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Exploring the relationship between metal exposure, BDNF, and behavior in adolescent males

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ABSTRACT

Background: Brain-derived neurotrophic factor (BDNF) plays an important role in brain development by regulating multiple pathways within the central nervous system. In the Human Biomonitoring for Europe Project (HBM4EU), this neurotrophin is being implemented as a novel effect biomarker to evaluate the potential threats of environmental chemicals on neurodevelopment.

Objectives: To explore the relationships among exposure to environmental metals, BDNF biomarkers at two levels of biological complexity, and behavioral function in adolescent males.

Methods: Data were gathered from 125 adolescents on: spot urine sample total concentrations of the neurotoxic metal(oid)s arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb); serum BDNF protein concentrations; and concurrent behavioral functioning according to the Child Behavior Check List (CBCL/6–18). In 113 of the participants, information was also collected on blood BDNF DNA methylation at six CpGs. Associations were evaluated by multivariate linear regression analysis adjusted for confounders.

Results: As, Cd, Hg, and Pb were detected in 100%, 98.5%, 97.0%, and 89.5% of urine samples, respectively. Median serum BDNF concentration was 32.6 ng/mL, and total percentage of BDNF gene methylation was 3.8%. In the adjusted models, urinary As was non-linearly associated with more internalizing problems and Cd with more externalizing behaviors. The percentage BDNF DNA methylation at CPGs #5 and the mean percentage CpG methylation increased across As tertiles (p-trend = 0.04 and 0.03, respectively), while 2nd tertile and 3rd tertile of Cd concentrations were associated with lower serum BDNF and higher CpG3 methylation percentage. Additionally, when BDNF was categorized in tertiles, serum BDNF at the 3rd tertile was associated with fewer behavioral problems, particularly withdrawn (p-trend = 0.04), social problems (p-trend = 0.12), and thought problems (p-trend = 0.04).

Conclusion: Exposure to As and Cd was associated with BDNF gene DNA methylation BDNF gene and serum BDNF, respectively. Associations with DNA methylation may be attributable to a higher variability over time in circulating BDNF concentrations than in the methylation status of this gene. Caution should be taken when interpreting the results relating postnatal Pb and Hg to behavioral functioning. Further studies are needed to verify these findings.

1. Introduction

The human brain develops from week eight of gestation up to late adolescence and even early adulthood (Rice and Barone, 2000; Stiles and Jernigan, 2010), being considered fully developed at around 25 years of age (Stiles and Jernigan, 2010). Hence, children are especially

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vulnerable to environmental neurotoxic compounds, including certain metals (Zhou et al., 2019). Current evidence suggests that exposure to environmental chemicals plays a major role in the so-called "silent pandemic of neurodevelopmental toxicity", *i.e.,* the rising incidence of behavioral and cognitive problems in children and adolescents, including autism spectrum disorders (ASDs) and attention-deficit hyperactivity disorder (ADHD) (Bellinger, 2009; Grandjean and Landrigan, 2014). However, although developmental susceptibility to environmental chemicals may extend into adolescence, the potential adverse health effects of environmental exposure in this age group have not been fully elucidated (Mustieles et al., 2020; Pfeifer and Allen, 2021).

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophic family, has been associated with a wide range of neuropsychological processes, including neuro- and glio-synaptogenesis, synaptic plasticity, and neurite growth, among others (Kowiański et al., 2018; Sasi et al., 2017). This is largely explained by the characteristic pattern of BDNF synthesis, in which several biologically active isoforms interact with multiple receptors, thereby triggering, upregulating, or downregulating numerous signaling pathways (Kowiański et al., 2018). BDNF has been proposed as a biomarker of effect for brain functioning, allowing the exploration of potential causal pathways between exposure to particular endocrine disruptors (e.g., metals, bisphenol A, polycyclic aromatic hydrocarbons) and neurobehavioral outcomes in epidemiological studies (Kalia et al., 2017; Kundakovic et al., 2015; Mustieles et al., 2020; Perera et al., 2015; Tang et al., 2014). Exposure of humans to the neurotoxic environmental metals mercury (Hg) (particularly methyl-Hg), cadmium (Cd), lead (Pb), and arsenic (As) has been associated with disturbances in the pattern of BDNF synthesis, mainly detected as alterations in serum concentrations of total BDNF (Karim et al., 2019; Spulber et al., 2010; Y. Wang et al., 2016; Zhou et al., 2019; Zou et al., 2014). However, the biological meaning of BDNF gene DNA methylation patterns remain poorly understood. In a mouse study, Kundakovic et al. reported that blood BDNF gene methylation levels at six CpGs reflected the methylation profile and transcription levels in the hippocampus; they suggested that blood BDNF DNA methylation levels might be a surrogate marker of brain BDNF expression in humans (Kundakovic et al., 2015).

Humans are simultaneously exposed to multiple environmental chemicals. There are particular concerns about metallic/metalloid elements, which are ubiquitous in the environment, given that some of them are known to be neurodevelopmental toxicants, even at very low doses (Grandjean and Landrigan, 2006; Jakubowski, 2011; Rodríguez-Barranco et al., 2016; Schoeman et al., 2009). These elements are frequently detected in human urine, blood, and hair samples (Gil and Hernández, 2015). Chronic exposure of humans to As, Cd, Hg, and Pb has been implicated in various adverse effects (ATSDR 2020; 2016, 2012, 1999), and epidemiologists have increasingly addressed the effects of this exposure on neurodevelopment in relation to anxiety, depression, Alzheimer's disease, and ASD, among others (Freire et al., 2018; Jaishankar et al., 2014; Long et al., 2019; Mravunac et al., 2019; Sanders et al., 2014; Shah-Kulkarni et al., 2020; Yousef et al., 2011; Zhou et al., 2019). However, the behavioral effects of environmental metal exposure remain controversial, in part because fully standardized instruments are not available to assess the behavioral functioning of children and adolescents (Ciesielski et al., 2012; Khan et al., 2011; Lucchini et al., 2012; Roberts et al., 2013; Sanders et al., 2015).

Effect biomarkers based on toxicologic findings have been identified by the Human Biomonitoring for Europe Project (HBM4EU) after comprehensive searches of the literature (Baken et al., 2019; Mustieles et al., 2020; Steffensen et al., 2020). The most promising biomarkers are being tested in several European cohorts to assess their value as indicators of the potential adverse effects of environmental chemicals. BDNF has been highlighted as a brain development marker that might complement neuropsychological tests (Mustieles et al., 2020). The hypothesis of the present study was that BDNF is involved in the causal pathway between metal exposure and adverse effects on behavioral function and therefore serves as an adequate epidemiological biomarker to evaluate exposure-mediator-effect relationships. The study objectives were therefore: to assess the relationship between exposure to As, Cd, Pb, and/or Hg and behavioral functioning in adolescent males; and to investigate the role of the BDNF biomarker measured at two levels of biological organization (BDNF gene DNA methylation and serum protein concentration).

2. Material and methods

2.1. Study population

This study is part of the *INMA*-INfancia y Medio Ambiente (Environment and Childhood) Project, a multicenter population-based birth cohort study designed to investigate the effects of environmental exposures and diet during pregnancy and early life on fetal, child, and adolescent development in different parts of Spain (Guxens et al., 2012). The INMA-Granada cohort recruited 668 mother-son pairs in Granada, Southern Spain, in 2000–2002 (Fernandez et al., 2007). Randomly selected adolescents from the baseline cohort were re-contacted to seek their participation in clinical follow-ups at the ages of 4–5 (n = 220, 32.9%) and 9–11 years (n = 298, 44.6%). Participants who attended both follow-up sessions (n = 269) were invited to participate in the most recent follow-up at the age of 15–17 years (2017–2019) Agreement was obtained from 151 (56.13%) of these, who underwent physical examinations at the Pediatrics Unit of our third-level university hospital in Granada (Castiello et al., 2020). All 151 participants provided a urine sample, and 135 of them also provided a blood sample. The present study included the adolescents with available data on urinary metal concentrations, behavioral outcomes, serum total BDNF protein concentrations, and relevant covariates (n = 125); information on BDNF gene DNA methylation patterns was also available for 113 of these adolescents (see Fig. 1). The parents/guardians of the adolescents signed informed consent to their participation in the study, which was approved by the Biomedical Research Ethics Committee of Granada (Spain).

2.2. Analysis of urinary metal concentrations

A single spot urine sample was collected from the first morning void of each participant on the day of their hospital visit. Samples were stored at −80 °C until analysis. Urinary concentrations of total (both organic and inorganic) As, Cd, Hg, and Pb were measured at the laboratory of the Department of Legal Medicine, Toxicology and Physical Anthropology, University of Granada, using inductively coupled plasma mass spectrometry with an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA) as previously described (Castiello et al., 2020). Quality control and quality assessment procedures included spiked samples with 400 μg/L of a multielement internal standard solution with Sc, Ge, Ir, and Rh; intermediate calibration standards; blanks; and the following certified reference materials (US National Institute of Standards and Technology): Trace Elements in Natural Water Standard Reference Material SRM 1640a and Seronorm (Sero, Billingstad, Norway), and Trace Elements Urine L1 and L2 (references 210605 and 210705, respectively)]. Limits of detection (LODs) were 0.60 μg/L for As, 0.01 μg/L for Cd, 0.05 μg/L for Hg, and 0.16 μg/L for Pb (Supplementary Material, Table S1). Urinary creatinine was measured by the Jaffle method in a Roche Cobas C-311 system using a commercial kit (Creatinine Jaffé Gen 2, CREJ2) and expressed as mg/dL.

2.3. Serum BDNF and whole blood BDNF gene DNA methylation

Peripheral venous blood samples were drawn from participants under non-fasting conditions between 5 p.m. and 7 p.m. on the same day as the collection of the urine sample. Blood samples were immediately processed to obtain serum and whole blood aliquots, which were

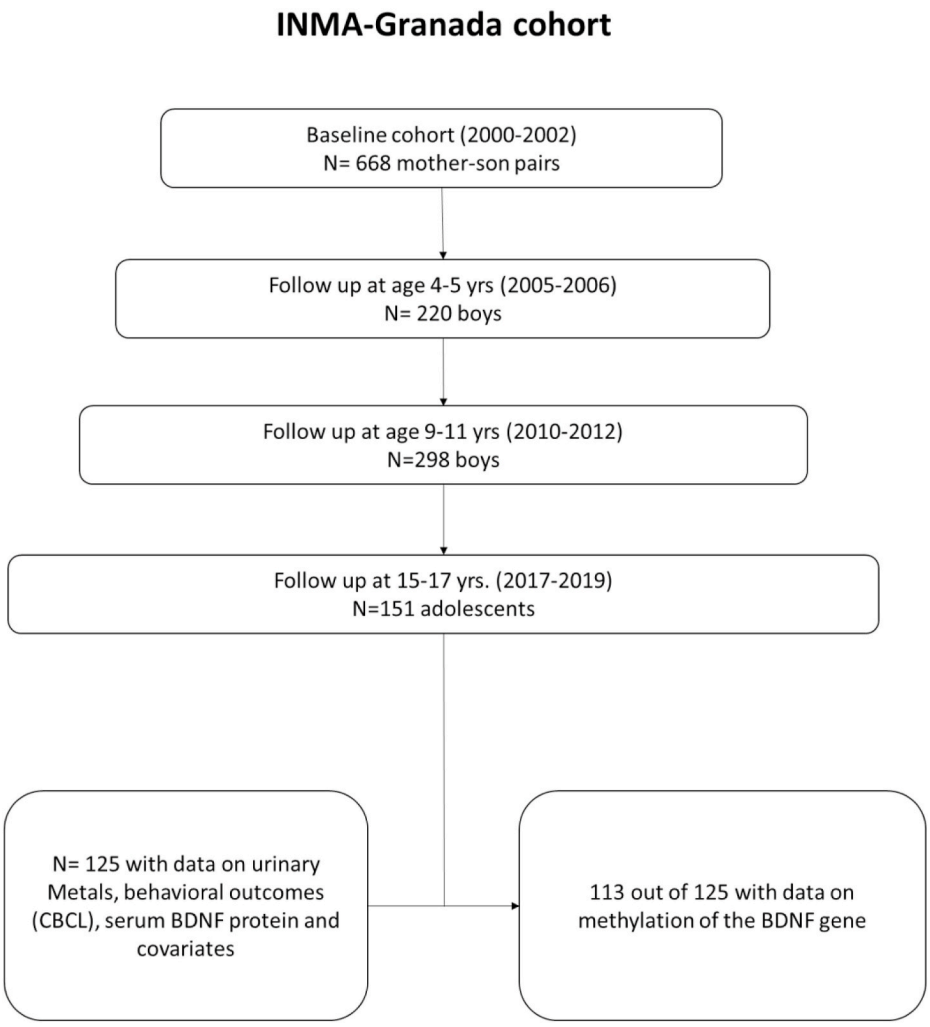

Fig. 1. Flow-chart showing the time-line of follow-ups conducted in the INMA-Granada cohort and the final sample of 15–17-year-old adolescents in the present study.

subsequently stored at −80 C°. Whole blood was sent in dry ice to the Human Genotyping Laboratory at the Spanish National Cancer Research Center, where genomic DNA was extracted using Maxwell® RSC equipment, quantified by PicoGreen assay, and diluted to 50 ng/μL. Extracted DNA was always stored at −80 °C until use.

Total serum BDNF concentrations (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 Biomedical Research Center (CIBM), Granada, Spain. Briefly, samples were defrosted, vortexed, aliquoted in 10 μL, and diluted 100-fold. Next, 50 μL of diluted sample was tested in duplicate, placed in a 96-well plate coated with an anti-BDNF monoclonal antibody, and incubated at room temperature for 2 h. The plate was then washed four times with 400 μL wash buffer solution, followed by the addition of 200 μL BDNF-specific monoclonal antibody in each well. The plate was incubated for 1 h at room temperature and then washed as described above. Finally, 200 μL of a mixture containing stabilized hydrogen peroxide and tetramethylbenzidine was added to each well, and the plate was incubated for 30 min at room temperature protected from light. Then, 50 μL of Stop Solution (sulfuric acid) was added to each well, and samples were immediately read by luminometry at 450 nm wavelength. Serum total BDNF protein concentrations had intra- and inter-assay coefficients of variation of <5% and 15%, respectively.

DNA methylation of the BDNF gene was determined by bisulfite pyrosequencing analysis at the IRSET (Institut de Recherche en Santé, Environnement et Travail - INSERM UMR1085) in Rennes (France), as described in detail elsewhere (Mustieles et al., 2022). Genomic DNA levels were quantified using the QuantiFluor dsDNA system (Promega E2670). Successively, 500 ng of genomic DNA was bisulfite converted (BS) with Epitect Fast Bisulfite Conversion kit (Qiagen, 59826), and the concentration and purification were then remeasured with NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode). Downstream PCR amplification (Biometra TProfessional Thermoycler, France) was performed using BDNF primers, 20 ng of BS-converted DNA, and Takara EpiTaq hot-start DNA polymerase at a final concentration of 0.6 U/25 μL (Takara, R110A) under the following conditions: initial denaturation at 98 °C for 30 s denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, running a total of 40 cycles. Primers used for BDNF amplification (0.4 μM final concentration) are reported in Table S1, and the reverse primer was biotinylated. Exon IV of BDNF was the target region (genomic coordinates: chr11:27,723,070–27,723,280 retrieved from UCSC Genome Browser Human February 2009 (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). After PCR amplification, the products were purified using the MinElute PCR purification kit (Qiagen, 28006) and then loaded on a 2% agarose gel to ensure amplification of a single BDNF product. Samples were sent to the LIGAN (Lille Integrated Genomics Advanced Network for personalized medicine) Genomic Platform in Lille (France) for pyrosequencing using

Pyromark Q4 Advanced Pyrosequencing technology. The methylation level at each CpG was expressed as percentage DNA methylation.

2.4. Behavioral functioning assessment

The validated Spanish version of the Child Behavior Checklist (CBCL/6–18) was used to evaluate the behavioral function of the participants (Sardinero García et al., 1997). This questionnaire was completed by the parents/guardians of each participant in relation to his behavior during the previous six months (Achenbach and Rescorla, 2013; Sardinero García et al., 1997). The CBCL contains 118 items rated on a three-point scale (not true, somewhat true, very/often true) and grouped in the following eight syndrome scales: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. These scales are summarized in three composite scales: internalizing problems (sum of anxious/depressed, withdrawn/depressed, and somatic complaints scale scores); externalizing problems (sum of rule-breaking behavior and aggressive behavior scale scores); and total problems (reported as sex and age-normalized T-scores). A higher scale score always indicates more behavioral problems (Achenbach and Rescorla, 2013).

2.5. Covariates

Data on sociodemographic characteristics and lifestyle factors were collected by administering *ad hoc* questionnaires to the participants and their parents/guardians. The weight, height, and body mass index (BMI) of participants were measured following standardized procedures, extensively detailed in Castiello et al. (2020). Covariates used in the present study included data collected at the 15- to 17-year-old follow up visit on the characteristics of the adolescents: age (in months, continuous), area of residence (categorized as urban or sub-urban/rural), annual family income (<25000, 25000–35000, or >35000 €), and passive smoking (yes or no); on the characteristics of their mothers: age (in years, continuous), intelligence (verbal reasoning measured by the similarities subtest of WAIS-III at the 9- to 10-year-old follow-up), marital status (stable partner: yes or no), schooling (up to primary, secondary, or university), current employment status (employed or unemployed) and alcohol consumption (yes or no). The adolescents also completed a validated food frequency questionnaire to obtain information on their overall fish consumption (monthly intake of <3 portions, 3–5 portions, or >5 portions) (Notario-Barandiaran et al., 2020).

2.6. Statistical analysis

Descriptive analyses were performed to summarize the sociodemographic and lifestyle characteristics of the study participants. Urine samples with undetected levels of As, Cd, Hg, and Pb were assigned a value of LOD/$\sqrt{2}$. Detection frequencies and/or percentiles were calculated for raw urinary metal concentrations (μg/L) and the effect biomarkers, i.e., serum BDNF and percentage DNA methylation at 6 CPIs of Exon-IV from the BDNF gene. Spearman's correlation analysis was conducted to assess relationships between metal levels concentrations, expressed as μg/L.

Multivariate linear regression models were performed for: i) the association of metal exposure with behavioral outcomes; ii) the association of metal exposure with the BDNF biomarkers of effect (serum BDNF and methylation profile of the BDNF gene) and iii) the association of these biomarkers with behavioral outcomes.

Urinary metal concentrations were left-skewed and therefore modeled as (natural) log-transformed variables. Associations with metals and effect biomarkers were considered as continuous variables and also categorized in tertiles to investigate possible non-linear relationships. Next, generalized additive models (GAM) were constructed for a more precise assessment of non-linear associations between metal exposure and behavioral outcomes. Confounders were carefully selected based on: i) substantive knowledge supporting their relevance for neurodevelopment and/or metals exposure; ii) their use in previous epidemiological studies; and iii) change in regression coefficient (beta) by more than 10%. Thus, two adjusted models were performed for all exposure-effects analyses. First model (Model 1) was adjusted for the age and BMI of adolescents, given that the age determines the stage of brain development and BMI is known to have an important impact on children's behavior (Hughes et al., 2020; Richards and Xie, 2015). We used unadjusted urinary metal concentrations and urinary creatinine concentrations as separate independent variables in accordance with previous observations reporting that this is a better approach to control for measurement error bias due to variability of urine concentrations (Barr et al., 2005; O'Brien et al., 2016). Because multiple metals may simultaneously affect behavioral functioning, regression models were mutually adjusted for all metals. In models with continuous exposure variable, all metals were introduced as continuous variables, whereas in models with categorical exposure variable, all metals were introduced categorized into tertiles. This approach was performed given that our sample size was not large enough to conduct advanced analysis of mixture effects. Model 1 was further adjusted by maternal schooling and intelligence, since these variables have their own influence on neurodevelopment and have been also extensively used in epidemiological studies evaluating neurodevelopmental outcomes (Patra et al., 2016; Wirt et al., 2015). Model 2 (fully-adjusted) was additionally controlled for adolescents' passive smoking and fish intake. Second-hand tobacco is a potential source of exposure to heavy metals, especially for Cd (Campbell et al., 2014; Navas-Acien, 2018; Spulber et al., 2010), while tobacco smoke has been negatively associated with the neurodevelopment of children (Chen et al., 2013; Lee et al., 2011; Spulber et al., 2010). Fish consumption was included because it is a major source of exposure to As, Hg, and Pb, although it has also been positively associated with neurodevelopment due to its content of fatty acids such as omega-3 (Gil and Gil, 2015; Mozaffarian and Rimm, 2006). Finally, multicollinearity was assessed in all regression models by calculating the variance inflation factor (VIF). Additionally, a sensitivity analysis was performed including one single element at a time to test the consistency across models. Associations showing $p < 0.05$ were considered significant. Nevertheless, given the relative small sample size, statistical significance was additionally evaluated based on internal validity, coherence and previous toxicological and epidemiological evidence of the observed associations (Amrhein et al., 2019). SPSS v26.0 (IBM, Chicago, IL) and R statistical software version 3.4.3 were used for data analyses.

3. Results

3.1. Descriptive analyses

Table 1 displays the general characteristics of the study participants and their mothers. The mean (standard deviation - SD) age of the adolescents was 16.9 (0.4) years and their mean BMI was 23.33 (4.99) kg/m^2. Almost three-quarters of the participants lived in urban areas, just under half were passive smokers, around one-third reported a monthly fish intake of less than 3 portions, and just over one-third had a family income of 25000–35000 €/year. Their mothers had a mean age of 39.5 years, almost all had a stable partner, just under one-third had a university education, more than three-quarters were employed, and around half of them regularly consumed alcohol (Table 1).

All urine samples contained quantifiable concentrations of As (median = 24.20 μg/L), 98.5% contained concentrations of Cd (median = 0.08 μg/L), 97.0% concentrations of Hg (median = 0.76 μg/L), and 89.5% concentrations of Pb (median = 0.42 μg/L) (Table 2). Significant positive correlations were found between As and Cd, Hg and Pb concentrations (Spearman's rho = 0.22, 0.52 and 0.19, respectively) and between Cd with Hg and Pb concentrations (Spearman's rho = 0.52 and

Table 1
General characteristics of study participants (n = 125).

Variables	Mean ± SD or n (%)
Adolescents	
Age (years)	16.6 ± 0.4
BMI (kg/m^2)	23.6 ± 5.2
Creatinine (mg/dL)	184.6 ± 57.6
Area of residence	
Urban	96 (72.2)
Sub-urban/rural	37 (27.8)
Passive smoking	
Yes	55 (41.4)
No	76 (57.1)
Fish consumption	
<3 portions per month	45 (33.8)
3–5 portions per month	40 (30.1)
>5 portions per month	43 (32.3)
Mothers	
Age (years)	39.6 ± 4.7
Schooling	
Up to primary	50 (40.0)
Secondary	44 (35.2)
University	31 (24.8)
Occupational status	
Employed	78 (62.4)
Unemployed	47 (37.6)
Marital status	
Stable partner	115 (92.0)
No stable partner	10 (8.0)
Alcohol consumption	
Yes	65 (52.0)
No	60 (48.0)
Annual family income (euros)	
<25000	48 (36.1)
25000-35000	59 (44.1)
>35000	29 (21.8)
Verbal reasoning*	15.6 ± 5.1

SD: Standard deviation; BMI: Body mass index.
*Verbal reasoning measured by similarities subtest of WAIS-III at 9-11-year follow-up.

0.36, respectively) but not between Hg and Pb concentrations (Supplementary Material, Table S2). The median concentration of serum BDNF was 32.6 ng/mL and the median percentage DNA methylation values for CpGs 1 to 6 were: 4.5%, 3.2%, 3.2%, 5.7%, 3.2%, and 2.4%, respectively; the median percentage total CpG methylation was 3.8% (Table 2). The distribution of CBCL T-scores is exhibited in Supplementary Material (Table S3). Globally, there was a lower prevalence of externalizing problems (16%) than of internalizing problems (32%) in this study population.

3.2. Metal exposure and adolescents' behavior

Table 3 displays the associations between tertiles of urinary metal concentrations and CBCL T-scores. The overall patterns pointed towards a non-linear relationship of urinary As and Cd concentrations with behavioral problems, with As exposure being associated with more internalizing problems, such as anxiety, somatic and thought problems; and Cd with more externalizing problems, such as social, attention problems and aggressive behavior. Further, second and third tertiles (intermediate and high levels) of urinary As and Cd levels were associated with greater anxiety and more somatic complaints, aggressive behaviors, and social and internalizing problems; however, some of these associations did not reach the statistical significance. These associations persisted but some were attenuated after adjustment for passive smoking and fish intake (Table 3). GAM analyses confirmed the presence of non-linear relationships for As and Cd (Supplementary Material, Figs. S1 and S2). Both Hg and Pb exposure showed associations with lower CBCL scores in several subscales, especially withdrawn, somatic complaints, and social and internalizing problems, which remained after adjustment for passive smoking and fish intake (Table 3).

Models considering continuous urinary metal concentrations showed associations of Cd with more social problems and aggressive behavior, although these relationships did not reach the statistically significance. However, Hg concentrations were significantly associated with fewer social problems (Table S4). The VIF was below 1.5 for each independent variable, ruling out multicollinearity. Finally, sensitivity analyses not showed substantial differences between models, neither in the direction of associations, and neither in overall patterns (Table S7).

3.3. Metal exposure and BDNF

The overall pattern showed lower serum BDNF levels across tertiles of urinary As and Cd concentrations, with significant associations between Cd and serum BDNF and between As and CpG5 and total CpGs methylation percentages. However, these associations were not observed for Hg and Pb (Table 4).

In relation to the BDNF gene methylation profile, concentrations of As in the third tertile (higher level) were associated with higher percentage methylation at CpGs #4, 5, and 6 and for total CpGs, with statistically significant results for CpG 5 and total DNA methylation. Associations were also found between urinary Cd in the second *versus* first tertile and lower BDNF gene methylation at CpGs #2 and 3 (Table 4). In the fully-adjusted model, the aforementioned associations remained and some became stronger (Table 4). Urinary Pb concentrations were positively but non-significantly associated with higher DNA methylation patterns, while urinary Hg showed associations with decreasing BDNF gene DNA methylation (Table 4). Models with continuous data showed non-significant associations of As with higher total CpG methylation and of Pb with higher methylation at CpG 1, whereas Cd was negatively but also non-significantly associated with methylation at CpG3 (Table S5). VIF values for each independent variable in all models were <1.5, ruling out multicollinearity. Sensitivity analyses showed an attenuation of associations between As and BDNF DNA methylation percentages, however, direction and overall tendencies were still observed (Table S8).

3.4. BDNF and adolescents' behavior

Continuous serum BDNF concentrations suggested association with a

Table 2
Distribution of urinary metal concentrations (μg/L), serum BDNF concentrations (ng/mL), and percentage of BDNF gene DNA methylation values at six CpGs (%).

		As		Cd		Hg		Pb	
% Detection (n = 125)		100		98.5		97.0		89.5	
Percentiles	**25**	8.26		0.05		0.29		0.27	
	50	24.20		0.08		0.76		0.42	
	75	44.07		0.12		1.02		0.70	
		Serum BDNF	**CpG1**	**CpG2**	**CpG3**	**CpG4**	**CpG5**	**CpG6**	**Total CpGs**
n		125	111	113	113	105	108	101	112
Percentiles	**25**	25.41	3.82	2.90	2.84	5.35	2.69	2.02	3.45
	50	32.59	4.46	3.20	3.21	5.70	3.16	2.34	3.77
	75	39.40	4.87	3.51	3.64	6.32	3.67	3.12	4.06

Table 3
Adjusted models for the association between tertiles of urinary metal concentrations and CBCL scores (n = 125).

CBCL scores	Model 1				Model 2			
	As tertiles (μg/g)							
	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	53.9 (4.9)	3.38 (0.39;6.37)*	1.71 (−1.34;4.76)	0.32	54.0 (4.9)	4.0 (0.87;7.13)**	1.77 (−1.31;4.84)	0.34
Withdrawn	57.0 (6.1)	0.83 (−2.64;4.29)	1.15 (−2.38;4.69)	0.52	57.0 (6.2)	0.95 (−2.68;4.57)	1.44 (−2.12;4.99)	0.43
Somatic complaints	55.2 (6.1)	5.77 (2.08;9.46)**	3.06 (−0.70;6.82)†	0.15	55.3 (6.2)	5.58 (1.66;9.50)**	2.86 (−0.99;6.70)†	0.20
Social problems	55.0 (5.8)	0.79 (−2.2;3.78)	1.34 (−1.71;4.39)	0.38	54.8 (5.8)	1.1 (−2.05;4.24)	1.49 (−1.59;4.57)	0.34
Thought problems	53.3 (4.3)	2.61 (−0.25;5.46)†	1.43 (−1.49;4.34)	0.37	53.2 (4.3)	2.56 (−0.45;5.57)†	1.36 (−1.60;4.31)	0.42
Attention problems	54.6 (4.6)	2.48 (−0.36;5.31)†	0.65 (−2.25;3.54)	0.72	54.5 (4.6)	2.58 (−0.43;5.60)†	0.60 (−2.36;3.55)	0.79
Rule-breaking behavior	54.6 (5.8)	−0.53 (−3.16;2.11)	−1.29 (−3.98;1.41)	0.34	54.7 (5.9)	−0.31 (−2.95;2.34)	−1.34 (−3.94;1.25)	0.29
Aggressive behavior	55.7 (6.3)	0.22 (−2.81;3.24)	0.18 (−2.90;3.27)	0.91	55.5 (6.3)	1.01 (−2.09;4.12)	0.35 (−2.7;3.40)	0.85
Composite scores								
Internalizing problems	52.4 (10.4)	5.28 (0.25;10.31)*	4.16 (−0.98;9.29)†	0.13	52.4 (10.6)	5.87 (0.52;11.22)*	4.43 (−0.82;9.68)†	0.12
Externalizing problems	52.2 (9.9)	0.59 (−4.14;5.32)	−0.54 (−5.37;4.29)	0.81	52.0 (10.0)	1.96 (−2.82;6.74)	−0.22 (−4.91;4.47)	0.87
Total problems	52.3 (9.7)	3.12 (−1.52;7.76)	1.98 (−2.76;6.71)	0.43	52.1 (9.8)	3.88 (−0.97;8.73)†	2.20 (−2.56;6.96)	0.41
CBCL scores	Cd tertiles (μg/g)							
	1st (0.03–0.05)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend	1st (0.01–0.03)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.2 (5.2)	0.29 (−2.91;3.48)	0.89 (−2.22;4.01)	0.56	55.2 (5.2)	0.67 (−2.66;4.0)	1.37 (−1.88;4.61)	0.40
Withdrawn	56.9 (6.8)	1.71 (−1.89;5.32)	0.94 (−2.58;4.45)	0.63	56.9 (6.8)	1.57 (−2.15;5.28)	1.37 (−2.25;4.99)	0.47
Somatic complaints	59.6 (8.1)	−0.05 (−4.05;3.94)	−3.42 (−7.31;0.47)†	0.07	59.6 (8.1)	0.48 (−3.67;4.63)	−2.78 (−6.83;1.26)	0.160
Social problems	53.4 (4.8)	4.85 (1.85;7.85)*	3.05 (0.13;5.98)*	0.06	53.4 (4.8)	4.50 (1.37;7.63)**	2.85 (−0.20;5.90)†	0.10
Thought problems	53.7 (4.9)	1.83 (−1.19;4.85)	1.98 (−0.97;4.92)	0.06	53.7 (4.9)	2.38 (−0.75;5.50)†	2.47 (−0.57;5.51)†	0.12
Attention problems	54.5 (5.1)	2.84 (−0.11;5.79)†	0.66 (−2.22;3.53)	0.19	54.5 (5.1)	2.94 (−0.15;6.03)†	0.77 (−2.23;3.78)	0.69
Rule-breaking behavior	54.4 (5.6)	−1.10 (−3.83;1.67)	−0.56 (−3.24;2.12)	0.71	54.4 (5.6)	−0.70 (−3.42;2.01)	0.12 (−2.52;2.76)	0.90
Aggressive behavior	53.9 (5.61)	2.28 (−0.88;5.44)	3.97 (0.89;7.05)*	0.01	53.9 (5.6)	2.56 (−0.64;5.76)†	4.26 (1.14;7.38)**	0.01
Composite scores								
Internalizing problems	55.6 (10.3)	1.54 (−3.77;6.84)	−0.82 (−5.99;4.36)	0.72	55.6 (10.3)	1.65 (−3.92;7.22)	−0.19 (−5.62;5.24)	0.91
Externalizing problems	50.6 (9.4)	1.87 (−3.08;6.82)	3.18 (−1.64;8.01)	0.19	50.6 (9.4)	2.37 (−2.57.7.32)	3.87 (−0.95;8.68)†	0.11
Total problems	52.5 (9.0)	3.57 (−1.26;8.40)	1.66 (−3.05;6.37)	0.53	52.5 (9.0)	3.84 (−1.15;8.83)†	2.21 (−2.65;7.07)	0.41
CBCL scores	Hg tertiles (μg/g)							
	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.9 (5.8)	−1.02 (−4.16;2.13)	−2.38 (−5.83;1.07)	0.17	56.3 (5.8)	−1.29 (−6.29;0.95)	−2.67 (−6.29;0.95)	0.14
Withdrawn	58.8 (8.2)	−3.04 (−6.59;0.51)†	−4.15 (−8.25;-0.26)*	0.04	59.0 (8.4)	−2.94 (−6.58;0.70)†	−4.57 (−8.62;-0.53)*	0.03
Somatic complaints	59.3 (7.8)	−3.36 (−7.22;0.51)†	−0.51 (−4.75;3.73)	0.87	59.5 (8.0)	−3.79 (−7.79;0.21)†	−1.25 (−5.70;3.20)	0.65
Social problems	56.6 (6.7)	−2.56 (−5.63;0.51)†	−5.37 (−8.73;-2.00)**	<0.001	56.3 (6.8)	−1.9 (−5.06;1.25)	−4.68 (−8.20–1.17)**	0.01
Thought problems	54.4 (5.1)	−0.73 (−3.71;2.25)	−1.61 (−4.88;1.64)	0.32	54.5 (5.3)	−1.10 (−4.17;1.97)	−2.35 (−5.76;1.07)	0.17
Attention problems	55.8 (5.3)	0.28 (−2.67;3.23)	−1.65 (−4.89;1.59)	0.29	55.5 (5.4)	0.49 (−2.58;3.56)	−1.51 (−4.92;1.91)	0.35
Rule-breaking behavior	54.9 (5.96)	−1.85 (−4.55;0.84)	−1.31 (−4.27;1.64)	0.40	55.1 (6.1)	−1.99 (−4.63;0.65)†	−1.77 (−4.71;1.17)	0.25
Aggressive behavior	56.0 (6.1)	−1.06 (−4.16;2.05)	−3.26 (−6.67;0.14)†	0.05	56.0 (6.2)	−0.94 (−4.06;2.19)	−3.28 (−6.76;0.20)†	0.06
Composite scores								
Internalizing problems	56.4 (10.9)	−3.18 (−8.39;2.04)	−2.51 (−8.23;3.21)	0.40	56.8 (11.1)	−3.20 (−8.64;2.25)	−2.95 (−9.01;3.11)	0.35
Externalizing problems	52.7 (9.66)	−1.57 (−6.43;3.30)	−2.47 (−7.81;2.87)	0.36	53.0 (9.7)	−1.47 (−6.30;3.37)	−2.74 (−8.12;2.64)	0.31
Total problems	54.6 (9.5)	−2.78 (−7.58;2.01)	−3.79 (−9.05;1.47)	0.15	54.7 (9.7)	−2.58 (−7.51;2.35)	−3.99 (−9.47;1.49)	0.15
CBCL scores	Pb tertiles (μg/g)							
	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
Syndrome scores								
Anxious depressed	55.5 (6.3)	0.31 (−2.7;3.31)	−2.28 (−5.25;0.69)†	0.14	55.7 (6.3)	0.20 (−2.92;3.33)	−2.30 (−5.42;0.82)	0.15
Withdrawn	57.4 (5.1)	−0.86 (−4.28;2.56)	−1.04 (−4.42;2.35)	0.54	57.4 (5.1)	−0.69 (−4.20;2.83)	−1.26 (−4.77;2.25)	0.48
Somatic complaints	60.3 (7.6)	−4.54 (−8.27;-0.81)*	−3.31 (−7.01;0.38)†	0.07	60.5 (7.6)	−4.62 (−8.50;-0.74)*	−3.37 (−7.24;0.51)†	0.09
Social problems	55.0 (5.7)	−0.17 (−3.12;2.78)	−0.76 (−3.68;2.16)	0.60	54.8 (5.7)	0.58 (−2.47;3.62)	−0.43 (−3.48;2.61)	0.78
Thought problems	54.8 (5.0)	−0.17 (−3.03;2.70)	−0.94 (−3.77;2.01)	0.51	54.8 (5.1)	−0.06 (−3.02;2.90)	−0.74 (−3.70;2.22)	0.62
Attention problems	55.9 (5.5)	−1.14 (−3.99;1.71)	−1.55 (−4.37;1.28)	0.27	55.8 (5.5)	−0.75 (−3.73;2.23)	−1.34 (−4.32;1.64)	0.37
Rule-breaking behavior	53.6 (4.4)	−0.27 (−2.88;2.33)	0.91 (−1.67;3.48)	0.49	53.7 (4.5)	0.31 (−2.25.2.88)	1.48 (−1.08;4.04)	0.25
Aggressive behavior	55.6 (5.7)	−0.11 (−3.10;2.88)	−0.84 (−3.8;2.12)	0.57	55.4 (5.7)	0.67 (−2.35;3.68)	0.01 (−3.00;3.03)	0.99
Composite scores								
Internalizing problems	57.5 (8.2)	−4.59 (−9.59;0.41)†	−4.19 (−9.14;0.76)†	0.09	57.7 (8.2)	−4.50 (−9.75;0.74)†	−4.32 (−9.57;0.92)†	0.10
Externalizing problems	52.7 (7.5)	−1.89 (−6.56;2.78)	−1.30 (−5.92;3.33)	0.57	52.5 (7.5)	−0.70 (−5.36;3.97)	0.05 (−4.62;4.71)	0.98
Total problems	55.3 (7.2)	−3.3 (−7.89;1.30)	−3.24 (−7.79;1.31)	0.15	55.2 (7.3)	−2.43 (−7.20;2.32)	−2.55 (−7.30;2.19)	0.28

Model 1: adjusted for adolescent's age and BMI, maternal schooling and intelligence, and for all metals simultaneously.
Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.
For all subscales, higher score indicates more behavioral problems.
**p < 0.0; *p < 0.05; †p < 0.10.

lower score for the withdrawn subscale (β = −0.12, 95%CI = −0.27, 0.03) (Fig. 2A). Lower scores were obtained by adolescents in the second and third tertiles of serum BDNF concentrations than by those in the first (lowest) tertile in the withdrawn [(β_{T2} = −3.77, 95% CI = −7.00;-0.53), (β_{T3} = −3.49, 95% CI = −6.95; −0.02)], social problems (β_{T3} = −2.52, 95% CI = −5.69,0.65, and thought problems (β_{T3} = −2.88, 95% CI = −5.78; 0.01) subscales, observing a significant linear trend for both withdrawn (p-trend = 0.04) and thought problems (p-trend = 0.04) (Table S6). A lower score in the total problems scale was observed in participants in the second *versus* first tertile of serum BDNF concentrations (β = −3.85, 95% CI = −8.28; 0.58).

When total DNA methylation of the BDNF gene was considered as a continuous variable, no significant association was found with the behavior of the adolescents, although the percentage BDNF gene methylation appeared in general to be inversely related to the behavioral scores (Fig. 2). Similar results were obtained when tertiles of total BDNF gene DNA methylation were considered (data not shown).

4. Discussion

The results of this exploratory study among Spanish adolescent males (aged 15–17 years) suggest a relationship between urinary As and Cd exposure and behavioral problems, possibly through their effects on BDNF secretion patterns (serum BDNF protein levels and BDNF gene DNA methylation percentage). In these adolescents, intermediate urinary As and Cd concentrations were associated with more internalizing and externalizing problems, respectively. Furthermore, results suggest that serum BDNF protein concentrations were lower in adolescents exposed to moderate and high As and Cd levels. High As concentrations were also associated with increased percentage BDNF gene DNA methylation and moderate urinary Cd concentrations suggested associations with decreased BDNF gene DNA methylation percentages. Interestingly, increased serum BDNF levels were associated with fewer behavioral alterations (*i.e.,* withdrawn and social, thought, and total problems). Hg and Pb concentrations were found to be inversely related to behavioral functioning. No statistically significant relationships were found between Hg or Pb concentrations and percentage BDNF gene DNA methylation or serum BDNF protein concentrations.

4.1. Epidemiological evidence on the association of As and Cd exposure with neurobehavior

Urinary Cd concentrations were within the range reported for adolescents by the National Health and Nutrition Examination Survey (NHANES, 2009–2014) and the German Human Biomonitoring Commission (Sanders et al., 2019; Schulz et al., 2011). However, urinary As concentrations were higher in the present population. Previous epidemiological studies have assessed the potential harmful effects of postnatal exposure to As and Cd on neurobehavioral function, but the results have not been conclusive. On the one hand, two systematic reviews found no association between As exposure and behavioral outcomes in children between 5 and 15 years of age (Rodríguez-Barranco et al., 2013; Tolins et al., 2014). On the other hand, two epidemiological studies in children aged between 6 and 12 years reported that urinary As (total and inorganic) was associated with poorer attention (Rodríguez-Barranco et al., 2016) and with depressive problems (Lin et al., 2017), more in line with the present findings. In other epidemiological studies, postnatal newborn hair concentrations of Cd were associated with withdrawn and social and attention problems in 7- to 16-year-old Chinese children (Bao et al., 2009), and urinary Cd was related to worse prosocial behavior in 10-year-old children (Gustin et al., 2018). However, no significant association was found between blood Cd concentrations and more behavioral problems in children at 2, 5, or 7 years of age (Cao et al., 2009). In the present study, urinary Cd concentrations were associated with CBCL subscales for externalizing behaviors (*i.e.*, social problems and aggressive behavior) and for somatic and thought problems. These patterns seem to point towards an association of As and Cd exposure with altered behavioral functioning in adolescents.

The above comparisons with the present findings should be interpreted with caution. First, because most previous studies measured As and Cd prenatally or during early or late childhood, whereas the present study focused on adolescence. Neurological mechanisms and the susceptibility of behavioral functions to these compounds differ among developmental periods (Gore et al., 2018; Spear, 2000; Stiles and Jernigan, 2010); which may explain the absence of evidence on the association between metal exposure and behavioral domains during this period of development (Rodríguez-Barranco et al., 2013; Spear, 2000). Second, data on metal concentrations may differ according to the matrix used (*e.g.*, urine, blood, hair, or drinking water). Urine is a useful matrix for assessing chronic exposure to Cd in biomonitoring studies because of its long half-life, reflecting long-term exposure, whereas concentrations of As in urine correspond to acute exposure (Gil and Hernández, 2015). Finally, wide variations in the instruments used to assess behavioral functioning may also explain discrepancies among studies (Rodríguez-Carrillo et al., 2019).

4.2. Possible effects of As and Cd on neurobehavior through alteration of BDNF expression patterns

The suggestive association of As and Cd exposure with behavioral functioning might be explained by their binding to N-methyl-D-aspartate (NMDA) receptors in the hippocampus (Karri et al., 2016). This would lead to a reduction in BDNF concentrations and consequent behavioral and cognitive impairments, consistent with the adverse outcome pathways (AOPs) described by Mustieles et al. (2020) (Fig. 3). The hippocampus is responsible for the formation of emotional responses and the acquisition of memory and learning, which are both associated with social behavior (Ciranna, 2006). It is especially susceptible to exogenous and endogenous stressors, and the resulting changes in its structure and function can play a crucial role in the development of mood disorders (Zaletel et al., 2017).

Exposure to As may affect behavioral function through a direct action on the BDNF gene, given that As can alter DNA methylation patterns, possibly by interacting with transcription factor binding sites (TFBS) and inhibiting DNA repair mechanisms (Demanelis et al., 2019; Karim et al., 2019) (mechanisms of action shown in Fig. 3, numbers 1 and 2). This may explain the present findings of increased BDNF gene methylation in adolescents with higher urinary As concentrations (Fig. 3). The present mechanism is also consistent with experimental findings of an association between memory deficits and decreased hippocampal BDNF and CAMP responsive element binding protein 1 (CREB) in mice exposed to As (Sun et al., 2015). As can also exert an indirect effect on BDNF *via* the following pathways: first, by the inhibition of NMDA receptors, which play a key role in Ca^{+2} influx mechanisms, leading to reduced BDNF concentrations (Wang et al., 2016) (Fig. 3, number 3); second, through an imbalance of the oxidative stress homeostasis, thereby increasing reactive oxygen species (ROS) and reducing glutathione (GSH) (Karri et al., 2016; Mimouna et al., 2018), which favors cell injury or death and leads to neuroinflammation and ultimately to the degeneration of hippocampal brain cells, reducing the expression of BDNF (Karri et al., 2016) (Fig. 3, number 4); and, finally, by altering the metabolism of neurotransmitters such as GSH or serotonin, which play an important role in the expression and production of BDNF (Htway et al., 2019; Ramos-Chávez et al., 2015). For example, adult male mice prenatally exposed to As exhibited a down-regulation of BDNF expression and social isolation-like behavior, possibly mediated by an As-induced alteration of the serotonergic system (Htway et al., 2019). The present results indicate that greater exposure to As could be associated with higher DNA BDNF gene DNA methylation percentage at several CpGs. If so, it would reduce BDNF gene expression levels and protein concentrations, potentially generating more behavioral problems. Some of the associations found with BDNF gene methylation and

Table 4
Adjusted models for the association of tertiles of urinary metal concentration with serum BDNF (n = 125) and BDNF gene methylation (n = 113).

	Model 1				Model 2			
	As tertiles (μg/g)							
	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend	1st (0.58–6.19)	2nd (6.47–16.18)	3rd (16.35–465.4)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β (95% CI)	
sBDNF	35.2 (10.1)	−1.15 (−5.97;3.66)	−2.91 (−7.87;2.05)	0.25	35.1 (10.3)	−0.77 (−5.87;4.34)	−2.69 (−7.64;2.26)	0.27
metBDNF								
CpG 1	4.5 (0.6)	−0.09 (−0.37;0.55)	−0.10 (−0.58;0.37)	0.66	4.5 (0.6)	0.12 (−0.35;0.60)	−0.08 (−0.56;0.40)	0.73
CpG 2	3.1 (0.5)	0.17 (−0.11;0.45)	0.20 (−0.09;0.50)	0.16	3.1 (0.5)	0.12 (−0.18;0.41)	0.18 (−0.11;0.48)	0.22
CpG 3	3.2 (0.5)	0.15 (−0.17;0.47)	0.22 (−0.11;0.54)	0.18	3.2 (0.5)	0.10 (−0.22;0.42)	0.19 (−0.14;0.51)	0.24
CpG 4	5.8 (0.9)	0.25 (−0.39;0.90)	0.62 (−0.05;1.28)†	0.06	5.8 (0.9)	0.20 (−0.47;0.87)	0.60 (−0.10;1.27)†	0.07
CpG 5	3.0 (0.6)	0.33 (−0.09;0.76)†	0.54 (0.11;0.98)*	0.01	3.0 (0.6)	0.23 (−0.19;0.65)	0.49 (0.07;0.91)*	0.02
CpG 6	2.4 (0.5)	0.25 (−0.37;0.87)	0.74 (0.09;1.39)*	0.02	2.4 (0.8)	0.12 (−0.48;0.72)	0.67 (0.05;1.29)*	0.03
CpG t	3.6 (0.5)	0.25 (−0.09;0.58)	0.41 (0.06;0.75)*	0.02	3.7 (0.5)	0.19 (−0.15;0.52)	0.38 (0.04;0.72)*	0.02
	Cd tertiles(μg/g)							
	1st (0.01–0.03)	2nd (0.04–0.05)	3rd (0.05–0.55)	p-trend	1st (0.01–0.03)	2nd (0.04–0.05)	3rd(0.05–0.55)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β(95% CI)	
sBDNF	34.6 (9.0)	−0.78 (−5.80;4.20)	−2.14 (−7.14;2.87)	0.38	34.6 (9.0)	−1.20 (−6.42;4.02)	−3.00 (−8.25;2.25)	0.25
metBDNF								
CpG 1	4.6 (1.0)	−0.29 (−0.76;0.19)	−0.13 (−0.63;0.36)	0.62	4.6 (1.0)	−0.36 (−0.84;0.13)	−0.17 (−0.67;0.33)	0.55
CpG 2	3.4 (0.5)	−0.39 (−0.67;-0.10)*	−0.14 (−0.43;0.16)	0.43	3.4 (0.5)	−0.41 (−0.69;-0.20)*	−0.14 (−0.43;0.16)	0.46
CpG 3	3.4 (0.5)	−0.37 (−0.69;-0.10)*	−0.14 (−0.48;0.19)	0.46	3.4 (0.5)	−0.41 (−0.72;-0.10)*	−0.18 (−0.51;0.15)	0.34
CpG 4	6.1 (1.0)	−0.43 (−1.07;0.22)	0.06 (−0.62;0.74)	0.82	6.1 (1.0)	−0.43 (−1.09;0.24)	0.04 (−0.66;0.73)	0.85
CpG 5	3.3 (0.7)	−0.21 (−0.65;0.22)	0.17 (−0.28;0.62)	0.42	3.3 (0.7)	−0.22 (−0.65;0.21)	0.14 (−0.30;0.31)	0.48
CpG 6	2.6 (0.8)	0.00 (−0.67;0.67)	0.46 (−0.24;1.15)	0.16	2.6 (0.8)	0.04 (−0.62;0.69)	0.41 (−0.26;1.07)	0.20
CpG t	3.9 (0.5)	−0.29 (−0.62;0.10)†	−0.00 (−0.36;0.35)	0.95	3.9 (0.5)	−0.32 (−0.70;0.00)†	−0.05 (−0.40;0.29)	0.83
	Hg tertiles(μg/g)							
	1st (0.02–0.23)	2nd (0.23–0.48)	3rd (0.49–3.24)	p-trend	1st (0.02–0.23)	2nd(0.23–0.48)	3rd(0.49–3.24)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β(95% CI)	β(95% CI)	
sBDNF	34.9 (9.5)	−0.14 (−5.06;4.79)	−0.65 (−5.96;4.65)	0.80	34.7 (9.8)	0.34 (−4.79;5.47)	0.28 (−5.33;5.88)	0.92
metBDNF								
CpG 1	4.6 (1.0)	−0.28 (−0.73;0.17)	0.29 (−0.22;0.80)	0.31	4.6 (10.0)	−0.21 (−0.68;0.25)	0.37 (−0.17;0.91)	0.20
CpG 2	3.3 (0.5)	−0.14 (−0.43;0.14)	−0.13 (−0.45;0.20)	0.41	3.3 (0.5)	−0.17 (−0.47;0.12)	−0.15 (−0.49;0.19)	0.35
CpG 3	3.4 (0.6)	−0.16 (−0.48;0.16)	−0.20 (−0.56;0.17)	0.27	3.3 (0.6)	−0.19 (−0.51;0.14)	−0.17 (−0.54;0.20)	0.34
CpG 4	6.1 (1.1)	−0.35 (−0.99;0.29)	−0.42 (−1.16;0.32)	0.24	6.1 (1.1)	−0.43 (−1.08;0.23)	−0.46 (−1.23;0.31)	0.21
CpG 5	3.4 (0.8)	−0.39 (−0.72;0.14)	−0.46 (−0.95;0.0)†	0.06	3.3 (0.8)	−0.34 (−0.76;0.10)†	−0.44 (−0.93;0.0)†	0.06
CpG 6	2.7 (1.0)	−0.32 (−0.96;0.32)	−0.64 (−1.39;1.1)†	0.09	2.7 (1.0)	−0.40 (−1.03;0.20)	−0.56 (−1.30;0.2)†	0.12
CpG t	3.9 (0.6)	−0.19 (−0.53;0.14)	−0.20 (−0.59;0.18)	0.27	3.8 (0.6)	−0.24 (−0.57;0.09)	−0.21 (−0.59;0.17)	0.26
	Pb tertiles(μg/g)							
	1st (0.01–0.18)	2nd (0.18–0.31)	3rd (0.31–2.64)	p-trend	1st (0.01–0.18)	2nd (0.18–0.31)	3rd(0.31–2.64)	p-trend
	Mean (SD)	β (95% CI)	β (95% CI)		Mean (SD)	β (95% CI)	β(95% CI)	
sBDNF	34.1 (9.8)	−1.01 (−5.78;3.75)	0.36 (−4.34;5.06)	0.87	33.9 (9.9)	−0.72 (−5.70;4.27)	0.81 (−4.16;5.78)	0.73
metBDNF								
CpG 1	4.4 (0.7)	0.35 (−0.15;0.84)	0.18 (−0.28;0.64)	0.48	4.4 (0.7)	0.38 (−0.12;0.90)†	0.18 (−0.29;0.65)	0.51
CpG 2	3.1 (0.5)	0.23 (-0.08;0.5)†	−0.02 (−0.30;0.26)	0.78	3.1 (0.5)	0.23 (−0.07;0.50)†	−0.04 (−0.32;0.25)	0.70
CpG 3	3.2 (0.5)	0.18 (−0.17;0.52)	0.06 (−0.26;0.38)	0.75	3.2 (0.5)	0.12 (−0.22;0.46)	0.00 (−0.31;0.32)	0.96
CpG 4	5.7 (0.8)	0.46 (−0.24;1.16)	0.29 (−0.35;0.93)	0.39	5.7 (0.8)	0.42 (−0.29;1.13)	0.26 (−0.40;0.91)	0.46
CpG 5	3.2 (0.6)	0.16 (−0.31;0.62)	−0.1 (−0.52;0.33)	0.60	3.2 (0.6)	0.10 (−0.36;0.55)	−0.17 (−0.58;0.24)	0.38
CpG 6	2.5 (0.7)	0.31 (−0.40;1.01)	−0.01 (−0.64;0.63)	0.55	2.5 (0.7)	0.18 (−0.51;0.87)	−0.13 (−0.74;0.49)	0.62
CpG t	3.7 (0.4)	0.30 (−0.05;0.70)†	0.07 (−0.26;0.40)	0.78	3.7 (0.4)	0.26 (−0.09;0.60)†	0.02 (−0.30;0.34)	0.99

Model 1: adjusted for adolescent's age and BMI, maternal schooling and intelligence, and for all metals simultaneously.
Model 2: additionally adjusted for passive tobacco smoking and total fish intake of adolescents.
For all subscales, a higher score indicates more behavioral problems.
sBDNF: serum BDNF; metBDNF: BDNF gene methylation.
*$p < 0.05$; †$p < 0.10$.

protein levels may be in line with the effects described in the above animal models.

As in the case of As, the neurotoxic activity of Cd has also been implicated in the disruption of various pathways. It has been found to cross the blood-brain barrier, enter the CNS, and disrupt the hippocampal membrane function (Kumar et al., 1996; Wang and Du, 2013) (Fig. 3, number 5). In murine studies, Cd exposure was reported to inhibit acetylcholine esterase (AchE) and Na^{+}/K^{+}-ATP-ase pump, reducing neuronal activity in pups (Gupta et al., 1991), Cd-induced redox homeostasis imbalance increased neuronal death in rats (Wang and Du, 2013) (Figs. 3 and 5), and Cd was found to mimic the ubiquitous intracellular ion Ca^{+2}, thereby inhibiting its influx pathways (Xu et al., 2011) (Figs. 3 and 6). However, inadequate information is available to accurately determine whether these pathways have a direct or indirect effect on hippocampal BDNF expression. Some animal studies also found a downregulation of BDNF expression after Cd exposure (Kadry and Megeed, 2018; Mimouna et al., 2018). In the present investigation, adolescents with urinary Cd concentrations in the second tertile (intermediate level) showed associations with decreased serum BDNF concentrations and a tendency towards reduced BDNF gene DNA.

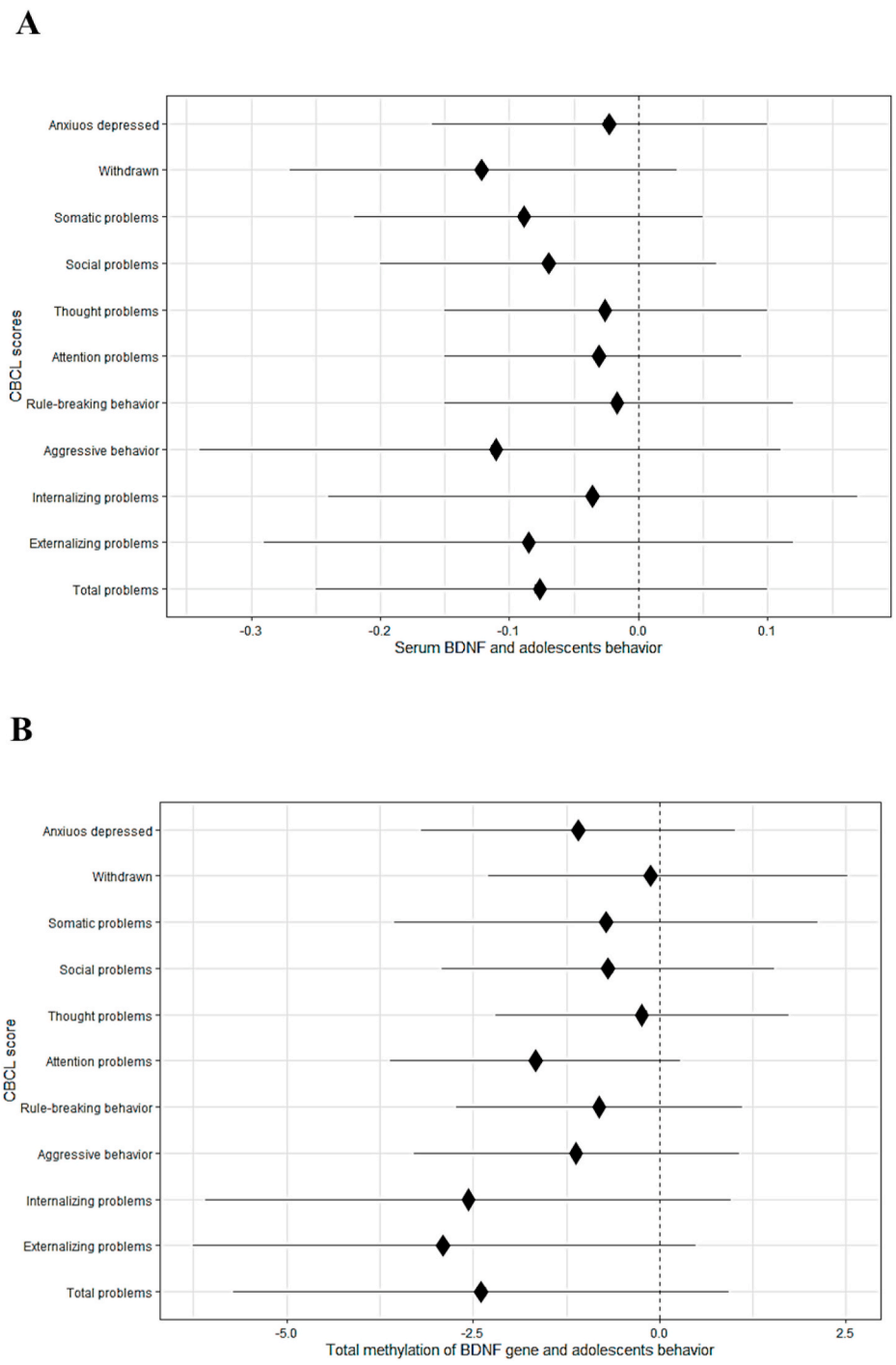

Fig. 2. Forest plot showing associations of serum BDNF concentrations (Fig. 2A) and total BDNF gene DNA methylation at six CpGs (Fig. 2B) with behavioral outcomes.

4.3. Hg and Pb exposure, adolescents' behavior and BDNF effect biomarker

Urinary Hg and Pb concentrations were also within the range described for adolescents by the National Health and Nutrition Examination Survey (NHANES, 2009–2014) and German Human Biomonitoring Commission (Sanders et al., 2019; Schulz et al., 2011). Unexpectedly, Hg and Pb concentrations were not associated with neurobehavioral problems in these adolescents. As anticipated, moderate urinary Pb concentrations tended to be associated with higher percentage DNA methylation at CpGs #1, 4 and with total CpG methylation, while urinary Hg concentrations were associated with lesser BDNF gene DNA methylation. No association was observed between serum BDNF protein concentrations and the studied metals.

Adverse effects of prenatal and postnatal exposure to Pb and Hg on cognitive function and intelligence are well documented in humans (Canfield et al., 2003; Cecil et al., 2008; Debes et al., 2006; Freire et al., 2018; Hu et al., 2006; Jusko et al., 2008; Lanphear et al., 2005; Llop et al., 2012; Wright et al., 2008). However, the potential impact of Hg and Pb on behavioral functioning remains unclear, although some studies found associations of postnatal exposure to Pb and Hg with anxiety, social problems, and ADHD (Debes et al., 2006; Liu et al., 2014;

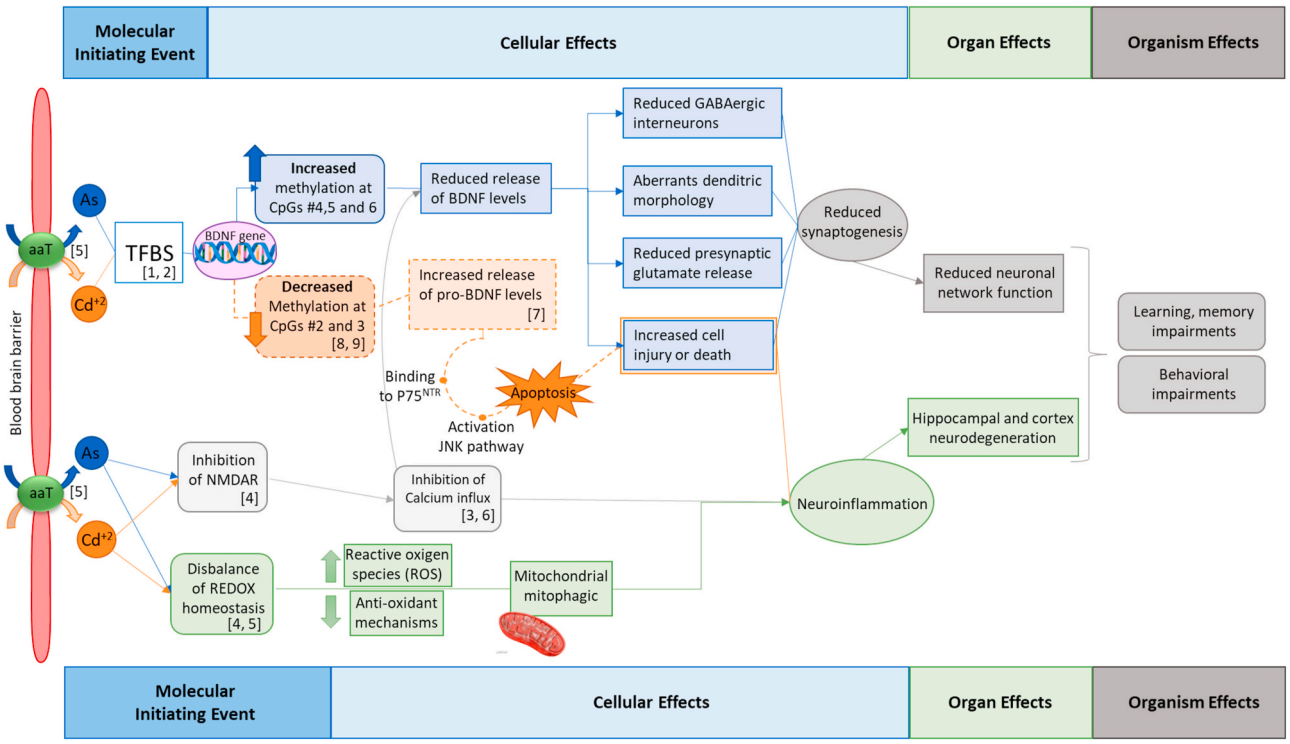

Fig. 3. Hypothesized adverse outcome pathway (AOP) based on the AOPs published by Mustieles et al. (2020) and other specific toxicological references for As (Demanelis et al., 2019; Karim et al., 2019; Karri et al., 2016; Wang et al., 2016) and Cd (Guan et al., 2019; Wang and Du, 2013; Xia et al., 2020; Xu et al., 2011; Zaletel et al., 2017). AOP followed by As and Cd in the hippocampus after crossing the blood brain barrier. As: Arsenic; aaT: amino acid transporter; BDNF: brain-derived neurotrophic factor; Cd: Cadmium; JNK: c-Jun N-terminal kinase; NMDAR: N-Methyl-D-aspartate receptors; pro-BDNF: immature isoform of BDNF; TFBS: Transcription factor binding sites. The observed downregulation of BDNF methylation might lead to higher concentrations of the immature BDNF isoform (pro-BDNF), known to activate cellular apoptosis by binding to P75 neurotrophin receptor (NTR) (Zaletel et al., 2017) [7], possibly explaining the suggested adverse association of Cd with behavior (Fig.3). Similar results were found in a zebrafish model showing increased BDNF expression after Cd exposure alongside locomotor alterations (Xia et al., 2020) [8] and in a genome-wide study finding that Cd exposure reduced global DNA methylation in drosophila melanogaster (Guan et al., 2019) [9]. However, further research is needed to verify this hypothesis, given the absence of published data on the effects of Cd on pro-BDNF secretion and fact that this BDNF form was not measured in the present study.

Roy et al., 2009). Caution should be taken in interpreting the present results on postnatal Pb and Hg and behavioral functioning, given that urinary concentrations of Hg and Pb may reflect short-term rather than long-term exposure (Gil and Hernández, 2015) and may not serve as appropriate biomarkers to evaluate potential effects on behavior. In addition, some of these apparently protective associations may be explained by dietary and lifestyle confounders. For instance, fish consumption is a potential source of toxic metals as well as beneficial nutrients for brain development (Cano-Sancho and Casas, 2021; Gil and Gil, 2015). Although fish consumption was controlled for in the present study, residual confounding or dietary misclassification cannot be ruled out.

4.4. Strengths and limitations

Study limitations include the small sample size, reducing the statistical power of analyses and preventing the assessment of the mixture effect of the selected metals on BDNF and behavioral function, as well as potential interactions among them. Instead, we simultaneously adjusted the models for all metals in order to assess the effect exerted by a single metal while accounting for the influence of the remaining elements. Future studies in larger populations would be needed to address the combined effect of metals mixtures on BDNF and neurodevelopment. The cross-sectional design also means that causal relationships could not be inferred. Furthermore, the study investigated the concentration of total As and Hg, with no speciation procedure. Recent data from the Environment and Childhood study show that the primary source of Hg exposure is fish (Signes-Pastor et al., 2017), where Hg is present as methyl-Hg, the most neurotoxic form. The source of exposure of As, however, remains unknown, although rice (inorganic As) and seafood (organic As) consumption seem to be major sources of As exposure in the Spanish population (Signes-Pastor et al., 2017). Therefore, it is not clear whether our study population is mostly exposed to inorganic or organic As. Nevertheless, this lack of specificity would tend to underestimate rather than overestimate As effects on neurodevelopment. Additionally, previous studies have also reported associations between urinary total As and behavioral function (Rodríguez-Barranco et al., 2016). While urinary Cd and As levels appear to be a straightforward choice, it may not be the best biomarker for Pb and Hg exposure, since their short biological half-lives makes them suitable biomarkers for current or recent exposure (Gil and Hernández, 2015). Conversely, urinary Cd levels are a suitable biomarker of long-term and lifetime exposure to this metal (Gil and Hernández, 2015; Järup and Åkesson, 2009). Urinary As is also considered as adequate biomarker of short-term exposure, since it concentrations remains relatively stable among individuals with consistent dietary patterns (Hughes, 2006; Marchiset-Ferlay et al., 2012). Therefore, results for Cd and As could be more reliable compared to those of Pb and Hg. Finally, spurious associations may have been identified due to the application of multiple analyses, although several significant associations are supported by toxicological and epidemiological studies and are unlikely to be the result of chance. Moreover, the estimated coefficients and confidence intervals should be taken as a global representation of the pattern of relationships between the study variables. Study strengths include the novel exploration approach of BDNF as biomarker of neurodevelopment, assessed at different levels of biological organization (DNA methylation and serum protein). In future

epidemiological studies, this approach could contribute to elucidate the neurodevelopmental effects of metals and metalloids, especially As and Cd. Another strength is the effort to characterize the effect of As and Cd on behavioral functioning, given the scant available evidence on the impact of these pollutants. Finally, there has been inadequate research of this type in adolescence, which is characterized by important changes in neurological mechanisms.

5. Conclusion

Within an epidemiological context, serum BDNF protein levels and BDNF gene DNA methylation profile might serve as effect biomarkers to characterize the relationship of postnatal exposure to toxic metals, such as As and Cd, with behavioral problems in adolescents. However, due to study limitations, our results need to be verified in future larger epidemiological studies on metal exposures during this and other critical windows of neurodevelopment. Biomarkers of brain function are needed in human biomonitoring studies to better address current gaps in knowledge between environmental exposures and neurodevelopmental disorders.

Declaration of Competing interest

The authors declare no actual or potential conflicts of interest. The funders had no role in the study design, data collection or analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary data

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