Science of the Total Environment 868 (2023) 161668

Contents lists available at ScienceDirect

Science of the Total Environment

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

Kisspeptin as potential biomarker of environmental chemical mixture effect on reproductive hormone profile: A pilot study in adolescent males

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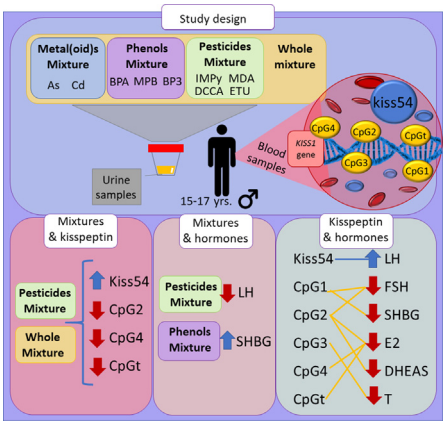
HIGHLIGHTS

- Kisspeptin was evaluated as a biomarker of effect in male adolescents.
- Mixtures of chemicals were associated with higher kisspeptin protein levels.
- Mixtures of chemicals were associated with lower *KISS1* DNA methylation.
- Kisspeptin markers were associated with adolescent's hormonal levels.
- Exposure to mixtures of chemicals altered reproductive hormone profile.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Lidia Minguez Alarcon

Keywords:
Kisspeptin
Effect biomarker
Xenobiotic mixtures
Endocrine disrupting chemicals
HBM4EU

ABSTRACT

Background: Kisspeptin has been proposed as an effect biomarker to understand the mechanisms by which some environmental chemicals adversely affect the human reproductive system.
Objective: To ascertain whether kisspeptin serum protein and DNA methylation levels are associated with exposure to several environmental chemicals (individually and as a mixture) and serum reproductive hormone levels in adolescent males.
Methods: Three phenols (bisphenol A [BPA], methyl-paraben [MPB], and benzophenone-3 [BP3]); two toxic metals (arsenic and cadmium); and four metabolites of non-persistent pesticides, including insecticides (2-isopropyl-6-methyl-4-pyrimidinol [IMPy], malathion diacid [MDA], and dimethylcyclopropane carboxylic acid [DCCA]) and fungicides (ethylene thiourea [ETU]) were measured in first-morning urine samples of 133 adolescent males aged 15–17 years from the INMA-Granada cohort. In blood samples collected on the same day, *KISS1* gene DNA methylation was measured at four CpGs from the Exon IV, as well as serum levels of kiss54 protein, total testosterone (T), estradiol (E_2),

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http://dx.doi.org/10.1016/j.scitotenv.2023.161668
Received 4 November 2022; Received in revised form 13 January 2023; Accepted 13 January 2023
Available online 16 January 2023

sex hormone binding-globulin, dehydroepiandrosterone sulfate, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Multiple linear regression and mixture (quantile g-computation) models were fit.
Results: Urinary MDA and DCCA concentrations were associated with higher kiss54 levels [% change (95%CI) for each log-unit increase in concentration = 2.90 (0.32;5.56), and 1.93 (0.45,3.43), respectively]; IMPy with lower DNA methylation percentage at CpG1 and total CpGs [% change (95%CI) = −1.15 (−1.96;-0.33): −0.89 (−1.73;-0.01), respectively]; and BP3 and DCCA with lower total CpGs methylation [−0.53 (−1.04;-0.01) and − 0.69 (−1.37;-0.01), respectively]. The pesticide mixture and the whole chemical mixture were associated with higher kiss54 [% change (95%CI) = 9.09 (3.29;15.21) and 11.61 (3.96;19.82), respectively] and lower methylation levels at several CpGs. Additionally, serum kiss54 in the third tertile was associated with higher LH levels [% change (95%CI) = 28.69 (3.75–59.63)], and third-tertile CpG1, CpG2, and total CpG methylation percentages were associated with lower FSH and E_2.
Conclusion: The findings of the present study and the negative correlation between serum kiss54 levels and *KISS1* DNA methylation percentages suggested that kisspeptin may be a promising effect biomarker.

1. Introduction

Adolescence is an understudied but susceptible period of development, orchestrated by the hypothalamus-pituitary-gonadal (HPG) axis, in which genetics and hormones regulate the final maturation of individuals (Howard, 2021). The maturation procedure is triggered by pulsatile secretion of the gonadotrophin-releasing hormone (GnRH), which stimulates the secretion of pituitary hormones: luteinizing hormone (LH), and follicle-stimulating hormone (FSH); and subsequently steroid hormones: estradiol (E_2), dehydroepiandrosterone sulfate (DHEAS), testosterone (T), and their transporter sex hormone binding globulin (SHBG) (Comninos and Dhillo, 2018; Pinilla et al., 2012). Hormones regulate themselves through a complex net of negative feedbacks that should be maintained to reach adequate reproductive health. Alterations in this cascade of events during childhood may increase the risk of reproductive adverse effects later in life, such as poor sperm quality and infertility (Howard, 2021; Bräuner et al., 2020; Parent et al., 2003).

The worldwide increase in the incidence of male reproductive disorders is thought to be caused in part by exposure to endocrine-disrupting chemicals (EDCs) since the reproductive function is highly sensitive to exogenous stressors able to alter the hormonal homeostasis (De Falco et al., 2015; Kasper-Sonnenberg et al., 2017; La Merrill et al., 2020; Lagos-Cabre and Moreno, 2012). Environmental phenols such as bisphenol A (BPA), benzophenone-3 (BP3), and methyl-paraben (MPB) are common environmental chemicals that elicit estrogenic and antiandrogenic effects *in vitro* and *in vivo* (Krzyżanowska et al., 2018; La Merrill et al., 2020; M.A. et al., 2017). Moreover, BPA has been associated with alterations in serum levels of T, E_2, SHBG, and FSH in children, adolescents, and adults (Liu et al., 2015; Miao et al., 2015; Mustieles et al., 2018; Wang et al., 2021). Other environmental chemicals such as non-essential metals and metalloids have also been associated with altered levels of reproductive hormones in adolescents and adults (Ashrap et al., 2019; De Craemer et al., 2017; Tao et al., 2021). In addition, alterations in pituitary hormone and sex steroid levels have been reported in adult males occupationally and non-occupationally exposed to insecticides, including organophosphates (OPs) and pyrethroids (Han et al., 2008; Meeker et al., 2009; Yang et al., 2019). However, the mechanisms through which these compounds elicit hormonal imbalance and adverse reproductive effects are complex and not fully understood.

In the European Human Biomonitoring Initiative (HBM4EU), effect biomarkers were proposed as additional markers to elucidate the potential adverse effect triggered by environmental chemicals (Mustieles et al., 2020). These types of biomarkers could help to increase the confidence of exposure-effect associations since they could be indicators of early biological effects exerted by chemical exposure (Mustieles et al., 2020; Zare Jeddi et al., 2021). In this regard, experimental studies have already assessed the role of kisspeptin in reproduction, placing its signaling at the apex of the HPG axis (Pinilla et al., 2012; Roepke and Sadlier, 2021).

Kisspeptin refers to a family of peptide hormones cleaved from the product of the *KISS1* gene in primates. The precursor of kisspeptin (pre-prokisspeptin) comprises 145 amino acids which include a putative 19-amino acid signal sequence, two dibasic cleavage sites, and one site for terminal cleavage and amidation, on which the biological activity of kisspeptins depends. All kisspeptin peptides share a common carboxy-terminal sequence through which they bound to their receptor, encoded as *KISS1R/Kiss1r*. This neuropeptide is secreted by kisspeptin neurons in the hypothalamus. There, they bind to KISS1R upon GnRH neurons, stimulating the production of GnRH into the local hypophyseal-portal circulation, triggering the secretion of pituitary and steroids hormones (Pinilla et al., 2012; Roepke and Sadlier, 2021; Skorupskaite et al., 2014). The kisspeptin isoform 54 (kiss54, named after the number of amino acids in its chain) is generated after the cleavage of pre-prokisspeptin, thus being the first biologically active protein isoform of kisspeptin (Roepke and Sadlier, 2021). Moreover, it is considered the major product of the *KISS1* gene and is easier to measure than their smaller isoforms (kiss10, or kiss14) (Pinilla et al., 2012; Roepke and Sadlier, 2021).

In the context of the HBM4EU Project, kisspeptin was proposed as an effect biomarker of reproductive function (Mustieles et al., 2020). To our knowledge, only a few epidemiological studies have evaluated its relationship with exposure to relevant environmental chemicals (Chen et al., 2013; Özgen et al., 2016). Previous results from our research group revealed associations between urinary 2-isopropyl-6-methyl-4-pyrimidinol (IMPy) concentrations, the metabolite of the OP insecticide diazinon, and increased levels of E_2, DHEAS, and FSH in adolescent males from the Environment and Childhood (INMA)-Granada cohort (Freire et al., 2021; Suárez et al., 2021), while no relationship was found between urinary arsenic (As) and cadmium (Cd), and serum reproductive hormone levels (Castiello et al., 2020). With this background, this is the first epidemiological study assessing the role of kisspeptin at two levels of biological organization in the association between exposure to environmental chemicals and reproductive hormones.

Thus, the aims of this study were: i) to evaluate the relationship between urinary concentrations of three environmental phenols (BPA, MPB, and BP3), As and Cd, and four metabolites of non-persistent pesticides (IMPy, malathion diacid [MDA], dimethylcyclopropane carboxylic acid [DCCA], and ethylene thiourea [ETU]), individually, per chemical class, and as a whole mixture, with serum kisspeptin protein levels, DNA methylation of *KISS1* gene, and serum reproductive hormone levels; and ii) the association between serum kisspeptin protein levels, the DNA methylation of *KISS1* gene, and reproductive hormone levels in the same sample of boys.

2. Material and methods

2.1. Study population

This study included 133 boys belonging to the INMA-Granada cohort who participated in a follow-up visit at the age of 15–17 years in 2017–2019. The INMA Project is a multi-center birth cohort study performed in different parts of Spain to investigate the impact of environmental exposure and diet on developmental health (Guxens et al., 2012). The INMA-Granada birth cohort was established between 2000 and 2002 by

recruiting 668 mother-son pairs at delivery in Granada province, Spain (Fernandez et al., 2007). Randomly selected pairs from the baseline cohort were contacted to request their participation in different clinical follow-ups at 4–5 (n = 220, 32.9 %) and 9–11 years (n = 298, 44.6 %). Those who attended both follow-up sessions (n = 269) were re-contacted and asked to participate in the most recent follow-up at the age of 15–17 years (2017–2019), from which 151 agreed to participate and underwent physical examination (Castiello et al., 2020). Study participants agreed to undergo a clinical examination at the San Cecilio University Hospital, Granada, Southern Spain. Anthropometric measurements included weight, height, and waist and hip circumferences, and Tanner staging and genital measurements were performed (Castiello et al., 2020). Each adolescent provided a first-morning urine sample collected under fasting conditions, and non-fasting blood samples were obtained from 135 out of 151 participants (89 %). The current analysis included 133 boys with available data on urinary phenols, metals, non-persistent pesticide metabolite concentrations, and serum reproductive hormone levels. From this population, measurements of serum kisspeptin 54 (kiss54) protein levels and DNA methylation of the *KISS1* gene of the Exon IV at 4 CpGs were available for 104 and 117 adolescents, respectively. Thus, missing values were imputed according to the statistical analyses section. The informed consent was signed by the parents' participants and the study protocol was approved by the Biomedical Research Ethics Committee of Granada (protocol number 0509-N17, date of approval March 28, 2017).

2.2. Collection and analysis of urine samples

First-morning spot urine samples were collected at the participants' residences and kept at −80 °C after arrival at the Hospital. The selection of phenols, metals, and pesticides was based on their endocrine disrupting properties, their toxicological and regulatory relevance (ECHA, 2022), the availability of measurements in the INMA-Granada cohort, and their frequency of detection. Metabolites of non-persistent pesticides included IMPy and MDA, specific metabolites of the organophosphate (OP) insecticides diazinon and malathion, respectively; DCCA (sum of *cis* and *trans* isomers), metabolite of pyrethroids cypermethrin, cyfluthrin, and cyhalothrin; and ETU, the major metabolite of ethylene-bis-dithiocarbamate (EBDC) fungicides such as mancozeb. All chemicals were detected in at least 70 % of the samples.

Concentrations of BPA, MPB, and BP3 were ascertained using dispersive liquid–liquid microextraction (DLLME) and ultra-high performance liquid chromatography in tandem with mass spectrometry detection (UHPLC-MS/MS) at the University of Granada (Spain), according to Adoamnei et al. (2018). Centrifuged urine samples were spiked with 50 μL of enzyme solution consisting of 10 mg of β-glucuronidase/sulfatase in 1.5 mL of 1 M ammonium acetate/acetic acid buffer solution at pH 5. After 24 h of incubation at 37 °C, treated urines were spiked with 30 μL of standard solution (1.25 mg/L of EP-$^{13}C_6$) and diluted to 10 mL with an aqueous solution (5 % NaCl, w/v) at pH 2. Afterward, the sample was then injected with a mixture of acetone and trichloromethane (0.75 mL, respectively), which was shaken, centrifuged, and evaporated. The resulting extract was dissolved in 100 μL of water (0.1 % ammonia)/acetonitrile (0.1 % ammonia), 70:30 (v/v). Finally, 10 μL was injected into the UHPLC-MS/MS system. For the quality control procedure, calibration curves were performed by plotting the analyte/surrogate peak area ratio against the analyte concentration.

Urinary concentrations of total As and Cd were measured with inductively coupled plasma mass spectrometry with an Agilent 8900 triple quadrupole ICP-MS (Agilent Technologies, Santa Clara, CA, USA), following a previously described methodology (Castiello et al., 2020), at the Department of Legal Medicine and Toxicology, University of Granada. Samples were spiked with a multielement standard solution (400 μg/L) including Ge, Ir, Rh, and Sc; blanks; intermediate calibration standards; and certified reference materials according to the US National Institute of Standards and Technology [Trace Elements in Natural Water Standard Reference Material SRM 1640a] and Seronorm (Sero, Billingstad, Norway) Trace Elements Urine L1 and L2 (references 210,605 and 210,705, respectively] were used for quality control and quality assessment.

To measure urinary IMPy and ETU, ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) was used at the "UNETE Research Unit" of the Biomedical Research Center (CIBM), University of Granada, Spain. MDA and DCCA were quantified by liquid chromatography coupled to mass spectrometry (LC-MS/MS) at the facilities of the MEDINA Foundation, Granada (Spain). These procedures were extensively explained previously (Freire et al., 2021; Rodríguez-Carrillo et al., 2022; Suárez et al., 2021). Non-persistent pesticide metabolites were extracted and calibrated without deconjugation, adding internal standards (IS) and standards solutions to 1 mL of urine samples (Suárez et al., 2021).

Limits of detection (LOD) were 0.1 μg/L for BPA, MPB, and BP3, 0.60 μg/L for As, 0.01 μg/L for Cd, 0.117 μg/L for IMPy, 0.052 μg/L for MDA, 0.055 μg/L for DCCA, and 0.072 μg/L for ETU (Supplementary Material, Table S1). To correct urine dilution, urinary creatinine levels were ascertained with the commercial kit (CREJ2) using the Jaffe method in a Roche Cobas C-311 system (mg/dL).

2.3. Biochemical measurements

Serum and whole blood aliquots were obtained from non-fasting (4–8 p.m.) peripheral blood samples and kept at −80 °C. Serum levels of T, DHEAS, E_2, SHBG, LH, and FSH were measured by electrochemiluminescence immunoassay with a Roche® kit (Elecsys System, Roche Diagnostics) at the *Instituto de Investigación Biosanitaria de Granada* (ibs.GRANADA), Granada, Spain. Serum kiss54 protein levels were measured using the Human KISS-54 kit (Biotek Synergy HT, Mybiosource, San Diego, CA) enzyme-like immunosorbent assay (ELISA) at the CIBM, University of Granada, Spain. Briefly, 10 μL of serum together with 50 μL of standards were placed in 96-well plates. Samples were diluted by adding 40 μL/well of sample diluent, and subsequently, 100 μL/well of Horseradish Peroxidase-Conjugate (HRP) was added and kept at 37 °C for 1 h. Then, plates were washed five times according to manufacturer's instructions, and 50 μL/well of reagents A and B were added. Plates were then sealed, protected from light, and kept at 37 °C for 15 min. Finally, absorbance was read using a luminometer at 450 nm.

2.4. KISS1 gene DNA methylation

DNA methylation of the *KISS1* gene was measured in whole blood samples using the gold standard bisulfite pyrosequencing technique at IRSET (Institut de Recherche en Santé, Environment et Travail - INSERM UMR1085), Rennes, France. Genomic DNA concentration and purity were assessed using a NanoDrop (Thermo Scientific NanoDrop 8000; DNA50 mode). Samples showed a 1.8–1.9 ratio at 260/280 absorbance, indicating optimal purity of extracted DNA. DNA concentration was re-analyzed using the QuantiFluor dsDNA system (Promega E2670). Subsequently, the Epitect Fast Bisulfite Conversion kit (Qiagen, 59,826) was used to bisulfite convert 500 ng of genomic DNA (500 ng). The concentrations of bisulfite converted DNA (BSDNA) were assessed using NanoDrop (Thermo Scientific NanoDrop 8000; RNA40 mode) and 20 ng DNA was used for downstream PCR amplification (Biometra TProfessional Thermoycler, France) of the *KISS1* using Takara EpiTaq hot-start DNA polymerase (Takara, R110A; 0.6 U/25 μL final concentration). Primers information is provided in Supplementary Materials (Table S2, reverse primer was biotinylated). The targeted region was the promoter of *KISS1* gene previously reported in human placentas (Avila et al., 2010). After PCR amplification, product purification was performed using 2 % agarose gel (MinElute PCR purification kit, Qiagen, 28,006). Finally, samples were pyrosequenced using Pyromark Q24 Advanced Pyrosequencing technology. The degree of DNA methylation was expressed as CpG percentage. Quality of DNA percentage measurement was done using the Pyromark Q24 software, samples not passing the quality control were pyrosequenced again. Thus, errors from technical handling were discarded.

2.5. Statistical analyses

The LOD/√2 was used to impute urinary concentrations of environmental chemicals and metabolites below the assay LOD. Multiple imputations by chained equation were conducted using the MICE package in R (×20) for participants with missing data on serum kiss54 (n = 29) and *KISS1* DNA methylation (n = 16). Detection frequencies and 25th, 50th, and 75th percentiles were calculated to describe creatinine-standardized urinary chemicals, kisspeptin effect biomarkers (serum protein level and blood DNA methylation), and serum hormone levels. The Spearman's test was conducted to evaluate correlations between environmental chemicals.

Multivariate linear regression analysis was performed to evaluate the relationship of i) individual urinary environmental chemicals/metabolites with serum kiss54 levels and blood *KISS1* gene DNA methylation; and ii) kisspeptin effect biomarkers with serum hormone levels. To reduce distribution skewness, urinary exposure biomarkers, kisspeptin biomarkers, and serum hormone levels were natural log-transformed. Kisspeptin biomarkers were further categorized into tertiles to evaluate potential non-monotonic associations.

Models were adjusted for adolescents' age, waist-to-height ratio (ratio between waist circumference and height measured in cm), and Tanner genital stage [sexual maturation not fully reached (<G5) *vs.* sexual maturation fully reached (G5)], since these are factors related to the maturation of the HPG axis (Bromberg and O'Donohue, 2013; Decaroli and Rochira, 2017; Seth et al., 2013; Sidhu et al., 2017). Annual household income (<25,000, 25,000–35,000, or >35,000 euros) was included in the models as an indicator of socioeconomic status. The season of biological sample collection (winter, spring, summer, or autumn) was included since it may have an influence on hormone levels and exposure to environmental chemicals, such as pesticides and BP3 (Tendler et al., 2021). Models were additionally adjusted for urinary dilution by introducing urinary creatinine (mg/dL) to correct for potential measurement error bias (O'Brien et al., 2016). The following covariates were also examined but were not found to confound the associations under study: body mass index (BMI, kg/m^2, continuous), area of residence (urban areas, suburban, or rural areas), passive smoking (whether parents or friends are smokers), alcohol consumption (never or <1 beverage or ≥1 beverage per month), and the timing of blood samples collection, since all were collected in the afternoon, within a narrow time frame (5–7 p.m.), thus discarding the potential influence of hormonal diurnal variation. To enhance the interpretation of results, linear regression estimates were expressed as the percentage of change in the dependent variable (*e.g.,* kisspeptin effect biomarker or hormone level) for each one-log unit increase in the concentration of the environmental chemical/kisspeptin biomarker [(exp(β)-1)*100] or for 2nd/3rd *versus* 1st tertile.

Quantile g-computation was used to assess the combined effect of environmental chemicals on kisspeptin biomarkers and reproductive hormones. First, the combined effect of each chemical family (phenols, metals-metalloid, pesticides) on the outcomes was estimated. Second, the combined effect of the overall chemical mixture on the outcomes was estimated. Quantile g-computation estimates the joint effect using a parametric generalized linear model which increases simultaneously all exposures by one tertile (Keil et al., 2020). Advantages of this method include the possibility of assessing individual exposure-effect relationships within the mixture in opposite directions, producing an unbiased estimate of the overall joint effect, and its use in small sample sizes (Eick et al., 2021; Keil et al., 2020). The functioning of the quantile g-computation is based on the categorization of urinary biomarkers of exposure to phenols, metals, and pesticides into quartiles. Each biomarker is given a negative or positive weight. If the individual compound shows a different direction of the effect, the weight is interpreted as the proportion of the partial effect in the negative or positive direction.

As Tanner stage may be in the exposure-effect biomarker-outcome pathway, sensitivity analyses excluding the Tanner stage from the models were conducted. Additionally, to account for the potential influence of imputed kisspeptin data, sensitivity analyses without imputed data were also developed. The significance level was established at $p < 0.05$; however, the internal validity and previous toxicological and epidemiological evidence were also taken into account when interpreting the results (Amrhein et al., 2019). SPSS v28.0.1 (IBM, Chicago, IL) and R statistical software version 4.2.0, package qgcomp (https://cran.r-project.org/web/packages/gWQS/index.html) were used for data analyses.

3. Results

3.1. Characteristics of study population

Table 1 summarizes the general characteristics of the study participants. The mean (standard deviation, SD) age of adolescents was 16.2 years, and the mean (SD) body mass index (BMI) and waist-to-height ratio were 23.1 (4.8) kg/m^2 and 0.47 (0.06), respectively. Nearly 60 % of boys had not reached full genital maturation. Most of them lived in urban areas and had an annual family income ≥25,000, and less than half were passive smokers and consumed more than one alcoholic beverage per month, respectively.

Median creatinine-unadjusted urinary concentrations were 3.46 μg/L for BPA, 2.41 μg/L for MPB, 1.06 μg/L for BP3, 20.97 μg/L for As, 0.08 μg/L for Cd, 0.25 μg/L for IMPy, 0.31 μg/L for MDA, 1.06 μg/L for DCCA, and 0.30 μg/L for ETU (Table 2). BPA was moderately positively correlated with MPB, BP3, and Cd (Spearman's rho, ρ = 0.30, 0.44, and 0.03, respectively); MPB was moderately positively correlated with BP3 (ρ = 0.50) and Cd (ρ = 0.22); and weak positive correlations were found between As and Cd (ρ = 0.21), and between DCCA and BP3 (ρ = 0.17), As (ρ = 0.18), and Cd (ρ = 0.20). No significant correlations were found for the remaining compounds (Fig. 1; Supplementary Material, Table S3).

Serum hormone levels were within normal reference ranges (Howard, 2021; Karbasy et al., 2016) according to participants' age and sex (Table SM1), with almost 13 % of them showing low T levels (<6.20 nmol/L) and around 30 % low E_2 levels (<26.80 pmol/L) (Table 2, Table SM1). Median serum kiss54 level was 2.65 ng/mL and median *KISS1* DNA methylation percentage was 87.69 %, 63.41 %, 77.54 %, 83.19 %, and 77.28 % for CpG1, CpG2, CpG3, CpG4, and total methylation percentage (CpGt), respectively (Table 2). Additionally, serum kiss54 levels were negatively correlated with *KISS1* DNA methylation levels, showing significant results with CpG4 (Supplementary Material, Fig. S1).

3.2. Association between individual chemicals and kisspeptin biomarkers

In single chemical exposure models, As, MDA and DCCA were associated with a slight increase in kiss54 levels, whereas Cd was associated

Table 1
General characteristics of study participants (n = 133).

		Mean ± SD/n (%)
Age (years)		16.2 ± 0.4
BMI (kg/m^2)		23.1 ± 4.8
Waist to height ratio		0.47 ± 0.06
Tanner genital stage	<G5	79 (59.4)
	=G5	54 (40.6)
Area of residence	Urban	95 (71.5)
	Suburban/rural	30 (28.5)
Passive smoking	Yes	55 (41.2)
	No	78 (58.8)
Alcohol consumption	Never or < 1 beverage per month	78 (60.0)
	≥1 beverage per month	55 (40.0)
Season of urine collection	Spring	29 (21.8)
	Summer	16 (12.0)
	Autumn	60 (45.1)
	Winter	28 (21.1)
Annual family income (euros)	<25,000	48 (36.1)
	25,000-35,000	52 (39.1)
	>35,000	33 (24.8)

SD: standard deviation; BMI: Body mass index; G5 = sexual maturation fully reached.

Table 2
Distribution of urinary environmental chemicals/metabolites (μg/g creatinine), serum hormone levels, and kisspeptin effect biomarkers (n = 133).

Chemicals		BPA	MPB	BP3	As	Cd	IMPy	MDA	DCCA	ETU
% detection		87	67	76	100	99	75	83	100	75
LOD (μg/L)		0.10	0.10	0.10	0.60	0.01	0.12	0.05	0.06	0.07
Percentiles (μg/g)	25	0.85	0.06	0.24	5.01	0.03	0.10	0.08	0.09	0.05
	50	1.97	0.54	1.27	10.18	0.04	0.20	0.16	0.60	0.14
	75	3.03	1.89	3.81	25.51	0.06	0.42	0.27	1.67	0.52

Hormone levels		T (nmol/L)	DHEAS (nmol/L)	E_2 (pmol/L)	SHBG (nmol/L)	LH (mU/mL)	FSH (mU/mL)
Percentiles	25	7.68	6986.84	9.17	24.92	3.02	2.17
	50	10.94	9596.28	27.44	32.59	4.3	3.57
	75	14.86	11,937.66	52.88	44.26	5.73	6.95

Kisspeptin markers		Serum kiss54 (ng/mL)	CpG1 (%)	CpG2 (%)	CpG3 (%)	CpG4 (%)	CpGt (%)
Percentiles	25	2.53	85.73	61.62	75.97	79.47	76.03
	50	2.65	87.69	63.41	77.54	82.19	77.28
	75	2.81	89.70	64.41	78.95	85.25	79.29

LOD: Limit of detection; kiss54: kisspeptin 54; CpGt: Total DNA methylation [(CpG1 + CpG2 + CpG3 + CpG4)/4]; BPA: Bisphenol A; MPB: methylparaben; BP3: benzophenone 3; As: arsenic (total), Cd: cadmium; IMPy: 2-isopropyl-6-methyl-4-pyrimidinol; MDA: malathion diacid; DCCA: dimethylcyclopropane carboxylic acid; ETU: ethylene thiourea.

with lower levels [% change (95 % CI): 1.72 (−0.11;3.58), 2.90 (0.32;5.56), 1.93 (0.45;3.43), and − 3.82 (−7.30;-0.22)], respectively, for each log-unit increase in urinary concentration of each chemical compound] (Table 3). IMPy and BP3 were also associated with lower DNA methylation percentages at CpG 1 and 2, respectively [% change (95 % CI): −1.15 (−1.96;-0.33) and − 1.08 (−2.08;-0.06) for each log-unit increase in methylation percentage]. Moreover, DCCA was associated with lower DNA methylation at CpG2 [% change (95 % CI): −1.83 (−3.14;-0.50], CpG4 [−1.35 (−2.65;-0.03)] and total CpGs methylation percentages [−0.69 (−1.37;-0.01)] (Table 3). Further, no substantial differences were found in the sensitivity analyses excluding the Tanner stage or the multiple imputation (Tables S6 and S9).

3.3. Chemical mixture effect on kisspeptin biomarkers and serum hormones

In the mixture effect analysis per chemical family (phenols, metal-metalloid, or pesticides), it was observed that each tertile increment in urinary concentration of pesticides was significantly associated with higher serum kiss54 levels [% change (95 % CI): 9.09 (3.29;15.21)], and lower CpG2 [−5.26 (−9.96;-0.32)], CpG4 [−6.03 (−10.63;-1.19)], and CpGt [−3.34 (−5.81;-0.81)]. Neither phenols nor metals mixture was associated with any kisspeptin biomarker. When assessing the total mixture effect, a significant positive association with serum kiss54 levels [% change (95 % CI): 0.066 (0.027;0.105)] and with lower CpG2 [−7.19 (−13.26;-0.71)], CpG4 [−7.87 (−13.81;-1.52)], and CpGt [−4.48 (−7.68;−1.16)] were found (Table S4, Fig. 2).

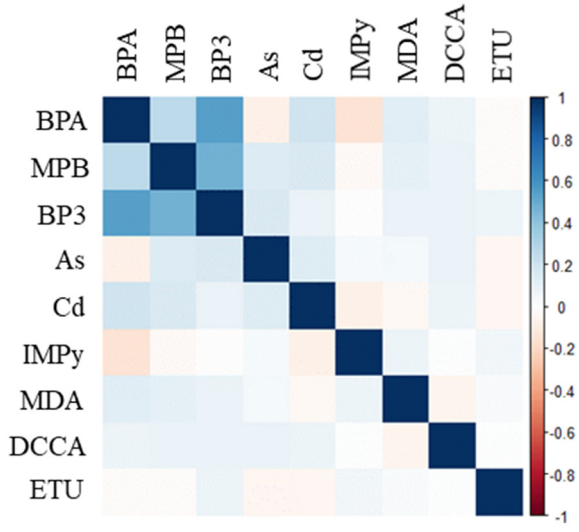

Fig. 1. Heatmap for the correlation between urinary exposure biomarkers.

Regarding serum hormones, the phenol mixture was significantly associated with higher SHBG levels [% change (95 % CI): 15.67 (4.89;27.56)] per each tertile increment in urinary phenols concentrations (Table S4, Fig. 2); and the pesticide mixture with lower LH [−23.34 (−38.63;-4.24)] (Table S4, Fig. 2). No significant association was found when assessing the remaining mixture effect on hormone levels (Table S4).

Sensitivity analyses excluding the Tanner stage from the models and kisspeptin imputed data did not show substantial differences in associations or trends (Tables S7 and S10).

3.4. Kisspeptin biomarkers and hormone levels

Adolescents with kiss54 level in the 3rd tertile had significantly higher LH [% change (95 % CI): 29.03 (2.50;62.43)]. Moreover, higher DNA methylation at CpG1 and 2 were associated with lower FSH [% change (95 % CI): −47.59 (−66.19;-18.76), and − 49.88 (−67.93;-21.68), respectively] (Table 4). Significant associations with lower E_2 levels were found for adolescents with higher DNA methylation (3rd tertile) at CpG2 [% change (95 % CI): −36.59 (−54.96;-10.73)], CpG4, [−40.42 (−57.25;-16.95)], and CpGt [−33.68 (−52.49;-7.42)]. Finally, higher methylation levels at CpG3 were associated with lower T levels [% change (95 % CI): −20.37 (−34.92;-2.55)] (Table 4). Analyses with kisspeptin biomarkers as continuous variables showed the same patterns, except for the significant association between CpG1 with higher T levels [% change (95 % change): 429.72 (27.31;2104.10)] (Table S5).

Results of models not adjusted for the Tanner stage were similar to those of models adjusted for the Tanner stage (Table S8); exclusion of imputed data led to stronger associations between kiss54 and TT and E2, whereas associations with LH were attenuated, although all trends remained in both models (Table S11).

4. Discussion

Results of this pilot study suggest that exposure to mixtures of several environmental chemical groups, including phenols, metals, and non-persistent pesticides, is associated with higher serum kiss54 levels in male adolescents. When only exposure to the pesticide mixture was considered, associations with higher serum kiss54 levels and lower DNA methylation

Table 3
Associations between individual environmental chemicals and kisspeptin biomarkers (n = 133).

	Kiss54			*KISS1* gene methylation														
				CpG1			CpG2			CpG3			CpG4			CpGt		
	%Change	95%CI		%Change	95%CI		%Change	95%CI		%Change	95%CI		%Change	95%CI		%Change	95%CI	
		LL	UL		LL	UL		LL	UL		LL	UL		LL	UL		LL	UL
BPA	0.25	−1.16	1.68	−0.54	−1.18	0.11	−0.51	−1.80	0.79	−0.27	−0.91	0.39	−1.00	−2.24	0.25	**−0.58**†	−1.22	0.08
MPB	0.80	−0.20	1.81	0.03	−0.43	0.49	−0.47	−1.39	0.46	0.09	−0.38	0.55	−0.16	−1.06	0.75	−0.09	−0.56	0.38
BP3	0.54	−0.58	1.68	−0.28	−0.79	0.24	**−1.08***	−2.08	−0.06	−0.27	−0.79	0.25	−0.81	−1.80	0.18	**−0.53***	−1.04	−0.01
As	**1.72**†	−0.11	3.58	−0.32	−1.16	0.53	−0.52	−2.19	1.19	−0.47	−1.31	0.38	0.04	−1.60	1.71	−0.24	−1.09	0.62
Cd	**−3.82***	−7.30	−0.22	−0.21	−1.91	1.52	0.07	−3.33	3.58	0.32	−1.40	2.06	−0.53	−3.81	2.86	−0.02	−1.75	1.74
IMPy	0.73	−1.12	2.60	**−1.15***	−1.96	−0.33	−0.99	−2.66	0.71	−0.33	−1.17	0.53	−1.30	−2.91	0.34	**−0.89***	−1.73	−0.05
MDA	**2.90***	0.32	5.56	0.68	−0.51	1.88	**2.48***	0.09	4.92	**−1.09**†	−2.25	0.08	0.62	−1.70	2.98	0.45	−0.76	1.67
DCCA	**1.93***	0.45	3.43	−0.47	−1.15	0.21	**−1.83***	−3.14	−0.50	0.45	−0.24	1.14	**−1.35***	−2.65	−0.03	**−0.69***	−1.37	−0.01
ETU	0.82	−0.75	2.42	0.36	−0.36	1.09	−0.67	−2.11	0.78	0.50	−0.23	1.23	−0.10	−1.51	1.33	0.09	−0.64	0.83

LL: Lower limit; UL: upper limit of the confidence interval (CI); kiss54: kisspeptin 54; CpGt: Total DNA methylation [(CpG1 + CpG2 + CpG3 + CpG4)/4]; BPA: Bisphenol A; MPB: methylparaben; BP3: benzophenone 3; As: arsenic (total), Cd: cadmium; IMPy: 2-isopropyl-6-methyl-4-pyrimidinol; MDA: malathion diacid; DCCA: dimethylcyclopropane carboxylic acid; ETU: ethylene thiourea. All models were adjusted for age (continuous), waist-to-height ratio (continuous), Tanner Stage (<G5/G5), urine creatinine concentrations (continuous), annual family income (<25,000/25,000-35,000/>35,000), season of biological sample collection (spring/summer/autumn/winter). *p < 0.05; †p < 0.10.

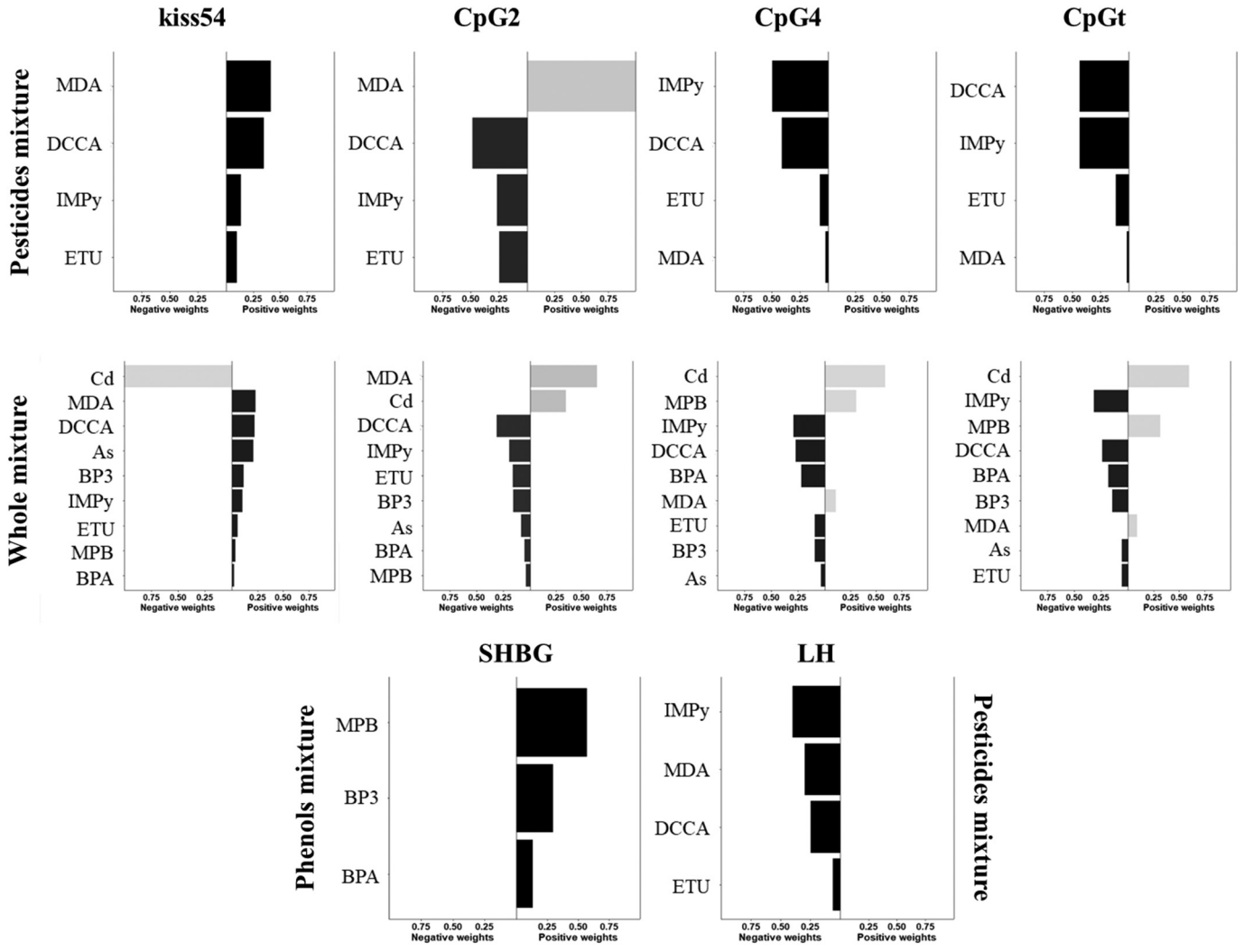

Fig. 2. Quantile G-computation model showing the most relevant effects of the chemical mixture on kisspeptin biomarkers and reproductive hormones. kiss54: kisspeptin 54; CpGt: Total DNA methylation [(CpG1 + CpG2 + CpG3 + CpG4)/4]; BPA: Bisphenol A; MPB: methylparaben; BP3: benzophenone 3; As: arsenic (total), Cd: cadmium; IMPy: 2-isopropyl-6-methyl-4-pyrimidinol; MDA: malathion diacid; DCCA: dimethylcyclopropane carboxylic acid; ETU: ethylene thiourea; SHBG: sex hormone binding-globulin; and LH: Luteinizing hormone.

Table 4
Associations between kisspeptin biomarkers and serum hormone levels (n = 133).

		T			DHEAS			E_2			SHBG			LH			FSH		
		%Change	95% CI		%Change	95% CI		%Change	95% CI		%Change	95% CI		%Change	95% CI		%Change	95% CI	
			LL	UL		LL	UL		LL	UL		LL	UL		LL	UL		LL	UL
Kiss54	T2	−3.62	−21.88	18.90	−5.78	−21.04	12.44	−0.90	−30.07	40.44	7.91	−7.06	25.29	**23.50**†	−1.49	54.83	−26.64	−53.53	15.82
	T3	22.72	−0.90	51.98	−4.40	−20.14	14.44	23.79	−13.20	76.55	7.91	−7.31	25.63	**29.03***	2.50	62.43	**−35.67**†	−59.59	2.39
CpG1	T2	7.96	−12.73	33.56	−4.63	−19.93	13.61	−4.05	−32.19	35.78	**−12.75**†	−24.62	0.99	**39.81***	12.08	74.39	**−41.73***	−62.57	−9.30
	T3	7.93	−12.58	33.25	−7.59	−22.29	9.90	−14.98	−39.72	19.92	−0.89	−14.25	14.57	16.20	−6.65	44.65	**−47.59***	−66.19	−18.76
CpG2	T2	5.19	−14.66	29.67	**−17.59***	−30.38	−2.44	−18.48	−41.58	13.75	−9.91	−22.04	4.11	9.20	−12.75	36.67	**−34.91**†	−57.86	0.56
	T3	−2.00	−20.94	21.48	−13.99	−27.67	2.27	**−36.59***	−54.96	−10.73	−10.50	−22.85	3.82	7.63	−14.52	35.51	**−49.88***	−67.93	−21.68
CpG3	T2	1.05	−17.66	24.00	−11.08	−25.12	5.60	2.30	−27.46	44.28	0.75	−13.01	16.68	4.40	−16.63	30.74	0.20	−35.86	56.54
	T3	**−20.37***	−34.92	−2.55	−7.54	−21.96	9.54	−2.79	−30.75	36.45	−1.79	−15.03	13.51	−7.04	−25.54	16.06	**46.47**†	−5.67	127.43
CpG4	T2	0.54	−18.28	23.69	−0.73	−16.33	17.78	−21.57	−43.56	8.98	−5.71	−18.47	9.05	10.85	−11.28	38.52	−0.54	−36.45	55.65
	T3	−12.07	−28.67	8.38	−8.65	−23.13	8.56	**−40.42***	−57.25	−16.95	−1.55	−14.99	14.01	−3.98	−23.31	20.23	−19.22	−48.60	26.94
CpGt	**T2**	8.82	−11.82	34.28	−0.31	−16.20	18.59	−4.64	−31.87	33.45	−9.86	−22.13	4.35	0.30	−20.07	25.86	−13.87	−45.22	35.42
	T3	−4.47	−22.46	17.70	−6.28	−21.11	11.35	**−33.68***	−52.49	−7.42	−2.17	−15.40	13.12	−4.15	−23.49	20.07	−26.04	−52.80	15.89

LL: Lower limit; UL: upper limit of the confidence interval (CI); T: Total testosterone; DHEAS = dehydroepiandrosterone sulfate; E_2 = estradiol; SHBG = sex hormone binding-globulin; LH = luteinizing hormone; FSH = follicle stimulation hormone; kiss54: kisspeptin 54; CpGt: Total DNA methylation [(CpG1 + CpG2 + CpG3 + CpG4)/4]. Model was adjusted for adolescents' age (continuous), waist to height ratio (continuous), Tanner Stage (<G5/>G5), urine creatinine concentrations (continuous), annual family income (<25,000/25,000-35,000/>35,000), season of biological sample collection (spring/summer/autumn/winter). *p < 0.05; †p < 0.10.

of *KISS1* were also found. Exposure to phenols was only associated with lower *KISS1* DNA methylation. On the other hand, kiss54 levels were associated with higher serum reproductive hormones, and *KISS1* DNA methylation with lower serum reproductive hormone levels. This is the first population-based study exploring the role of kisspeptin protein and its DNA methylation patterns in the association between exposure to environmental chemicals and reproductive hormones.

In general, urinary concentrations of environmental chemicals measured in adolescent males were lower or within ranges previously described for children and adolescents. Regarding phenols, urinary MPB concentrations were lower, but BPA and BP3 concentrations were higher than previously reported in adolescents from Belgium, and Germany (Murawski et al., 2021; Tschersich et al., 2021). Regarding pesticides, MDA concentrations were generally lower than those found in children in Spain, the US, and Costa Rica (Roca et al., 2014; van Wendel de Joode et al., 2016a); ETU was within the ranges of those found in French and Latin American children living near agricultural fields (Raherison et al., 2019; Wilhelm et al., 2008); and IMPY and DCCA concentrations were higher than reported for Spanish and US children (Hernández et al., 2019; Oulhote and Bouchard, 2013). Urinary Cd concentrations were within the range and total (organic and inorganic) As concentrations were higher than those reported in North American and German adolescents (Sanders et al., 2019; Schulz et al., 2011). Additionally, serum kiss54 levels were within the range of those previously reported (0.16–4.65 ng/mL) (Özgen et al., 2016; Gorkem et al., 2018). However, concentrations are not entirely comparable since no epidemiological studies have measured kiss54 in the male population.

Single exposure models showed that total As and pesticide metabolites MDA, and DCCA were positively associated with serum kiss54 levels, IMPy was also inversely associated with CpG1 and CpGt methylation, BP3 and DCCA with CpG2, and BPA was borderline associated with lower CpGt. The few experimental studies analyzing the effect of single environmental chemicals on kisspeptin *in vivo* found increased kisspeptin expression after exposure (Li et al., 2018; Overgaard et al., 2013; Qiu et al., 2020; Roepke and Sadlier, 2021; Wang et al., 2014). For example, mancozeb-exposed rats experimented increased kisspeptin mRNA levels in the arcuate nucleus (ARC) (Overgaard et al., 2013); perinatal exposure to As and BPA increased hypothalamic kisspeptin protein and reproductive serum hormones levels in female mice (Li et al., 2018; Qiu et al., 2020); and BPA increased *KISS1* mRNA and kisspeptin protein at the anteroventral periventricular nucleus (AVPVN) in adult female mice (Wang et al., 2014). These studies may partially support our findings, although differences between animal and human populations (*e.g.*, exposure concentrations and/or metabolism) should be taken into account when comparing these results.

Experimental studies have shown that BPA may act centrally in the hypothalamus by altering estrogenic positive and negative feedbacks on GnRH, which are mediated by AVPV and ARC kisspeptinergic neurons, respectively (Kurian et al., 2015; Ruiz-Pino et al., 2019). Furthermore, increased kisspeptin expression was enhanced by estrogen-dependent activation of *KISS1* promoter histone 3 (H3) acetylation in rodents (Tomikawa et al., 2012; Uenoyama et al., 2021). Therefore, it could be plausible that BPA up-regulates ARC/AVPV kisspeptin expression through its binding to ERα, initiating kisspeptin positive/negative feedback on GnRH, and subsequently up/down-regulating hormonal concentrations. Due to the brain's dimorphic nature, males show lower kisspeptinergic neurons in AVPVN (Uenoyama et al., 2021), suggesting higher susceptibility to BPA central effects in boys, as previously reported in several epidemiological studies (Caporossi and Papaleo, 2015; Rehman et al., 2018; Rodríguez-Carrillo et al., 2019).

Two recent epidemiological studies evaluated the relationship between BPA exposure and kisspeptin. Thus, gestational exposure to BPS and BPA were associated with higher total kisspeptin levels in Chinese children (Wang et al., 2022) and with higher serum kiss54 levels in peripubertal girls diagnosed with central precocious puberty or premature thelarche, respectively (Özgen et al., 2016). Although their results were in line with the present findings, they are not entirely comparable, since this study included

only boys, Wang et al. (2022) study focused on prenatal exposure, and girls in the study of Özgen et al. (2016) had particular endocrine conditions. To the best of our knowledge, epidemiological studies assessing the joint effect of environmental chemicals on kisspeptin during or after puberty are not available in the literature. Nonetheless, some contradictory results have been described in experimental studies. For instance, sheep fetuses exposed to a cocktail of environmental chemicals extracted from sewage showed decreased *KISS1* mRNA levels at the hypothalamus (Bellingham et al., 2009), whereas a study in female rats perinatally exposed to a mixture of pesticides (epoxiconazole, mancozeb, prochloraz, tebuconazole, and procymidone) reported no additive effect on hypothalamic kisspeptin neurons (Overgaard et al., 2013). However, these studies were focused on perinatal exposure, while the present study was conducted in adolescents.

The present study showed that 2 out of 4 pesticide metabolites and the pesticide mixture were associated with lower kiss54 levels, which may suggests an additive effect. Due to the novelty of our study, no epidemiological comparisons could be made for pesticides since no previous studies assessed the role of the selected pesticides on kisspeptin. However, there are some mechanisms that may lead to kisspeptin up or down-regulation. First, by the aforementioned interaction with ERα (Uenoyama et al., 2021). In this way, diazinon, cypermethrin, and deltamethrin, parent compounds of IMPy and DCCA, exert estrogenic activities *in vitro* (Chen et al., 2011; Manabe et al., 2006; Mnif et al., 2011). However, whether this effect could be produced centrally, as observed for BPA, remains unknown. Secondly, by external interaction with Leydig cells, which decreases T circulating levels and activates the release of kisspeptin through the negative feedback triggered by the hypothalamic-pituitary-testicular (HPT) axis (Babakhanzadeh et al., 2020; Moreira et al., 2021). In this regard, dimethoate-exposed Leydig cells of male rats led to decreased T levels (Babakhanzadeh et al., 2020). Third, through alteration of the AMP-activated protein kinase (AMPRK), another key regulator for T secretion in Leydig cells (Moreira et al., 2022). For instance, exposure to some pesticides such as chlorpyrifos, ziram, or acetamiprid triggered the transformation of cAMP into AMP, activating AMPRK and repressing steroidogenesis *in vitro* and *in vivo*, which subsequent effects on central kisspeptinergic levels (Chen et al., 2018; Eze et al., 2019; Kong et al., 2017; Liu et al., 2018).

Interestingly, the present study found that the pesticide mixture was associated with decreasing levels of LH, a key regulator of spermatogenesis initiation and maintenance (Moreira et al., 2021). This association could be explained based on the negative-feedback mechanism on the HPG axis. Lower LH would lead to lower T levels, thus increasing kisspeptin expression in order to increase this pituitary hormone and reach homeostasis (Moreira et al., 2021). A Mexican study found decreased LH levels among men occupationally exposed to dimethylthiophosphate (DMTP), whereas Finnish men environmentally exposed to pesticides showed increased LH levels (Palaniswamy et al., 2021; Recio et al., 2005). Due to the cross-sectional nature of the present study and the current knowledge, results should be interpreted with caution.

Further, the association between kisspeptin biomarkers and reproductive hormones was explored to understand the role of kisspeptin as effect biomarker in the HPG axis. Our results indicated that higher kiss54 levels were associated with increased LH, and higher DNA methylation of *KISS1* with decreased T, E_2, DHEAS, and FSH levels. These associations were within expected, since the lower the *KISS1* DNA methylation, the higher kiss54 serum levels and subsequently higher reproductive hormones levels (Pinilla et al., 2012; Semaan and Kauffman, 2013; Tomikawa et al., 2012). Moreover, serum kiss54 levels were negatively correlated with DNA methylation patterns, especially at CpG4. These results are also partially supported by a clinical trial that found that the administration of kiss54 increased plasma LH and T in males (Dhillo et al., 2005). In this line, experimental studies reported that central or peripheral administrations of kisspeptin stimulated the HPG axis in mice models, resulting in increased LH levels (Castellano et al., 2006; Gottsch et al., 2004; Thomson et al., 2004). However, whether these associations are unidirectional remains controversial, since reproductive hormones can play a role in regulating hypothalamic KISS1 DNA methylation and kiss54 levels through both negative and positive feedback (Semaan and Kauffman, 2013). Additionally, experimental studies assessing the correlation between central and peripheral kisspeptin levels (*e.g.* DNA methylation of the KISS1 gene for instance) may provide support to these results.

Interestingly, the phenols mixture was associated with an overall increase of serum reproductive hormonal levels, especially with SHBG, together with lower methylation of *KISS1* DNA at several CpGs. Moreover, the whole mixture (all families together) showed trends towards increased T, DHEAS, E2, and SHBG levels. Whether these results highlight an increment in disease risk due to increased hormonal levels within a long-time period remains unknown. Nevertheless, increased T levels in adult males have been associated with an increased risk of prostate cancer and hypertension (Mohammadi-Shemirani et al., 2020). Additionally, higher levels of SHBG may be associated with cardiovascular diseases, a higher risk of erectile dysfunction, or infertility in males (Gyawali et al., 2019; Li et al., 2021; Wan et al., 2021).

Several limitations need to be addressed in this study. First, its cross-sectional design only allows us to highlight potential associations and does not allow exploring the temporal link between exposure and outcomes. Second, the small sample size may have limited the statistical power and therefore the ability to detect some associations. In addition, the INMA-Granada cohort only includes males, which could be seen as a limitation to understand the effect of environmental chemicals on females. The possibility of exposure misclassification cannot be excluded, since phenols and pesticides have short biological half-lives (urinary metabolites likely reflect exposure within the previous 24–48 h). Proper measurement of these compounds requires repeated urine sample measurements to improve exposure assessment and reduce exposure misclassification. The inclusion of creatinine levels in adjustment models has probably contributed to reducing exposure misclassification (Wang et al., 2016, 2019). Another limitation to consider is the possibility of interindividual variability of urinary chemical concentrations over time (Bravo et al., 2020; Casas et al., 2018; van Wendel de Joode et al., 2016b). Nevertheless, given that the INMA-Granada cohort includes mother-child pairs from the general population with similar lifestyles, interindividual variability could be mitigated (Barr, 2008; Kim et al., 2017; Park et al., 2017). Potential selection bias may be also considered, given that study participants were more likely to reside in an urban area and belong to a higher-income family than those in previous follow-ups of the INMA-Granada cohort (Table S12). Moreover, this study measured peripheral rather than central *KISS1* DNA methylation, which should be taken into account when interpreting the results described. Studies assessing the correlation between central and peripheral concentrations of KISS1 DNA methylation are needed to overcome this issue. Type I errors and spurious associations due to the performance of multiple tests performance (*e.g.*, 109 comparisons) cannot be ruled out. Nevertheless, our hypothesis falls within toxicological studies (Mustieles et al., 2020), although due to the scarce knowledge available further studies are needed to confirm these results.

Despite study limitations, this is the first epidemiological study assessing the mixture effect of relevant environmental contaminants on kisspeptin at two levels of biological organization. The present results are supported by previous toxicological and epidemiological evidence, although due to the novelty of this study few comparisons could be made. Additionally, this study was focused on adolescents, an understudied period of development. Furthermore, reproductive hormones were included as a next step to physiologically validate kisspeptin as a valuable effect biomarker for adverse reproductive outcomes triggered by environmental exposures. However, mediation analyses cannot be performed due to a lack of associations between environmental chemicals and hormones. Finally, due to the small sample size and the interindividual variability of urinary environmental chemicals, further larger studies are needed to confirm our results.

5. Conclusion

Environmental chemical exposure may increase serum kisspeptin protein levels, altering the homeostasis and up-down regulating HPG axis

hormone concentrations. In this study, it seems that exposure to both individual chemicals and mixtures was associated with higher kiss54 levels and lower *KISS1* DNA methylation. Additionally, serum kiss54 levels and DNA methylation showed associations towards higher or lower reproductive hormone levels, respectively. Due to the inherent limitations of this cross-sectional study, caution should be taken when interpreting these results. Thus, the measurement of kisspeptin should be implemented in large longitudinal studies to validate our findings, since it may provide mechanistic information to understand how environmental chemicals alter the reproductive function.

Funding and acknowledgements

The authors greatly acknowledge the support received by Manuel Tena-Sempere. Also, the European Union's Horizon 2020 research and innovation program HBM4EU for the financial support under Grant Agreement #733032. The study was further supported by the ISCIII with grants no. CP16/00085 and PI 17/01526, and the Human Genotyping Laboratory at the Spanish National Cancer Research Centre, CeGen-PRB3, which was supported by grant no. PT17/0019, of the PE I + D + i 2013-2016, funded by the Instituto de Salud Carlos III (ISCIII) and ERDF. The funding of the open access fee to the University of Granada / CBUA is also gratefully acknowledged. This research would not have been achieved without the selfless collaboration of the INMA-Granada adolescents and families who took part in the study. The authors also thank the support of the Biomedical Research Networking Center-CIBER de Epidemiología y Salud Pública (CIBERESP), and the University of Granada (Grant "UNETE," UCE-PP2017-06). A. Rodríguez-Carrillo received a postdoctoral fellowship granted by the Ramon Areces Foundation, and Shereen Cynthia D'Cruz were under contract with the HBM4EU project. C. Freire received a grant under the Miguel Servet Type II program of the ISCIII "Fondo Europeo de Desarrollo Regional" (CPII21/00014; ISCIII/FEDER).

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Andrea Rodriguez-Carrillo: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Sylvie Remy:** Writing – review & editing. **Shereen Cynthia D'Cruz:** Investigation, Writing – review & editing. **Elena Salamanca-Fernandez:** Writing – review & editing. **Fernando Gil:** Writing – review & editing. **Pablo Olmedo:** Investigation, Writing – review & editing. **Vicente Mustieles:** Investigation, Writing – review & editing. **Fernando Vela-Soria:** Investigation, Writing – review & editing. **Kirsten Baken:** Writing – review & editing. **Nicolás Olea:** Writing – review & editing. **Fátima Smagulova:** Writing – review & editing. **Mariana F. Fernandez:** Supervision, Funding acquisition, Project administration, Writing – review & editing. **Carmen Freire:** Supervision, Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161668.

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