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BDNF as a potential mediator between childhood BPA exposure and behavioral function in adolescent boys from the INMA-Granada cohort

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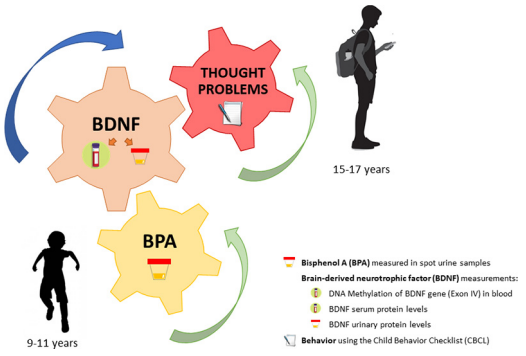
HIGHLIGHTS

- Childhood BPA exposure was linked to higher BDNF DNA methylation at adolescence.
- Childhood BPA was associated with thought and somatic problems at adolescence.
- BDNF may mediate BPA-behavior associations and should be further investigated.
- Brain derived neurotrophic factor-BDNF seems a promising neurologic effect biomarker.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Bisphenol A (BPA) exposure has been linked to altered behavior in children. Within the European Human Biomonitoring Initiative (HBM4EU), an adverse outcome pathway (AOP) network was constructed supporting the mechanistic link between BPA exposure and brain-derived neurotrophic factor (BDNF).
Objective: To test this toxicologically-based hypothesis in the prospective INMA-Granada birth cohort (Spain).
Methods: BPA concentrations were quantified by LC-MS/MS in spot urine samples from boys aged 9–11 years, normalized by creatinine and log-2 transformed. At adolescence (15–17 years), blood and urine specimens were collected, and serum and urinary BDNF protein levels were measured using immunoassays. DNA methylation levels at 6 CpGs in Exon IV of the BDNF gene were also assessed in peripheral blood using bisulfite-pyrosequencing. Adolescent's behavior was parent-rated using the Child Behavior Checklist (CBCL/6-18) in 148 boys. Adjusted linear regression and mediation models were fit.
Results: Childhood urinary BPA concentrations were longitudinally and positively associated with thought problems ($\beta = 0.76$; 95% CI: 0.02, 1.49) and somatic complaints ($\beta = 0.80$; 95% CI: −0.16, 1.75) at adolescence. BPA concentrations were positively associated with BDNF DNA methylation at CpG6 ($\beta = 0.21$; 95% CI: 0.06, 0.36) and mean CpG methylation ($\beta = 0.10$; 95% CI: 0.01, 0.18), but not with total serum or urinary BDNF protein

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levels. When independent variables were categorized in tertiles, positive dose-response associations were observed between BPA-thought problems (p-trend = 0.08), BPA-CpG6 (p-trend ≤ 0.01), and CpG6-thought problems (p-trend ≤ 0.01). A significant mediated effect by CpG6 DNA methylation was observed (β = 0.23; 95% CI: 0.01, 0.57), accounting for up to 34% of the BPA-thought problems association.
Conclusions: In line with toxicological studies, BPA exposure was longitudinally associated with increased BDNF DNA methylation, supporting the biological plausibility of BPA-behavior relationships previously described in the epidemiological literature. Given its novelty and preliminary nature, this effect biomarker approach should be replicated in larger birth cohorts.

1. Introduction

Bisphenol A (BPA) is a highly produced synthetic monomer used in polycarbonate plastics and epoxy resins. Among many consumer products, BPA is found in the inner lining of cans and tins (Cao et al., 2009; González et al., 2020; Kim et al., 2020), polycarbonate plastic bottles (Carwile et al., 2009), thermal receipts (Ehrlich et al., 2014; Molina-Molina et al., 2019), medical equipment (Iribarne-Durán et al., 2019), and textiles (Freire et al., 2019). Human BPA exposure is ubiquitous and more than 90% of the European population still has detectable concentrations in their urine (Covaci et al., 2015; Tschersich et al., 2021), despite the fact that BPA analogues have been recently introduced as replacements (Wu et al., 2018). BPA has also been measured in serum, placenta, breastmilk and amniotic fluid, demonstrating internal exposure (Vandenberg et al., 2010).

As a paradigmatic endocrine disrupting chemical (EDC), BPA is known to interfere with diverse aspects of hormone signaling at low doses (Heindel et al., 2020; Ma et al., 2019). Apart from its reprotoxic (Peretz et al., 2014) and metabolism disrupting activities (Akash et al., 2020), BPA is a developmental neurotoxicant in experimental animals (Nesan et al., 2018; Patisaul, 2019). The human literature appears increasingly consistent for altered behavior in children (Ejaredar et al., 2017; Mustieles et al., 2015; Mustieles and Fernández, 2020), although the potential mechanisms underlying observational associations remain poorly investigated.

Research on novel effect biomarkers is among the aims of the European Human Biomonitoring for Europe (HBM4EU) Initiative. Effect biomarkers are measurable biological changes that allow the evaluation of dose–response relationships and may provide a mechanistic link between exposure, early health impairment and health outcomes, consequently improving HBM and risk assessment of environmental chemicals (Baken et al., 2019; Mustieles et al., 2020). We have recently reviewed all the effect biomarkers used in epidemiological studies in relation to BPA exposure, identifying brain-derived neurotrophic factor (BDNF) as a promising biomarker of brain function (Mustieles et al., 2020). An adverse outcome pathway (AOP) network was also constructed, supporting that BPA may interfere with BDNF signaling through different but converging biological mechanisms (thyroid, estrogenic and glutamatergic-related pathways), potentially leading to behavioral and cognitive impairments (Fig. 1).

Discovered in 1982, BDNF is a member of the neurotrophin family of growth factors (Binder and Scharfman, 2004). Although it can be found throughout the brain, its expression is particularly high in the hippocampus, amygdala, cerebellum and cerebral cortex in both rodents

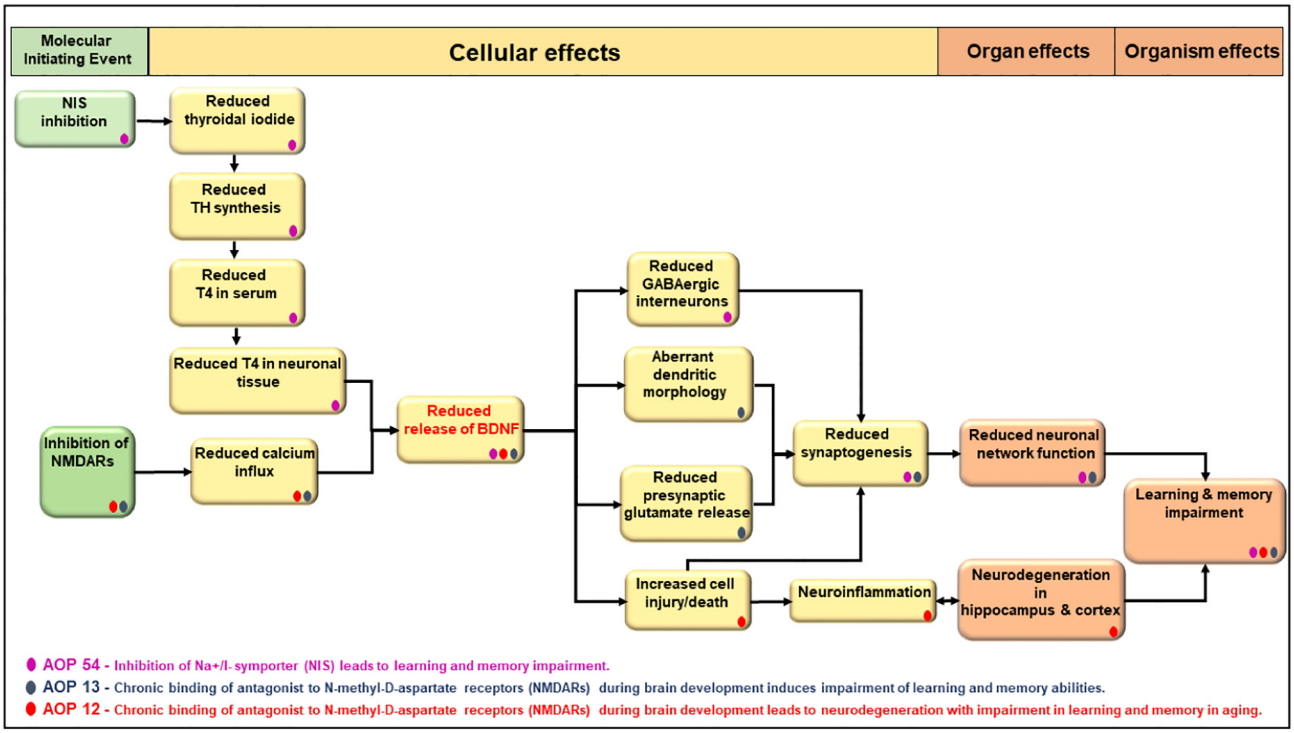

Fig. 1. Adverse outcome pathway network leading to a reduced release of BDNF.
Modified from Mustieles et al. (2020). Integration of 3 fully-developed AOPs (12, 13 and 54) from the AOP wiki (https://aopwiki.org/aops). Boxes represent molecular initiating events (MIEs) and key events (KEs) leading to learning and memory impairment. BPA has been shown to interfere with most of these key events in toxicological studies. In addition to thyroid and *N*-methyl-D-Aspartate (NMDAR) pathways, BPA can also interfere with estrogenic pathways to influence BDNF regulation and behavioral outcomes including anxiety and depression (Mustieles et al., 2020).

and humans (Miranda et al., 2019). The precursor pro-BDNF is synthesized and stored in dendrites or axons, and then is used to produce the mature BDNF protein. Of note, the pro- and mature-BDNF forms show opposite actions on neuronal function, providing an additional level of regulation. While pro-BDNF preferentially binds the p75 neurotrophin receptor leading to apoptosis, the mature BDNF form activates tyrosine kinase receptors promoting cell survival and synaptic plasticity (Miranda et al., 2019).

Epigenetic mechanisms, especially DNA methylation, influence BDNF expression and regulation (Ikegame et al., 2013). Patients with psychiatric disorders generally show decreased neural BDNF levels, often associated with increased DNA methylation at specific BDNF promoters (Ikegame et al., 2013). Importantly, DNA methylation changes in the BDNF gene are consistent across tissues including brain and blood, supporting its use as a peripheral biomarker of psychiatric disorders based on both rodent (Kundakovic et al., 2015) and human postmortem studies (Stenz et al., 2015). On the other hand, serum total BDNF levels have been previously associated with depression and other psychiatric disorders as shown by different meta-analyses (Polyakova et al., 2015; Rodrigues-Amorim et al., 2018; Toll, 2015). Although less studied, urinary total BDNF levels have also been proposed as a biomarker of executive function in adults (Koven and Collins, 2014).

The current work aimed to test our previous toxicologically-based hypothesis (Mustieles et al., 2020) focusing on the BPA exposure – BDNF – behavior triad in the Environment and Childhood (INMA)-Granada birth cohort of boys by investigating: i) whether childhood BPA exposure (9–11 years) is longitudinally associated with behavioral function at adolescence (15–17 years); ii) the longitudinal relationship between childhood BPA exposure and BDNF biomarkers measured at adolescence (protein levels in serum and urine, and blood DNA methylation); iii) the cross-sectional relationship between BDNF biomarkers and behavior in adolescents; and iv) whether BDNF biomarkers may mediate BPA-behavior associations.

2. Methods

2.1. Study population

This study forms part of the INMA Project, a multicenter population-based birth cohort study formed by seven cohorts designed to investigate the effect of environmental exposures and diet during pregnancy on fetal, child and adolescent development in different geographical areas of Spain (Guxens et al., 2012). The INMA-Granada cohort initially recruited 668 mother-son pairs with the aim to investigate associations between prenatal exposure to environmental chemicals and male urogenital malformations (Fernandez et al., 2007). A random sample of the initial cohort was re-contacted and asked to participate in follow-up clinical visits at the ages of 4–5 years (N = 220) and 9–11 years (N = 300). In the last follow-up (2017–2019), all boys that participated in the two previous visits were re-contacted. Of these, 155 boys aged 15–17 years agreed to participate and their parents signed the informed consent (Castiello et al., 2020). The principles of the declaration of Helsinki were followed, and the initial study and all follow-ups were approved by the Biomedical Research Ethics Committee of Granada. The physical and neuropsychological evaluation was performed at the Pediatric Unit of San Cecilio University Hospital (HUSC) in Granada.

The current analysis included 130 boys with available urinary BPA concentrations at 9–11 years of age and behavioral data at 15–17 years completed by parents. Between 107 and 121 boys were included in BPA-BDNF biomarker associations. Between 103 and 116 boys were included in BDNF-behavior associations. Finally, 103 children with complete data on exposure, BDNF and outcome were included in the mediation analysis (Fig. 2). Although no significant differences in socio-demographic or clinical characteristics were observed between the children included in this analysis (n = 130) and the remaining who also participated in the previous follow-up at 9–11 years of age (n = 139), childhood BPA concentrations tended to be higher and maternal education lower in the current analysis (Table S1).

2.2. Childhood BPA exposure assessment

Children provided a single non-fasting spot urine sample at the 9–11 year-old visit, between 17:00 and 20:00 h. Urine was collected in 10-mL polypropylene tubes and immediately stored at −20 °C. Total BPA (free plus conjugated) was determined by liquid chromatography-mass spectrometry at the laboratory of the Department of Analytical Chemistry of the University of Cordoba (Spain) as previously described in detail (Perez-Lobato et al., 2016). The limits of detection (LOD) and quantification (LOQ) were, respectively, 0.1 μg/L and 0.2 μg/L. Extended analytical information and procedures, including quality control and assurance (QA/QC) followed are provided in Perez-Lobato et al. (2016). The collection, storage, and processing of urine biospecimens was performed under controlled conditions, and account was taken for potential BPA external contamination from collection containers, equipment or labware. Urinary creatinine concentrations (mg/dL) were assessed at the Public Health Laboratory of the Basque Country (Spain) to account for urine dilution. BPA concentrations were normalized by urinary creatinine and expressed as μg of BPA/g of creatinine.

2.3. BDNF biomarkers at adolescence

On the day of their hospital visit at 15–17 years of age, each adolescent collected the first morning urine void and peripheral venous blood was collected from participants under non-fasting conditions between 17:00–19:00 h. Blood samples were immediately processed to obtain serum and whole blood aliquots. Urine and processed blood samples were subsequently stored at −80 °C. Whole blood was sent on dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Centre, where genomic DNA was extracted with the Maxwell® RSC equipment, quantified using the PicoGreen assay and normalized to 50 ng/μL. The extracted DNA was always stored at −80 °C until use.

Total serum BDNF levels (ng/mL) were measured using the commercial Quantikine® enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Serum samples were defrosted, vortexed, separated in two aliquots of 10 μL, diluted 100 times and tested according to manufacturer's instructions at the Biomedical Research Center (CIBM) of the University of Granada (Spain). Each sample was tested in duplicate in different plates and the mean of these two values was calculated in order to reduce measurement variation. Intra- and inter-assay coefficients of variability were <5% and <15%, respectively.

Total urinary BDNF levels were measured using the commercial RayBio® ELISA kit (Raybiotech, Norcross, GA, USA). Urine samples were defrosted, vortexed, and pre-treated following the protocol established by Koven and Collins (2014), with minor modifications. Samples were assessed at the Biomedical Research Center (CIBM) of the University of Granada (Spain) following manufacturer's instructions. Each sample was assessed in duplicate and the mean value was calculated. Intra- and inter-assay coefficients of variability were <5% and <15%, respectively. Creatinine concentrations (mg/dL) in the urine of adolescents were assessed at the Instituto de Investigación Biosanitaria de Granada (ibs.Granada, Spain) to account for urine dilution. Urinary BDNF concentrations were normalized by creatinine and expressed as μg of BDNF/g of creatinine.

DNA methylation was analyzed using the bisulfite pyrosequencing technique at IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085), Rennes, France. Briefly, genomic DNA concentration and purity was measured using NanoDrop (Thermo Scientific NanoDrop 8000; DNA50 mode). All the samples had approximately 1.8–1.9 ratio at 260/280 absorbance indicating that the extracted DNA

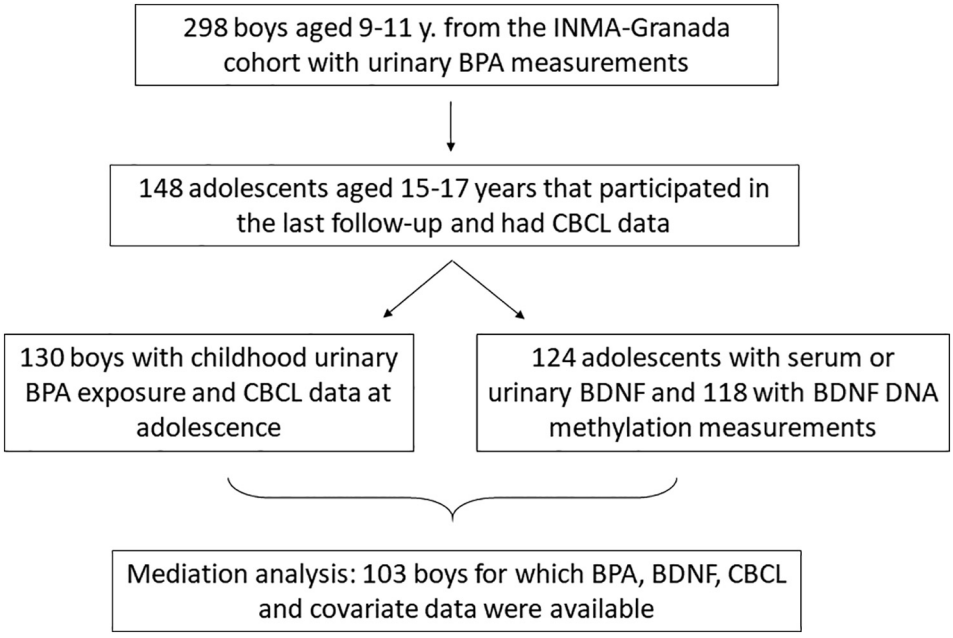

Fig. 2. Participant flow-chart in the Environment and Childhood (INMA)-Granada cohort follow-up visits from the age of 9–11 years to 15–17 years. BPA (Bisphenol A); CBCL (Child Behavior Checklist); BDNF (Brain-derived neurotrophic factor).

was pure. Since accurate quantification of DNA is extremely important for epigenetic studies, DNA concentration was further verified using QuantiFluor dsDNA system (Promega E2670) which is a highly sensitive system for measuring only double-stranded DNA (dsDNA). Subsequently, 500 ng of genomic DNA was bisulfite converted (BS) using Epitect Fast Bisulfite Conversion kit (Qiagen, 59826) following manufacturer's protocol. The concentration after bisulfite conversion and purification was remeasured using NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode) as recommended for BS-DNA. 20 ng of BS-converted DNA was used for downstream PCR amplification (Biometra TProfessional Thermoycler, France) of BDNF by using Takara EpiTaq hot-start DNA polymerase (Takara, R110A; 0.6 U/25 μl final concentration) that could amplify BS-converted DNA, under the following conditions: initial denaturation 98 °C for 30 s followed by denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C at 30 s, for a total of 40 cycles. The primers used for BDNF amplification (0.4 μM final concentration) are provided in Table S2, of which the reverse primer was biotinylated. The targeted region was Exon IV of BDNF (genomic coordinates: chr11:27,723,070–27,723,280 retrieved from UCSC Genome Browser Human February 2009 (GRCh37/hg19), which has been previously validated in rodents and humans (Kundakovic et al., 2015) and contains 6 CpGs including a CREB-binding site (cAMP response element-binding site). Following PCR amplification, the products were purified using MinElute PCR purification kit (Qiagen, 28,006) and were loaded on a 2% agarose gel and a single BDNF product (210 bp size) was observed indicating BDNF primer specificity and absence of primer-dimers. The samples were sent to the Genomic Platform LIGAN (Lille Integrated Genomics Advanced Network for personalized medicine), Lille (France), and were pyrosequenced using Pyromark Q24 Advanced Pyrosequencing technology. The degree of methylation at each CpG was expressed as percentage of DNA methylation.

The Pyromark Q24 software measures the percentage of methylation at each CpG and has a built-in quality control system for each run. The software uses non-CpG peaks as reference peaks and determines how well they match with the theoretical pyrogram generated based on the original BDNF sequence to be analyzed. CpG sites that deviate from an expected peak size are highlighted by the software. Since this could happen due to variations in PCR efficiency at certain regions, samples with CpGs that did not pass the quality control were pyrosequenced again to discard an error from technical handling. The number of CpGs that did not finally pass the quality control was small (<4% of all CpG measurements performed). Given that the quality control was CpG-specific, one individual could for example have data quantified for CpGs 1-to-5 but lack data on CpG6 or any other CpG. This small percentage of missing CpGs was multiple imputed (see Statistical analysis section). Extended details on the fine-tuning and quality controls performed for the measurement of BDNF biomarkers can be found elsewhere, as part of the HBM4EU project (Fernández et al., 2021).

2.4. Behavioral assessment

Adolescent's behavioral function was evaluated using the parent-reported Child Behavior Checklist (CBCL/6-18), a validated questionnaire that evaluates the parental perception about the behavior of their children and/or adolescents during the previous six months (Achenbach and Rescorla, 2001). The CBCL includes 118 items rated on a three-point Likert scale (0 = "Not True", 1 = "Somewhat or Sometimes True", or 2 = "Very/Often True"), that are grouped into eight syndrome scales (anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior). These scales are grouped into two empirically-derived composite scales: i) the internalizing domain as a measure of emotional problems (sum of scores on the anxious/depressed, withdrawn/depressed, and somatic complaints scales); and ii) the externalizing domain as a measure of behavioral problems (sum of scores on the rule-breaking behavior and aggressive behavior scales). Three other scales are considered mixed-syndrome scales that do not belong to either domain: social, thought, and attention problems (Achenbach and Rescorla, 2001). The total problems composite scale finally quantifies general impairment and corresponds to the sum of scores from all eight syndrome scales, together with a group of 17 "Other problems" items that do not belong to any specific syndrome scale. Raw scores for each scale were converted to sex- and age-normalized t-scores, which were used to evaluate behavior as a continuous outcome in the main analyses. Higher scores mean more behavioral problems in all scales. Children with CBCL/6–18 T-scores ≥60 on internalizing or externalizing problem scales and T-scores ≥65 on

diagnostic scales are considered as borderline/clinical cases (Achenbach and Rescorla, 2001). Parents assessed the behavioral functioning of adolescents under the supervision of a trained psychologist (AM) blinded to the BPA exposure status of the children.

2.5. Covariates

Information on sociodemographic, lifestyle factors and anthropometric data were obtained from validated questionnaires and physical examinations by trained stuff during the follow-ups of the children and from their clinical records. Maternal education (categorized as up to primary, secondary school or university) and age of boys (months) were gathered from the questionnaires. Pediatricians measured the weight (kg) and height (cm) of the adolescents without shoes and in light clothing using an electronic scale (TANITA model 354, Seca Corporation, Hamburg, Germany), and age- and sex-specific body mass index (BMI) z-scores were calculated using the 2007 World Health Organization (WHO) growth reference standards (de Onis, 2007). Urinary cotinine levels were measured at 9–11 years of age to account for second-hand smoke exposure. Cotinine was determined by competitive enzyme immunoassay (EIA) using commercial EIA microplate kits at the Public Health Laboratory of the Basque Country (Fernández et al., 2015). Alcohol consumption frequency and type of beverage (fermented vs. distilled drinks) was self-reported by adolescents, and classified as never or less than 1 drink/month vs. more than 1 drink/month.

2.6. Statistical analysis

Study participant characteristics were described using measures of central tendency and dispersion for numerical variables and frequencies for categorical variables. Creatinine-normalized urinary BPA concentrations were log2-transformed to minimize the skewness of the distribution. For BDNF DNA methylation measures, there was a small percentage of specific CpGs that did not pass the quality control (<4%) among individuals that had the remaining CpGs adequately quantified. Given that CpGs showed varying degrees of correlation among them (Table S3), missing CpG data were multiple imputed (20 imputations) using the regression method to avoid potential selection bias issues (i.e., slight differences in sample size for each CpG investigated).

Covariates were chosen a priori based on previous knowledge and/or those that modified the estimate (regression coefficient) of the exposure variable by >10%. To avoid an overadjustment, and to improve the comparability of exposure-mediator-outcome associations, all models were adjusted for the same set of covariates: adolescent's age (months) and BMI (z-scores) at behavioral assessment, since age at assessment predicted CBCL scores in this population and childhood adiposity is known to play a relevant role in neurodevelopment (Steegers et al., 2021); maternal education (primary, secondary or higher) as a well-known measure of socioeconomic status and parenting environment (Koutra et al., 2012; Patra et al., 2016), children's urinary cotinine levels (mg/dL) at 9–11 years of age, as tobacco exposure during childhood is an important predictor of neurobehavior (Chen et al., 2013); and alcohol consumption at adolescence since it has been identified as a relevant predictor of BDNF regulation in this period (Miguez et al., 2020).

Multivariable linear regression models were fit to assess: i) Longitudinal associations between childhood log2-transformed creatinine-normalized BPA concentrations and continuous t-scores for each behavioral scale at adolescence; ii) Longitudinal associations between childhood log2-transformed BPA concentrations and continuous values of BDNF biomarkers at adolescence; and iii) Cross-sectional associations between selected log2-transformed BDNF biomarkers and t-scores of selected CBCL scales, both assessed at adolescence. Beta coefficients and 95% CIs represent the mean change in the dependent variable, for each doubling in the independent variable. In order to explore potential dose-response associations within positive findings, we additionally categorized independent variables (BPA concentrations and BDNF biomarkers) in tertiles, taking the lowest tertile as the reference. Statistical tests for trend across tertiles were calculated by entering the independent variable as an ordinal level indicator (1, 2, 3) of each tertile in the regression model.

To determine whether selected BDNF biomarkers are potential mediators of the longitudinal association between BPA exposure and adolescent's behavior, mediation analysis was performed to calculate the total, direct and indirect effects. To reduce the number of comparisons, mediation analysis was guided by associations previously observed in multivariable regression models. Beta coefficients and 95% CIs were estimated after 10,000 bootstrapped replications. The total effect represents the relationship between the exposure (i.e., BPA) and outcome (i.e., behavior) without accounting for any mediator. The natural direct effect represents the proportion of the statistical relationship between exposure and outcome that is not attributable to the mediator (i.e., BDNF). The mediational or natural indirect effect represents the proportion of the statistical relationship between exposure and outcome that is driven by the mediator. The percentage mediated was calculated as: indirect effect / (direct effect + indirect effect) × 100.

SPSS v25.0 (IBM, Chicago, IL) was used for data analyses. Mediation analysis was performed using the PROCESS macro v3.5 for SPSS (http://processmacro.org/index.html). The significance level was set at P-value <0.05 and all tests were two-tailed. A P-value between 0.05 and 0.10 was considered as being suggestive of statistical significance. Notwithstanding, results were interpreted considering their internal validity and coherence, as well as the existing toxicological and epidemiological support, rather than solely depending on statistical significance (Amrhein et al., 2019). Given the targeted and predefined toxicological hypothesis investigated in this work (Mustieles et al., 2020), and the moderate number of comparisons tested, we did not perform a *post-hoc* correction for multiple comparisons to avoid a disproportionate increase in the frequency of type II errors (Rothman, 2014).

3. Results

3.1. Characteristics of the study population

Mean (standard deviation - SD) age of children at urine collection was 9.90 (0.32) years. Children's mean (SD) urinary concentrations of creatinine and cotinine were 100 (39.8) mg/dL and 15.9 (32.8) ng/mL, respectively. Adolescents completed the follow-up with a mean (SD) age and BMI of 16.6 (0.38) years and 23.6 (5.08) kg/m^2, respectively, and 38.5% consumed alcoholic beverages more than once per month. Regarding mothers, more than two-thirds had completed primary (37.7%) and secondary (36.2%) education, while 26.2% had completed university studies (Table 1). The distribution of CBCL behavior t-scores of adolescents is presented in Table S4. Internalizing problems (30.0%) were more prevalent than externalizing behaviors (13.8%) (Table S4).

BPA concentrations were quantified in all urine samples at the 9–11 year-old visit, showing a large range of concentrations (between 0.46 and 76.4 μg/g), and a median and interquartile range (IQR) of 5.41 (3.05, 10.6) μg/g (Table 1). Serum and urinary total protein BDNF levels measured in adolescents showed a median (IQR) of 31.5 (25.4, 38.8) ng/mL and 2.14 (1.56, 3.09) μg/g, respectively (Table 1). The mean percentage of BDNF DNA methylation in the six CpGs investigated in blood samples from the adolescents showed a median (IQR) of 3.70 (3.45, 4.04), with a minimum value of 2.70% and a maximum of 5.54%. The range and distributions of methylation percentages for each individual CpG are presented in Fig. S1. Pearson correlation coefficients were assessed between CpG's percentage of DNA methylation and serum and urinary BDNF levels (Table S3). Most CpGs tended to positively correlate among them (suggesting the possibility of co-methylation), while CpG1 showed a different pattern of correlation (Table S3). A higher percentage of methylation in most CpGs tended to correlate with lower serum protein BDNF levels, with the exception

Table 1
Descriptive analysis of BPA concentrations, BDNF biomarkers, and sociodemographic characteristics of boys evaluated at both 9–11 and 15–17 years of age (n = 130) from the Spanish INMA-Granada cohort.

Percentiles	Min	p10	p25	p50	p75	p90	Max
Child BPA (μg/g)	0.46	1.71	3.05	5.41	10.6	18.9	76.3
Adolescent serum BDNF protein levels (ng/mL)	17.2	20.3	25.4	31.5	38.8	47.4	56.0
Adolescent urinary BDNF protein levels (μg/g)[a]	0.16	1.09	1.56	2.14	3.09	4.31	15.2
Adolescent urinary BDNF protein levels (ng/mL)[a]	0.15	2.15	2.68	4.52	5.45	6.15	7.40
Adolescent BDNF mean CpG methylation (%)	2.70	3.20	3.45	3.70	4.04	4.64	5.54

Characteristics	N (%)/mean (SD)
Maternal education	
Primary	49 (37.7%)
Secondary	47 (36.2%)
University	34 (26.2%)
Adolescent alcohol intake (Yes)	50 (38.5%)
Child age at urine collection (years)	9.90 (0.32)
Child urinary creatinine (mg/dL)	100 (39.8)
Child urinary cotinine (ng/mL)	15.9 (32.8)
Adolescent age at follow-up (years)	16.6 (0.38)
Adolescent BMI at follow-up (kg/m^2)	23.6 (5.08)
Adolescent BMI z-scores	0.58 (1.33)

BPA (Bisphenol A); BDNF (Brain-derived neurotrophic factor); BMI (body mass index); p: percentile.
[a] Adolescent raw urinary BDNF protein levels were expressed as ng/mL, while creatinine-corrected urinary BDNF levels were expressed as μg/g.

of CpG1. On the contrary, urinary protein BDNF levels were not correlated with most CpGs, with the exception of CpG1, for which a positive borderline-significant correlation was observed (Table S3). Serum and urinary BDNF protein levels were not significantly correlated, although an inverse relationship was observed between both biomarkers.

3.2. Longitudinal associations of childhood BPA exposure with behavior at adolescence

Childhood urinary BPA concentrations tended to be associated with poorer behavior in most CBCL scales at adolescence, except for social and attention problems (Table 2). Each doubling in urinary BPA concentration was associated with a 0.76-point (95% CI: 0.02, 1.49) increase in t-scores for the thought problems scale (Table 2). When BPA concentrations were categorized in tertiles, a dose-response function with thought problems was confirmed (Fig. 3.A, p-trend = 0.08). Children in the upper BPA tertile showed a mean increase of 2 points in thought problems t-scores (range 50–82 points, Table S4) compared to those in the lowest tertile (Fig. 3.A). BPA exposure was additionally associated with increased somatic (β = 0.80; 95% CI: −0.16, 1.75) and total problems (β = 0.80; 95% CI: −0.13, 1.73), although confidence intervals included the null value, and a dose-response shape was not observed for total problems (Fig. S2).

Table 2
Longitudinal associations between childhood urinary BPA concentrations (9–11 years) and parent-reported behavior of adolescent boys aged 15–17 (n = 130).

Behavioral functions (CBCL)	BPA (μg/g of creatinine)*	
	β (95% CI)	P-value
Syndrome scores		
Anxious/depressed	0.34 (−0.44, 1.11)	0.392
Withdrawn	0.17 (−0.66, 0.99)	0.690
Somatic complaints	**0.80 (−0.16, 1.75)**	**0.102**
Social problems	−0.11 (−0.87, 0.66)	0.779
Thought problems	**0.76 (0.02, 1.49)**	**0.045**
Attention problems	−0.35 (−1.16, 0.46)	0.394
Rule-breaking problems	0.42 (−0.29, 1.13)	0.245
Aggressive behavior	0.00 (−0.77, 0.77)	0.998
Composite scores		
Internalizing problems	0.39 (−0.50, 1.28)	0.382
Externalizing problems	0.23 (−0.35, 0.81)	0.433
Total problems	**0.80 (−0.13, 1.73)**	**0.092**

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). Higher Child Behavior Checklist (CBCL) t-scores mean more behavioral problems for all scales. * Continuous BPA concentrations normalized by urinary creatinine were log2-transformed and treated as the independent variable.

3.3. Longitudinal associations of childhood BPA exposure with BDNF biomarkers at adolescence

Childhood urinary BPA concentrations tended to be associated with a higher percentage of BDNF DNA methylation at adolescence in all CpGs investigated (Table 3). BPA concentrations were positively and significantly associated with higher DNA methylation at CpG6 (β = 0.21; 95% CI: 0.06, 0.36) and the mean methylation of the six CpGs assessed (β = 0.10; 95% CI: 0.01, 0.18). Suggestive associations with CpG3 and CpG5 were also observed (β = 0.09; 95% CI: −0.00, 0.17; β = 0.11; 95% CI: −0.00, 0.22). Notably, the magnitude of the association observed for CpG6 doubled those of CpGs 3, 5 and CpG mean. When BPA concentrations were categorized in tertiles, dose-response associations were observed for these three CpGs, with CpG6 showing again the most robust association (Figs. 3.B and S2). Childhood BPA exposure was not associated with total BDNF protein levels measured in either serum or urine at adolescence (Table 3).

3.4. Cross-sectional associations between BDNF biomarkers and behavior at adolescence

Given that BPA exposure was more clearly associated with thought problems compared to the remaining CBCL scores (Table 2 and Fig. S2), we decided to focus on this scale. When BDNF DNA methylation was considered as the independent variable, most CpGs tended to be positively associated with thought problems, being CpG1 the unique exception (Table 4). CpG6 was significantly associated with thought problems (β = 2.59; 95% CI: 0.31, 4.87), and a suggestive positive association was also observed for CpG5 (β = 3.42; 95% CI: −0.22, 7.05) [Table 4]. When the CpGs previously associated with BPA (Table 3) were categorized in tertiles, dose-response associations were observed for CpGs 5 and 6 and the mean CpG methylation, but not CpG3 (Fig. S2). This dose-response association was stronger and especially evident for CpG6. Thus, boys in the upper tertile of CpG6 BDNF DNA methylation showed a mean increase of 4 points in thought problems t-scores (range 50–82 points, Table S4) compared to those in the lowest tertile (Fig. 3.C). Regarding serum and urinary total BDNF levels, no cross-sectional associations were observed with thought problems (Table 4).

3.5. Mediation analysis

As summarized in Fig. 3, BPA was longitudinally and dose-dependently associated with increased thought problems (Fig. 3.A), and with a higher percentage of BDNF DNA methylation, especially at CpG6 (Fig. 3.B) at adolescence. Additionally, CpG6 methylation was cross-sectionally and dose-dependently associated with increased thought problems (Fig. 3.C). Based on these results, we decided to evaluate whether there was a mediation effect in the subset of 103 boys with available data for the exposure, mediator and outcome. In adjusted models, a significant indirect effect of CpG6 DNA methylation (β = 0.23; 95% CI: 0.01, 0.57) was observed, accounting for up to 34% of the BPA-thought problems association (Fig. 4). No significant indirect effects were observed for CpG5 and the mean CpG methylation in relation to thought problems, or somatic complaints (Table S5).

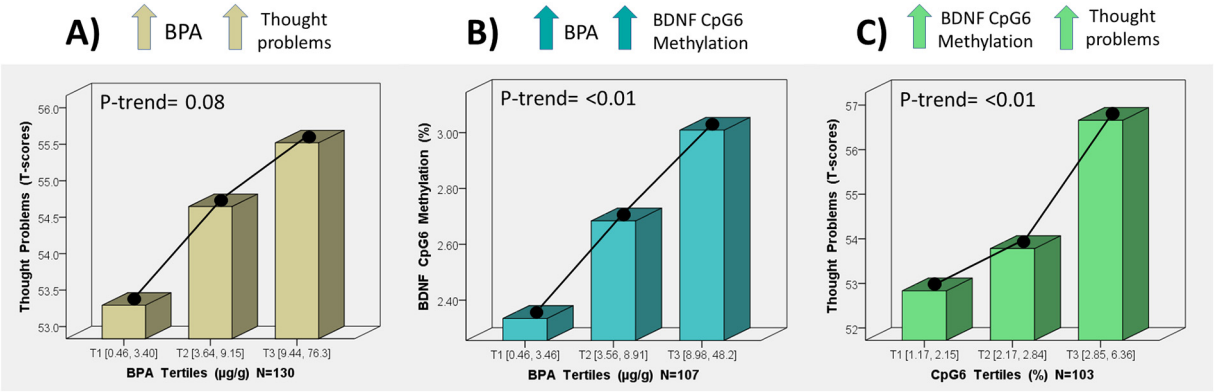

Fig. 3. Relationships among BPA exposure, CpG6 DNA methylation and thought problems categorizing the independent variable in tertiles to assess dose-response trends within the most robust findings.
Childhood urinary BPA concentrations categorized in tertiles were longitudinally, dose-dependently and positively associated with thought problems (A) and with the percentage of BDNF DNA methylation at CpG6 of Exon IV (B). Additionally, CpG6 DNA methylation percentage categorized in tertiles was cross-sectionally, dose-dependently and positively associated with thought problems (C). Models were adjusted for age and BMI z-scores (continuous) at neuropsychological evaluation (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). Higher t-scores mean more behavioral problems. Note: The scales of Y-axes represent the range of mean values for tertiles. The full range is 50–82 points for thought problems t-scores, and 1.17%–6.36% in the case of CpG6 BDNF DNA methylation.

4. Discussion

In the INMA-Granada birth cohort, higher childhood urinary BPA concentrations were longitudinally associated with increased behavior problems at adolescence, especially thought problems. Childhood BPA exposure was also longitudinally associated with a higher percentage of DNA methylation at the promoter region IV of the BDNF gene measured at adolescence, especially evident at CpG number 6. Moreover, increased BDNF DNA methylation predicted the occurrence of thought problems, and CpG6 mediated the association between BPA and thought problems. Our findings suggest that BPA exposure may alter BDNF epigenetic regulation, leading to altered neurobehavior during a critical and understudied period of development such as adolescence (Fuhrmann et al., 2015; Pfeifer and Allen, 2020).

In this same cohort, we previously found that higher urinary BPA concentrations were cross-sectionally associated with increased thought, somatic and social problems in 269 boys at the age of 9–11 years (Perez-Lobato et al., 2016). Although in the current work the number of adolescent boys assessed at 15–17 years was lower due to attrition during the follow-up (n = 130), we prospectively confirmed previous associations with thought and somatic problems, which may signal greater vulnerability to the subsequent development of a mental disorder in adulthood (Paus et al., 2008). The thought problems scale includes obsessive thoughts, compulsive behaviors and strange ideas among other items, and has been linked to psychosis during adulthood (Salcedo et al., 2018). Moreover, the co-occurrence of high scores in both the thought problems and somatic complaints scales has been related to mania (Morgan and Cauce, 1999). Nevertheless, the interpretation of our results must be done at a population instead of a clinical level (Bellinger, 2012, 2004).

Previous studies have reported associations between prenatal BPA exposure and child internalizing problems (Braun et al., 2017, 2011; Grohs et al., 2019; Harley et al., 2013; Perera et al., 2016; Philippat et al., 2017), including somatic complaints (Evans et al., 2014; Li et al., 2020). Regarding thought problems, higher prenatal BPA exposure was associated with increased scores in this scale at 7–9 years in children from the Columbia Center for Children's Environmental Health (CCCEH) cohort, although postnatal BPA exposure in the same children was not cross-sectionally associated with this scale (Roen et al., 2015). Postnatal studies have been more scarce, although our findings may be compatible with those previously described by Hong et al. (2013) and Harley et al. (2013) who found cross-sectional associations between postnatal BPA exposure and CBCL total problems and

Table 3
Longitudinal associations between child urinary BPA concentrations (9–11 years) and BDNF biomarkers in adolescent boys (15–17 years) from the INMA-Granada cohort.

BDNF measurements	BPA (µg/g of creatinine)*		
	β (95% CI)	P-value	N
BDNF protein levels			
Serum BDNF (ng/ml)	−0.19 (−1.47, 1.10)	0.773	120
Urinary BDNF (µg/g)	0.13 (−0.09, 0.35)	0.254	121
Blood BDNF DNA methylation			
CpG1 (%)	0.03 (−0.09, 0.14)	0.635	107
CpG2 (%)	0.06 (−0.02, 0.13)	0.142	107
CpG3 (%)	**0.09 (−0.00, 0.17)**	**0.056**	107
CpG4 (%)	0.09 (−0.08, 0.26)	0.290	107
CpG5 (%)	**0.11 (−0.00, 0.22)**	**0.055**	107
CpG6 (%)	**0.21 (0.06, 0.36)**	**0.006**	107
CpG mean (%)	**0.10 (0.01, 0.18)**	**0.027**	107

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). * Continuous BPA concentrations normalized by urinary creatinine were log2-transformed and treated as the independent variable.

Table 4
Cross-sectional associations between BDNF biomarkers and behavior in adolescent boys (15–17 years) from the INMA-Granada cohort.

BDNF measurements*	Thought problems (CBCL)		
	β (95% CI)	P-value	N
BDNF protein levels			
Serum BDNF (ng/ml)	−0.79 (−3.55, 1.97)	0.571	115
Urinary BDNF (µg/g)	−0.48 (−1.82, 0.86)	0.481	116
Blood BDNF DNA methylation			
CpG1 (%)	−2.22 (−7.03, 2.60)	0.363	103
CpG2 (%)	0.85 (−4.11, 5.81)	0.735	103
CpG3 (%)	0.88 (−3.70, 5.46)	0.704	103
CpG4 (%)	2.64 (−2.02, 7.31)	0.263	103
CpG5 (%)	**3.42 (−0.22, 7.05)**	**0.065**	103
CpG6 (%)	**2.59 (0.31, 4.87)**	**0.026**	103
CpG mean (%)	3.52 (−2.07, 9.11)	0.214	103

Data are presented as Beta estimates and 95% Confidence Intervals [β (95% CIs)]. Models were adjusted for age and BMI z-scores (continuous) at behavioral assessment (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous) and alcohol consumption at adolescence (yes/no). * Continuous BDNF biomarkers were log2-transformed and treated as the independent variable.

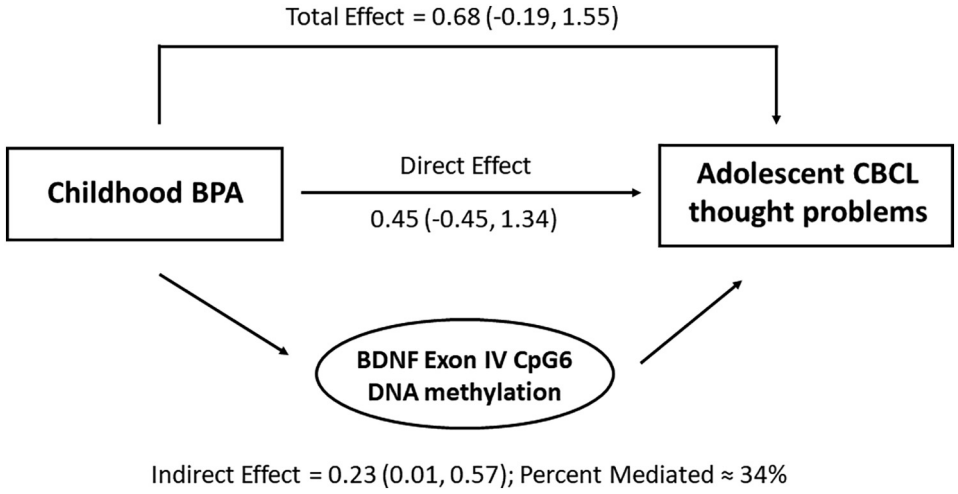

Fig. 4. Mediation model showing associations between childhood urinary BPA concentrations, BDNF DNA methylation and thought problems t-scores at adolescence (n = 103). Beta coefficients and 95% CIs are reported for the total, direct and indirect (i.e., mediated) effects. Continuous log2-transformed BPA concentrations and CpG6 methylation percentage, and continuous thought problems t-scores were used. Models were adjusted for age and BMI z-scores (continuous) at neuropsychological evaluation (15–17 years), maternal education (primary, secondary or university), urinary cotinine at 9–11 years (continuous), and alcohol consumption (yes/no) at adolescence.

internalizing problems, respectively. Noteworthy, BPA exposure has been related to both internalizing and externalizing behaviors in toxicological and observational studies (Mustieles and Fernández, 2020), hindering comparisons but suggesting that poorer emotional regulation and executive function may underlie these altered behavioral phenotypes. In this context, the effect biomarker approach followed in this work complemented the information provided by neuropsychological scales, increasing the internal coherence and validity of our findings.

Similar to toxicological studies in rodents (Kundakovic et al., 2015; Mustieles et al., 2020), our findings support that childhood BPA exposure promotes a higher degree of DNA methylation at the promoter region IV of the BDNF gene. Kundakovic et al. (2015) orally treated pregnant BALB/c mice with either BPA (200 μg/kg per day) or vehicle throughout gestational days 0–19, demonstrating lasting DNA methylation changes in Exon IV of the BDNF gene in the hippocampus and blood of the exposed offspring, which is in line with a CLARITY-BPA toxicological report in adult rats (Cheong et al., 2018). Kundakovic et al. (2015) additionally tested their hypothesis in a subset of children from the abovementioned CCCEH cohort. The authors found that boys -but not girls- born to mothers in the highest category of prenatal urinary BPA concentrations showed a significantly higher cord blood DNA methylation of BDNF Exon IV at CpG sites 1 and 2 compared to boys in the lowest exposure group (n = 40 boys with either low or high prenatal BPA exposure). Importantly, prenatal BPA was previously associated with behavioral problems in 198 children from the CCCEH cohort (Perera et al., 2012) including thought problems at 7–9 years of age (Roen et al., 2015). Although in the current study BPA exposure was more robustly associated with CpGs 5 and 6, a possible association towards higher methylation at CpG 2 -but not CpG 1- was also noticed (Table 3). Our findings point to the same direction as Kundakovic et al. (2015), although BDNF DNA methylation was evaluated at different developmental periods (neonates vs. adolescents). Indeed, this may explain the divergence in the involvement of specific CpGs, since BDNF regulation varies throughout development (Kowiański et al., 2018).

The mediational effect observed for CpG6 DNA methylation between BPA exposure and thought problems is supported by: i) the strong and predefined toxicological hypothesis (Kundakovic et al., 2015; Mustieles et al., 2020); and ii) the consistent dose-response associations between the BPA – CpG6 – thought problems triad (Fig. 3). Our results highlight BDNF epigenetic regulation as a plausible key event in BPA-neurobehavior associations that should be further investigated in larger birth cohorts.

All CpGs investigated showed a methylation status below 10% (Fig. S1), supporting previous reports describing BDNF as a low-methylated gene in absolute terms (Cattaneo et al., 2016). Indeed, due to this characteristic, microarray-based DNA methylome measurements do not seem a reliable method for BDNF methylation assessment compared to bisulfite-pyrosequencing (Forest et al., 2018; Sugden et al., 2020). Despite low absolute methylation status in the BDNF gene, slight variations in methylation at Exon IV have been linked to functional gene expression changes in experimental animals and human in vitro models (Kundakovic et al., 2015; Martinowich et al., 2007; Pruunsild et al., 2011; Zheleznyakova et al., 2016). Our results support this point, since in the case of CpG6, even small mean methylation differences of around 2% between extreme tertiles (T3: 3.8% vs. T1: 1.8%) predicted a difference of almost 4-points in thought problems t-scores (T3: 56.7 vs. T1: 52.8) [Fig. 3.C].

Serum total protein BDNF levels measured in INMA-Granada adolescents (median: 31.5 ng/mL) were in line with a previous report in 223 male teenagers around 14 years of age showing a mean of 27.0 ng/mL (Pedersen et al., 2017). While no previous study has assessed urinary BDNF concentrations in adolescents, current urinary BDNF levels (median: 4.52 ng/mL) were higher compared to young adults (0.6 ng/mL) (Koven and Collins, 2014). Despite serum total BDNF levels have been related to psychiatric diseases (Polyakova et al., 2015; Rodrigues-Amorim et al., 2018) and that urinary BDNF has been proposed as a biomarker of executive function in adults (Koven and Collins, 2014), we did not find associations with BPA exposure.

The fact that we found longitudinal associations between BPA exposure and blood BDNF DNA methylation but not with serum or urinary total BDNF levels may be due to several reasons. First, we expect DNA methylation to be more stable over time (months or even years) compared to circulating protein levels (Kundakovic et al., 2015), facilitating the detection of prospective associations. However, the temporal variability of BDNF biomarkers is unknown for DNA methylation and scarce in the case of urinary and serum BDNF protein levels (Molendijk et al., 2012). Second, total BDNF protein levels were measured, not differentiating between the pro- and mature forms, which may have reduced the ability to detect associations (Jiang et al., 2017; Lin et al., 2021). Finally, while peripheral BDNF DNA methylation seems to be well correlated with its methylation status in the brain (Stenz et al., 2015), serum BDNF may be influenced by peripheral sources such as platelets (Gejl et al., 2019), and urinary BDNF by local production in the bladder (Antunes-Lopes and Cruz, 2019), which would tend to mask associations. Further research is needed to confirm our findings and identify

the most predictive BDNF biomarkers, as well as to assess their stability over time through repeated measures.

A higher percentage of DNA methylation in most CpGs correlated with lower serum protein BDNF levels, with the exception of CpG1 (Table S3). This is in line with our initial hypothesis that a higher percentage of DNA methylation would reduce BDNF gene expression and protein synthesis. However, we were not able to elucidate whether this negative correlation was mainly accounted by reduced levels of the mature BDNF form, the pro-BDNF form, or both. Interestingly, urinary BDNF levels showed and inverse relationship with CpG1 DNA methylation, and with serum BDNF levels, suggesting that serum and urinary BDNF levels may have a different biological meaning.

BPA exposure in INMA-Granada boys is higher compared to some studies (Covaci et al., 2015; Tschersich et al., 2021), but similar to others (Braun et al., 2011; Calafat et al., 2008; Perera et al., 2012). Timing of urine collection in the present study (i.e., evening), but also food intake and lifestyle patterns may partially explain the higher levels, since a subset of the same boys at 4–5 years of age showed similar urinary BPA concentrations compared to the 9–11 years-old visit (Casas et al., 2011).

Overall, our findings suggest that BDNF methylation status at Exon IV is a physiologically valid molecular effect biomarker of children's behavior that may mediate some of the well-known toxicological effects of BPA exposure on brain and behavior (Nesan et al., 2018; Patisaul, 2019). Given that many previous epidemiological studies have reported associations between prenatal/postnatal BPA exposure and neurodevelopment (reviewed in Mustieles et al., 2015; Mustieles and Fernández, 2020), BDNF biomarkers, as well as other neurological effect biomarkers (Cediel Ulloa et al., 2021), could be implemented in future biomonitoring studies to improve the inference of causal relationships. Moreover, effect biomarkers of brain function will be useful for the timely assessment of BPA structural analogues such bisphenol S and F which show similar neuroendocrine disruption potential (Rosenfeld, 2017; Tanner et al., 2020).

Among the strengths of this work are the predefined hypothesis based on toxicological data organized following the AOP framework (Mustieles et al., 2020), together with the assessment of BDNF at complementary levels of biological organization. For BDNF DNA methylation, the gold standard (bisulfite-pyrosequencing) was used. The need for a more systematic implementation of effect biomarkers has been recently highlighted (Zare Jeddi et al., 2021), and together with our previous theoretical work (Mustieles et al., 2020), this study exemplifies how to go from toxicological knowledge to the implementation and validation of novel effect biomarkers in HBM studies. Another strength is that BPA exposure was evaluated during late childhood and behavior during adolescence, which are important but understudied periods of brain development (Konrad et al., 2013). The longitudinal design confirmed previous cross-sectional associations between BPA and behavior in the same cohort (Perez-Lobato et al., 2016), reducing the possibility of reverse causality issues. Additionally, we observed dose-response relationships among the exposure-BDNF-behavior triad, and indications of potential mediation by BDNF DNA methylation. Overall, this effect biomarker approach grounded on toxicological data helped to establish dose–response and mechanistic relationships, increasing the biological plausibility and internal consistency of the findings.

Regarding limitations, BPA exposure was assessed in one spot urine sample, which may lead to exposure misclassification due to its non-persistent nature and short-term viability. However, this would likely result in attenuation bias, rather than an overestimation of effects (Vernet et al., 2019). The sample size was small, reducing our ability to detect potential associations, and limiting the number of covariates to be controlled for in the models. Notwithstanding, this dataset was sufficient to observe interrelated associations coherent with the hypothesized toxicological pathway. A limitation of our mediation analysis is that BDNF methylation was measured in samples collected at the same time that adolescent's behavior was assessed. Notwithstanding, we do not expect a substantial alteration in the temporal ordering of the exposure-mediator-outcome (Gelfand et al., 2009), since DNA methylation constitutes the most stable "omics" signature over time (Gallego-Paüls et al., 2021), probably providing information on the past months before the measurement. Future studies testing the temporal variability of BDNF biomarkers will help to improve the interpretation of exposure-BDNF associations. Another limitation is that our study design only included boys and sex-dependent associations could not be tested (Mustieles and Fernández, 2020). Apart from anxiety, depression and other psychiatric diseases, BDNF has also an important role in long-term memory and learning (Cunha et al., 2010). Although we investigated behavioral outcomes, unfortunately no evaluation of cognitive abilities was performed during the last INMA-Granada follow-up when boys were aged 15–17 years. While adolescence is an important and understudied period of brain development, BPA exposure during pregnancy was not available in this cohort, being unable to compare how prenatal and postnatal BPA exposures interact to influence adolescent's neurobehavior. In addition to BPA, other environmental chemicals such as phthalates (Ponsonby et al., 2016), lead (Sachana et al., 2018) and arsenic (Karim et al., 2019), are also known to alter BDNF regulation in experimental animals, and future works should consider the influence of chemical mixtures. Finally, residual confounding arising from unmeasured or uncontrolled covariates including lifestyle patterns (e.g., physical activity, diet, etc.) cannot be ruled out.

5. Conclusions

Childhood BPA exposure was longitudinally associated with a higher percentage of BDNF DNA methylation at adolescence, partially accounting for BPA-behavior associations. Our results highlight the role of BDNF as a promising and toxicologically-supported effect biomarker of brain function that may help to improve the inference of causal relationships in observational studies dealing with environmental exposures and children's neurodevelopment. Given the modest sample size analyzed in this pilot study and the novelty of these findings, future studies should replicate them under different settings, windows of development, and in the context of chemical mixtures.

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CRediT authorship contribution statement

Vicente Mustieles: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Andrea Rodríguez-Carrillo:** Validation, Writing – original draft, Visualization, Writing – review & editing. **Fernando Vela-Soria:** Investigation, Data curation, Writing – review & editing. **Shereen Cynthia D'Cruz:** Investigation, Writing – review & editing. **Arthur David:** Writing – review & editing. **Fatima Smagulova:** Writing – review & editing. **Antonio Mundo-López:** Writing – review & editing. **Alicia Olivas-Martínez:** Investigation, Writing – review & editing. **Iris Reina-Pérez:** Writing – review & editing. **Nicolás Olea:** Funding acquisition, Writing – review & editing. **Carmen Freire:** Resources, Project administration, Writing – review & editing. **Juan P. Arrebola:** Supervision, Methodology, Writing – review & editing. **Mariana F. Fernández:** Supervision, Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

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.scitotenv.2021.150014.

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