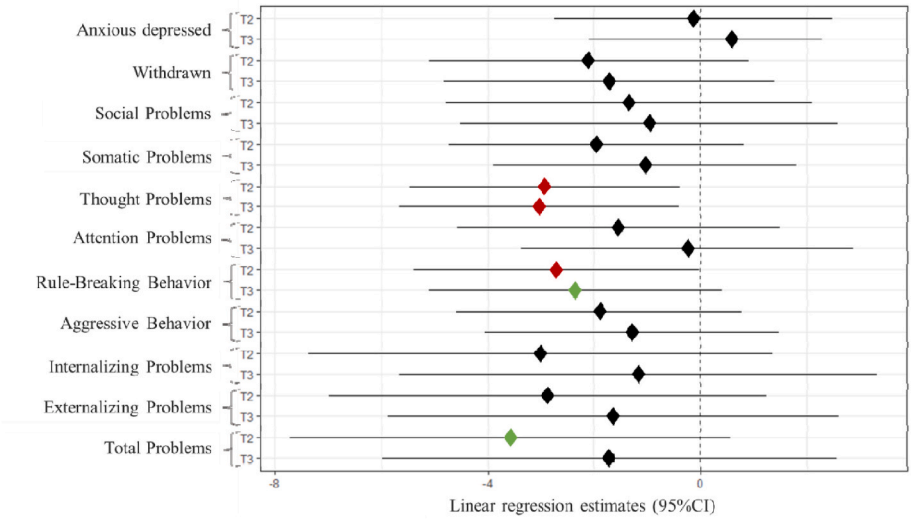

Fig. 2. Forest plot showing linear regression estimates (95%CI) for the association between tertiles of serum BDNF levels (T2 and T3) and adolescents' behavior (n = 130). Model was adjusted for adolescent's age (months), BMI (continuous), alcohol consumption (>1 beverage/month; ≤1 beverage/month), and maternal education (primary/secondary/university). Red diamonds represent statistical associations with p-values < 0.05; green diamonds represent p-values < 0.1; black diamonds represent p-values above 0.1.

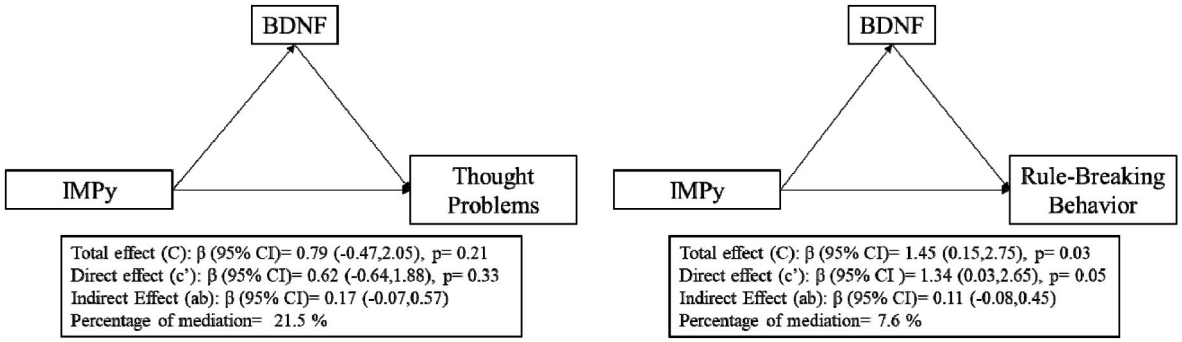

Fig. 3. Analysis exploring the role of serum BDNF protein as a potential mediator of the association between urinary IMPy concentrations and thought and rule-breaking problems in adolescents (n = 130). Beta coefficients are displayed for total, direct, and indirect effects. BDNF: Brain-derived neurotrophic factor; IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine. Both IMPy and BDNF levels were modeled based on tertiles and thought and rule-breaking behavioral problems were normalized using t-scores. Model was adjusted for adolescents' age (months), BMI, alcohol consumption (>1 beverage/month; ≤1 beverage/month), season of urine collection (spring/summer/autumn/winter), urinary creatinine (mg/dL), and maternal education (primary/secondary/university). *p < 0.05; **p ≤ 0.01; †p < 0.10.

DNA methylation suggests that exposure to pyrethroids, even at low levels, could alter BDNF gene methylation patterns. There are many mechanisms by which pyrethroids could exert their neurotoxic potential, fully described in Fig. 4. First, permethrin interacts with DNA methyltransferases (DNMT), altering DNA methylation patterns *in vivo* (Bordoni et al., 2015) (Fig. 4; #10). Second, via a compensatory mechanism for retardation of sodium channels voltage-dependent (Nav) activation through down-regulation of Nav expression, linked to decreased BDNF expression, as reported in two studies with deltamethrin-exposed mice (Imamura et al., 2006; Magby and Richardson, 2017) (Fig. 4; #6 and #7). Third, inhibition of NMDAR after permethrin and deltamethrin exposure using *in vivo/vitro* models led to a down-regulation of the cAMP response element-binding protein (CREB), involved in BDNF expression, resulting in hippocampal BDNF mRNA repression (Imamura et al., 2000; Zhang et al., 2018) (Fig. 4; #8 and #9). In addition, cypermethrin and bifenthrin exposure increased ROS leading to neuroinflammation and long-lasting behavioral impairments in murine models (Gargouri et al., 2018; Nasuti et al., 2007) (Fig. 4, #11 and #12). This AOP like-network builds up a potential mechanism pathway supporting our findings regarding 3-PBA and alteration of BDNF gene DNA methylation.

4.3. Carbamates and dithiocarbamates

In this study, urinary 1-N concentrations were lower than previously reported among German children aged 5–7 years from the general population; urinary ETU concentrations were, however, within the range of those reported for French and Latin American children, aged 5–9 years and living near agricultural fields (Raherison et al., 2019; van Wendel de Joode et al., 2016; Wilhelm et al., 2008). The observed associations between urinary ETU levels with more social and fewer anxiety problems do not support the results of a Costa Rican study that found no association between ETU and behavioral problems in children assessed at 6–9 years (van Wendel de Joode et al., 2016); although are partially supported by the results of a Mexican study that showed an association between prenatal ETU and more social problems in 1-year--old infants (Mora et al., 2018). Differences in windows of exposure (i.e., prenatal *versus* postnatal exposure) and age at behavioral assessment (infants *versus* adolescents), among others, impair the comparison of findings. Experimental studies have nonetheless found that prenatal exposure to mancozeb elicited worse behavioral outcomes, which was mechanistically supported by decreased hippocampal proteins, such as calcium/calmodulin-dependent kinases II (CaMKII), glutamate receptor 1 (GluR1), and synaptophysin (Lee et al., 2015). Mancozeb and other dithiocarbamate fungicides are widely used for the growth of fruits and vegetables, but there is very limited information regarding their effects on neurodevelopment (Ekman et al., 2013). In this regard, a major common metabolite of these fungicides, ETU, is a known thyroid inhibitor, since it prevents the ionization of thyroglobulin, thus inhibiting the synthesis of T3 and T4 (Mutic et al., 2017), which ultimately could

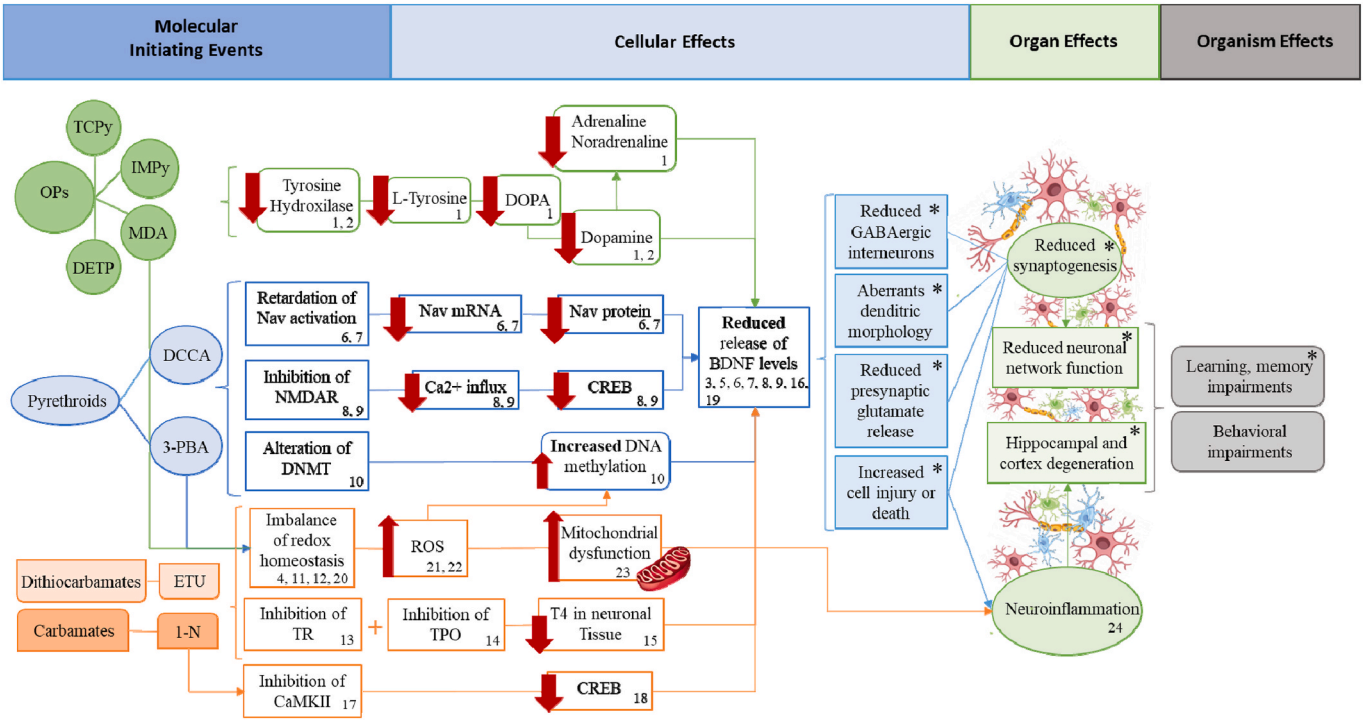

Fig. 4. Proposed Adverse Outcome Pathway-like through which pesticide exposure could impair behavioral functioning based on results from this epidemiological study and results from mechanistic studies published previously. 1-N = 1-naphthol; 3-PBA = 3-phenoxybenzoic acid; BDNF = brain-derived neurotrophic factor; CaMKII = calcium/calmodulin-dependent kinases II; CREB = cAMP response element binding protein; DETP = diethyl thiophosphate; DCCA = 2,2,dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid; DNMT = DNA methyl transferases; DOPA = dihydroxyphenylalanine; ETU = ethylenethiourea; IMPy = 2-isopropyl-4-methyl-6-hydroxypyrimidine; MDA = Malathion dicarboxylic acid; Nav = Sodium channels voltage-dependent; NMDAR= N-methyl D-aspartate receptor; Ops = Organophosphates; ROS = reactive oxygen species; T4 = Thyroxine; TCPy = 3,5,6-trichloro-2-pyridinol; TH = Tyrosine hydroxilase; TPO = Thyroid peroxidase; TR = Thyroid receptor. **OP pesticides** inhibit tyrosine hydroxilase, this enzyme anabolizes L-tyrosine, precursor of the dihydroxyphenylalanine (DOPA), leading to decreasing dopamine levels and consequently adrenaline and noradrenaline [1]. Decreased dopaminergic neurotransmitters leads to reduced hippocampal BDNF levels, converging in behavioral impairments [2, 3]. Additionally, MDA increases ROS, leading to reduction of BDNF [4]. Retardation of Nav activation due to **pyrethroids** exposure induced a compensation mechanism where Nav expression (Nav mRNA and Nav protein) in reduced, decreasing BDNF levels [5, 6, 7]. Pyrethroids can inhibit NMDARs, thus decreasing calcium influx and decreasing activation of CREB, leading to lower BDNF secretion and possibly subsequent behavioral impairments [8, 9]. Additionally, pyrethroids can also induce alterations of DNMT, thus increasing BDNF gene DNA methylation and decreasing serum BDNF protein [10]. In addition, pyrethroids imbalance the redox homeostasis, increasing ROS and leading to neuroinflammation, with deleterious consequences for brain development [11, 12]. **1-N** and especially **ETU** are known inhibitors of thyroid receptor (TR) and thyroid peroxidase (TPO), which lead to impaired thyroid hormone secretion, thus decreasing thyroxine (T4) concentrations in brain tissue, reducing BDNF synthesis and ultimately leading to neurodevelopmental alterations [13, 14, 15, 16 and *]. 1-N inhibits CaMKII, which decreases activation of CREB and therefore BDNF gene transcription [17, 18, 19]. Finally, 1-N and ETU imbalance redox homeostasis by uncoupling the mitochondrial electron transport chain, leading to mitochondrial dysfunction and generation of ROS and neuroinflammation, impairing learning, memory and behavioral functioning [22, 23, 24]. *(Mustieles et al., 2020).

lead to decreasing BDNF levels, as proposed by Mustieles et al. (2020). However, in this cohort, ETU was not associated with thyroid hormones (Freire et al., 2021).

Urinary 1-N was also associated with less serum BDNF levels and urinary ETU with more BDNF DNA methylation percentage at several CpGs. Different pathways supporting these findings have been fully described in Fig. 4 (Bjørling-Poulsen et al., 2008; Maranghi et al., 2013). First, inhibition of thyroid receptor (TR) and thyroid peroxidase (TPO) was observed in murine models after exposure to 1-N and ETU, resulting in impaired thyroid hormone secretion and reduction of hippocampal BDNF synthesis, ultimately leading to neurodevelopmental alterations (Maranghi et al., 2013; Marinovich et al., 1997; Shafiee et al., 2016; Sun et al., 2008) (Fig. 4; #13, #14, #15, #16). Second, *in vivo/in vitro* models observed CaMKII inhibition after 1-N exposure, impairing the phosphorylation of CREB and the transcription of BDNF, thus decreasing BDNF-mediated neurite growth (Islam et al., 2019; Lee et al., 2015; Saito et al., 2013) (Fig. 4; #18, #17, #19). Finally, 1-N and ETU can uncouple the mitochondrial electron transport chain, leading to mitochondrial dysfunction, generating ROS, and ultimately promoting neuroinflammation and subsequent behavioral impairments (Bjørling-Poulsen et al., 2008; Domico et al., 2007; Gupta et al., 2007; He et al., 2020; Muthaiah et al., 2013) (Fig. 4; #20, #21, #22, #23 and #24).

4.4. Mixture effect

When the combined effect of IMPy, MDA, DCCA, and ETU was considered using WQS analysis a possible association with withdrawal, social, and thought problems was found, with MDA and IMPy accounting with the highest weights. Remarkably, when MDA was assessed individually, no statistical association was found. This may be due to an additive effect of the OP compounds present in the mixture. However, further results would be needed to confirm these observations. The mixture effect also revealed associations with higher BDNF gene DNA methylation and lower BDNF protein levels. MDA showed to be the highest contributor for increased DNA methylation in the mixture model, but again when assessed individually, no associations were found. Thus, the mixture approach may have unmasked MDA effects on the adolescents' neurodevelopment, highlighting the importance of addressing mixture effects.

In previous epidemiological studies, prenatal exposure to mixtures of environmental chemicals, including pesticides, was adversely associated with different aspects of brain development, such as lower IQ, cognitive

functioning, and higher risk of ADHD in children, but pesticides presented low weights in these studies (Guo et al., 2020; Kalloo et al., 2021; Lenters et al., 2019; Vuong et al., 2020). None of the aforementioned studies included IMPy, MDA, DCCA, or ETU in their mixtures assessment exclusively. Regarding mixture effects on BDNF, an *in vitro* study found an up-regulation of BDNF mRNA after exposure to a mixture of diverse chemical compounds, including chlorpyrifos, cyfluthrin, and deltamethrin (Özdemir et al., 2018). Due to the novelty of this assessment, results should be interpreted with caution and further studies are needed to confirm our results.

4.5. Strengths and limitations

Study limitations include its cross-sectional design, which limits causal inference, the small sample size, and the impossibility of assessing potential effect modification by gender. Additionally, some spurious associations may have been identified due to the performance of multiple tests, although our results are supported and contextualized by previous toxicological and epidemiological evidence. Our results are unlikely to be the result of chance, particularly for IMPy and ETU. Due to the short biological half-lives of the analyzed pesticides (urinary metabolites likely reflect exposure within the previous 24–48 h), their urinary metabolites have a relatively high inter-individual variability over time, increasing the risk of exposure misclassification. Nevertheless, as our study included adolescents from general population, for whom the main source of pesticide exposure is through diet (Kim et al., 2017), exposure to non-persistent pesticides can be assumed to be rather continuous (Barr, 2008). In this regard, Thiphom et al. (2013) indicated that in cases of continuous low-dose exposure to non-persistent pesticides such as pyrethroids, their metabolites can be considered as biomarker of long-term exposure. Furthermore, the inclusion of creatinine in the models may reduce exposure misclassification since study participants have the same age and sex, major factors influencing creatinine excretion rates (Wang et al., 2016, 2019). To address the potential exposure misclassification, measurement of repetitive urine samples should be performed in future studies to improve the exposure assessment. Additionally, TCPy and 3-PBA were measured as conjugated, thus no deconjugation process was developed. However, this would lead to an underestimation rather than an overestimation of pesticides' effect on BDNF and behavior. Among the strengths, we should highlight the novel approach exploring the role of BDNF as a biomarker of brain function at different levels of the biological complexity on the association between non-persistent pesticides exposure and neurodevelopment. To the best of our knowledge, this is also the first study to explore the combined effect of several pesticides' exposure on BDNF and behavioral functioning among adolescents, an understudied period of development, and to conduct a mediation analysis to further deepen our knowledge into exposure-effect associations. However, the relatively high interindividual variability of urinary pesticide metabolites should be considered when interpreting these results.

5. Conclusion

Urinary concentrations of IMPy and ETU, metabolites of the (banned) insecticide diazinon and dithiocarbamate fungicides, respectively, were associated with more behavioral problems among Spanish adolescent males. In addition, a possible combined effect of the IMPy, MDA, DCCA, and ETU mixture on increasing behavioral problems and BDNF gene DNA methylation was found. These associations could be due to alterations in BDNF levels and/or in the DNA methylation of the BDNF gene. The use of biomarkers of effect in epidemiological studies could have an added value since they provide additional information that could help to elucidate and understand exposure-effect relationships of a given hypothesis. This study is the first to present a complex panel of exposure-effect associations on a very relevant topic. However, longitudinal studies with larger sample sizes are needed to confirm these results.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113115.

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