TESIS DOCTORAL

UNIVERSIDAD DE GRANADA

EXPOSICIÓN A PESTICIDAS NO PERSISTENTES Y OTROS DISRUPTORES ENDOCRINOS, DESARROLLO PUBERAL Y SUSCEPTIBILIDAD GENÉTICA EN NIÑOS Y NIÑAS DE LA COHORTE INFANCIA Y MEDIO AMBIENTE (INMA)

Programa de doctorado en Medicina Clínica y Salud Pública

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RESUMEN

Diversos pesticidas no persistentes, como insecticidas organofosforados y piretroides, fungicidas y herbicidas, y otros compuestos químicos presentes en productos de consumo de uso cotidiano, como los ftalatos, poseen actividad estrogénica y/o anti-androgénica, siendo clasificados como disruptores endocrinos (DEs). Los procesos implicados en el desarrollo de la pubertad son potencialmente vulnerables a la acción de los DEs y la exposición en momentos críticos del desarrollo podría ser una de las causas atribuidas a la tendencia hacia el adelanto de la pubertad observada en las últimas décadas, especialmente en las niñas. Además, determinadas variantes genéticas podrían conferir mayor susceptibilidad a los potenciales efectos adversos en salud de los pesticidas y otros contaminantes ambientales.

El primer objetivo específico de esta Tesis Doctoral fue realizar una revisión sistemática de la evidencia epidemiológica de asociación entre la exposición temprana a pesticidas no persistentes y desarrollo puberal (edad de inicio o estado de maduración sexual). Para ello, se realizó una búsqueda sistemática en las bases de datos MEDLINE y SCOPUS de los artículos publicados hasta noviembre de 2020 y se excluyeron los estudios no epidemiológicos, no originales, publicados en idiomas diferentes al inglés, español, italiano o portugués y los estudios en los que el desarrollo puberal o la maduración sexual no era la variable respuesta principal. En la metodología se siguieron las directrices de la guía PRISMA y la calidad de las evidencias se evaluó mediante la escala GRADE. Trece estudios cumplían los criterios de inclusión y ocho de ellos describían asociaciones significativas con alteraciones en la edad de inicio de la pubertad. Así, la exposición *in utero* a herbicidas tipo atrazina se relacionó con menarquia más temprana en un estudio; la exposición infantil a insecticidas organofosforados (OPs) se asoció con retraso en el desarrollo puberal en ambos sexos en otro estudio, mientras que la exposición a insecticidas piretroides se asoció con retraso en la maduración sexual en niñas y adelanto puberal en niños en dos estudios, respectivamente. La exposición prenatal a múltiples pesticidas se relacionó con adelanto puberal en niñas y retraso puberal en niños en dos estudios, respectivamente, mientras que el residir en áreas rurales durante la infancia se relacionó con retraso en la edad de la menarquia en niñas en un estudio y con adelanto puberal en niños en otro.

El segundo objetivo fue evaluar la asociación entre la exposición a pesticidas no persistentes y el desarrollo puberal de niños y niñas con edad entre 7 y 11 años. Para ello se realizó un estudio transversal en el que se cuantificaron cuatro metabolitos de insecticidas (TCPy, metabolito de clorpirifós; IMPy, metabolito de diazinón; DETP,

metabolito no específico de insecticidas OPs y 3-PBA, metabolito genérico de piretroides) y el metabolito de fungicidas bisditiocarbamatos de etileno, etilentiourea (ETU), en muestras urinarias de 939 niños y 606 niñas que participan en el estudio de cohortes multicéntrico Infancia y Medio Ambiente (INMA) en Asturias, Gipuzkoa, Granada, Sabadell y Valencia. Para la evaluación puberal de los niños se empleó la escala de Tanner y la Escala de Desarrollo Puberal (PDS). Mediante regresión logística multivariante, se examinó la asociación de cada metabolito con la probabilidad de estar en estadío ≥2 para cada hito puberal. Se exploró además el efecto modificador del índice de masa corporal (IMC) mediante análisis estratificado (peso bajo/normal *versus* sobrepeso/obesidad). En las niñas, se observó que mayores concentraciones urinarias de DETP y ETU se asociaban con mayor probabilidad de haber iniciado la pubertad según la PDS, y que las concentraciones de ETU también se asociaban con mayor probabilidad de haber iniciado el desarrollo mamario, especialmente en niñas con peso bajo/normal. En los niños, la detección de TCPy, ETU y 3-PBA se asoció con mayor probabilidad de haber iniciado el desarrollo genital, siendo la asociación con ETU y 3-PBA significativa solo en niños con peso bajo/normal y sobrepeso/obesidad, respectivamente. En cambio, DETP se asoció con retraso puberal en niños con sobrepeso/obesidad.

El tercer objetivo fue evaluar la asociación entre la exposición prenatal a ftalatos y el desarrollo puberal de niños y niñas de las cohortes INMA de Gipuzkoa, Sabadell y Valencia en la misma ventana temporal (7-10 años). Un total de 409 niñas y 379 niños participaron en el estudio. Se recogieron muestras de orina de las madres durante el embarazo, en las que se analizaron los metabolitos de 6 ftalatos (DEP, DiBP, DnBP, BBzP, DEHP y DiNP) y el plastificante no ftalato DINCH®. Se examinó la asociación entre la exposición prenatal a ftalatos y el desarrollo puberal evaluado mediante la PDS cumplimentada por los padres. Para ello, se utilizó la regresión de Poisson con varianza robusta. Para evaluar el posible papel del IMC como factor modificador se realizó análisis estratificado. En niños, la exposición prenatal a DEHP, DEP y DnBP estaba asociaba con un mayor riesgo de haber iniciado la pubertad, y en niñas, la exposición a DEHP se asoció con mayor riesgo de adrenarquia. En cambio, BBzP y DINCH® se asociaron con menor riesgo de adrenarquia en niños con sobrepeso/obesidad. En niñas con sobrepeso, DiBP, DnBP y DINCH® se asociaron con un riesgo ligeramente mayor de gonadarquia. El efecto combinado de la exposición a la mezcla de ftalatos, evaluado mediante regresión de suma de cuantiles ponderada (WQS), no se asoció con la pubertad en ninguno de los dos sexos.

El cuarto objetivo de esta Tesis Doctoral fue examinar la asociación entre la exposición a pesticidas no persistentes y el estado de maduración sexual en 201 adolescentes varones de 14 a 17 años de edad, participantes de las cohortes INMA de Granada y Menorca. Se cuantificaron los metabolitos urinarios TCPy, IMPy, DETP y malatión diácido (MDA), metabolito de malatión; los metabolitos inespecíficos de los piretroides 3-PBA y ácido dimetilciclopropano carboxílico (DCCA); el 1-naftol (1-N), metabolito de carbarilo y ETU. La maduración sexual se evaluó mediante la escala de Tanner, la PDS y medición de volumen testicular (VT). Se empleó regresión logística multivariante para examinar la asociación de cada metabolito con la probabilidad de estar en estadío 5 de Tanner, estadío ≥4 de la PDS y de presentar VT ≥25 mL. La exposición a DETP, TCPy y MDA se asoció con una menor maduración sexual (gonadal para DETP y TCPy y adrenal para MDA), mientras que 1-N se asoció con mayor maduración adrenal pero menor VT.

El quinto y último objetivo fue evaluar la relación entre la exposición a pesticidas no persistentes y las concentraciones séricas de hormonas sexuales en adolescentes varones de la cohorte INMA-Granada y la posible interacción con polimorfismos (SNPs) en genes involucrados en el metabolismo de pesticidas. Para ello se realizaron dos estudios transversales en adolescentes de la cohorte de Granada que participaron en visita de seguimiento a la edad de 15-17 años: en el primero se cuantificaron las concentraciones urinarias de los metabolitos de insecticidas OPs: TCPy, IMPy, DETP y DEDTP, y en el segundo se cuantificaron los metabolitos urinarios ETU, 3-PBA y 1-N. Para ambos estudios se cuantificaron las concentraciones séricas de las principales hormonas sexuales y tiroideas y se examinaron las frecuencias de los SNPs de PON1, CYP2C19 y CYP2D6. La exposición a TCPy se asoció con un aumento de los niveles de dehidroepiandosterona sulfato (DHEA-S) y disminución de estradiol (E_2), hormona folículo estimulante (FSH) y hormona anti-Mülleriana (AMH); la detección de IMPy con aumento de los niveles de E_2, DHEA-S, FSH, AMH y prolactina y con menores niveles de hormona luteinizante (LH) y de la proteína transportadora de hormonas sexuales (SHBG); la detección de DETP se asoció, aunque de forma no significativa, con aumento de los niveles de testosterona y triiodotironina (T3) y disminución de FSH, AMH y prolactina. El efecto de IMPy y DETP sobre los niveles de DHEA-S y testosterona, respectivamente, fue mayor en los adolescentes portadores del genotipo PON1 55MM, asociado a menores concentraciones de PON1, mientras que el efecto de TCPy, IMPy y DETP sobre los niveles de hormona tiroidea fue mayor en los adolescentes con menor capacidad de

metabolización, portadores de los genotipos PON1 192QR/RR o 55MM. En el segundo estudio, la exposición a 3-PBA se asoció con mayores niveles de T3, mientras que 1-N con aumento de DHEA-S y disminución de E_2 y FSH. La magnitud de las asociaciones observadas para 1-N y DHEA-S era más fuerte en los portadores de las variantes de pérdida de función CYP2C19*2 y CYP2D6*4. Además, se observó una asociación entre la detección de ETU y niveles de cortisol solo en los portadores de los genotipos 1846GA y AA del gen CYP2D6.

En conclusión, los resultados de esta Tesis Doctoral sugieren que la exposición a determinados pesticidas no persistentes y otros DEs como los ftalatos en etapas de especial vulnerabilidad, como la infancia y el embarazo, podría estar asociada con alteraciones en el desarrollo puberal y en las concentraciones de hormonas sexuales, siendo el estado nutricional y la susceptibilidad genética individual factores que pueden modificar dichas asociaciones.

ABSTRACT

Numerous non-persistent pesticides, such as organophosphate and pyrethroid insecticides, fungicides or herbicides, and other chemical compounds such as phthalates, widely present in household items, have shown pro-estrogenic and anti-androgenic effects, being classified as endocrine disruptors chemicals (EDCs).

The processes involved in pubertal timing are potentially vulnerable to EDCs, and exposure at critical windows of development could be implicated in the secular trend of puberty observed in boys and girls in recent decades. Interindividual genetic variations could confer greater susceptibility to potential adverse health effects of hormonally active compounds.

The first specific objective of this doctoral thesis was to conduct a systematic review of epidemiological studies assessing the association between early exposure to non-persistent pesticides and pubertal development (age at the onset and/or state of sexual maturation). A systematic search was carried out using MEDLINE and SCOPUS databases, including original articles published up to November 2020. The PRISMA guidelines were used, and the quality of the evidence was assessed using the GRADE scale. Thirteen studies were selected after excluding non-original and non-human studies. Exposure to different types of pesticides has been associated with altered puberty timing in girls and/or boys in eight studies. In utero exposure to atrazine has been related to earlier age of menarche in girls; exposure to organophosphate (OP) pesticides has been related to delayed sexual development in boys and girls in one study; childhood pyrethroid exposure has been associated with pubertal delay in girls and pubertal advancement in boys in two studies, respectively; prenatal/ exposure to multiple pesticides has been linked to earlier puberty onset in girls and pubertal delay in boys childhood, in two studies respectively, while living in rural area during childhood was associated to a greater age at menarche in one study and to an earlier puberty onset in boys in other study.

The second objective was to evaluate the association between exposure to non-persistent pesticides and pubertal development in 7-11-years-old boys and girls. A cross-sectional study was carried out. Four insecticide metabolites (TCPy, chlorpyrifos metabolite; IMPy, diazinon metabolite; DETP, non-specific organophosphate metabolite and 3-PBA, pyrethroid metabolite) and the fungicide metabolite ethylene-bis-dithiocarbamate (ETU) were quantified in urinary samples of 939 boys and 606 girls from the Childhood and Environment (INMA) cohort study carried out in Asturias, Gipuzkoa,

Granada, Sabadell and Valencia. As pubertal assessment, the Tanner scale and the Pubertal Development Scale (PDS) were used. The association between each metabolite and the probability of being stage ≥2 for each pubertal milestone was examined using multivariate logistic regression. Effect modification by BMI was explored by interaction terms and stratified analysis. In girls, DETP and ETU exposure was associated with higher odds of overall pubertal development on the PDS, and ETU was also associated with higher odds of breast development in non-overweight girls. In boys, detection of TCPy, ETU, and 3-PBA were associated with higher odds of genital development, and the association for ETU and 3-PBA were significant only in normal/underweight and overweight/obese children, respectively. In contrast, DETP was associated with delayed puberty in overweight/obese children.

The third objective was to evaluate the effect of prenatal exposure to phthalates on the pubertal development of boys and girls from Gipuzkoa, Sabadell and Valencia INMA cohorts. Urinary metabolites of six different phthalate diesters (DEP, DiBP, DnBP, BBzP, DEHP, and DiNP) and non-phthalate plasticizer DINCH® were quantified in two urine samples collected during pregnancy from mothers participating in the INMA Spanish cohort study. Pubertal assessment of their children at age 7-10 years (409 boys, 379 girls) was conducted using the parent-reported PDS. Modified Poisson and Weighted Quantile Sum (WQS) regression was employed to examine associations between prenatal phthalates and risk of puberty onset, adrenarche, and gonadarche. Effect modification by child weight status was explored by stratified analysis. Prenatal exposure to DEHP, DEP, and DnBP was associated with a higher risk of puberty onset in boys, and DEHP with a higher risk of adrenarche in girls. In contrast, exposure to BBzP and DINCH® was associated with a lower risk of adrenarche in overweight/obese boys. In overweight girls, DiBP, DnBP, and DINCH® were associated with a slightly increased risk of gonadarchia. Exposure to phthalate mixture, assessed by quantile sum regression, was not associated with puberty in boys or girls.

The fourth objective was to examine the association between urinary biomarkers of non-persistent pesticides and sexual maturation in 14 to 17 years old boys from the INMA cohorts of Granada and Menorca. Urinary metabolites were measured in single spot urine samples, including: TCPy, IMPy, DETP and diacid malathion (MDA), malathion metabolite; the non-specific pyrethroid metabolites 3-PBA and dimethylcyclopropane carboxylic acid (DCCA); the 1-naphthol (1-N), carbaryl metabolite and ETU. Sexual maturation was assessed using Tanner stages, self-reported

Pubertal Development Scale, and testicular volume (TV). Multivariate logistic regression was employed to examine associations between urinary pesticide metabolites and the odds of being in Tanner stage 5 of genital development (G5) and pubic hair growth; stage ≥4 of overall pubertal development, gonadarche, and adrenarche; or having mature TV (≥25 mL). Exposure to DETP, TCPy, and MDA was associated with a decrease in pubertal maturation (gonadal for DETP and TCPy and adrenal for MDA), while 1-N was associated with an increase in adrenal maturation and decreased testicular volume. Exposure to 3-PBA was associated with smaller testicular size.

The fifth objective was to evaluate the relationship between exposure to pesticides and serum concentrations of sex hormones in male adolescents and the possible interaction with polymorphisms in genes involved in pesticide metabolism. For this purpose, two cross-sectional studies were carried out in 16 to 17 years old boys from the INMA Granada cohort. In the first study, urinary concentrations of organophosphate metabolites: TCPy, IMPy, DETP and DEDTP were measured in the urine sample of 134 boys, and in the second study, the urinary metabolites ETU, 3-PBA and 1-N were quantified in 117 boys. For both studies, the serum concentrations of the main sex and thyroid hormones were measured and alterations in the PON1, CYP2C19 and CYP2D6 genes were examined. Exposure to TCPy was associated with increased level of dehydroepiandrosterone (DHEA-S) and decreased oestradiol (E2), FSH, and anti-mullerian hormone (AMH); the detection of IMPy with increased E_2, DHEA-S, FSH, AMH and prolactin and with lower levels of SHBG and LH; DETP detection was marginally associated with elevations in testosterone and triiodothyronine (T3) and decreases in FSH, AMH, and prolactin levels. The effect of IMPy and DETP on DHEA-S and TT levels, respectively, was higher in subjects that carried the PON1 55MM genotype, while the effect of TCPy, IMPy, and DETP on thyroid hormone levels was higher in PON1 192QR/RR or 55MM genotype carriers. In the second study, exposure to 3-PBA was associated with elevated T3, while 1-N with elevated DHEA-S and decreased E_2 and FSH. Poor CYP2C19 and CYP2D6 metabolizers (GA and AA genotype carriers) showed a greater increase in DHEA-S for detected versus undetected 1-N compared with GG genotype carriers. Poor CYP2D6 metabolizers (1846GA and AA genotypes) evidenced increased cortisol for detected versus undetected ETU. The magnitude of some of the associations was stronger for the carriers of the mutations in the analysed genes.

In conclusion, the results of this doctoral thesis suggest that exposure to different classes of non-persistent pesticides and other endocrine disruptors, during critical window

such as childhood and pregnancy, could be associated with variations in the concentrations of sexual hormones, in pubertal development and sexual maturation of boys and girls and that nutritional status and individual genetic susceptibility can modify these associations.

LISTA DE ABREVIACIONES

1-N:1-naftol

3-PBA: Ácido 3-fenoxibenzoico

ACTH: Corticotropina

ADN: Ácido desoxiribonucleico

ALSPAC: *Avon Longitudinal Study of Parents and Children*

AMH: Hormona anti-Mülleriana

AR: Receptor de andrógenos

ARN: Ácido ribonucleico

BBzP: Butil bencil ftalato

BPA: Bisfenol A

CAR: Receptor constitutivo de androstano

CIBM: Centro de Investigación Biomédica

CNIO: Centro Nacional de Investigaciones Oncológicas

COP: Compuesto orgánico persistente

CRH: Hormona liberadora de corticotropina

CYP450: Citocromo P450

DCCA: Ácido carboxílico de 2,2-dimetilciclopropano

DDE: Dicloro difenil dicloroetileno

DDT: Dicloro difenil tricloroetano

DE: Disruptor endocrino

DEDTP: Dietilditiofosfato

DEHP: Dietihexil ftalato

DEP: Dietil ftalato

DETP: Dietiltiofosfato

DHEA: Dehidroepiandrosterona

DHEA-S: Sulfato de dehidroepiandrosterona

DiBP: Diisobutil ftalato

DINCH: Éster diisononílico del ácido 1,2-ciclohexano dicarboxílico

DiNP: Dinonil ftalato

DnBP: Di-n-butil ftalato

E_2: Estradiol

EBDC: Bisditiocarbamato de etileno

ECHA: *European Chemical Agency*

ECLIA: Inmunoensayo de electroquimioluminiscencia

EEUU: Estados Unidos

EFSA: *European Food Safety Authority*

ER: Receptor de estrógenos

ETU: Etilentiourea

FLEHS: *The Flemish Environment and Health Study*

FSH: Hormona folículo estimulante

GABA: Ácido gamma-aminobutírico

GALP: Péptido similar a galanina

GnRH: Hormona liberadora de gonadotropinas

GRADE: *Grading of Recommendations, Assessment, Development and Evaluations*

GSA: *Global Screening Array*

GWAS: Estudio de asociación del genoma completo

HCB: Hexaclorobenzeno

HHA: Hipotálamo-hipófisis-adrenal

HHG: Hipotálamo-hipófisis-gonadal

HUSC: Hospital Universitario San Cecilio

IC: Intervalo de confianza

IGF-1: Factor de crecimiento insulínico tipo 1

IMC: Índice de masa corporal

IMIM: Instituto de Investigación del Hospital del Mar

IMPy: 2-isopropil-6-metil-4-pirimidinol

INMA: Infancia y Medio Ambiente

LD: Límite de detección

LH: Hormona luteinizante

NE: Norepinefrina

NHANES: *National Health and Nutrition Examination Survey*

NIPH: *Norwegian Institute of Public Health*

NPY: Neuropéptido Y

OMS: Organización Mundial de la Salud

OP: Organofosforado

OR: Odds Ratio, razón de probabilidad

PBDEs: Éteres de difenilo polibromados

PCBs: Bifenilos policlorados

PDS: Escala de desarrollo puberal

PECO: Población, Exposición, Comparación, Outcome

PFAS: Sustancia perfluoroalquilada

PHV: Pico de velocidad de crecimiento

PON1: Paraoxonasa 1

PPAR: Receptor activado por proliferadores peroxisomales

PP: Pubertad precoz

PPC: Pubertad precoz central

PPP: Pubertad precoz periférica

PRISMA: *Preferred Reporting Items for Systematic reviews and Meta-Analyses*

PRL: Prolactina

PVC: Policloruro de vinilo

PXR: Receptor X de pregnano

RR: Riesgo relativo

SHBG: Proteína transportadora de hormonas sexuales

SNP: Polimorfismo de nucleótido único

StAR: Proteína reguladora aguda esteroidogénica

STROBE: *Strengthening the Reporting of Observational studies in Epidemiology*

T3: Triiodotironina

T4: Tiroxina

TCPy: 3,5,6-tricloropiridinol

TSH: Tirotropina

UE: Unión Europea

UHPLC-MS/MS: Cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas

WQS: Suma de cuantiles ponderados

LISTA DE FIGURAS

1. INTRODUCCIÓN

1.1 Pubertad

La pubertad es la etapa del desarrollo en la que ocurren los cambios físicos que llevan al individuo a alcanzar la madurez reproductiva y la adultez. Durante esta etapa el individuo experimenta los procesos de maduración cognitiva y psico-social propios de la adolescencia.

Los eventos cardinales de la pubertad son representados por la gonadarquia, que consiste en la maduración y activación de los testículos y de los ovarios para la producción de hormonas sexuales y de los gametos, y la adrenarquia, que es el aumento de producción de andrógenos por parte de la glándula suprarrenal. Desde un punto de vista fenotípico, estos eventos se traducen en la adquisición de caracteres sexuales secundarios: en respuesta a la activación gonadal, el aumento de esteroides sexuales se refleja en la aparición del botón mamario y en la maduración de útero, vagina y vulva en las niñas y en el agrandamiento de los testículos, el alargamiento del pene y la profundización de la voz en los niños, mientras que la adrenarquia se manifiesta en la aparición del vello púbico (pubarquia) y axilar en ambos sexos y del vello facial en el varón, y en la maduración de las glándulas sudoríparas apocrinas con el característico cambio en el olor corporal y la aparición de acné. Los esteroides sexuales también contribuyen a la maduración esquelética que lleva a alcanzar la talla adulta y a los cambios en la composición corporal propios de cada sexo, con prevalencia de masa muscular en el varón y de masa grasa en la mujer.

1.1.1 Fisiología de la pubertad

La gonadarquia es impulsada por la producción y la liberación de forma pulsátil de hormona liberadora de gonadotropinas (GnRH) por el hipotálamo anterior, la cual estimula, en las células gonadotropas de la hipófisis, la producción de las gonadotropinas denominadas hormona foliculoestimulante (FSH) y hormona luteinizante (LH) alimentos desecados (1,2)(3). La hipófisis se compone de un lóbulo posterior o neurohipófisis y de un lóbulo anterior o adenohipófisis donde se localizan, entre otros tipos celulares diferenciadas, las células gonadotropas productoras de FSH y de LH. La circulación portal hipotálamo-hipofisaria consiste en una red de vasos sanguíneos que se originan en un plexo capilar próximo a la eminencia media del hipotálamo y que, pasando por el tallo

hipofisario, se dirigen hacia la adenohipófisis. Así, las hormonas procedentes del hipotálamo llegan a las células hipofisarias sin una dilución sistémica significativa, lo que permite una regulación muy precisa de la producción de gonadotropinas (4). Un aspecto muy característico de la unión hipotálamo-hipófisis en la producción de gonadotropinas es que el patrón de señalización de GnRH determina la cantidad y calidad de la secreción de gonadotropinas. Así, la liberación pulsátil de GnRH es capaz de estimular la producción de FSH y LH, mientras que la secreción continua de GnRH (o la administración exógena de análogos de GnRH de acción prolongada) inhibe la liberación de gonadotropinas; además, según la amplitud y la frecuencia de los picos de GnRH, se secreta FSH o LH, proporcionando un mecanismo por el cual dos gonadotropinas funcionalmente distintas pueden ser reguladas diferencialmente por una sola hormona liberadora del hipotálamo (5).

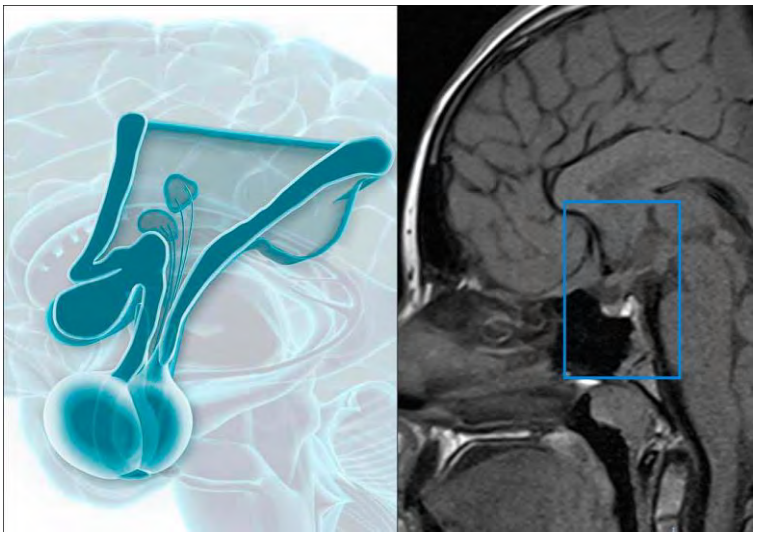

Fig. 1a Representación esquemática de la unión hipotálamo-hipofisaria.
Fig. 1b RMN cerebral en secuencia T1 en la que se aprecian adenohipófisis y neurohipófisis.

En niñas, la FSH estimula la maduración de los folículos ováricos y actúa sobre las células de la granulosa ovárica activando la enzima aromatasa que convierte en estradiol (E_2) los precursores andrógenos producidos en las células de la teca ovárica bajo estímulo de la LH. Al inicio de la pubertad, la producción de E_2 estimula la maduración de los genitales femeninos, la aparición del botón mamario y promueve la fusión de las placas de crecimiento, que se traduce en el "estirón" puberal. En fases más avanzadas de la pubertad, la producción hipofisaria de FSH y LH junto con la liberación ovárica de E_2 conduce a la ovulación y los ciclos menstruales.

En niños, la FSH estimula las células de Sertoli en el testículo induciendo la maduración tisular de los túbulos seminíferos, lo que se traduce en un aumento del volumen testicular. Paralelamente, La LH estimula la maduración de las células de Leydig para la producción de testosterona. El aumento de la concentración de testosterona contribuye a la maduración de los túbulos seminíferos y al crecimiento del pene,

profundización de la voz y aumento de la musculatura. Parte de la testosterona es convertida por el enzima aromatasa en E_2 que induce el crecimiento óseo, al igual que ocurre en las niñas.

La adrenarquia es el resultado de la maduración de la zona reticular de las glándulas suprarrenales en la que ocurre un aumento de la actividad de la 17-hidroxilasa (17,20 liasa), de la enzima P450c17 y un aumento de la actividad del citocromo b, resultando en un aumento de la producción de dehidroepiandrosterona (DHEA), sulfato de dehidroepiandrosterona (DHEA-S) y androstendiona que inducen, en ambos sexos, el crecimiento del vello púbico y axilar y la maduración de las glándulas sudoríparas apocrinas.

1.1.2 Evaluación clínica del desarrollo puberal

En la mayoría de los casos, los cambios puberales se producen según una secuencia predecible de eventos visibles que se inician en las niñas con la aparición del botón mamario (telarquia) y en los niños con el aumento del volumen testicular. Estos eventos ocurren, en media, en torno a los 10,5 años en niñas y los 11,5 años en niños. El instrumento más utilizado y el *"patrón oro"* para la evaluación clínica de la pubertad es la escala descrita en 1979 por Marshall y Tanner (6,7). Se trata de cuatro escalas compuestas cada una por 5 categorías sucesivas en las que se describen respectivamente los cambios secuenciales del crecimiento mamario femenino, del crecimiento de pene y testículos masculinos y del vello púbico en ambos sexos desde la etapa prepuberal (estadío 1) hasta la madurez completa (estadío 5). En las niñas, la maduración de los ovarios es en realidad el primer evento puberal que puede ser observado ecográficamente. El indicador ecográfico de la activación ovárica es la presencia de al menos 6 folículos de 4 mm de diámetro. Aunque la situación retroperitoneal del ovario puede dificultar su reconocimiento ecográfico, el aspecto multiquístico del ovario y los cambios morfológicos del útero ofrecen una evaluación fidedigna del comienzo de la pubertad en niñas. El útero prepuberal presenta una forma

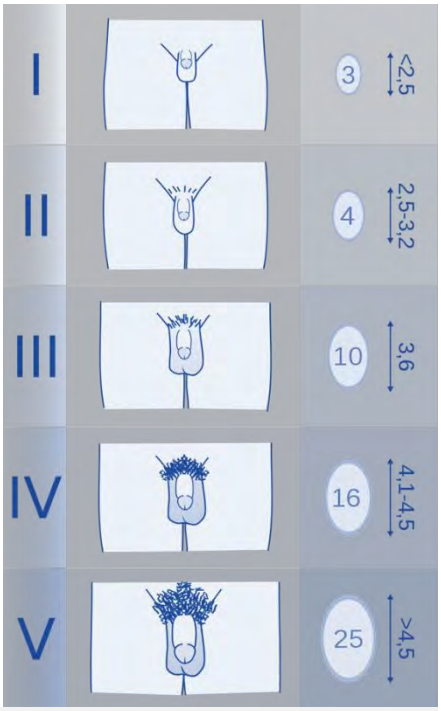

Fig. 2 Escala de Tanner para niños

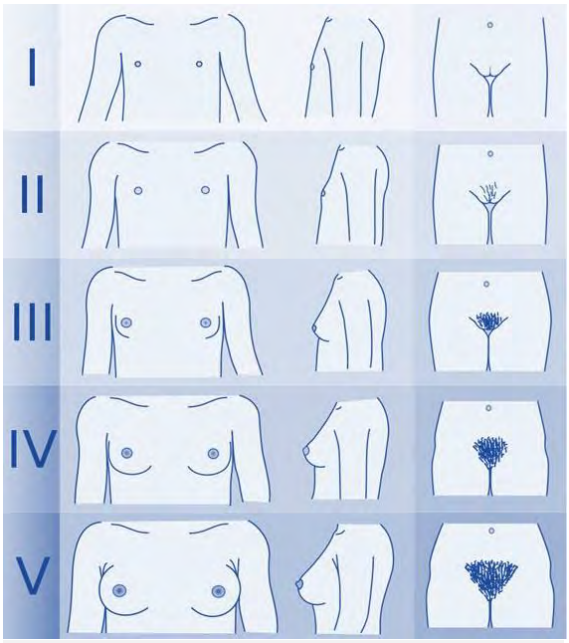

Fig. 3 Escala de Tanner para niñas

Fig. 4 Orquidómetro de Prader

tubular con una longitud de 2-3 cm, mientras que durante la progresión puberal aumenta de tamaño, hasta unos 5-8 cm de longitud, asume una conformación bulbosa y la relación cuerpo-útero (longitud del cuerpo/longitud del cuello uterino) alcanza una relación 2:1. En los niños, la situación extra-abdominal de los testículos permite una fácil evaluación de su volumen, mediante comparación con el orquidómetro de Prader, una cadena de 12 cuentas volumétricas numeradas de 1 a 25 mL. Cuando el volumen testicular alcanza los 4 mL se considera que se ha iniciado el crecimiento testicular puberal. La pubarquia refleja el aumento de producción de andrógenos (adrenarquia) y suele iniciarse pocos meses después de la telarquia y del aumento del volumen testicular, aunque hasta un 15% de los/as niños/as experimentan la pubarquia como primer evento puberal, siendo considerada una variante de la normalidad (8).

El "estirón" o aumento de la velocidad de crecimiento tiene relación con la secreción de esteroides sexuales. En los niños, ocurre paralelamente al aumento del volumen testicular y alcanza su pico máximo entre los estadíos 3 y 4 de desarrollo genital, con un volumen testicular de alrededor de 15 mL. En esta etapa del desarrollo masculino, en respuesta a la elevación de las concentraciones de testosterona, también ocurre la profundización de la voz y empieza a observarse el crecimiento del vello facial, que alcanza su máximo en el estadío 5 de Tanner. En las niñas, el estirón ocurre previamente a la telarquia, reflejando el aumento de esteroides sexuales producidos por los ovarios ya activados, y presenta su pico máximo alrededor del estadío 3 de desarrollo mamario. La menarquia, primer sangrado menstrual, marca el inicio de los ciclos menstruales y suele ocurrir aproximadamente a los dos años de la telarquia. La espermarquia es la primera

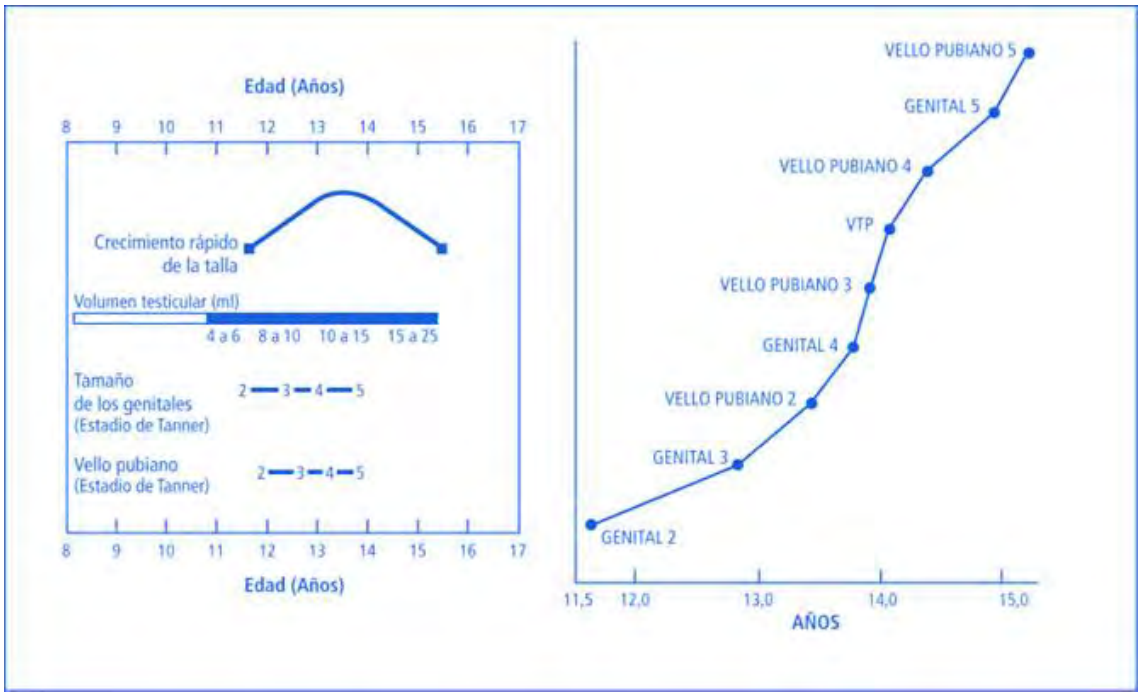

Fig. 5 Evolución del desarrollo puberal en el niño. Adaptado de *Marshall and Tanner, (1970)*

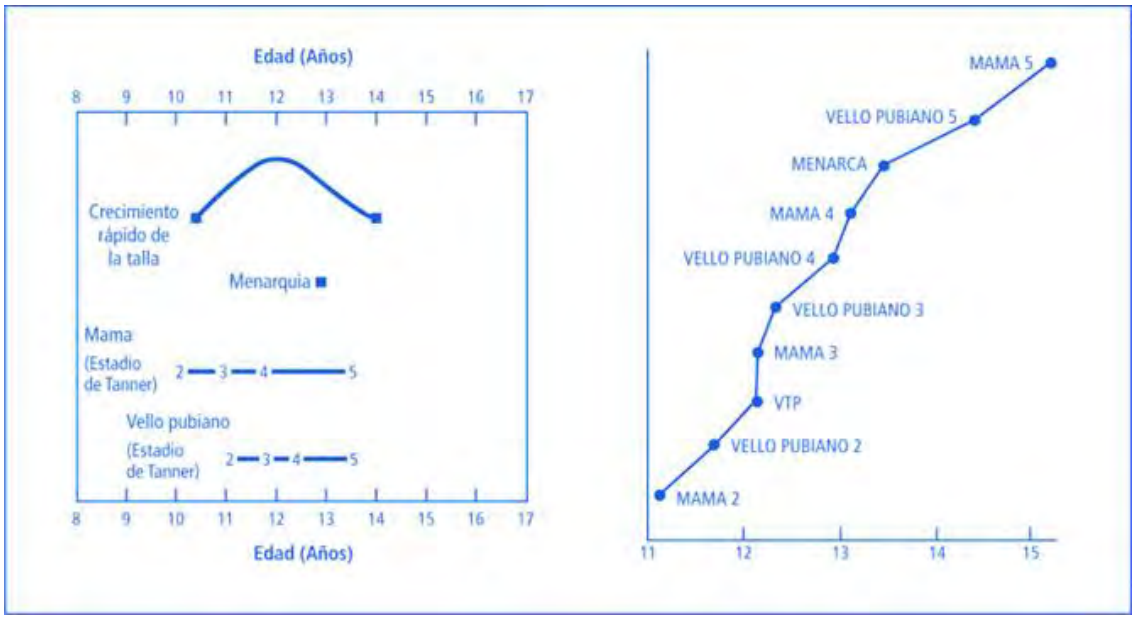

Fig. 6 Evolución del desarrollo puberal en la niña. Adaptado de *Marshall and Tanner. (1969)*

eyaculación del hombre, suele ocurrir de forma involuntaria en emisiones nocturnas y se presenta generalmente alrededor del estadío 3 de Tanner, paralelamente al pico máximo de velocidad de crecimiento. Tanto la menarquia como la espermarquia no suelen representar el inicio de la fertilidad, debido a que la gametogénesis precisa la maduración completa del sistema hormonal reproductivo.

La evaluación del desarrollo puberal en estudios epidemiológicos puede suponer un desafío, sobre todo en estudios que involucran amplias poblaciones. De hecho, las

escalas de Tanner y la medición del volumen testicular, que actualmente son los instrumentos más utilizados en la práctica clínica, requieren la participación de profesionales sanitarios entrenados y pueden ser poco aceptados por los niños o adolescentes. La escala de desarrollo puberal o PDS (del inglés: *Pubertal Development Scale)* ha resultado ser una válida alternativa a la escala de Tanner, sobre todo en estudios epidemiológicos. Los adolescentes, los padres o tutores legales son invitados a rellenar un cuestionario sobre crecimiento, vello corporal y cambios en la piel para ambos sexos, y caracteres específicos para cada sexo, con preguntas sobre vello facial y cambio de voz para los niños y sobre el desarrollo mamario y menarquia para las niñas (9). Hay cuatro opciones de respuesta (1-4): todavía no iniciado; apenas iniciado; definitivamente iniciado; parece completo. Siguiendo el algoritmo definido por Carskadon y Acebo (1993) (10) la puntuación continua obtenida se transforma en una escala ordinal de cinco categorías: 1) Pre-pubertad, 2) Pubertad temprana, 3) Pubertad media, 4) Pubertad tardía y 5) Post-pubertad. Además, Shirtcliff et al. (2009) desarrollaron un segundo algoritmo en el que se diferencia el desarrollo gonadal del adrenal de modo que el desarrollo mamario y la menarquia en niñas y el crecimiento testicular, la profundización de la voz y el crecimiento del vello facial en niños, así como el "estirón" de talla en ambos sexos, se consideran indicadores de gonadarquia, mientras que la aparición de vello púbico y de acné se consideran reflejo de la adrenarquia (11). La PDS ha demostrado ser un instrumento confiable y válido para evaluar el desarrollo puberal en niños/as, y tanto el cuestionario de PDS auto-cumplimentado como el cumplimentado por los padres han mostrado una fuerte consistencia interna y fiabilidad test-retest (12). Entre los otros métodos de valoración puberal, también se ha descrito el pico de velocidad de crecimiento (PHV, del inglés: *Peak Height Velocity*) como indicador indirecto de pubertad (13–15). Por ejemplo, en una cohorte de niños con diabetes tipo 1, estableciendo un valor para el pico de velocidad de crecimiento de ≥5,9 cm/año en varones y ≥6,6 cm/año en mujeres, se obtuvo una sensibilidad de 87% en la determinación del inicio de la pubertad (13).

1.1.3 Cronología de la pubertad e implicaciones en salud pública

La pubertad se considera normal cuando ocurre dentro de un rango de ±2 desviaciones estándar respecto a la media poblacional, que en países occidentales se encuentra entre los 8 y 13 años en niñas y entre los 9 y 14 años en niños. La pubertad precoz se define como la aparición de los caracteres sexuales secundarios antes de los 8

años en niñas y de los 9 años en niños, mientras que la pubertad retrasada se define por la ausencia de caracteres sexuales secundarios a los 13 años para las niñas y a los 14 años en niños. La pubertad precoz puede clasificarse en central (PPC) y periférica (PPP). La primera implica la activación de las neuronas hipotalámicas GnRH y, en consecuencia, de todo el eje hipotálamo-hipofisario-gonadal, mientras que la PPP no está mediada por la activación del sistema nervioso central y de dicho eje. Existen otras entidades clínicas consideradas variantes de la normalidad como la adrenarquia y la telarquia precoces idiopáticas que consisten en la aparición aislada del carácter sexual secundario antes de los límites inferiores de edad sin que haya activación del eje hipotálamo-hipófisis-gonadal y, por lo tanto, no progresan hacia una verdadera pubertad precoz.

Durante el último siglo se ha documentado ampliamente un descenso progresivo en la edad de inicio de la pubertad, sobre todo en niñas, fenómeno conocido como "tendencia secular de la pubertad" (16,17). La edad media de presentación de la menarquia parece haber disminuido de 2 a 3 meses por década desde el inicio del siglo XIX hasta mitad del siglo XX en Europa (18) y aproximadamente 2 meses por década en

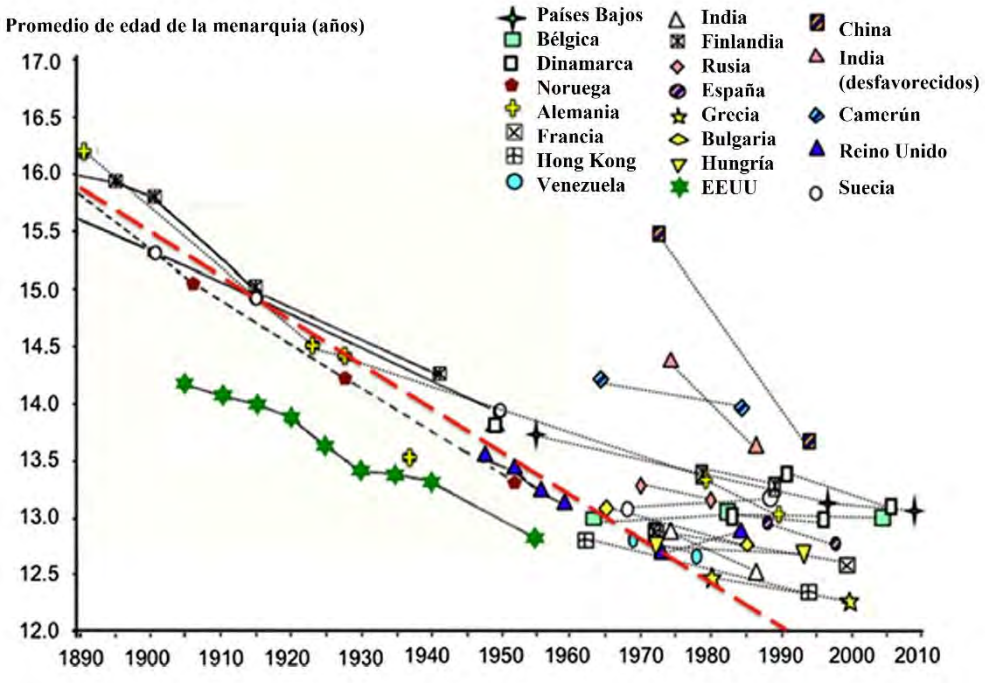

Fig. 5 Tendencia Secular de la menarquia. Adaptado de *Parent et al. (2015)*

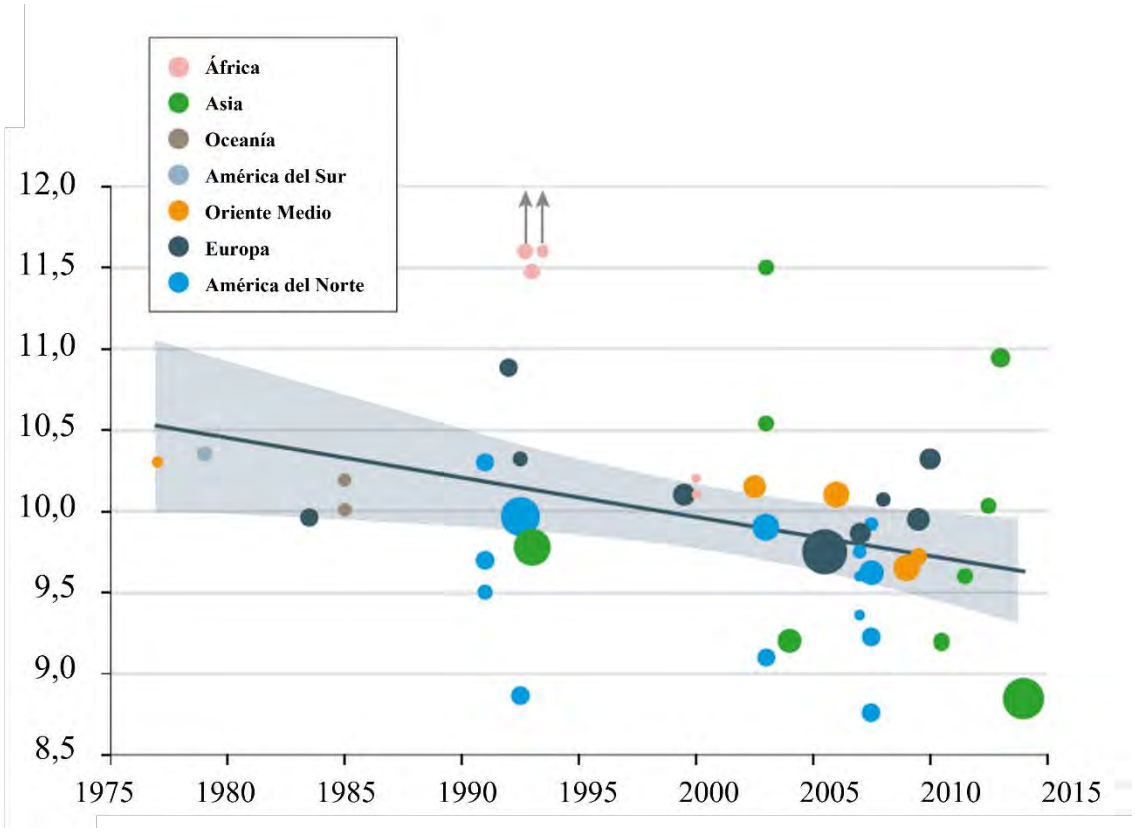

Fig. 6 Tendencia secular de la telarquia. Adaptado de *Ecker-Lind C. et al. (2020)*

la primera mitad del siglo XX en Estados Unidos (EEUU) (19). Esta tendencia parece haberse estabilizado en los países occidentales desde los años '60 a los '80 del siglo pasado (20,21), para luego observarse nuevamente un descenso, aunque más leve, en los últimos 20-30 años (22–24). Un fenómeno parecido también se ha observado en países occidentales para la edad media de presentación de la telarquia, con un descenso a partir de la década de los '90 más marcado que el observado para la menarquia y especialmente en niñas de raza negra de EEUU (20,25–27). En niños existe controversia debido a que, si bien se ha observado un leve descenso en la edad de inicio del desarrollo genital en las décadas más recientes, los estudios que han abordado este tema presentan como principal limitación la falta de datos históricos rigurosos con los que comparar los hallazgos más recientes (25,28–30).

En cuanto a las causas, la tendencia secular de la pubertad, y especialmente de la menarquia, ha sido atribuida principalmente a la mejora de las condiciones nutricionales y socioeconómicas de la población (31–34). El hecho de que se continúe observando un descenso en la edad de inicio de la pubertad en las décadas más recientes, cuando posiblemente las condiciones de salud pública se hayan mantenido estables, es actualmente objeto de un intenso interés científico, no solo por tratar de identificar los

factores determinantes de este fenómeno, sino también por las posibles consecuencias en salud pública de un desarrollo sexual cada vez más precoz de la población infantil (21,35,36). En este sentido, las alteraciones en la edad de inicio de la pubertad se han relacionado con diferentes comorbilidades en la adolescencia y en la vida adulta. La adolescencia representa una importante etapa en el desarrollo comportamental y social del individuo, y la pubertad temprana se ha relacionado con mayor riesgo de problemas psicosociales como ansiedad, depresión, trastornos conductuales y comportamientos de riesgo como consumo de tabaco, alcohol e inicio temprano de relaciones sexuales (37–40). Las variaciones en la edad de presentación de la pubertad están asociadas también con alteraciones musculoesqueléticas, así los niños y las niñas con pubertad precoz experimentan un pico en la velocidad de crecimiento más temprano y una talla final más baja con respecto a su talla genética, mientras que el retraso puberal está asociado con reducción en la mineralización ósea y con un mayor riesgo de fracturas en la adultez temprana (41–43). También se han documentado diferentes efectos a largo plazo de las variaciones en la pubertad. Por ejemplo, la pubertad precoz en mujeres es un factor de riesgo conocido para la aparición de cáncer de mama (44,45) y en varones se ha asociado con aumento de riesgo de cáncer testicular y de próstata en algunos estudios epidemiológicos (46–48), habiéndose identificado la pubertad tardía como un posible factor protector frente a la aparición de cáncer de testículo (49). En mujeres, además, la aparición precoz de la menarquia o de la pubarquia se ha asociado con mayor riesgo de hipertensión, dislipemia, síndrome metabólico y alteraciones cardiovasculares, aunque está en discusión el papel del sobrepeso y la obesidad en dichas asociaciones (50–54).

1.1.4 Factores implicados en la cronología de la pubertad

La amplia variabilidad de la edad de inicio de la pubertad, sea esta fisiológica o patológica, es el reflejo de múltiples factores individuales y ambientales que intervienen en este proceso madurativo, como se describe a continuación.

1.1.4.1 Factores moleculares y genéticos

Como se ha mencionado anteriormente, el evento molecular desencadenante del inicio de la pubertad es la secreción pulsátil de GnRH hipotalámico. El avance en el conocimiento de la fisiología molecular y de la genética de la pubertad ha

permitido reconocer diversos mecanismos de señalización que influyen en la activación hipotalámica.

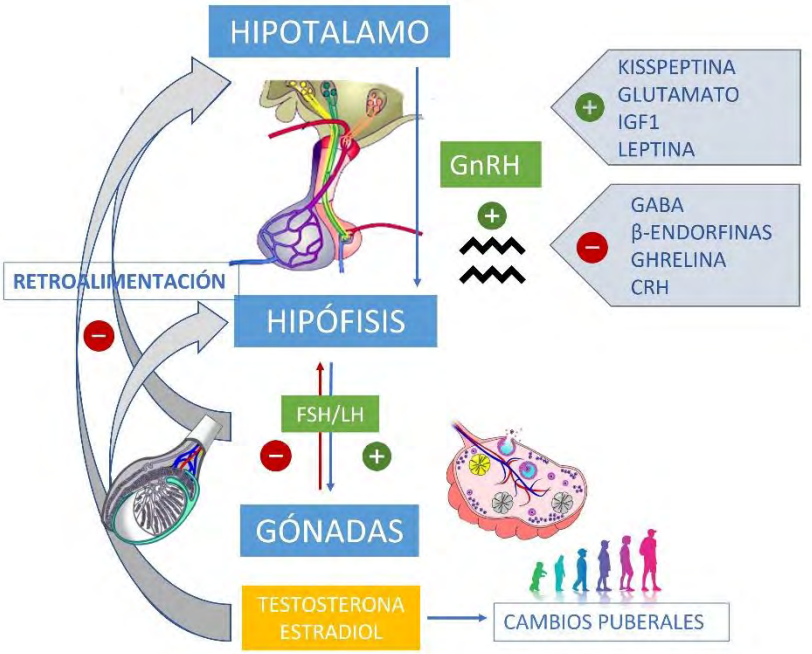

Fig. 7 Regulación molecular del eje hipotálamo-hipófisis-gónadas

La kisspeptina, la neuroquinina B y la dinorfina son moléculas sintetizadas en las neuronas KNDy del hipotálamo anterior que regulan la secreción pulsátil de GnRH (55–57). Mutaciones con pérdida de función de los genes KISS1 y TAC3 que codifican, respectivamente, kisspeptina y neuroquinina B, o de los genes que codifican sus receptores (KISS1R y TAC3R), están relacionadas con casos de hipogonadismo hipogonadotropo idiopático, mientras que en modelos animales la dinorfina disminuye la frecuencia de los picos pulsátiles de GnRH (58–61). La leptina producida en el tejido adiposo periférico, el factor de crecimiento insulínico tipo 1 (IGF-1) y neurotransmisores como la norepinefrina (NE), el neuropéptido Y (NPY), el glutamato y el péptido similar a la galanina (GALP) ejercen un estímulo activador sobre las neuronas GnRH, mientras que el ácido gamma-aminobutírico (GABA), las beta-endorfinas, la ghrelina y la hormona liberadora de corticotropina (CRH) inhiben la secreción pulsátil de GnRH. La FSH y LH hipofisarias y la testosterona y la progesterona producidas por las gónadas ejercen una retroalimentación negativa sobre el eje. La inhibina (la forma A en mujeres, la forma B en varones) estimulada por la FSH es liberada en el torrente sanguíneo y ejerce un efecto de retroalimentación sobre el hipotálamo y las células gonadotropas reduciendo la secreción de GnRH, LH y FSH, siendo la inhibina selectiva para la FSH y los esteroides sexuales para la secreción de LH. El E_2 parece actuar de forma bimodal en los sistemas de retroalimentación, siendo capaz de activar, en modelos animales, las neuronas productoras de kisspeptina localizadas en el núcleo periventricular antero-ventral del hipotálamo, mientras que inhibe las neuronas productoras de kisspeptina localizadas en el núcleo arcuato (62).

Se ha hipotetizado que hasta un 50-75% de la variabilidad interindividual en la edad de inicio de la pubertad se debe a la combinación de diversos factores genéticos (63). En niñas, se ha observado una mayor correlación de la edad de la menarquia entre gemelas monocigóticas y entre parejas madre-hija, respecto a la población general (64). Los estudios de asociación del genoma completo (GWAS, del inglés: *Genome Wide Association Study*) son estudios genéticos que buscan variaciones genéticas a lo largo del genoma humano y evalúan su asociación con determinados rasgos fenotípicos. Se caracterizan por la búsqueda de pequeñas variaciones como microdeleciones, repeticiones o variaciones de nucleótidos únicos (SNPs, del inglés: *Single Nucleotide Polymorphisms*) en el genoma de amplias poblaciones de individuos para valorar la influencia de estas variaciones sobre la aparición de ciertas enfermedades o caracteres. En cuanto a la pubertad, estudios de GWAS han permitido identificar 389 *loci* genéticos relacionados con la edad de la menarquia en niñas y 76 *loci* genéticos asociados con la edad de inicio de la pubertad en niños (65,66). Gran parte de estos *loci* además están relacionados, especialmente en niñas, con el índice de masa corporal (IMC) y la talla, lo que explicaría la estrecha relación entre el estado nutricional y el desarrollo puberal.

Recientemente, el estudio de la epigenética ha puesto en evidencia mecanismos moleculares heredables que intervienen en la regulación de la expresión genética, mediante la metilación de secuencias de ADN o la modificación de las histonas. En modelos animales, se han identificado dos complejos moleculares que podrían ejercer un papel activador (el grupo Trithorax) o represor (grupo Polycomb) de la actividad transcripcional en las neuronas involucradas en la liberación pulsátil de GnRH (67,68).

1.1.4.2 Factores Nutricionales

Un adecuado estado nutricional y de salud condiciona una edad de inicio de la pubertad más temprana, mientras que la desnutrición y otras enfermedades crónicas no tratadas determinan un retraso en la maduración puberal (31).

La relación entre balance energético y desarrollo puberal ha sido documentada por numerosos estudios epidemiológicos y del análisis de los *loci* genéticos de la pubertad también se evidencia una fuerte asociación entre el IMC y la edad de la menarquia (65). Una hipótesis muy aceptada es la que relaciona la tendencia secular de la pubertad con la creciente prevalencia de obesidad infantil en los países desarrollados. En niñas, el aumento de grasa corporal o del IMC se ha asociado con una edad más precoz del inicio de la pubertad y de la menarquia en varios estudios longitudinales (69–72) y transversales

(73–76), aunque un metaanálisis que incluía 11 estudios y 4841 sujetos no encontró una diferencia significativa en la edad de la menarquia entre niñas obesas y aquellas con peso normal (71). En niños, los resultados son contradictorios, con algunos estudios que observaron que los niños obesos presentaban un desarrollo genital más precoz (77) y otros que reflejaban lo contrario (78,79). Un estudio realizado en una amplia muestra de niños estadounidenses racialmente diversa evidenció una pubertad más temprana en niños con sobrepeso en comparación con niños con peso normal u obesidad, y una pubertad más tardía en los niños con obesidad en comparación con los que tenían peso normal y sobrepeso (80).

En cuanto a los mecanismos subyacentes a la relación entre adiposidad y desarrollo puberal, la leptina y la insulina se encuentran entre los factores metabólicos mayormente implicados (81,82) y, por otro lado, los factores nutricionales pre- y perinatales también parecen tener una influencia sobre la edad de inicio de la pubertad. Así, los niños y niñas que al nacimiento son pequeños para la edad gestacional presentan, en general, una pubertad más temprana sobre todo si en la primera infancia han experimentado un crecimiento acelerado y una ganancia de peso más pronunciada (83,84). Se ha hipotetizado que esto se debe a que el feto que ha estado sujeto a una restricción energética tiende a desarrollar, de forma compensatoria, un fenotipo ahorrador energético en la vida extrauterina, siendo más propenso a desarrollar resistencia a la insulina y síndrome metabólico (85). La hiperinsulinemia y la composición corporal prevalentemente adiposa estarían nuevamente en la base de una pubertad más temprana.(86,87).

1.1.4.3 Factores psico-sociales

El estrés crónico durante la infancia ejerce un papel supresor en el inicio de la pubertad, habiéndose descrito retrasos en la aparición de los primeros caracteres sexuales y de la menarquia en caso de enfermedades crónicas, conflictos bélicos o situaciones familiares adversas o desestructuración del núcleo familiar (88,89). La activación del eje adreno-corticoide, con niveles elevados de hormona liberadora de corticotropina (CRH), parece estar a la base de la respuesta inhibitoria de la pubertad en situaciones de enfermedades crónicas o de estrés crónico, paralelamente al componente de malnutrición que frecuentemente se da en estos contextos (90,91). Las situaciones familiares adversas, socioeconómicas o emocionales como la ausencia del padre o historias de abusos en la infancia se han relacionado con un inicio más tardío de la pubertad (92,93).

La adopción internacional ha sido especialmente estudiada en los últimos años como causa de adelanto puberal, sobre todo en niñas. Según el registro español de (PPC idiopática, publicado en 2010, el riesgo de desarrollar PPC en las niñas adoptadas de otros países es de hasta 25 veces mayor que el de las niñas nacidas en España (94). Los mecanismos biológicos que explicarían esta asociación pueden ser múltiples, incluyendo el *background* genético, mejores condiciones nutricionales y socioeconómicas y reducción del estrés crónico (31,95). En un estudio retrospectivo realizado en Bélgica en 2001 se analizaron las características de una cohorte de niñas diagnosticadas de PPC idiopática, de las cuales aproximadamente 30% provenían de otros países, con características auxológicas (peso y altura) normales en el momento de la migración (96). Esto llevó a sospechar de factores no nutricionales como posible causa del adelanto puberal. Además, los resultados mostraron concentraciones séricas de metabolitos de pesticidas organoclorados con actividad estrogénica significativamente mayores en las niñas provenientes de otros países, sentando las bases para la hipótesis de la disrupción endocrina como uno de los factores implicados en las variaciones en la edad de inicio de la pubertad, no solo en poblaciones migrantes, sino también en la población general.

1.2 Disruptores endocrinos

Según la definición de la Organización Mundial de la Salud (OMS), se considera disruptor endocrino (DE) cualquier sustancia exógena o mezcla de sustancias que altera la función del sistema endocrino y, por tanto, causa efectos adversos sobre la salud en un organismo, en su progenie o en una población (97). Se trata de sustancias químicas que, por sus características moleculares, son capaces de alterar la homeostasis hormonal del organismo humano y animal mediante diferentes mecanismos. Un documento reciente de consenso internacional ha dilucidado las diez principales vías mediante las cuales los DEs llevan a cabo esta (98):

1. Activación de receptores hormonales: los DEs, ligándose a receptores específicos de ciertas hormonas, simulan su actividad, desvinculándola de los mecanismos fisiológicos que la regulan.
2. Antagonismo con receptores hormonales: los DEs pueden actuar de forma antagónica a la actividad hormonal ligándose a receptores de membrana o intranucleares de las hormonas.

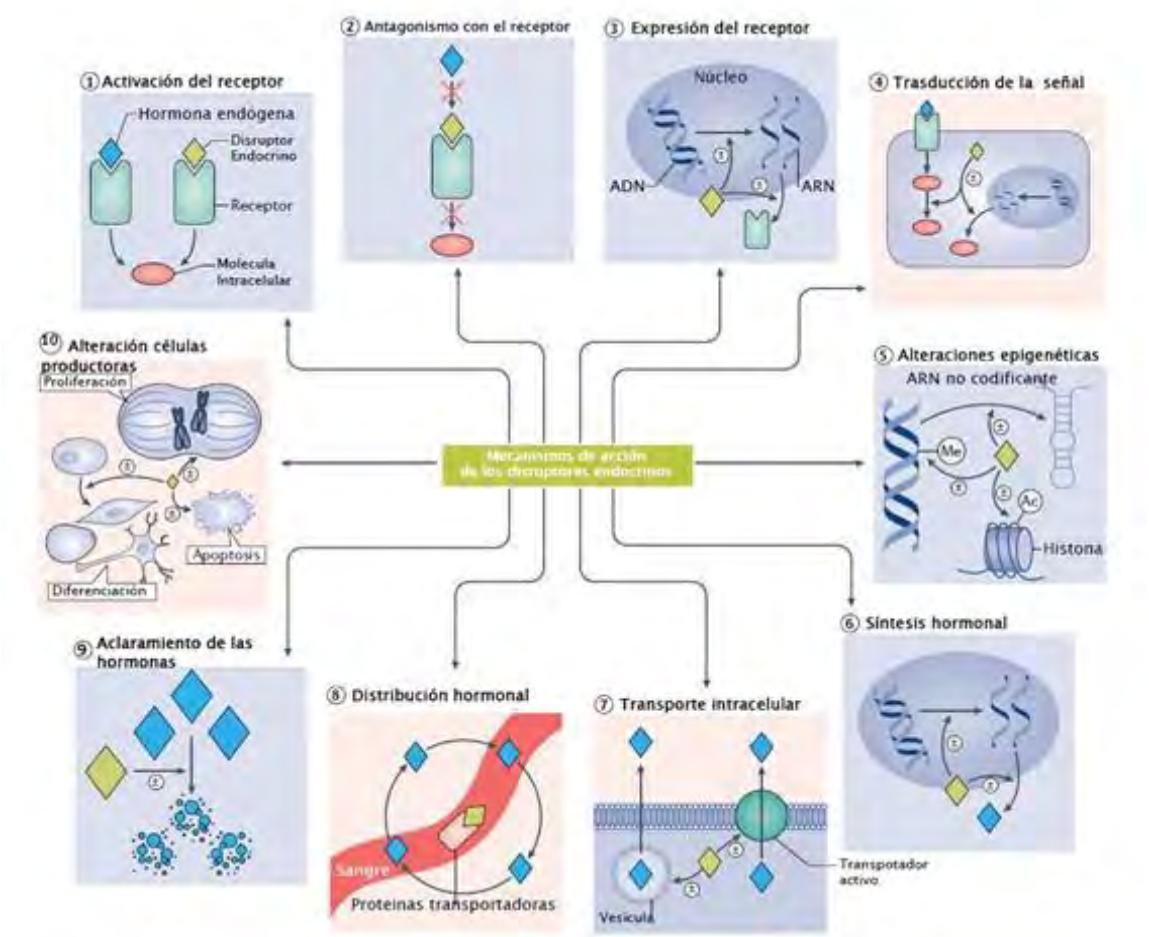

Fig. 8 Las 10 características de los DEs. Adaptado de *La Merrill et al. (2020)*

3. Alteración de la expresión de receptores, modificando el patrón de distribución de los receptores hormonales.
4. Modificación de las señales de transducción intracelular en células reguladas por hormonas. La unión de una hormona con su receptor activa un complejo molecular de enzimas, coenzimas y cofactores que tienen como finalidad la transducción de la señal hormonal y que puede ser alterado por los DEs.
5. Interacciones epigenéticas. Alterando los procesos de metilación del ADN, modificaciones de la cromatina y expresión del ARN no codificante, los DEs pueden determinar cambios heredables en la expresión y función hormonal.
6. Alteración de la síntesis de hormonas, mediante interacción en la expresión de enzimas claves para la hormonogénesis.
7. Alteración del transporte de las hormonas a través de las membranas celulares, interfiriendo en la dinámica de las vesículas y en la secreción celular.

8. Interacción con las moléculas de transporte en el torrente circulatorio de las hormonas, alterando la biodisponibilidad de las hormonas para los órganos diana.
9. Alteración del anabolismo y catabolismo de las hormonas. Los DEs interfieren en su inactivación, degradación, aclaramiento, excreción o eliminación.
10. Alteración del crecimiento de las células productoras de hormonas o de las células reguladas por hormonas, modificando los patrones de atrofia o hiperplasia/hipertrofia, diferenciación, migración, proliferación o apoptosis de las células diferenciadas para la producción de hormonas.

Para comprender los mecanismos de acción y las repercusiones en salud pública de la disrupción endocrina hay que tener en cuenta ciertas características comunes a los DEs (99):

- La exposición puede ocurrir en cualquier etapa de la vida, aunque se han reconocido periodos de especial vulnerabilidad como las etapas pre- y perinatales y la primera infancia, en las que el sistema hormonal en desarrollo es especialmente susceptible a la influencia de los DEs (100).

- Por lo general, existe cierta latencia entre el momento de la exposición y sus efectos adversos, de manera que la exposición en la infancia o durante el embarazo puede tener consecuencias en la vida adulta.

- Se trata de un grupo muy heterogéneo de moléculas, de origen natural o sintética, generalmente pequeñas, de masa molecular inferior a 1000 Dalton, que actúan a baja dosis y de forma combinada con las hormonas endógenas, lo cual impide determinar una dosis mínima de efecto (101).

- Las curvas dosis-respuesta muestran, en la mayoría de los casos, un patrón no lineal, sin que exista una relación proporcional entre la dosis de exposición y la magnitud del efecto (102).

- Además, por lo general, los individuos están simultáneamente expuestos a múltiples contaminantes ambientales, por lo que los efectos de la exposición a mezclas de DEs son difícilmente predecibles debido a posibles acciones sinérgicas, antagónicas o aditivas de los diferentes compuestos (103).

1.2.1 Clasificación de los disruptores endocrinos

Dependiendo de la resistencia a la degradación ambiental y biológica, los DEs pueden clasificarse en persistentes y no persistentes. Los compuestos orgánicos persistentes (COPs) resisten a la degradación fotolítica, biológica y química y, por lo tanto, permanecen en el ambiente y en los organismos expuestos durante largos periodos de tiempo. En el ser humano, la principal ruta de exposición es la dieta y, por su naturaleza lipofílica, los COPs se almacenan en el tejido adiposo (104). Por su lento metabolismo tienden a bioacumularse, con lo cual su efecto se magnifica debido a que las concentraciones en los organismos vivos aumentan conforme se va ascendiendo en la cadena alimentaria (105). Pertenecen a este grupo los pesticidas organoclorados como diclorodifeniltricloroetano (DDT) y hexaclorobenceno (HCB), las dioxinas, los bifenilos policlorados (PCBs), los retardantes de llama (o éteres de difenilo polibromados [PBDEs]) y las sustancias perfluoradas (PFAS), aunque estas últimas no tienden a acumularse en el tejido adiposo. A lo largo de las últimas décadas se han observado múltiples efectos adversos en salud ligados a la exposición a COPs, como un mayor riesgo de cáncer hormono-dependiente (testículo, mama y próstata), así como efectos adversos en la función reproductiva (infertilidad, peor calidad seminal, endometriosis, etc.) tanto masculina como femenina (106–110). Además, la exposición prenatal a COPs se asociado con bajo peso al nacer (111,112), disminución de la distancia anogenital en varones (113), mayor riesgo de obesidad y alteraciones cardio-metabólicas (114,115), disfunción tiroidea, efectos adversos en el neurodesarrollo y alteraciones inmunitarias, entre otros (116). El Convenio de Estocolmo entró en vigor en 2004 con la participación de 151 países que se comprometían a reducir o eliminar la producción y el uso de COPs (117) dejando espacio para la aparición en la industria de un gran número de compuestos, más modernos y con vida media más corta, denominados por eso no persistentes.

Los DEs no persistentes se caracterizan por su vida media corta en el organismo, debido a un menor peso molecular que los COPs y una menor afinidad por el tejido adiposo. Son, en general, compuestos hidrosolubles que son metabolizados en pocas horas a nivel hepático mediante conjugación con grupos glucurónidos y con ésteres de sulfato, lo que permite su eliminación por vía renal (1,118,119). Pertenecen a este grupo una gran variedad de grupos de DEs como los bisfenoles, los ftalatos, los parabenos, las benzofenonas y los pesticidas modernos.

Los ftalatos son contaminantes DEs omnipresentes en la sociedad actual, ampliamente utilizados en productos de uso cotidiano. Se dividen en ftalatos de bajo peso molecular, como el dietil ftalato (DEP), el diisobutil ftalato (DiBP) y el di-n-butil ftalato (DnBP), utilizados en adhesivos y en cosméticos y productos de higiene personal como disolventes y fijadores de fragancias, y los ftalatos de alto peso molecular, como el butil bencil ftalato (BBzP), dietilhexil ftalato (DEHP) y el dinonil ftalato (DiNP), que suelen usarse como aditivos del policloruro de vinilo (PVC), confiriendo al plástico flexibilidad y durabilidad, y por ende están presentes en una inmensa variedad de productos, incluyendo materiales de construcción y eléctricos, textiles, envoltorios de alimentos, juguetes y dispositivos médicos (120,121). Los estudios de biomonitorización humana muestran que la exposición a ftalatos en la población general es ubicua (120–122), habiéndose identificado entre los grupos más expuestos las mujeres en edad fértil y los niños, probablemente debido a un mayor uso de productos cosméticos y juguetes de plástico y mayor exposición a través del polvo doméstico (123–126). Tras la exposición, los ftalatos se hidrolizan en pocas horas a sus respectivos monoésteres, que pueden transformarse sucesivamente en metabolitos secundario, y tras conjugación con grupos glucurónidos se eliminan por vía urinaria o digestiva; la cuantificación de los metabolitos, primarios o secundarios, en orina es actualmente el método más utilizado para evaluar la exposición (127).

Los ftalatos son DEs conocidos que ejercen actividad anti androgénica *in vitro*, y estudios en modelos animales sugieren que también interfieren con receptores estrogénicos y con los mecanismos moleculares implicados en la esteroidogénesis (128–133). Existe una amplia literatura acerca del impacto de la exposición humana a ftalatos durante los primeros años de vida y la edad adulta sobre el desarrollo y la función reproductiva (134–136). Así, la exposición a ftalatos se ha asociado con reducción de la distancia anogenital, malformaciones urogenitales, peor calidad espermática y niveles reducidos de testosterona en los hombres (135,136); con disfunción placentaria, resultados adversos del embarazo (parto prematuro y bajo peso al nacer), insuficiencia ovárica, endometriosis, desequilibrio hormonal e infertilidad en las mujeres (134,135); y con alteraciones en el desarrollo puberal en niños y niñas, como se describe más adelante (apartado 5).

1.3 Pesticidas no persistentes

Los pesticidas no persistentes incluyen insecticidas organofosforados (OP, del inglés: *organophosphate*), piretroides, neonicotinoides y carbamatos, fungicidas como los ditiocarbamatos y herbicidas como el glifosato, y son ampliamente utilizados en la actualidad, tanto en la producción alimentaria como para uso doméstico. España es el mayor consumidor de pesticidas de uso agrícola en la Unión Europea (UE), habiendo utilizado en 2020 aproximadamente 75 millones de kilogramos de pesticidas, de los cuales 38 millones eran fungicidas (137).

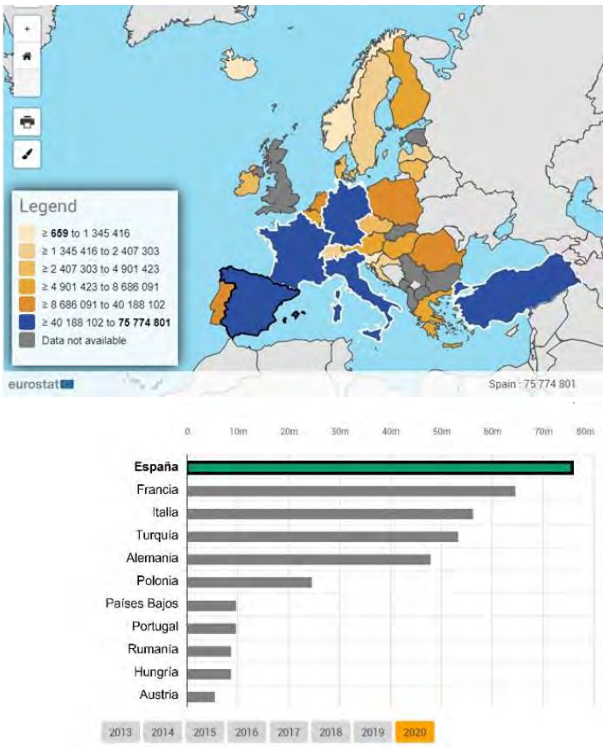

Fig. 9 Consumo de pesticidas por año y país europeo. *Fuente Statistics | Eurostat [Internet]*.

La principal ruta de exposición a pesticidas no persistentes en la población general es la dieta, a través de la ingesta principalmente de vegetales y frutas procedentes de la agricultura convencional. Poblaciones residentes en zonas rurales, además, pueden sufrir los efectos de la deriva de pesticidas agrícolas, a través de la inhalación o el contacto dérmico con partículas dispersas en el ambiente y la ingesta de agua contaminada (138). Una vez en el organismo, los pesticidas no persistentes son rápidamente metabolizados y sus metabolitos son excretados en la orina a partir de las 4 horas y hasta las 48 horas tras la exposición, dependiendo del compuesto y la vía de exposición (139). De hecho, la medición de los metabolitos urinarios de pesticidas es actualmente el método mayormente empleado para evaluar la exposición a estos compuestos (140–142). Debido a su corta vida media biológica, tras la ingesta con la dieta, las concentraciones urinarias son un reflejo de la exposición reciente; de hecho, se ha observado que la instauración de una dieta orgánica, tanto en adultos como en niños, reduce rápidamente, en pocos días, las concentraciones urinarias de pesticidas (143,144).

Los insecticidas OPs y los carbamatos desempeñan su función a través de la inhibición de la acetilcolinesterasa que es responsable, en la placa neuromotora, de la

degradación del neurotransmisor excitador, la acetilcolina, bloqueando así la transmisión de los impulsos nerviosos en las sinapsis colinérgicas. Pertenecen a este grupo insecticidas muy utilizados mundialmente en las últimas décadas como el clorpirifós, el diazinón, el malatión y el paratión. No obstante, debido al riesgo de intoxicación aguda humana, el uso de OPs en el ámbito doméstico está prohibido actualmente. El clorpirifós fue clasificado en 2019 como tóxico para la reproducción debido a sus efectos adversos sobre el neurodesarrollo, y, en consecuencia, prohibido en la UE en 2020 (145). Otros como el diazinón y el paratión han demostrado potencial cancerígeno (146), lo que condujo a la desautorización del uso de diazinón y paratión en la UE en 2006 y 2009, respectivamente (147).

Los piretroides son derivados sintéticos de la piretrina (*Chrysanthemum cinerariaefolum*) y presentan como estructura básica un ácido y un alcohol con enlace éster, siendo en la mayoría de los casos moléculas presentes en las formulaciones comerciales como mezcla de enantiómeros. Los piretroides se encuentran entre los pesticidas más utilizados tanto en agricultura como en el ámbito doméstico para el control de plagas (148). Se encuentran también en antiparasitarios tópicos para mascotas, medicamentos utilizados para tratar la escabiosis y en tratamientos tópicos para la pediculosis (149). A concentraciones ambientalmente relevantes, se han descrito efectos adversos especialmente en el crecimiento y desarrollo respira(150–152), motivo por el cual los piretroides están sujetos a una creciente presión reguladora en la UE (153).

Otra clase de pesticidas muy común son los fungicidas, y entre ellos los ditiocarbamatos, que son derivados del ácido carbámico en los que los átomos de oxígeno son sustituidos por átomos de azufre. Los bisditiocarbamatos de etileno (EBDC, del inglés: *ethylene-bis-dithiocarbamates*), en forma de complejos con manganeso (maneb), zinc (zineb, ziram) o una combinación de manganeso y zinc (mancozeb), se han utilizado extensamente como fungicidas en la agricultura desde la década de 1940. Los fungicidas ditiocarbamatos se degradan fácilmente en ambientes acuosos y suelos para formar etilentiourea (ETU) y otros productos de transformación. La ETU también es el principal producto del metabolismo de los ditiocarbamatos en plantas y mamíferos y sus concentraciones en orina son utilizadas en estudios de biomonitorización humana como biomarcadores de exposición (154). Por el potencial teratógeno y carcinógeno de la ETU, conocido desde hace décadas (155), y por su efecto disruptor tiroideo (156), el uso de algunos fungicidas de esta familia ha sido restringido en la UE, como el mancozeb y el

maneb prohibidos a partir de 2017 y 2021, respectivamente, mientras que siguen autorizados otros como el ziram y el metiram (147).

Variaciones genéticas en las secuencias que codifican enzimas claves para el metabolismo de xenobióticos pueden conferir una mayor susceptibilidad a los potenciales efectos adversos de sustancias tóxicas como los pesticidas no persistentes. La esterasa paraoxonasa-1 (PON1) participa en la hidrólisis de los metabolitos de OPs como el clorpirifós y el diazinón (157). En la secuencia codificante del gen PON1 se han identificado dos SNPs en las posiciones 192 y 55 relacionados con variaciones en su actividad: el polimorfismo L55M (sustitución metionina/leucina) asociado a bajas concentraciones de PON1 (157) y el polimorfismo Q192R (sustitución glutamina [Q]/arginina[R]) que da lugar a la expresión de isoformas de PON1 con diferente capacidad catalítica según el sustrato, siendo la isoforma R más eficaz para hidrolizar el clorpirifós y la Q para el diazinón (158). Los citocromos CYP2C19 y CYP2D6 están involucrados en el metabolismo de numerosos pesticidas incluidos carbamatos y piretroides (159–161). Los polimorfismos con pérdida de función CYP2C19 G681A y CYP2D6 G1846A (ambos por sustitución glicina/alanina) se encuentran entre los SNPs más investigados en relación con el metabolismo de los pesticidas (162). Existen evidencias que sugieren que la variabilidad interindividual en la respuesta a los pesticidas es en parte explicable por estas variaciones; por ejemplo, un estudio mexicano examinó la interacción entre la exposición ocupacional a OPs y los polimorfismos PON1 sobre los niveles de hormonas tiroideas (163). Los resultados mostraron que en los portadores del genotipo 192RR el aumento de los niveles de hormonas tiroideas (tirotropina [TSH] y triiodotironina [T3] total) asociados con las concentraciones urinarias de metabolitos de insecticidas OPs era mayor que en los portadores de los genotipos 192QQ y QR (163).

1.3.1 Pesticidas no persistentes como disruptores del eje gonadal

Estudios experimentales sugieren que diversos pesticidas no persistentes pueden actuar como DEs e interferir en los procesos fisiológicos implicados en la pubertad. Así, estudios *in vitro* han demostrado que un gran número de insecticidas OPs, carbamatos y piretroides y de fungicidas EBDC presentan afinidad por los receptores estrogénicos (ERα, ERβ) y androgénicos (AR) (164). Los OPs clorpirifós y diazinón y los piretroides permetrina, cipermetrina y deltametrina han demostrado actividad proestrogénica; los OPs paratión y metil-paratión y la ciflutrina poseen actividad anti-andrógenica; y algunos

como los piretroides fenvalerato y ciflutrina y el carbamato metiocarb actúan de forma combinada activando los receptores estrogénicos y antagonizando los androgénicos (164–167).

El proceso de síntesis de las hormonas sexuales en las gónadas es otra posible diana para muchos pesticidas no persistentes con actividad como DEs. Por ejemplo, los insecticidas clorpirifós, piperifós, cipermetrina y metomilo y el fungicida ziram han demostrado, en modelos animales, capacidad de modular negativamente la actividad de enzimas implicados en la esteroidogénesis como las proteínas StAR, aromatasa y enzimas pertenecientes a la superfamilia del citocromo CYP450 (168–171).

Otros mecanismos son representados por la interferencia en la expresión de los receptores hormonales a múltiples niveles del eje hipotálamo-hipofisario-gonadal (172–174) y en los mecanismos moleculares de señalización que se desencadenan al interior de la célula diana tras la unión del receptor hormonal a su ligando (175–177).

1.3.2 Pesticidas no persistentes y niveles de hormonas sexuales

Si bien las evidencias experimentales sugieren que la exposición a pesticidas no persistentes pueda alterar el equilibrio del eje hipotálamo-hipofasario-gonadal, las evidencias que relacionan la exposición a los pesticidas modernos con alteraciones en los niveles circulantes de hormonas sexuales son escasas y provienen principalmente de estudios realizados en hombres adultos. Por ejemplo, la exposición a piretroides, valorada mediante la cuantificación de las concentraciones del metabolito urinario ácido 3-fenoxibenzoico (3-PBA), se ha relacionado con elevación de los niveles de FSH y LH en varones adultos y de 9 a 16 años de edad (178–180), mientras que las concentraciones urinarias de otro metabolito común a ciertos piretroides, el ácido carboxílico de 2,2-dimetilciclopropano (DCCA), se ha asociado con reducción de los niveles de testosterona, E_2 e inhibina B en adultos (179). También se han descrito descensos en las concentraciones de testosterona en relación con la exposición a insecticidas OPs en hombres adultos (181–185), aunque en algunos estudios esta asociación es nula o incluso positiva (186–188), mientras que no se han encontrado asociaciones consistentes entre los niveles de metabolitos de OPs y las concentraciones de FSH, LH, prolactina e inhibina B (181–183,186,188). Sólo un estudio epidemiológico ha examinado la relación entre la exposición a carbamatos y los niveles de hormonas sexuales, en el que se encontró una asociación inversa entre las concentraciones urinarias de 1-naftol (1-N), el principal

metabolito del carbarilo, y los niveles de testosterona, E_2 y LH en hombres adultos (182). En mujeres, las evidencias son aún más escasas, aunque dos estudios han relacionado el trabajo agrícola con aumento de las concentraciones de E_2 y progesterona (189,190) y de FSH y LH (190); y un estudio encontró asociación entre exposición a los OPs clorpirifós, diazinón y malatión en mujeres trabajadoras agrícolas y en sus hijos de 9 a 15 años de edad y descenso en los niveles de FSH y LH con una correlación significativa entre concentraciones de clorpirifós y E2 en las madres, mientras que no se observó asociación significativa con los niveles hormonales en los niños (191).

1.4 Influencia de los disruptores endocrinos en la cronología de la pubertad

Desde que Krstevska-Konstantinova et al. (2001) (96) observaron una relación entre la exposición a pesticidas organoclorados y el riesgo de desarrollar PPC en niñas adoptadas internacionalmente, crece el número de estudios que sugieren que la creciente exposición a DEs en las últimas décadas pueda ser uno de los factores responsables de la continua tendencia de adelanto de la pubertad tanto en niñas como en niños (192,193).

Entre los contaminantes persistentes, los pesticidas organoclorados, las dioxinas, los retardantes de llama y algunos metales pesados han sido objeto de interés por su potencial de inducir alteraciones en la pubertad (193). Así, algunos estudios epidemiológicos han relacionado la exposición al insecticida DDT y su metabolito dicloro difenil dicloroetileno (DDE) con adelanto de la pubertad en ambos sexos (96,194,195). En concreto, dos estudios caso-control reportaron que la exposición a DDE se asociaba con un mayor riesgo de pubertad precoz (196) y telarquia precoz en niñas (204) la exposición prenatal a DDE se relacionó con una edad de la menarquia más temprana en otro estudio (194). En la región de Flandes en Bélgica, Den Hond et al. (2011) (197) analizaron las concentraciones séricas de varios organoclorados, incluyendo DDE, el fungicida organoclorado HCB, y PCBs, y metales pesados como mercurio y cadmio, en una amplia muestra de adolescentes de 14-15 años y observaron que las concentraciones de HCB, DDE y PCBs se asociaban con un desarrollo genital y de vello púbico más precoz en niños, mientras que en las niñas las concentraciones de mercurio y PCBs se asociaron con retraso de la pubarquia y la menarquia, respectivamente. Un efecto acelerador en el desarrollo genital también se ha observado en hijos varones de madres expuestas a PCBs durante el embarazo en una cohorte de madres-hijos en Rusia (198)

mientras que dos estudios realizados en el marco de la tercera Encuesta Nacional de Examen de Salud y Nutrición (NHANES III, por su sigla en inglés) de Estados Unidos mostraron que la exposición a mercurio en niños de 8-16 años de edad estaba asociada con un mayor riesgo de presentar menarquia, telarquia y pubarquia a edades más tempranas (199,200).

Entre los DEs no persistentes, el bisfenol A (BPA) es uno de los más estudiados principalmente por sus efectos adversos para la reproducción, habiendo demostrado capacidad estrogénica en numerosos ensayos *in vitro* e *in vivo* (201,202). Si bien algunos estudios epidemiológicos apuntan a que existe una relación entre la exposición a BPA y retraso puberal tanto en niños como en niñas (203–207), en un reciente metaanálisis que incluía 10 estudios epidemiológicos, comprendiendo un total de 5621 sujetos, no se observó asociación entre la exposición a BPA y el desarrollo puberal, salvo por una débil relación con telarquia más tardía en niñas (208).

Como se ha mencionado anteriormente, evidencias *in vitro* e *in vivo* indican que los ftalatos actúan como disruptores del eje gonadal, actuando como anti-andrógenos (209,210) y una literatura emergente sugiere que la exposición temprana a este tipo de compuestos químicos podría estar asociada con alteraciones en la edad de inicio de la pubertad en niños y niñas (211–213). No obstante, la mayoría de los estudios son transversales y no han descrito resultados concluyentes (206,214–217). Las evidencias más consistentes provienen de unos pocos estudios longitudinales que examinan la exposición prenatal, observándose en algunos de ellos asociaciones con adelanto del desarrollo mamario y del vello púbico y adelanto de la menarquia en niñas (211,213,218,219), mientras otros muestran resultados inconsistentes u opuestos (212,220,221). En niños, la exposición prenatal a ftalatos de alto peso molecular como DEHP se ha asociado con adelanto de la pubarquia y la gonadarquia en un estudio (212), aunque otros dos encontraron asociación con retraso de la pubarquia (212) y asociación nula para diferentes ftalatos (211,219).

1.4.1 Pesticidas no persistentes y pubertad

Los estudios epidemiológicos que han investigado la relación entre la exposición a pesticidas no persistentes y variaciones en la edad de inicio y progresión de la pubertad son todavía pocos y metodológicamente muy dispares. Resumidamente, la exposición durante la infancia a insecticidas OPs se asoció con retraso en el desarrollo sexual de

niños y niñas en el estudio FLEHS, en Bélgica (222) mientras que la exposición a piretroides en áreas urbanas de la región de Hangzhou, China, se asoció con retraso puberal en niñas (223) y aceleración puberal en niños (180). En la cohorte ALSPAC de Reino Unido, un estudio encontró una asociación entre la exposición materna durante el embarazo al herbicida atrazina y una edad de la menarquia más temprana en las hijas (224) y un estudio danés vinculó la exposición ocupacional de mujeres embarazadas a múltiples pesticidas con un desarrollo mamario más temprano en las hijas (225) y un menor volumen testicular y longitud del pene en los hijos (226). En un estudio de muy reciente publicación, realizado en China con niños y niñas de 11 a 16 años de edad, la exposición a tiacloprid, un insecticida neonicotinoide, se asoció con retraso en el desarrollo genital en niños y adelanto en el desarrollo del vello axilar en las niñas (227). Otros estudios transversales, en los que se consideraba el residir en zonas rurales como indicador indirecto de la exposición a múltiples pesticidas, no encontraron asociaciones significativas con el desarrollo puberal (228–230).

1.5 Proyecto Infancia y Medio Ambiente (INMA)

Con el objetivo general de investigar el efecto de la exposición a contaminantes ambientales en la salud materno-infantil, nace en España a finales de los años noventa el proyecto multicéntrico Infancia y Medio Ambiente (INMA) (https://www.proyectoinma.org) . El proyecto se compone de 7 cohortes de nacimiento localizadas en Asturias, Gipuzkoa, Granada, Menorca, Ribera d'Ebre, Sabadell y Valencia. Las madres fueron reclutadas durante el embarazo entre 1997 y 1999 en Menorca y Ribera d'Ebre y entre 2004 y 2008 en Asturias, Gipuzkoa, Sabadell y Valencia, mientras que en Granada fueron reclutadas tras el parto en 2000-2002 (231). Para la mayoría de las cohortes se realizaron visitas de seguimiento al parto, al año, a los 2, 4, 7-8, 9-10, 11-12 y 14-16 años en las que se recogió información sobre el desarrollo intrauterino, variables sociodemográficas y antecedentes reproductivos y de enfermedad de la familia, así como

Fig. 10 Cohortes del proyecto Infancia y Medio Ambiente

dieta de la madre y del hijo, incluida la lactancia materna y posibles fuentes de exposición a contaminantes ambientales, atmosféricos, del agua, humo de tabaco y DEs. La recogida de información se realizó mediante cuestionarios en formato de entrevista, se realizó exploración física por parte de personal sanitario (pediatra o enfermero/a), recogida de muestras biológicas (sangre, placenta, orina, saliva, leche materna, cabello y uñas) y en ocasiones exploraciones complementarias como ecografía o pruebas funcionales.

En términos generales, el proyecto viene investigando el grado de exposición a contaminantes ambientales en población infantil en España, en diferentes contextos geográficos, durante la vida prenatal, el nacimiento y a lo largo de la infancia y la adolescencia, y evaluando el impacto de éstos sobre el desarrollo, crecimiento, y salud de los niños.

2. HIPÓTESIS

Diversos pesticidas no persistentes y ciertos componentes de bienes de consumo cotidianos como los ftalatos han demostrado capacidad de actuar como DEs y un gran número son sospechosos de presentar actividad hormonal. A nivel mundial, la producción alimentaria es altamente dependiente del uso intensivo de pesticidas, siendo España el mayor consumidor de pesticidas en la UE, especialmente de fungicidas. Se sospecha que la exposición a pesticidas no persistentes y otros DEs en etapas de especial vulnerabilidad, como el embarazo y la infancia, pueda tener un impacto negativo en la función de los ejes hormonales gonadal y adrenal que regulan el desarrollo puberal y la maduración sexual, acarreando efectos adversos en la salud reproductiva. A su vez, la pubertad precoz y otras alteraciones de la edad de inicio y cronología de la pubertad se han relacionado con el riesgo de diferentes problemas de salud en la adolescencia y la vida adulta, como cáncer hormono-dependiente y enfermedades metabólicas y cardiovasculares. Entre los factores relacionados con la cronología de la pubertad, la exposición a compuestos DEs en momentos críticos del desarrollo podría ser una de las causas atribuidas a la tendencia hacia el adelanto de la pubertad en niñas y niños observada en las últimas décadas. Determinadas variantes genéticas podrían conferir mayor susceptibilidad a los potenciales efectos adversos en salud de los pesticidas no persistentes y otros contaminantes ambientales.

3. OBJETIVOS

En base a la hipótesis enunciada, el objetivo principal de esta Tesis Doctoral es evaluar la exposición infantil a pesticidas no persistentes y a ftalatos durante el embarazo y su asociación con el desarrollo puberal y los niveles de hormonas sexuales en niños y niñas del Proyecto INMA, así como examinar posibles interacciones gen-ambiente. Para ello se han abordado los siguientes objetivos específicos:

OBJETIVO 1

Revisar la evidencia epidemiológica disponible en la literatura científica sobre la relación entre la exposición durante el embarazo, la infancia o la adolescencia a pesticidas no persistentes y el desarrollo puberal y/o el estado de maduración sexual.

OBJETIVO 2

Evaluar la asociación entre las concentraciones urinarias de metabolitos de pesticidas no persistentes y el desarrollo puberal de niños y niñas de las cohortes INMA a la edad de 7-11 años.

OBJETIVO 3

Evaluar la asociación entre las concentraciones urinarias de metabolitos de ftalatos durante el embarazo y el desarrollo puberal de niños y niñas de las cohortes INMA a la misma edad.

OBJETIVO 4

Evaluar la asociación entre las concentraciones urinarias de metabolitos de pesticidas no persistentes y el estado de maduración sexual de varones adolescentes (14-17 años) del Proyecto INMA.

OBJETIVO 5

Evaluar la asociación entre las concentraciones urinarias de metabolitos de pesticidas no persistentes y los niveles séricos de hormonas sexuales en varones adolescentes de la cohorte INMA-Granada, así como la posible interacción con variantes genéticas (SNPs) susceptibles de modificar el efecto de la exposición sobre los niveles hormonales.

4. MATERIALES Y MÉTODOS

4.1 Objetivo 1

Para llevar a cabo el primer objetivo propuesto se realizó una revisión sistemática de los estudios epidemiológicos disponibles en la literatura científica que investigan la relación entre exposición durante el embarazo o la infancia/adolescencia a pesticidas no persistentes y el desarrollo puberal y/o maduración sexual de niños y niñas.

4.1.1 Diseño del estudio

Revisión sistemática.

4.1.2 Formalización de la pregunta de investigación y estrategia de búsqueda en base de datos

La revisión sistemática se realizó de acuerdo con las directrices "PRISMA" (acrónimo del inglés: *Preferred Reporting Items for Systematic Reviews and Metaanalyses*) (232). Con la finalidad de factorizar la pregunta de investigación y justificar los criterios de inclusión y exclusión se empleó la siguiente estrategia PECO (acrónimo del inglés: *Population, Exposure, Comparator, Outcome*) (233):

Población: Niños y niñas desde el periodo prenatal hasta la adolescencia;

Exposición: Cualquier exposición a pesticidas no persistentes, incluyendo insecticidas, fungicidas y herbicidas (excluyendo los pesticidas organoclorados)

Grupo de comparación: Niños y niñas no expuestos o expuestos a dosis inferiores de pesticidas no persistentes.

Resultado (*outcome*): Inicio y/o progresión de la pubertad valorado mediante cualquiera de los siguientes métodos: edad de la menarquia, estadíos de Tanner (que describe el desarrollo mamario en niñas, el desarrollo genital en niños y el desarrollo de vello púbico en ambos sexos) y/o medición de hormonas sexuales, principalmente testosterona, E_2, FSH y LH.

La búsqueda bibliográfica se realizó consultando dos bases de datos de literatura médica: Medline (Pubmed) y Scopus. La estrategia de búsqueda se formuló para identificar en primera instancia toda publicación científica que incluyera en el título, en el resumen o en el texto los siguientes términos en inglés: "*pesticides*", "*non-persistent pesticides*", "*insecticides*", "*herbicides*", "*fungicides*", "*agrochemicals*" o "*biocidals*" en

combinación con "*puberty*", "*pubertal development*", "*sexual development*", "*sexual maturity*", "*sex hormones*", "*menarche*", "*thelarche*", "*gonadarche*", "*adrenarche*", "*pubarche*" o "*adolescents*".

4.1.3 Selección y extracción de datos

Del resultado inicial de la búsqueda en las bases de datos se seleccionaron los estudios epidemiológicos originales, publicados hasta abril de 2020 en inglés, español, italiano o portugués, en los que la variable de resultado (*outcome*) era la cronología o desarrollo puberal y/o el estado de maduración sexual y/o los niveles de hormonas sexuales. Se excluyeron los estudios experimentales (*in vitro* o *in vivo*), los relatos de caso y las series de casos. La selección de los trabajos elegibles y la extracción de datos fueron realizados independientemente por dos investigadores, y eventuales divergencias se resolvieron por consenso.

De los artículos seleccionados se extrajo la siguiente información: autores, año de publicación, país de realización, diseño del estudio, objetivos, número de participantes, edad, sexo y zona de residencia. En cuanto a la exposición se extrajo información sobre el método de evaluación (cuantificación de metabolitos de pesticidas en muestras biológicas y/o cuestionarios y/o zona de residencia de los participantes), el tipo(s) o grupo(s) de pesticida(s) o compuesto(s) específico(s) analizado(s) y la ventana temporal de exposición (prenatal, infancia, adolescencia). Para cada estudio, se extrajo el *outcome* principal y las asociaciones encontradas.

4.1.4 Evaluación de la calidad

Para evaluar la calidad de la evidencia proporcionada por los estudios se utilizó el modelo GRADE (acrónimo del inglés: *Grading of Recommendations Assessment, Development and Evaluation*) que evalúa la calidad en la evidencia, definida como la confianza en la estimación del efecto (234). De partida, según el sistema GRADE, se clasifican como estudios de calidad alta los estudios experimentales (ensayos clínicos aleatorizados) y de calidad baja los observacionales. En un segundo paso, el sistema establece unos criterios a evaluar que harían subir o bajar el nivel de calidad inicialmente asignado. Son criterios de mejor calidad:

- Fuerza de la asociación.
- Existencia de un gradiente dosis-respuesta.

- Evidencia de posibles factores de confusión o sesgos que podrían haber reducido el efecto observado.

Los criterios que bajarían la puntuación en la evaluación de calidad son:

- Limitaciones en el diseño del estudio que aumentan el riesgo de sesgo.
- Inconsistencia de los resultados, cuando los resultados muestran una variabilidad o heterogeneidad no explicada.
- Incertidumbre acerca de que la evidencia sea directa.
- Imprecisión: tiene lugar si los intervalos de confianza son amplios, las muestras pequeñas o los eventos escasos.
- Sospecha de sesgo de publicación.

De acuerdo con la puntuación obtenida, el sistema GRADE clasifica la calidad de la evidencia en: muy baja (⨁), baja (⨁⨁), moderada (⨁⨁⨁), alta (⨁⨁⨁⨁). Además, para valorar la calidad de la información reportada en los estudios incluidos en la revisión se aplicó el sistema STROBE (acrónimo inglés de: *Strengthening the Reporting of Observational Studies in Epidemiology Statement*) en el que se responde a 22 preguntas relacionadas con la calidad de la información recogida en los artículos en sus diferentes secciones (título, resumen, introducción, metodología, resultados y discusión).

4.2 Objetivo 2

Para valorar la posible asociación entre la exposición a pesticidas no persistentes y el desarrollo puberal, se llevó a cabo un estudio transversal con niños y niñas que participan en las diferentes cohortes del Proyecto INMA.

4.2.1 Diseño del estudio

Estudio transversal.

4.2.2 Población en estudio

En el estudio se incluyeron a todos los niños y niñas del Proyecto INMA que participaron en visitas de seguimiento a la edad de 7-11 años y que disponían de muestras de orina recogida en esta visita. Se incluyeron las cohortes de Asturias, Gipuzkoa,

Granada, Sabadell y Valencia. Estas cinco cohortes estaban constituidas inicialmente por 3238 mujeres que fueron reclutadas en el primer trimestre de embarazo entre los años 2003 y 2008, salvo en Granada donde fueron reclutadas al parto entre 2000 y 2002. Las cohortes estaban formadas por parejas madre-hijos de ambos sexos, salvo en Granada donde se reclutaron solo madres que dieron a luz hijos varones, y en todas ellas se excluyeron las madres que presentaban patologías crónicas como diabetes, hipertensión, patologías tiroideas y las que desarrollaron complicaciones durante el embarazo potencialmente prejudiciales para el desarrollo y el crecimiento fetal. De los 3238 niños inicialmente reclutados, un total de 1976 participaron en la visita de seguimiento a los 7-11 años de edad, en la cual se realizó una valoración del desarrollo puberal y, de estos niños, 1539 entregaron una muestra de orina en la que se determinó la concentración de metabolitos de pesticidas no persistentes. Los padres o tutores legales de los niños firmaron el consentimiento informado, y el protocolo de investigación contaba con la aprobación por el Comité de Ética local de cada una de las cohortes (Anexo I y II).

4.2.3 Biomarcadores de exposición

Las muestras de orina se recogieron a primera hora de la mañana en las cohortes de Asturias y Valencia; en Sabadell y Gipuzkoa fueron obtenidas a partir del *pool* de dos muestras de orina, una recogida a primera hora de la mañana del día de la visita y otra recogida la noche anterior antes de acostarse; y en Granada la muestra de orina se recogió por la tarde durante la misma visita (entre las 17:00 y las 20:00 horas). Todas las muestras fueron almacenadas a -80ºC hasta el momento del análisis y remitidas al Centro de Investigación Biomédica (CIBM) de la Universidad de Granada.

Para evaluar la exposición a pesticidas no persistentes se cuantificaron las concentraciones urinarias de los siguientes metabolitos: 3,5,6-tricloropiridinol (TCPy), metabolito específico del insecticida clorpirifós; 2-isopropil-6-metil-4-pirimidinol (IMPy), metabolito específico del insecticida diazinón; dos metabolitos inespecíficos de insecticidas OPs comunes: dietiltiofosfato (DETP) y dietilditiofosfoato (DEDTP); 3-PBA, metabolito genérico de insecticidas piretroides; y ETU, metabolito de fungicidas EBDCs como maneb y mancozeb. El análisis de los metabolitos urinarios se realizó mediante cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas (UHPLC-MS/MS) utilizando un cromatógrafo UHPLC Ultimate 3000 (Thermo Fischer) y el espectrómetro de masas Q Exactive Focus (Thermo Fischer). Los límites de

detección (LD) fueron: 0,039 µg/L para TCPy; 0,117 µg/L para IMPy; 0,116 µg/L para DETP; 0,142 µg/L para DEDTP; 0,072 µg/L para ETU; y 0,117 µg/L para 3-PBA. En todas las muestras de orina se midieron las concentraciones de creatinina, según el método Jaffé (235) con un analizador bioquímico Cobas C-311 (Roche) para poder corregir las concentraciones urinarias de metabolitos de pesticidas y así tomar en cuenta posibles variaciones en la dilución urinaria.

4.2.4 Evaluación del desarrollo puberal

Para evaluar el estado de desarrollo puberal de los niños y niñas de las cohortes INMA, se utilizaron las escalas de Tanner y la PDS (Anexo III). En las cohortes de Asturias y Valencia se utilizó la escala de Tanner de desarrollo genital (G) para los niños, mamario (S) para las niñas y del vello púbico (P) para ambos sexos, mientras que en Granada, cuya cohorte estaba compuesta por varones, se realizó solo la valoración del desarrollo genital (Tanner G). En Gipuzkoa y Sabadell se utilizó la escala PDS cumplimentada por los padres o tutores legales de los participantes y en Valencia se utilizó tanto la escala de Tanner como la PDS. La clasificación PDS se obtuvo aplicando a los cuestionarios los algoritmos descritos por Carskadon y Acebo (1993) (10) y por Shirtcliff et al. (2009) (11), obteniéndose 5 categorías de desarrollo global, gonadal y adrenal: 1) pre-pubertad, 2) pubertad temprana, 3) pubertad media, 4) pubertad tardía y 5) post-pubertad (9). Si bien la concordancia entre la clasificación obtenida a partir de la PDS y los estadíos de Tanner no es perfecta, la PDS se considera un instrumento válido y confiable para la evaluación puberal (12). La variable respuesta principal en este estudio fue haber iniciado la pubertad, considerándose como tal estar en un estadío 2 o superior según la escala PDS o Tanner.

4.2.5 Covariables

La información sobre otras covariables de interés se recogió a partir de cuestionarios a los cuales los padre o tutores legales respondieron en las visitas del embarazo o del seguimiento del niño. Se consideraron como posibles variables confusoras las siguientes variables maternas:

- Cohorte (Asturias, Gipuzkoa, Granada, Sabadell o Valencia);
- Edad de la madre al parto (continua, en años);
- Educación materna (primaria, secundaria o universitaria);

- Etnia (caucásica o no caucásica);
- Área de residencia durante el embarazo (urbana, sub-urbana o rural);
- Ser fumadora durante el embarazo (sí o no);
- Ganancia ponderal durante el parto (continua, en kilogramos).

Las variables relativas a los niños/as fueron:

- Peso al nacer (continua, en gramos);
- Edad gestacional al parto (continua, en semanas);
- Edad al momento de la evaluación puberal (continua, en años);
- Peso y altura (continuas, en kilogramos y centímetros, respectivamente);
- IMC (en kg/m^2).

El IMC fue transformado en puntuación *z-score* para edad y sexo en base a las curvas de referencia de la OMS (236) y posteriormente se categorizaron en bajo peso (<-1 desviación estándar [DE]), peso normal (±1 DE), sobre peso (>+1 DE) y obesidad (>+2 DE, equivalente a un IMC de 30 kg/m^2 a la edad de 19 años).

4.2.6 Análisis estadístico

Se realizó el análisis descriptivo de las concentraciones urinarias de los metabolitos de pesticidas (tanto ajustadas por creatinina como sin ajustar), de las características generales de los participantes y de las variables de desarrollo puberal. Se utilizaron percentiles para describir la distribución de las concentraciones urinarias de los metabolitos de pesticidas, asignando un valor correspondiente a LD/√2 a aquellas muestras en que la concentración del metabolito era inferior al LD. Las concentraciones urinarias de metabolitos y de creatinina fueron transformadas en el logaritmo natural para la aproximación normal. Se analizó la correlación entre las concentraciones urinarias de los distintos metabolitos de pesticidas mediante la prueba de Spearman.

Para investigar la asociación entre las concentraciones urinarias de metabolitos de pesticidas y el estar en un estadío de desarrollo puberal ≥ 2 se emplearon modelos de regresión logística de efectos mixtos. En los modelos, los metabolitos detectados en más del 60% de las muestras (IMPy y DETP) se introdujeron en forma continua y también categorizados en "exposición baja" (<LD), "exposición intermedia" (entre el LD y el percentil 75) y "exposición alta" (>percentil 75). La ETU, que fue detectada en un 50% de las muestras, fue clasificada en estas tres categorías, mientras que TCPy y 3-PBA, que fueron detectados en menos del 40% de las muestras, fueron introducidos como variables

dicotómicas (>LD o ≤LD). DEDTP se detectó en menos del 5% de las muestras y por lo tanto se excluyó del análisis de asociación. Se realizaron modelos separados para niños y niñas. Los modelos incluían las concentraciones urinarias de metabolitos de pesticidas y las concentraciones de creatinina como variables independientes separadas, con la finalidad de reducir el sesgo de error de medición debido a la variabilidad en las concentraciones de orina (237). Según estudios epidemiológicos previos (180,223,225,226) y siguiendo criterios biológicos, los modelos fueron ajustados por la edad en años de los niños/as, cohorte de origen, el IMC z-score y la talla de los niños en el momento de la visita, y el nivel de estudios de la madre. El resto de covariables consideradas en el estudio, incluidas el área de residencia, la época del año en que se realizó la visita de seguimiento y las características de la madre durante el embarazo, no cumplían el criterio estadístico para ser consideradas factores de confusión (cambio del coeficiente de regresión >10%). Para evaluar el posible efecto modificador del IMC, descrito en estudios previos como un importante predictor del desarrollo puberal (238,239), los modelos se estratificaron en dos grupos: niños/as con bajo peso/normo peso y niños/as con sobrepeso u obesidad. Las asociaciones se expresaron como probabilidad (*odds ratio* [OR] con su respectivo intervalo de confianza [IC] del 95%) de haber iniciado la pubertad (estadío ≥2) por cada incremento de unidad logarítmica en la concentración del metabolito o para concentraciones detectadas/moderadas/altas frente a no detectadas/bajas. Se utilizaron los programas informáticos R (versión 4.1) y SPSS (versión 26) para los análisis estadísticos y se estableció un valor de $p<0,05$ como punto de corte para la significación estadística.

4.3 Objetivo 3

Para evaluar la asociación entre la exposición intrauterina a ftalatos y el desarrollo puberal se realizó un estudio longitudinal en parejas madre-hijo/a de las cohortes INMA de Gipuzkoa, Sabadell y Valencia.

4.3.1 Diseño del estudio

Estudio longitudinal.

4.3.2 Población en estudio

Se incluyeron en el estudio a todas las parejas madre-hijo/a de las cohortes de Gipuzkoa, Sabadell y Valencia que participaron en la visita de seguimiento de los 7-10 años y que disponían de información sobre exposición prenatal a ftalatos. De los 2240 recién nacidos en estas cohortes (2003-2008), un total de 1298 niños/as participaron en el seguimiento a la edad de 7-10 años durante la cual se realizó una evaluación del desarrollo puberal. El estudio se realizó en 788 niños de dichos niños/as, 409 niños y 379 niñas, de los que se disponía de información sobre las concentraciones de metabolitos de ftalatos medidos en la orina materna durante el embarazo. Los participantes entregaron su consentimiento informado, tanto en el seguimiento del embarazo como en el seguimiento del niño/a y el protocolo de investigación fue aprobado por el Comité de Ética local de cada cohorte (Anexos I y II).

4.3.3 Biomarcadores de exposición

Las muestras de orina de las madres fueron recogidas en ayunas en el primer y en el tercer trimestre de embarazo. En Valencia y Gipuzkoa el análisis de ftalatos en orina se realizó en un *pool* de las dos muestras, mientras que en Sabadell se obtuvo la media de las concentraciones cuantificadas en las dos muestras de orina.

Se cuantificaron ocho metabolitos correspondientes a cinco ésteres de ftalatos, de los cuales tres son de bajo peso molecular (DEP, DiBP y DnBP) y dos de alto peso molecular (BBzP y DEHP). En una submuestra de 460 mujeres de las cohortes de Gipuzkoa y Valencia se analizaron además las concentraciones de los metabolitos de DiNP y del plastificante no-ftalato 1,2-ciclohexano dicarboxílico ácido diisononil éster (DINCH), conocido como Hexamoll® DINCH. Las muestras de orina de la cohorte de Sabadell se analizaron en el Instituto de Investigación del Hospital del Mar (IMIM) de Barcelona, mediante cromatografía líquida de ultra alta resolución acoplada a espectrometría de masa en tándem (240), mientras que las muestras de Valencia y Gipuzkoa se analizaron en el *Norwegian Institute of Public Health* (NIPH) en Oslo, Noruega, mediante cromatografía líquida acoplada a espectrometría de masa (241). Para comparar las dos técnicas, se enviaron al NIPH 10 muestras de orina de Sabadell ya analizadas en Barcelona con niveles variables de metabolitos de ftalatos y se compararon las concentraciones medidas en ciego en los dos laboratorios (242), obteniéndose una fuerte correlación entre los resultados (rho de Spearman entre 0,69 y 0,97). Se

cuantificaron las concentraciones urinarias de creatinina en las dos muestras obtenidas de cada mujer (primer y tercer trimestre de embarazo) mediante método Jaffé con un analizador AU5400 Beckman Coulter© (IZASA®) en el laboratorio Echevarne en Barcelona, y se utilizó la media de las dos determinaciones para ajustar los biomarcadores de exposición por el grado de dilución de la orina.

4.3.4 Evaluación del desarrollo puberal

El desarrollo puberal de los niños y niñas se evaluó mediante la escala PDS cumplimentada por los padres o tutores legales. Como ya se ha descrito, la puntuación obtenida se categorizó en 1) pre-pubertad, 2) pubertad temprana, 3) pubertad media, 4) pubertad tardía y 5) post-pubertad para el desarrollo global, adrenal y gonadal según los algoritmos de Carskadon y Acebo (1993) (10) y Shirtcliff et al. (2009) (11). Se consideró como variable respuesta principal el haber iniciado la pubertad al estar en estadío 2 o superior según las escalas PDS (Anexo III).

4.3.5 Covariables

Se recogió información sobre otras variables de interés durante las visitas del primer y tercer trimestre de embarazo y de seguimiento del niño. Las covariables maternas incluían:

- Cohorte (Gipuzkoa, Sabadell o Valencia);
- Edad al momento del embarazo (continua, en años),
- Educación materna (primaria, secundaria o universitaria);
- Paridad (nulípara, 1 parto previo o ≥2 partos previos);
- Área de residencia durante el embarazo (urbana o rural);
- Ser fumadora durante el embarazo (sí o no);
- Ser fumadora pasiva durante el embarazo (sí o no);
- Haber trabajado durante el embarazo (sí o no)
- IMC previo al embarazo (kg/m^2).

Las variables referidas al niño fueron:

- Edad al momento de la evaluación puberal (continua, en años);
- Convivencia con el padre (sí o no);

- Estado ponderal (bajo peso/normopeso/sobrepeso/obesidad), calculado como referido en secciones anteriores a partir de peso y altura medidos en la visita.

4.3.6 Análisis estadístico

Todos los metabolitos de ftalatos fueron detectados en más del 99% de las muestras de orina y los metabolitos de DINCH® se detectaron en 83-94% de las 460 orinas de Gipuzkoa y Valencia, asignando el valor LD/√2 a las muestras con valor inferior al LD. Para simplificar el análisis, se calcularon las sumas de las concentraciones molares de los metabolitos de DEHP, DiNP y de DINCH®. La suma (∑) se calculó en base a la concentración molar de los metabolitos, sumando las concentraciones divididas por el peso molecular de cada uno de los metabolitos y posteriormente multiplicando por el peso molecular de los compuestos padre. Se analizó la correlación entre las concentraciones urinarias de los distintos metabolitos de pesticidas mediante la prueba de Spearman.

Previo al análisis de regresión, las concentraciones urinarias de metabolitos de ftalatos y de creatinina fueron transformadas en el logaritmo natural para la aproximación normal. Las asociaciones entre los biomarcadores de exposición y el desarrollo puberal se evaluaron mediante regresión de Poisson con varianza robusta, separadamente para niños y niñas (243). Se realizaron dos modelos, un primer modelo ajustado por creatinina, cohorte de origen y edad del niño, y un segundo modelo que se ajustó además por las siguientes características maternas: edad, nivel de estudios, IMC previo al embarazo, paridad, y exposición al humo de tabaco durante el embarazo. Como descrito en las secciones anteriores, las concentraciones de creatinina, transformadas logarítmicamente, se introdujeron en los modelos como variable independiente, y otras covariables, como el área de residencia y el haber trabajado durante el embarazo y la convivencia con el padre al momento de la visita) se excluyeron del modelo por criterio estadístico. Las asociaciones se expresaron como riesgo relativo (*relative risk* [RR] con su respectivo IC del 95%) de haber iniciado la pubertad (estadío ≥2) por cada incremento de una unidad logarítmica en la concentración del metabolito/suma de metabolitos. Para evaluar el posible efecto modificador del IMC, los modelos se estratificaron en dos grupos: niños/as con bajo peso o peso normal y niños/as con sobrepeso u obesidad. Finalmente, se evaluó el posible efecto combinado de la exposición a la mezcla de ftalatos mediante modelos de regresión de suma de cuantiles ponderados (WQS, del inglés: *Weighted Quantile Sum*)

(244), utilizando las mismas variables de ajusto que en los modelos de Poisson, incluido el análisis de sensibilidad ajustado por IMC. Se utilizaron los programas informáticos R (versión 4.1) y SPSS (versión 26) para los análisis estadísticos y se estableció un valor de $p<0,05$ como punto de corte para la significación estadística.

4.4 Objetivo 4

Para investigar la asociación entre exposición a pesticidas no persistentes y el estado de maduración sexual en varones se llevó a cabo un estudio transversal en adolescentes de dos cohortes del proyecto INMA.

4.4.1 Diseño del estudio

Estudio transversal.

4.4.2 Población en estudio

Los participantes eran varones de las cohortes INMA de Granada y Menorca. Estas cohortes contaban inicialmente con 1150 parejas madre-hijo (668 en Granada y 482 en Menorca), reclutadas tras el parto en el Hospital Universitario San Cecilio (HUSC) de Granada en 2000-2002 y en el Hospital Mateu Orfila de Menorca en 1997-2000. A la edad de 14-17 años, 139 niños de Menorca y 151 niños de Granada participaron en una visita de seguimiento en la que se realizó una evaluación del estado de maduración sexual y se recogió una muestra de orina para cuantificación de metabolitos de pesticidas no persistentes. El presente estudio incluye 201 de estos niños de los cuales se disponía de las variables de interés. Los padres o tutores legales firmaron el consentimiento informado y el protocolo de investigación fue aprobado por los comités de ética locales de las dos cohortes (Anexos I y II).

4.4.3 Biomarcadores de exposición

En la visita de seguimiento, cada participante recogió una muestra de orina en ayunas, a primera hora de la mañana (6:00-8:00 horas), que fue almacenada a -80ºC y remetida al CIBM, Universidad de Granada, para su análisis. Mediante cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas (UHPLC-MS/MS), se cuantificaron las concentraciones urinarias de los siguientes metabolitos: TCPy, IMPy,

DETP, DEDTP, 3-PBA, ETU y 1-N. En una submuestra de 161 de los 201 niños del estudio se midieron también el ácido dicarboxílico de malatión (MDA), metabolito del insecticida OP malatión, y el DCCA, metabolito de los piretroides permetrina, cipermetrina y ciflutrina. Además de los LDs descritos en la sección 4.2.3, los LDs de 1-N, MDA y DCCA fueron 0,156 µg/L, 0,052 µg/L y 0,055 µg/L, respectivamente. En todas las muestras de orina se cuantificaron las concentraciones de creatinina, según el método descrito anteriormente.

4.4.4 Evaluación de la maduración sexual

Para evaluar el grado de maduración sexual de los adolescentes, se utilizó la escala de Tanner que fue valorada por un pediatra en la cohorte de Granada y auto-cumplimentada por los mismos adolescentes en la cohorte de Menorca, a los que se le ofreció un cuestionario con la descripción gráfica en imágenes de los estadíos de Tanner (Anexo III). Además, los adolescentes de ambas cohortes fueron invitados a cumplimentar la PDS (10), obteniéndose una clasificación en 5 estadíos para desarrollo global, gonadal y adrenal, como se ha descrito anteriormente. En total, se obtuvo información sobre desarrollo genital para 175 niños, sobre crecimiento de vello púbico para 181, mientras que 197 adolescentes contestaron la PDS. En Granada, el pediatra también midió el volumen testicular en una submuestra de 139 adolescentes, mediante palpación y comparación con el orquidómetro de Prader, registrándose el de mayor tamaño en caso de diferencia de tamaño entre los dos testículos.

4.4.5 Covariables

Se examinaron las siguientes variables como posibles factores de confusión:

- Cohorte (Granada o Menorca);
- Edad del niño al momento de la evaluación puberal (continua, en años);
- Estación del año al momento de la visita (primavera, verano, otoño o invierno);
- Z-score del IMC (continuo)
- Estado ponderal, calculado como anteriormente descrito (bajo peso/normopeso o sobrepeso/obesidad)
- Exposición al humo de tabaco (sí o no);
- Área de residencia (urbana o rural);

- Edad materna (continua, en años);
- Educación materna (primaria, secundaria o universitaria)
- Situación parental (madre con pareja estable: sí o no);
- Ingresos económicos familiares (<25.000, 25.000-35.000 o >35.000 €/año).

4.4.6 Análisis estadístico

Los metabolitos de pesticidas detectados en <50% de las muestras de orina (TCPy y 1-N) se transformaron en variables dicotómicas (detectado/no detectado) antes del análisis de regresión, mientras que los metabolitos detectados en ≥50% de las muestras se categorizaron en "baja exposición" (<LD), "exposición moderada" (LD-percentil 75) o "alta exposición" (>percentil 75). El DEDTP y el 3-PBA fueron detectados en <30% de los participantes y se excluyeron de los análisis de asociación. El estadío de Tanner y el volumen testicular se categorizaron en desarrollo sexual completo (Tanner G=5, Tanner PH=5, TV≥25 mL) o desarrollo incompleto (Tanner G<5, Tanner PH<5, TV<25 mL). Los estadíos obtenidos a partir de la PDS se clasificaron como pubertad tardía/post-pubertad (estadío ≥4) o pubertad pre/temprana/media (estadío <4), tanto para el desarrollo puberal global, como adrenal y gonadal.

La asociación entre la exposición a pesticidas y el grado de maduración sexual se evaluó mediante regresión logística, con un modelo para cada metabolito de pesticida (variable independiente) y cada *outcome* o variable respuesta (variable dependiente). En un primer paso, se creó un modelo básico ajustado por la cohorte de origen, la edad del niño y la concentración de creatinina urinaria. Las concentraciones de metabolitos de pesticidas y de creatinina se introdujeron en los modelos previa transformación en el logaritmo natural. En un segundo paso, se introdujo en los modelos la escolaridad de la madre, para controlar el nivel socioeconómico de la familia. Y en un tercer paso, los modelos se ajustaron por todas las variables anteriores y el IMC z-score del niño, para evaluar el posible papel mediador del IMC en las asociaciones a estudio. Otras covariables, como la edad y el nivel de educación de la madre, el área de residencia, los ingresos económicos familiares y la estación del año en que se realizó la visita de seguimiento, se excluyeron del modelo por no cumplir con el criterio estadístico para factor de confusión. Se realizó un análisis de sensibilidad, estratificando el modelo completo por cohorte de pertenencia, para tener en consideración eventuales diferencias

de clasificación del estadío de Tanner, que fue reportado por el adolescente en la cohorte de Menorca y valorado por un pediatra en la de Granada.

Se evaluó también el posible efecto de confusión de la co-exposición ajustando los modelos de regresión por todos los metabolitos de pesticidas (excepto DETP y DCCA para evitar sobrestimar la exposición, debido a que son metabolitos que comparten compuestos padre con otros metabolitos ya incluidos en el modelo). Las asociaciones se expresaron como probabilidad (OR con su respectivo IC95%) de haber alcanzado la madurez sexual en niños con concentraciones de pesticidas detectadas en comparación con niños con concentraciones no detectadas o con concentraciones moderadas/altas frente a bajas. El nivel de significancia estadística se fijó en $p<0,05$. Se utilizó IBM SPSS Statistics v.26 para el análisis de datos.

4.5 Objetivo 5

Para evaluar la asociación entre la exposición a pesticidas no persistentes y los niveles séricos de hormonas sexuales, así como la posible interacción con polimorfismos genéticos relacionados con el metabolismo de xenobióticos, se llevaron a cabo dos estudios, analizando pesticidas OPs y no OPs, respectivamente, en adolescentes varones de la cohorte de INMA-Granada. Para detallar la metodología empleada, se hará referencia a los dos estudios utilizando el nombre del primer autor y el año de publicación de los dos artículos resultado de éstos: Suarez et al. 2021 (245) y Freire et al. 2021 (246).

4.5.1 Diseño de los estudios

Estudios transversales.

4.5.2 Población en estudio

La cohorte INMA-Granada reclutó inicialmente 668 parejas madre-hijo (varón) nacidos en el HUSC de Granada en 2000-2002. Se excluyeron de la cohorte madres que presentaban patologías crónicas como diabetes, hipertensión, patologías tiroideas y las que desarrollaron complicaciones durante el embarazo potencialmente prejudiciales para el desarrollo y el crecimiento fetal, así como las mujeres no residentes en la provincia de Granada. Se realizaron visitas de seguimiento cuando los niños tenían 4-5 años y 9-11 años de edad, en las que participaron, respectivamente, 220 y 300 niños. Los niños que participaron en estas visitas de seguimiento fueron contactados e invitados a participar en

una nueva visita de seguimiento a la edad de 15-17 años, que se realizó también en el HUSC entre 2017 y 2019. De los 155 niños que aceptaron participar, se recogieron muestras de orina y suero en 135 niños. De los dos estudios realizados, el primero (Freire et al, 2021) (246) incluye una submuestra de 134 de estos niños y el segundo (Suarez et al. 2021) (245) de 117 niños. Los padres o tutores legales de los niños firmaron el consentimiento informado y el protocolo de estudio fue aprobado por el Comité Ético de Investigación Provincial de Granada (Anexos I y II).

4.5.3. Recogida y análisis de muestras biológicas

Cada participante recogió una muestra de orina en ayudas, a primera hora de la mañana (entre 6:00 y 8:00 horas), el mismo día de la visita de seguimiento, y se extrajo una muestra de sangre periférica por la tarde, en el hospital, que fue procesada para la obtención de suero en las primeras 4 horas tras la extracción; ambos especímenes fueron almacenados a -80ºC hasta el momento del análisis.

4.5.3.1 Biomarcadores de exposición

En el primer estudio (Freire et al. 2021 (246)) se evaluaron las concentraciones urinarias de ETU, 3-PBA y 1-N. En el segundo (Suarez et al. 2021 (245)), se evaluaron TCPy (clorpirifós), IMPy (diazinón), DETP y DEDTP. El análisis de los metabolitos urinarios se realizó mediante UHPLC-MS/MS, como se ha descrito anteriormente. En todas las muestras de orina se midieron las concentraciones de creatinina, según el método Jaffé (235) con un analizador bioquímico Cobas C-311 (Roche).

4.5.3.2 Análisis de hormonas

En las muestras de suero obtenidas se analizaron las concentraciones de las siguientes hormonas y proteínas: testosterona total, E_2, DHEA-S, FSH, LH, proteína transportadora de hormonas sexuales (SHBG), hormona anti-Mülleriana (AMH), prolactina (PRL), IGF-1, hormona adrenocorticótropa (ACTH) y cortisol, así como hormonas tiroideas: tiroxina libre (T4), T3 y TSH. El análisis de los parámetros hormonales se llevó a cabo mediante inmunoensayo de electroquimioluminiscencia (ECLIA) usando un kit Elecsys (Roche) en la Plataforma Científico-Técnica del ibs.GRANADA y los valores de referencia utilizados se reúnen en el Anexo VII, Tabla S3.

4.5.3.3 Extracción de ADN y genotipado

Se determinaron SNPs relacionados con la expresión de enzimas que intervienen en el metabolismo de los xenobióticos. A partir de las muestras de sangre total obtenidas en la visita, se realizó la extracción de ADN y genotipado GWAS en búsqueda de los SNPs en CYP2C19 G681A y CYP2D6 G1846A en Freire et al. 2021 (246) y PON1 Q192R y L55M en Suárez et al. 2021 (245), considerados como marcadores de mayor susceptibilidad genética a los efectos adversos de los pesticidas en base a estudios previos, como explicado en el apartado 3 El genotipado del genoma completo se realizó con la tecnología Infinium de Illumina con Global Screening Array (GSA) en el Laboratorio de Genotipado Humano del Centro Nacional de Investigaciones Oncológicas Carlos III (CNIO) de Madrid.

4.5.4 Covariables

Se han considerado como posibles variables confusoras:

- Edad del niño (16 ó 17 años);
- IMC (continua, en kg/m^2);
- Hora del día de la extracción de sangre venosa (continua);
- Estación del año al momento de la visita (primavera, verano, otoño o invierno);
- Área de residencia (urbana o rural);
- Ingresos económicos familiares (25.000, 25.000-35.000 o >35.000 €/año);
- Masa grasa medida por bioimpedancia (continua, porcentaje).

4.5.5 Análisis estadístico

Se realizaron análisis descriptivos de las características generales de los participantes, de las concentraciones urinarias de los pesticidas y de los niveles séricos de hormonas, así como de las frecuencias alélicas de los polimorfismos analizados. Se utilizaron percentiles para describir la distribución de las concentraciones urinarias de los metabolitos de pesticidas y a las concentraciones que estaban por debajo del LD se les asignó un valor correspondiente a $LD/\sqrt{2}$. Las concentraciones urinarias de metabolitos, tanto en su valor absoluto como corregidas por creatinina, y las concentraciones hormonales fueron transformadas en el logaritmo natural para la aproximación normal a

la distribución de estas variables. Previo a los análisis de asociación, las concentraciones urinarias de 3-PBA, 1-N, IMPy, TCPy, DETP y DEDTP se categorizaron en variables dicotómicas (detectado o no detectado), debido al bajo porcentaje de detección de estos metabolitos, mientras que las concentraciones de ETU fueron tratadas como una variable dicotómica (detectado o no detectado) y también de forma continua.

Para examinar las asociaciones entre las concentraciones urinarias de metabolitos de pesticidas y los niveles hormonales se empleó regresión lineal múltiple. Según estudios epidemiológicos previos (180,223,225,226) y siguiendo criterios biológicos, los modelos se ajustaron por las concentraciones urinarias de creatinina, la edad del niño, el IMC, la estación del año en que se realizó la visita y la hora de extracción de la muestra de sangre. El área de residencia, los ingresos económicos anuales familiares y la composición corporal del niño medida por bioimpedancia no cumplían el criterio estadístico para ser consideradas factores de confusión (cambio del coeficiente de regresión >10%) y fueron excluidas del estudio. Las asociaciones se expresaron como porcentaje de variación de los niveles hormonales en los niños expuestos (≥LD) en relación a los no expuestos (<LD), y en el caso de variable continua (ETU) como porcentaje de variación por cada aumento del doble en las concentraciones del metabolito.

Se evaluó la interacción entre la exposición y los polimorfismos CYP2C19 G681A y CYP2D6 G1846A (Freire et al. 2021 (246)) y PON1 Q192R y L55M (Suárez et al. 2021 (245)) mediante estratificación de los modelos de regresión lineal en función de presencia o ausencia de uno o ambos alelos alterados (por ejemplo, en el caso del gen CYP2C19, GG frente a GA o AA). Se estableció un valor de significación estadística con $p<0,05$ y se utilizó el programa R versión 3.4.3 para los análisis.

5. RESULTADOS

5.1 Resultado objetivo 1:

EXPOSURE TO NON-PERSISTENT PESTICIDES AND PUBERTY TIMING: A SYSTEMATIC REVIEW OF THE EPIDEMIOLOGICAL EVIDENCE

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Resumen

Antecedentes: Numerosos pesticidas no persistentes modernos han demostrado actividad estrogénica/antiandrogénica y han sido clasificados como disruptores endocrinos (DEs). Los procesos implicados en el desarrollo de la pubertad son vulnerables a los DEs, puesto que son compuestos que interfieren con el metabolismo o la actividad de los esteroides sexuales.

Objetivo: Realizar una revisión sistemática de los estudios epidemiológicos sobre la relación entre la exposición temprana a pesticidas no persistentes y el desarrollo puberal y/o la maduración sexual en niñas y niños.

Métodos: Se realizó una búsqueda sistemática utilizando las bases de datos MEDLINE y SCOPUS. Se incluyeron los artículos originales publicados hasta noviembre de 2020, y se excluyeron los estudios no originales, *in vitro* o en modelos animales, casos clínicos o series de casos, los trabajos publicados en idiomas diferentes al inglés, español, italiano o portugués y los estudios en los que el desarrollo puberal o la maduración sexual no era variable respuesta principal. En la metodología se siguieron las directrices de la guía PRISMA y la calidad de la evidencia proporcionada por los estudios seleccionados se evaluó mediante la escala GRADE

Resultados: Trece estudios cumplían los criterios de inclusión y ocho de ellos describían asociaciones significativas con alteraciones en la edad de inicio de la pubertad. Así, la exposición *in utero* a herbicidas tipo atrazina se relacionó con menarquia más temprana en un estudio; la exposición infantil a insecticidas organofosforados (OP) se asoció con retraso en el desarrollo puberal en ambos sexos en otro estudio, mientras que la exposición

a insecticidas piretroides se asoció con retraso en la maduración sexual en niñas y adelanto puberal en niños en dos estudios, respectivamente. La exposición prenatal a múltiples pesticidas se relacionó con adelanto puberal en niñas y retraso puberal en niños en dos estudios, respectivamente, mientras que el residir en áreas rurales durante la infancia se relacionó con retraso en la edad de la menarquia en niñas en un estudio y con adelanto puberal en niños en otro.

Conclusiones: La mayoría de los estudios revisados describen una relación entre la exposición a pesticidas y alteraciones en el desarrollo puberal o los niveles de hormonas sexuales, aunque la calidad de la evidencia es generalmente baja. Son necesarios más estudios longitudinales que investiguen el impacto de la exposición a diferentes clases de pesticidas sobre el desarrollo puberal y posibles interacciones entre diferentes compuestos.

Abstract

Background: Numerous modern non-persistent pesticides have demonstrated estrogenic/anti-androgenic activity and have been classified as endocrine disrupting chemicals (EDCs). Processes involved in puberty development are vulnerable to EDCs, such as compounds that interfere with the metabolism or activity of sex steroids.

Objective: To conduct a systematic review of epidemiological studies on the relationship between early-life exposure to non-persistent pesticides and puberty timing and/or sexual maturation in girls and boys.

Methods: A systematic search was carried out using MEDLINE and SCOPUS databases, including original articles published up to November 2020.

Results: Thirteen studies were selected after excluding non-original and non-human studies. Exposure to different types of pesticides has been associated with altered puberty timing in girls and/or boys in eight studies. In utero exposure to atrazine has been related to earlier age of menarche in girls; exposure to organophosphate (OP) pesticides has been related to delayed sexual development in boys and girls; childhood pyrethroid exposure has been associated with pubertal delay in girls and pubertal advancement in boys; and prenatal/childhood exposure to multiple pesticides has been linked to earlier puberty onset in girls and pubertal delay in boys.

Conclusions: Most of the reviewed studies describe a relationship between pesticide exposure and changes in the age of puberty onset or sex hormone levels, although the quality of the evidence is generally low. Further well-designed longitudinal studies are

warranted on specific classes of pesticides and on possible interactions between different types of compounds.

Keywords

Non-persistent pesticides; endocrine disruptors; puberty timing; sex hormones; epidemiological studies.

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Introduction

The age of puberty onset has progressively decreased among girls in developed countries over the past century (1). This has been attributed to improvements in the socio-economic conditions of children and in their nutritional and hygienic status (2). The age at menarche is the most widely used marker of puberty onset in girls but there is no equivalent indicator in boys. Researchers have associated earlier puberty onset, especially in girls, with a higher risk of adverse health effects, including hormone-dependent cancer, type 2 diabetes, cardiovascular disease, and psychological problems (3–5). The timing of puberty onset, which is highly variable among individuals, is strongly determined by genetic factors (6); however, there is increasing evidence that obesity is associated with a higher risk of precocious puberty in girls (7). Maturation of the reproductive system is also known to be affected by psychosocial factors, early-life nutrition, and anthropometric growth patterns during infancy and childhood (8).

Environmental factors have also been implicated in the decreasing age of puberty onset over the past few decades, including exposure to chemical compounds able to alter hormonal homeostasis, so-called endocrine-disrupting chemicals (EDCs) (9). These hormonally active substances are widely present in the environment and exert their effect by binding to specific receptors, changing intracellular signaling pathways, and altering hormonal synthesis and metabolism (10). Pubertal development depends on the production of sex steroids, and this process is regulated by activation of the hypothalamic-pituitary-gonad (HPG) axis through an increase in gonadotropin-releasing hormone (GnRH) pulse frequency in the hypothalamus, which promotes the pituitary production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These mechanisms are susceptible to disruption by EDCs, especially by those with estrogenic, anti-estrogenic and/or anti-androgen activity, including compounds that interfere with the metabolism or activity of sex steroids (11).

Organochlorine (OC) pesticides are persistent organic pollutants whose use has been severely restricted in industrialized countries since the 1990s due to their adverse effects on human health. Several studies have shown that human exposure to OC pesticides during critical windows of development can affect reproductive health through their well-studied anti-estrogenic and anti-androgenic activity (12). Researchers have described a link between earlier menarche in girls and exposure to dichlorodiphenyltrichloroethane (DDT) (13) and between delayed puberty in boys and exposure to OC pesticides, among other pollutants (14). Currently, most pesticides used in agricultural and domestic applications are non-persistent compounds, including insecticides such as organophosphates (OPs), carbamates, and synthetic pyrethroids, herbicides, and fungicides. *In vitro* studies

suggest that several OP pesticides, pyrethroids, and carbamates exert estrogenic and/or anti-androgenic activity by binding to specific receptors or by altering sex steroid synthesizing enzymes such as CYP19 (aromatase) and CYP17 (15,16). For instance, the OP pesticide chlorpyrifos demonstrated estrogenic activity *in vitro* (17), and its anti-androgenic activity was found to affect steroidogenesis by reducing testosterone production in Leydig cell cultures (18). Another OP pesticide, dimethoate, was found to reduce steroidogenic acute regulatory (StAR) protein expression and consequently testosterone production by Leydig cells *in vitro* (19) and *in vivo* (20). Pyrethroids are a large group of pesticides with well-documented estrogenic and anti-androgenic effects (15). They have also been shown to alter the expression of steroidogenic acute regulatory protein (StAR) and cytochrome P450 (CYP450) side chain cleavage enzyme (CYP450scc) genes (21). In addition, it has been proposed that pyrethroids and their metabolites may act as a substrate for xenobiotic nuclear receptors, such as peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (22), potentially interfering with steroidogenesis (23,24).

Fungicides such as the dithiocarbamate mancozeb have anti-androgenic effects that can alter the binding of dihydrotestosterone to the human receptor (25), while imidazole fungicides have demonstrated competitive inhibition of aromatase activity (9,16). Glyphosate, the most heavily used herbicide worldwide, is also suspected to have anti-estrogenic and anti-androgen activity and to act as an EDC (26). Thus, *in vivo* studies have shown that prenatal exposure to glyphosate triggers androgen-like effects that can alter reproductive development and lower the age of puberty onset in males (27).

Experimental studies have demonstrated the estrogenic/anti-androgenic activity and reproductive toxicity of numerous modern non-persistent pesticides, but only limited human data are available. An increasing number of epidemiological studies, mostly in agricultural workers, have examined changes in circulating levels of reproductive hormones in relation to exposure to non-persistent pesticides (28,29). However, there has been little human research on the relationship between exposure to these pesticides and puberty, and the findings have been controversial. In order to offer an overview of what has been done in this field, the objective of this study was to conduct a systematic review of epidemiological studies on the relationship between early-life exposure to modern non-persistent pesticides and alterations in the timing of sexual maturation and onset of puberty.

Methods

In order to provide an answerable question and to determine the rationale for the inclusion and exclusion criteria, the following

PECO (Population, Exposure, Comparator, and Outcomes) statement (30) was developed:

Population: Humans from prenatal stage to adolescence.

Exposure: Any exposure to modern non-persistent pesticides, including insecticides, herbicides and fungicides (not persistent organochlorine pesticides);

Comparator: Humans non-exposed or exposed to lower levels of non-persistent pesticides than more highly exposed humans.

Outcome: Timing (onset and/or progression) of puberty assessed as: a) age at menarche; b) pubertal development according to Tanner stage (breast development and pubic hair for girls, and genital development and pubic hair for boys); c) serum sex hormone levels (mainly estradiol and testosterone, follicle-stimulating hormones, and luteinizing hormone).

The PubMed/MEDLINE (https://www.ncbi.nlm.nih.gov/pubmed) and SCOPUS databases were used to systematically search the scientific literature up to November 2020 for original epidemiologic studies on the relationship between exposure to non-persistent pesticides and puberty timing and/or sexual maturation in girls and/or boys. Figure 1 depicts a flow chart of the review process (identification, selection, and exclusion of items), which followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (31).

A search was conducted for the terms “pesticides”, “non-persistent pesticides”, “insecticides”, “herbicides”, “fungicides”, “agrochemicals” OR “biocidals” in combination with “puberty”, “pubertal development”, “sexual development”, “sexual maturity”, “sex hormones”, “menarche”, “thelarche”, “gonadarche”, “adrenarche”, “pubarche” OR “adolescents” in the title, key words or abstract of journals. In addition, reference lists in retrieved studies were manually searched for relevant publications. Retrieved items were individually screened by two researchers (F.C. and C.F.) to exclude: non-original studies (*i.e.*, reviews, editorials), animal or *in vitro* studies, case reports or series; language other than English, Spanish, Italian, or Portuguese, and studies not assessing puberty timing (*i.e.*, pubarche, adrenarche, menarche, thelarche) or sexual maturity status (*e.g.*, sex hormone levels) as main outcome. Discrepancies in the selection of studies were resolved by consensus, consulting a third reviewer if necessary. The two researchers independently gathered data from the selected articles on: author(s), year of publication, country/region, study design, study objectives; sample size (number of participants); participant characteristics (age, sex, area of residence); exposure time window (prenatal, postnatal, or childhood); exposure assessment method; pesticide class and specific compounds/metabolites investigated; defined outcomes; and the results of association analysis. The quality of evidence

provided by the selected studies was assessed by using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) framework (32), which is especially useful for the assessment of environmental and occupational health studies. GRADE focuses on the assessment of the certainty in the evidence, defined as the confidence in the estimate (magnitude) of effect, and is based on the following criteria: a) downgrading criteria: risk of bias, inconsistency, indirectness, imprecision, publication bias; and b) upgrading criteria: large effect, dose-response effect, and plausible residual confounding would further support inferences regarding effect (33). Following the GRADE criteria, epidemiological studies were categorized according to the quality of the evidence as "very low" (⊕), "low" (⊕⊕), "moderate" (⊕⊕⊕), or "high" (⊕⊕⊕⊕). The researchers also independently assessed the reporting quality of each study according to the Strengthening the Reporting of Observational Studies in Epidemiology Statement (www.strobe-statement.org), using a checklist of 22 items related to the title, abstract, introduction, methods, results, and discussion sections of articles (see Table 1).

Results

The initial search retrieved 1284 articles. Out of these, 328 reviews/non-original studies, 622 animal or *in vitro* studies, 13 case reports/series, and 13 articles in a language other than English, Spanish, Italian or Portuguese were excluded. Out of the remaining 308 articles, the full text was not available for 2 and one was a study protocol article (34), leaving 282 to be screened. A total of 13 articles met all eligibility criteria for the review (Figure 1).

Table 2 displays the study characteristics. They were conducted in Belgium, Denmark, Mexico, United Kingdom, South Africa, China, or Korea. Nine had a cross-sectional design, two were birth cohort studies, and two were case-control studies. The sample sizes ranged from 30 to 463 participants. Environmental exposure to pesticides was assessed in six studies by measuring urinary concentrations of pesticide metabolites and in three by using the area of residence as proxy exposure measure. Work-related exposure was assessed in two studies of maternal occupational exposure during pregnancy and in one study of reproductive outcomes in the female spouses of men occupationally exposed to pesticides. Five studies evaluated exposure to mixtures of pesticides, including herbicides, fungicides, insecticides, and one study did not report the type/class of pesticide under investigation (Table 3).

Regarding the quality of evidence, seven studies (35–41) were classified as "very low", five (42–46) as "low", and one (47) as "moderate". The main reasons for downgrading the quality were risk of bias due to inadequate control of plausible confounders and imprecision due to small sample size. It should be noted that the GRADE framework

has been applied successfully to rate evidence from intervention studies within the field of clinical medicine, and evidence from observational studies has generally been classified as low. The exposure of girls to non-persistent pesticides was associated with puberty acceleration in three studies (36,44,47) and with delayed sexual maturation in three studies (35,43,45), with one study reporting no association with precocious puberty (40). In the Danish birth cohort study, breast development was earlier in girls born to women occupationally exposed to a mixture of pesticides during pregnancy, and they had higher androstenedione levels and lower aromatase levels at school age (47). Likewise, *Namulanda et al.* (44) reported a greater risk of early menarche among girls prenatally exposed to higher levels of diaminochlorotriazine, a metabolite of the herbicide atrazine. In another study, *Garry et al.* (36) observed a decline in the age at menarche from 1920 through 1949 that was statistically significant among women living in rural areas up to the age of 18 years. By contrast, *Ye et al.* (43) observed that girls aged 9-15 years with higher urinary levels of 3-phenoxybenzoic acid (3-PBA), a common metabolite of several pyrethroids, had a greater risk of delayed puberty, indicated by lower odds of having reached menarche and lower odds of being in breast Tanner stage 3 and pubic hair Tanner stage >2. In the same line, *Croes et al.* (45) found that higher urinary levels of OP metabolites diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyl dithiophosphate (DEDTP) were associated with lower Tanner breast stage at the age of 14-15 years. Furthermore, *Rodríguez-López et al.* (41) found higher urinary dimethylphosphate (DMP) and DEDTP levels in girls with alterations in serum LH levels, and *Graham et al.* (35) observed increased age at menarche in girls living in farming areas environmentally exposed to non-endocrine-disrupting pesticides in comparison to non-exposed girls. In other studies, *Suh et al.* (40) found no difference in the urinary levels of 320 compounds (including non-persistent pesticide metabolites) between 30 girls experiencing precocious puberty and 30 pre-pubertal controls, while *Guillette et al.* (37) found no difference in breast Tanner stage between peripubescent girls living in agricultural *versus* non-agricultural areas.

The exposure of boys to non-persistent pesticides was associated with delayed sexual maturation in two studies (45,46), whereas the exposure of male adolescents was found to have a puberty-promoting effect by another two (38,42), and one investigation found no significant difference in sexual maturation between boys living on and off farms (39). *Wohlfahrt-Veje et al.* (46) reported that boys prenatally exposed to several non-persistent pesticides had smaller testes and shorter penile length at school age than unexposed boys, although they did not differ in serum hormone levels. *Croes et al.* (45) found that urinary

concentrations of OP metabolites DMP, dimethyl thiophosphate (DMTP), and dimethyl dithiophosphate (DMDTP) were negatively associated with genital development in 14- to 15-year-old-boys; they also observed a negative relationship between ethyl-OP metabolites and free testosterone serum levels and between both ethyl- and methyl-OP metabolites and estradiol serum levels. By contrast, *Ye et al.*(42) reported that urinary 3-PBA levels were positively associated with genital development (higher Tanner stage and larger testicle size) and with serum FSH and LH levels. In a study by *Croes et al.* (38), higher Tanner stages and total and free estradiol levels were observed in boys living in a rural region with intensive fruit cultivation than in boys residing in other settings. Finally, *English et al* (39). reported that boys living on a farm had higher serum FSH and estradiol levels and lower LH levels than those who did not, but they observed no difference in genital development.

Discussion

A limited number of studies were eligible for this review; however, the recent epidemiological evidence identified suggests a relationship of early-life exposure to non-persistent pesticides, particularly OP and pyrethroid insecticides and the herbicide atrazine, with altered puberty timing. *In utero* exposure to atrazine has been related to earlier age of menarche in girls (44), exposure to OP pesticides has been related to delayed sexual development in boys and girls (45), childhood pyrethroid exposure has been associated with pubertal delay in girls (43) and pubertal advancement in boys (42), and prenatal/childhood exposure to multiple pesticides has been linked to earlier puberty onset in girls (36,47) and pubertal delay in boys (46) except for one study that reported higher age at menarche in exposed *versus* non-exposed girls (35).

OP pesticides

OP pesticides are widely used in agriculture as insecticides, and dietary intake is the main source of exposure for the general population. The restraining effect of OP exposure on male puberty observed by *Croes et al. (2015)* (45) is supported by *in vitro* and animal studies showing that OP pesticides can act at different levels of the hypothalamic-pituitary-gonad axis. Thus, OPs such as dimethoate, chlorpyrifos, dichlorvos, fenthion, fenitrothion, and tolclofos-methyl exert estrogenic and antiandrogenic activity *in vitro* (15), and OPs such as dimethoate, malathion, and acephate lead to downregulation of the physiological mechanisms of male puberty in animal models. For instance, dimethoate decreased androgen synthesis by reducing FSH and LH levels (15), blocking Leydig cell differentiation during puberty (48) and downregulating StAR gene expression (20). Malathion was also found to reduce testosterone and estradiol synthesis by modulating the expression of genes involved in

the secretion of hypothalamic, adrenal, and gonadal hormones (19). These effects on the mechanisms underlying puberty have also been observed for other OP compounds, including diazinon (49), acephate (50), and triazophos (51).

Epidemiological studies suggesting interference by OP pesticides in mechanisms regulating sex hormone production have mainly focused on men occupationally exposed to OPs, revealing decreased testosterone levels and elevated levels of FSH or LH or both gonadotrophins (52). These findings may indicate that OP pesticides can exert a blocking effect on testosterone production by gonads in humans, producing negative feedback on gonadotrophins.

Pyrethroids

Besides OPs, synthetic pyrethroids are among the most frequently used insecticides in agricultural and domestic settings. Two cross-sectional studies in this review associated exposure to pyrethroids with a puberty-promoting effect in boys and a puberty-retarding effect in girls (28,53). It is worth mentioning that urinary 3-PBA levels found by *Ye et al.* (42,43) in Chinese boys and girls are comparable to those reported in children and general from the general population in Europe, Asia, and the USA (54–56).

This effect on girls is supported by animal studies in which female rats pre- and perinatally exposed to fenvalerate exhibited pubertal delay consistent with a peripheral antiestrogenic effect (42,43) and a lack of LH response to GnRH stimulation (57), suggesting pituitary interference by pyrethroids. Several *in vitro* studies in ovarian granulosa cells showed that the pyrethroids fenvalerate and bifenthrin reduced the production of progesterone, prostaglandin 2, and estrogens by altering the expression of genes involved in steroidogenesis, such as StAR and CYP450scc (58). The puberty acceleration observed in boys with higher urinary 3-PBA levels (21) is supported by findings of early puberty in male rats exposed to cypermethrin through a pleiotropic effect on the HPG axis, with an increased production of testosterone in Leydig cells and of FSH and LH at pituitary level and, at high doses, an increase in the frequency of pulsatile GnRH secretion (42).

A few studies have evaluated the disruptive potential of pyrethroids on the HPG axis in adult males. Thus, *Meeker et al.* (59) observed that urinary 3-PBA concentrations were positively associated with FSH and LH levels and that urinary DCCA metabolite concentrations were negatively associated with testosterone levels. *Han et al.* (60) also found that urinary 3-PBA concentrations in non-occupationally exposed men were related to increased LH levels and decreased estradiol levels but had no effect on testosterone production. These observations suggest that the increase in pituitary hormones may be caused by a decreased production of steroid hormones *via* a negative feedback mechanism. Indeed, numerous *in vitro* and animal studies

have suggested that exposure to synthetic pyrethroids (e.g., fenvalerate, deltamethrin, cypermethrin, and lambda-cyhalothrin and their metabolites 3-PBA and DCCA) may have anti-androgenic effects (15) and may reduce testosterone production (61). This evidence appears to refute the hypothesis of a male puberty-accelerating effect proposed by *Ye et al.* (42). This discrepancy may be explained by recent data on the enantioselectivity of certain pyrethroid-derived compounds. Enantiomers are specular isomers that are often present, in varying amounts, in commercial pyrethroid insecticides. It was found that certain pyrethroid enantiomers (e.g., 1-R-trans-aS-cypermethrin, 1S-cis-bifenthrin and trans-permethrin) exert an androgenic and anti-estrogen effect (59) and lack an inhibitory effect on testosterone production (62). In other words, some pyrethroid enantiomers may have a greater accelerating effect on male puberty than others.

Herbicides

Atrazine is the only herbicide that has been studied in relation to puberty in humans. It was banned in Europe in 2004 and replaced for agricultural use by other herbicides such as glyphosate. The relationship observed between prenatal exposure to atrazine and precocious puberty in girls (44) is supported by several *in vitro* studies showing that atrazine increases estrogen levels by activating aromatase in ovarian granulosa cells and adrenal cortical carcinoma cells (44). In addition, animal studies reported that atrazine activates the HPA axis centrally at hypothalamic level, with an alteration in adrenal gland morphology (63). According to these experimental data, the mechanisms underlying the puberty-promoting effect of atrazine exposure in girls involve interference in the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes.

Caveats to interpretation

Nine out of the ten reviewed studies had a cross-sectional design (35–39,41–43,45) This type of investigation is easier to perform in comparison to a longitudinal study and allows the analysis of multiple outcomes; however, it is inadequate to evaluate causal relationships between exposure to non-persistent pesticides and puberty timing. This is because puberty is a complex physiological phenomenon that can be influenced by multiple factors, including the maternal BMI, anthropometrics of the newborn, duration of breastfeeding, prepuberal BMI, and psychosocial factors. In addition, modern non-persistent pesticides do not accumulate in the human organism; hence, longitudinal studies are necessary to estimate chronic exposure throughout early life and childhood. The ideal approach would be to estimate the accumulated exposure to non-persistent pesticides by the repeated measurement of biomarkers of recent exposure (e.g., urinary concentration of metabolites) alongside the administration of questionnaires to gather data

relevant to the history of potential exposure (e.g., place of residence, occupation of family members, lifetime environmental exposure to agricultural or domestic-use pesticides). A further difficulty is that the diet is the main source of pesticide exposure in the general population, who consume small doses of multiple compounds repeatedly throughout life. Importantly, few data are available on potential—interactions among pesticide compounds (64) and their long-term health effects. Epidemiological findings on exposure to multiple pesticides reflect the complex pressure of contamination on populations; however, given that some endocrine disrupting pesticides exert an agonist action but others an antagonist action, such studies may not offer reliable information on the impact of total pesticides exposure. For example, the lack of a significant effect reported by *English et al.* (39) in male adolescents exposed to multiple pesticides (chlorpyrifos and deltamethrin, among others) may be in part explained by the apparent antagonistic effects on boys of OP pesticides and pyrethroids suggested by *Croes et al.* (45) and *Ye et al.* (42), associated with pubertal delay and pubertal advance, respectively. Conversely, epidemiological studies that consider a specific chemical compound or group may underestimate the effects of other EDCs, with loss of internal validity. This is especially relevant for the selection of suitable control groups. For example, *Namulanda et al.* (44) conducted a nested case-control study to determine the association between early menarche and *in utero* exposure to atrazine-type herbicides, identifying the maternal BMI, the BMI of the girls at 7 years, and the duration of breastfeeding as potential confounders; however, concurrent exposure to other pesticides or EDCs was not taken into account.

The methods used to evaluate puberty onset and progression varied among the reviewed studies. The age of menarche offers an objective indicator of puberty onset for girls but there is no equivalent for boys. However, the appearance of secondary sexual characteristics such as breast buttons in girls, increased testicular size in boys, and pubic hair development in both sexes provide relevant information, with the Tanner scale as gold standard reference. This scale was used to assess sexual maturation in all except five studies in this review (35,36,40,41,44). It was administered by clinicians in all of these studies except for two, in which the scale was self-assessed by participants(42,43). Only one study measured testicular volume using ultrasound (46). In another investigation, a greater discrepancy was observed (37) between simple visualization and breast palpation in the evaluation of breast development in girls from an agricultural area than in those from a non-agricultural area, suggesting an effect of pesticide exposure on fat distribution that may be relevant to breast development assessment. The comparability of findings could be improved by obtaining not only Tanner scale results but also ultrasound

measurements of testicular size in boys and of ovarian and mammary size and uterine corpus/cervix ratio in girls.

The sex hormones selected for analysis also varied among the studies. Elucidation of the mechanisms underlying the effect of modern pesticides on puberty requires data on the hormones produced by organs involved in pubertal development, such as the hypothalamus, pituitary gland, gonads, and adrenal glands. Serum testosterone, estradiol, sexual hormone binding globulin (SHBG), FSH, and LH levels were investigated by *Croes et al.* (38,45)*, English et al.* (39) and *Rodríguez-López et al* .(41) while *Wohlfahrt-Veje et al.* (46,47) added the adrenal hormones androstenedione and dehydroepiandrosterone sulfate to this list. Urinary levels of FSH and LH were studied by *Ye et al.* (42,43) as a proxy of serum gonadotrophin levels. However, no study has investigated levels of hypothalamic hormones such as the gonadotropin-releasing hormone (GnRH), corticotropin, or corticotropin-releasing hormone (CRH).

Public health implications

The majority of reviewed studies offer some evidence of a relationship between exposure to non-persistent pesticides and changes in puberty timing. However, the quality of evidence provided by these studies is generally low, due to important methodological limitations. Children are exposed to pesticides worldwide, and biomonitoring studies in Europe, the United States, and Asia have reported comparable urinary levels of pesticide metabolites, including OPs, pyrethroids, and atrazine-type herbicides, in large samples of children or pregnant women (65–67). These compounds are widely used in food crops, and the main source of exposure is *via* the diet (54–56), including drinking water (65), followed by environmental exposure due to residence near areas of intensive agricultural production (66). Given the magnitude and ubiquity of exposure to modern pesticides, its impact on the age of puberty onset requires further investigation. This is an important public health issue because of the long-term health effects of precocious puberty, including hormone-related cancers and metabolic and cardiovascular disorders (67). Moreover, it has been found that exposure to modern pesticides increases the risk of metabolic and cardiovascular diseases and hormone-dependent cancers (68), and this association may be mediated in part by changes in the age of puberty onset induced by this exposure.

Conclusions

Exposure to non-persistent pesticides during critical windows of development and growth may alter the normal onset and progression of puberty. However, inadequate data have been published to establish a univocal relationship, and these effects likely depend on the type of pesticide and on the timing and frequency of exposure, among other factors. Given the ubiquity of these

compounds in the environment and the lack of evidence on their health effects in children and adolescents, further longitudinal epidemiological studies are needed to establish a basis for the regulation of their use and the prevention of potentially harmful effects.

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Competing financial interest declaration

The authors declare no actual or potential competing financial conflict of interests.

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Table 1. Reporting quality assessment of reviewed studies according to STROBE Statement-checklist for observational studies.

Item	Study												
Title and abstract	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Introduction													
Background/rationale													
Explain the scientific background and rationale for the investigation being reported	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Objectives													
State specific objectives, including any prespecified hypotheses	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Methods													
Study design													
Present key elements of study design early in the paper	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Setting													
Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	N	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Participants													
(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	NA	NA	NA	NA	NA	Y	Y	NA	NA	NA	NA	NA	NA
Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	NA	NA	NA	NA	NA	NA	NA	NA	Y	NA	NA	NA	N
Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	N	Y	N	Y	Y	NA	NA	Y	NA	N	Y	Y	NA
(*b*)*Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Case-control study—For matched studies, give matching criteria and the number of controls per case	NA	NA	NA	NA	NA	NA	NA	NA	¨N	NA	NA	NA	NA
Variables													
Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	N	Y	N	Y	Y	Y	Y	Y	Y	N	N	Y	Y
Data sources/ measurement													
For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Bias													
Describe any efforts to address potential sources of bias	N	N	N	Y	N	N	N	N	Y	N	N	N	N
Study size													
Explain how the study size was arrived at	Y	Y	N	Y	Y	Y	N	Y	Y	N	Y	Y	Y
Quantitative variables													

Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Statistical methods													
(*a*) Describe all statistical methods, including those used to control for confounding	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
(*b*) Describe any methods used to examine subgroups and interactions	N	N	N	N	Y	N	N	N	N	NA	NA	NA	NA
(*c*) Explain how missing data were addressed	N	N	N	N	N	N	N	N	Y	N	N	N	N
(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed	NA	NA	NA	NA	NA	Y	Y	NA	NA	NA	NA	NA	NA
Case-control study—If applicable, explain how matching of cases and controls was addressed	NA	NA	NA	NA	NA	NA	NA	NA	Y	NA	NA	NA	NA
Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	N	NA	Y	Y	Y	NA	NA	Y	NA	NA	NA	Y	NA
(*e*) Describe any sensitivity analyses	N	N	N	N	Y	N	N	N	N	N	Y	Y	N
Results													
Participants													
(a) Report numbers of individuals at each stage of study e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	N	N	N	Y	N	Y	N	Y	Y	N	Y	Y	N
(b) Give reasons for non-participation at each stage	N	N	N	Y	N	N	N	Y	N	N	Y	Y	N
(c) Consider use of a flow diagram	N	N	N	N	N	Y	N	N	N	N	N	N	N
Descriptive data													
(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	N	N	N	Y	Y	Y	Y	N	Y	Y	Y	Y	Y
(b) Indicate number of participants with missing data for each variable of interest	N	N	N	N	N	N	N	N	Y	N	Y	N	N
(c) *Cohort study*—Summarize follow-up time (e.g., average and total amount)	NA	NA	NA	NA	NA	N	N	NA	NA	NA	NA	NA	NA
Outcome data													
Cohort study—Report numbers of outcome events or summary measures over time	NA	NA	NA	NA	NA	Y	Y	NA	NA	NA	NA	NA	NA
Case-control study—Report numbers in each exposure category, or summary measures of exposure	NA	NA	NA	NA	NA	NA	NA	NA	Y	NA	NA	NA	N
Cross-sectional study—Report numbers of outcome events or summary measures	N	N	Y	Y	Y	NA	NA	Y	NA	Y	Y	Y	NA
Main results													
(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	N	N	N	Y	N	Y	Y	Y	Y	Y	Y	N	N
(*b*) Report category boundaries when continuous variables were categorized	NA	NA	NA	NA	NA	NA	Y	NA	Y	NA	Y	Y	NA
(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Other analyses													
Report other analyses done e.g., analyses of subgroups and interactions, and sensitivity analyses	N	N	N	N	Y	N	N	N	Y	N	Y	Y	N
Discussion													
Key results													
Summarize key results with reference to study objectives	Y	Y	Y	Y	N	Y	Y	N	Y	Y	Y	N	Y

Limitations													
Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	N	N	N	N	Y	Y	Y	N	Y	N	N	Y	Y
Interpretation													
Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	N	Y	Y	N	Y	Y	Y	N	Y	N	N	Y	N
Generalizability													
Discuss the generalizability (external validity) of the study results	N	N	N	Y	N	N	N	N	Y	N	N	Y	N
Other information													
Funding													
Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
(1) Graham et al., 1999; (2) Garry et al., 2002; (3) Guillette et al., 2006; (4) Croes et al., 2009; (5) English et al., 2012; (6) Wohlfahrt-Veje et al., 2012a; (7) Wohlfahrt-Veje et al., 2012b; (8) Croes et al., 2015; (9) Namulanda et al., 2017; (10) Ye et al., 2017a; (11) Ye et al, 2017b; (12) Rodriguez-López et al., 2020; (13) Suh et al., 2020. Y: Yes; N: No; N.A.: Not applicable.													

Table 2. Epidemiological studies on exposure to non-persistent pesticides and puberty timing.

Study	Location	Design	Study population	Sample size	Exposure timing	Exposure assessment	Pesticides	Outcome	Results	Quality*
Graham et al., 1999	China	Cross-sectional	Women living in farming areas born between <1949 and 1978	12,727	Pre-menarcheal	Questionnaire on pesticides exposure (pesticides used in cotton and rice farming areas)	Multiple pesticides: endocrine-disrupting (ED) pesticides, non-ED pesticides, and other pesticides	Age at menarche	↑ Age at menarche in women exposed to non-ED pesticides compared to non-exposed women. ↓ Mean age at menarche (2.8 years) over 40 years.	⊕
Garry et al., 2002	Minnesota, US	Cross-sectional	Females spouses of pesticide applicators	456	Childhood/adulthood	Pesticide use and living in a rural area	Multiple pesticides: herbicides, fungicides, insecticides, and fumigants	Age at menarche	↓ Age at menarche of women born in 1920-1949, and a modest increase in those born in 1950-1979, among those who lived in a rural setting before age 18 yrs.	⊕
Guillette et al., 2006	Mexico	Cross-sectional	Girls 8-10 yrs.	50	Childhood (peripubescent)	Area of residence: agricultural area (N=30) and non-agricultural area (N=20)	Multiple pesticides	Breast development: visual Tanner stage, and diameter (cm) of breasts and of palpable mammary gland.	No differences in Tanner stage between girls from agricultural and non-agricultural areas. Greater concordance between breast diameter and mammary diameter in non-exposed girls.	⊕

Croes et al., 2009	Belgium	Cross-sectional	Adolescents 14-15 yrs.		Adolescence	Area of residence, including rural area (N=190) and fruit growing area (N=184).	None reported	Tanner stages of genital development, pubic hair growth, and breast development. Serum sex hormones: testosterone (total and free), LH, SHBG, and E_2 (total and free).	↑ % of boys having reached pubic hair stage 3 or genital development stage 3 in the fruit area. ↑ Free and total E_2 among boys residing in the fruit growing area compared to other areas.	⊕
English et al., 2012	South Africa	Cross-sectional	Boys 5-19 yrs.	269	Childhood/adolescence	Living on a farm (177 boys living on a farm and 92 non-farm boys) Questionnaire: lifetime environmental exposure to agricultural pesticides, domestic pesticide use.	Multiple pesticides: OPs, pyrethroids, fungicides, etc.	Tanner stages of genital development and testicular volume (orchidometer). Serum sex hormones: testosterone, LH, FSH, SHBG, E_2.	↓ LH associated with living on a farm. ↑ FSH and E_2 associated with living on a farm.	⊕
Wohlfahrt-Veje et al., 2012a	Denmark	Longitudinal	Girls 6-11 yrs.	83	In utero	Girls born to women working in greenhouses during pregnancy: 53 exposed and 30 unexposed to non-persistent pesticides in early pregnancy.	Multiple pesticides: fungicides, insecticides, herbicides, and growth regulators.	Tanner stages of breast development and public hair growth. Serum sex hormones: testosterone, LH, FSH, SHBG, E_2, DHEA, DHT, androstenedione, prolactin, AMH, inhibin A, inhibin B.	Earlier age at breast development (stage B2 or higher) in exposed *versus* unexposed girls. ↑ Androstenedione and ↓ AMH levels in exposed *versus* unexposed girls.	⊕⊕⊕
Wohlfahrt-Veje et al., 2012b	Denmark	Longitudinal	Boys 6-11 yrs.	94	In utero	Boys born to women working in greenhouses during pregnancy: 59 exposed and 35 unexposed to non-persistent pesticides at early pregnancy.	Multiple pesticides: fungicides, insecticides, herbicides, and growth regulators.	Tanner stages of genital development, testicular volume (ultrasound), and penile length. Serum sex hormones: testosterone (free and total), LH, FSH, SHBG, E_2, DHEA, DHT, androstenedione, prolactin, AMH, inhibin B.	↓ Testicular volume and penile length in prepubertal boys of exposed mothers (reduction increased with maternal exposure level: high>medium>unexposed). Boys with genital malformations and prenatally exposed to pesticides had ↓ testis, ↓ penile length, and ↓ inhibin B than prepubertal boys without genital malformations.	⊕⊕
Croes et al., 2015	Belgium	Cross-sectional	Adolescents 14-15 yrs.	210	Adolescence	Urinary biomarkers	OP metabolites: DEPs (DEP, DETP, DEDTP) and DMPs (DMP, DMTP, DMDTP) *Para*-dichlorophenol (metabolite of	Tanner stages of genital development, pubic hair growth, and breast development. Serum sex hormones: testosterone (total and free), LH, SHBG, E_2 (total and free)	Boys: ↓ Free E_2 associated with ∑DEPs and ∑DMPs ↓ Free testosterone and ↓ odds for reaching adult testosterone levels associated with ∑DEPs Delayed genital development associated with ∑DMPs Girls: delayed breast development associated with ∑DEPs	⊕⊕

							para-dichlorobenzene)			
Namuladda et al., 2017	United Kingdom	Nested case-control (ALSPAC cohort)	Girls 8-17 yrs.	174 cases 195 controls	In utero	Maternal urinary biomarkers	Herbicide atrazine metabolites: DACT, desethyl atrazine, desethyl atrazine mercapturate, atrazine mercapturate, hydroxyl atrazine, atrazine, and desisopropyl atrazine.	Cases: menarche<11.5 yrs. (early) Controls: menarche≥11.5 yrs.	No significant association for girls with DACT ≥median (OR=1.13, 95%CI=0.82, 1.55) compared to girls with undetected levels. Exclusion of girls with missing data: significantly ↑ OR for girls with DACT level ≥median (OR=1.86, 95%CI=1.03, 3.38).	⊕⊕
Ye et al., 2017a	China	Cross-sectional	Boys 9-16 yrs.	463	Childhood/adolescence	Urinary biomarkers	Pyrethroid insecticides: 3-PBA and 4-F-3-PBA metabolites	Tanner stages of genital development (self-assessment) and testicular volume (orchidometer). Urinary levels of LH and FSH.	10% increase in urinary 3-PBA levels associated with ↑ LH (2.4%) and FSH (2.9%) levels. Increased urinary 3-PBA associated with ↑ odds of being in G2, G3, or G4, respectively, and with ↑ odds of having a testicular volume of 12-19 mL compared to <4 mL.	⊕⊕
Ye et al., 2017b	China	Cross-sectional	Girls 9-15 yrs.	305	Childhood/adolescence	Urinary biomarkers	Pyrethroid insecticides: 3-PBA and 4-F-3-PBA metabolites.	Tanner stages of breast development and pubic hair growth (self-assessment), and age at menarche.	Increased urinary 3-PBA levels associated with ↓ odds of being in B3 and P2 stage, respectively. Urinary 3-PBA associated with ↓ odds of having reached menarche.	⊕⊕
Rodriguez-López et al., 2020	Mexico	Cross-sectional	Girls 12-17 yrs. having reached menarche	29	Adolescence	Urinary biomarkers	OP metabolites: DEPs (DEP, DETP, DEDTP) and DMPs (DMP, DMTP, DMDTP)	Serum sex hormones: FSH, LH, E_2, progesterone, and prolactin.	Higher urinary DMP and DEDTP level in girls with alterations in serum LH levels	⊕

Suh et al., 2020	Korea	Case-control	Girls 5-9 yrs.	30 cases 30 controls	Childhood/adolescence	Urinary biomarkers	Multiple pesticides: 320 metabolites analyzed. Only dinotefuran (a neonicotinoid insecticide) was detected	Cases: precocious puberty (LH level >5 mIU/mL in a GnRH stimulation test) Controls: prepubertal girls	No apparent relationship between urinary pesticide metabolites and precocious puberty.	⊕

↑ Increase; ↓ decrease; OR: odds ratio; CI: confidence interval.

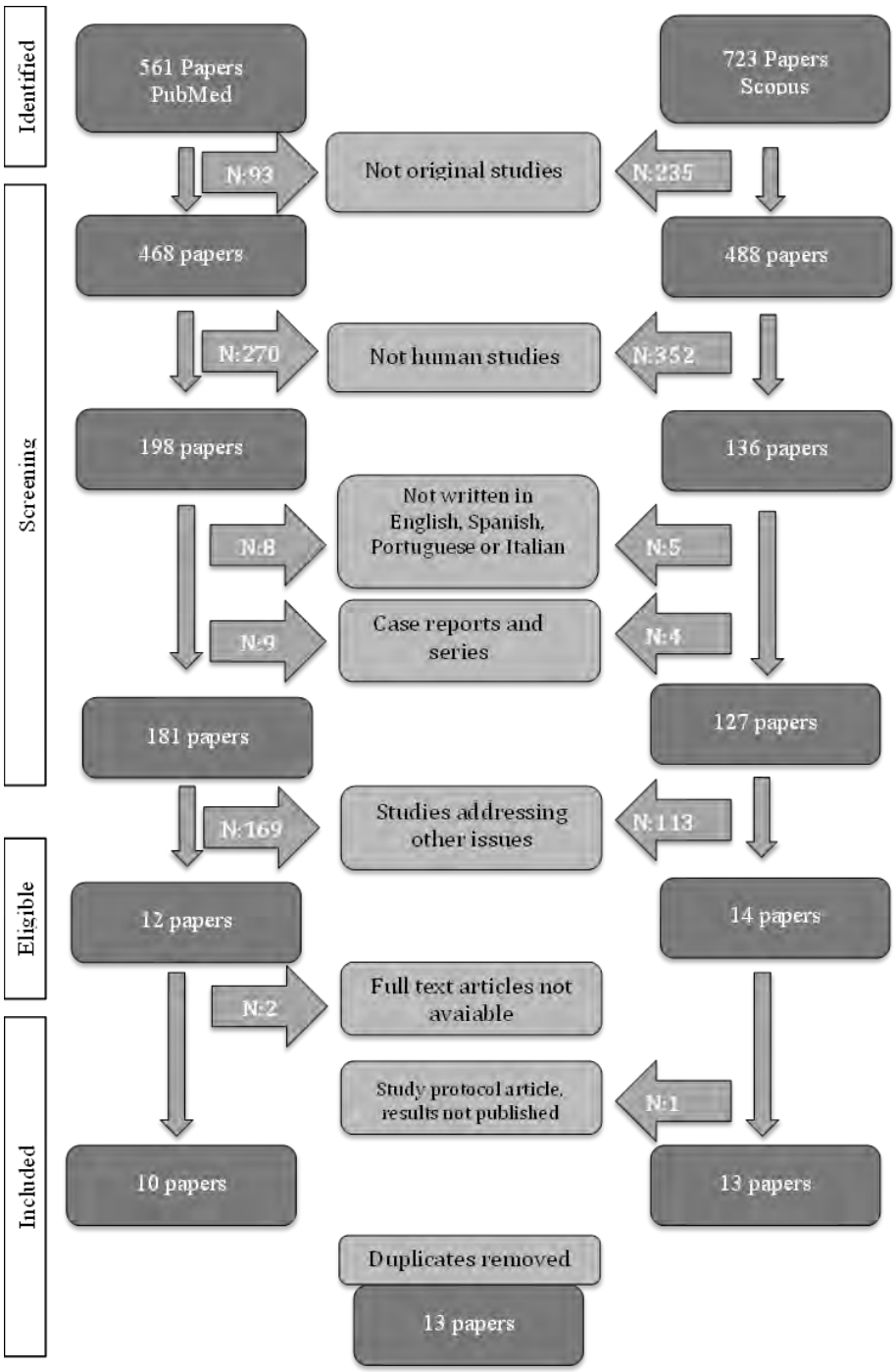
OPs: organophosphates; DEP: diethylphosphate; DETP: diethylthiophosphate; DEDTP; diethyldithiophosphate; DMP: dimethylphosphate; DMTP: dimethylthiophosphate; DMDTP: dimethyldithiophosphate; DACT: diaminochlorotriazine; 3-PBA: 3-phenoxybenzoic acid; 4-F-3-BPA: 4-fluoro-3-phenoxibenzoic acid.

*Quality: ⊕: very low; ⊕⊕: low; ⊕⊕⊕: moderate; ⊕⊕⊕⊕: high.

Table 3. Classes and individual pesticides assessed in the revised studies.

Functional class	**Chemical class**	**Urinary metabolites**	**Studies**
Insecticides	Organophosphates	Dialkylphosphates: Diethylphosphates: Diethylphopshate, Diethylthiophopshate, Diethyldithiophopshate Dimethyphosphates: Dimethythiophosphate, Dimethydithiophosphate.	*Croes et al. (2015)* *Rodríguez-López et al. (2020)*
	Pyrethroids	3- Phenoxybenzoic acid (3-PBA) 4- Fluoro-3-phenoxybenzoic acid (4F-3-PBA)	*Ye et al (2017a)* *Ye et al (2017b)*
Herbicides	Atrazine	Diaminochlorotriazine (DACT), Desethyl atrazine (DEA), Desethyl atrazine mercapturate (DEAM), Atrazine mercapturate (AM), Hydroxyl atrazine (ATZ-OH), Desisopropyl atrazine (DIA).	*Namulanda et al. (2017)*
Multiple pesticides			*Graham et al. (1999)* *Garry et al. (2002)* *Guillette et al. (2006)* *English et al. (2012)* *Wohlfahrt-Veje et al. (2012)* *Wohlfahrt-Veje et al. (2012b)* *Suh et al. (2020)*

Figure 1 Flowchart outlining the protocol adopted in this systematic review.



5.2 Resultado objetivo 2:

CHILDHOOD EXPOSURE TO NON-PERSISTENT PESTICIDES AND PUBERTAL DEVELOPMENT IN SPANISH GIRLS AND BOYS: EVIDENCE FROM THE INMA (ENVIRONMENT AND CHILDHOOD) COHORT

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Resumen

Estudio transversal en el que se evaluó la asociación entre los metabolitos urinarios de pesticidas no persistentes y el desarrollo puberal en niños y niñas de áreas urbanas y rurales en España, examinando la modificación del efecto por el índice de masa corporal (IMC). Se cuantificaron cuatro metabolitos de insecticidas (TCPy, metabolito de clorpirifós; IMPy, metabolito de diazinón; DETP, metabolito no específico de organofosforados; 3-PBA, metabolito de piretroides) y un metabolito de fungicidas bisditiocarbamato de etileno (etilentiourea, ETU) en orina de 606 niñas y 933 niños de 7 a 11 años que participaron entre 2010 y 2016 en el Proyecto Infancia y Medio Ambiente (INMA). El desarrollo puberal se determinó mediante la escala de Tanner y/o la escala de desarrollo puberal (PDS) cumplimentada por los padres. Se examinó la asociación entre cada metabolitos de pesticidas y la probabilidad de estar en estadío 2+ para cada hito puberal mediante regresión logística de efectos mixtos. La modificación del efecto por el IMC se exploró usando términos de interacción y realizando análisis estratificado. En las niñas, concentraciones de DETP y ETU superiores al percentil 75 (P75) se asociaron con mayor probabilidad de haber iniciado el desarrollo puberal según la PDS (OR [IC 95%]=1,86 [1,07-3,24] y 1,71 [1,03-2,83], respectivamente, para >P75 *versus* concentraciones no detectadas), mientras que concentraciones de ETU >P75 también se asociaron con mayor probabilidad de haber iniciado el desarrollo mamario (OR [IC 95%]=5,55 [2,83-12,91]), especialmente en niñas con bajo peso/peso normal (OR [IC 95%]=10,08 [2,62-38,76]). En los niños, la detección de TCPy (40%) y 3-PBA (34%) se asoció con mayor probabilidad de haber iniciado el

desarrollo genital (OR [IC 95 %]=1,97 [1,08-3,57] y 2,08 [1,15-3,81], respectivamente), siendo la asociación con 3-PBA significativa solo en niños con sobrepeso/obesidad. Además, una mayor exposición a ETU (>P75) se asoció con mayor probabilidad de desarrollo genital en niños con bajo peso/peso normal (OR [IC 95%]=2,89 [1,08-7,74]), y una mayor concentración de DETP con menor probabilidad de haber iniciado la pubertad según la PDS en niños con sobrepeso/obesidad (OR [IC 95%]=0,94 [0,89-0,99] por cada aumento de una unidad logarítmica en la concentración de DETP).

Abstract

This study assessed cross-sectional associations between urinary metabolites of non-persistent pesticides and pubertal development in boys and girls from urban and rural areas in Spain and examined effect modification by body mass index (BMI). Four metabolites of insecticides (TCPy, metabolite of chlorpyrifos; IMPy, metabolite of diazinon; DETP, non-specific metabolite of organophosphates; 3-PBA, metabolite of pyrethroids) and the metabolite of ethylene-bis-dithiocarbamate fungicides (ETU) were quantified in urine collected in 2010-2016 from 7-11-year-old children (606 girls, 933 boys) participating in the INMA Project. Pubertal development was ascertained by Tanner stages and/or parent-reported Pubertal Development Scale (PDS). Associations between pesticide metabolites and odds of being in stage 2+ for breast development (girls), genital development (boys), pubic hair growth (girls and boys), and/or overall puberty onset, gonadarche, and adrenarche (PDS for girls and boys) were examined by mixed-effect logistic regression. Effect modification by BMI was explored by interaction terms and stratified analysis. In girls, DETP and ETU concentrations>75th percentile (P75) were associated with higher odds of overall puberty development (OR [95% CI]=1.86 [1.07-3.24] and 1.71 [1.03-2.83], respectively, for >P75 vs. undetected concentrations), while ETU>P75 was also associated with higher odds of breast development (OR [95% CI]=5.55 [2.83-12.91]), particularly in girls with underweight/normal weight (OR [95% CI]=10.08 [2.62-38.76]). In boys, detection of TCPy (40%) and 3-PBA (34%) was associated with higher odds of genital development (OR [95% CI] =1.97 [1.08-3.57] and 2.08 [1.15-3.81], respectively), and the association with 3-PBA was observed in boys with overweight/obesity alone. In addition, ETU>P75 was associated with higher odds of genital development in boys with underweight/normal weight (OR [95% CI] = 2.89 [1.08-7.74]) but higher DETP with lower odds of puberty in boys with overweight/obesity (OR [95% CI] = 0.94 [0.89-0.99] per log-unit increase in concentration). Results suggest an association of childhood exposure to ETU and certain insecticides with earlier puberty in girls and boys that may be modified by child BMI.

Keywords

Pesticides; puberty; genital development; breast development; endocrine-disrupting chemicals

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1. Introduction

Contemporary pesticides are widely used in food production as well as in non-agricultural urban and domestic settings. The main exposure route for pesticides in the general population is the diet, especially through the consumption of conventionally grown fruits and vegetables (Fortes et al. 2013; Lu et al. 2008). Once in the human body, they are rapidly metabolized and excreted, mainly *via* urine (Barr 2008; Egeghy et al. 2011). Organophosphate (OP) and pyrethroid insecticides and certain herbicides are the most common pesticides in the European Union (EU) and worldwide. Dithiocarbamate fungicides are another extensively employed class of pesticides. Experimental evidence suggests that several currently used and banned non-persistent pesticides may act as endocrine-disrupting chemicals (EDCs) (Andersen et al. 2002; Kojima et al. 2004; Orton et al. 2011). For instance, *in vitro* studies have shown that OP insecticides such as chlorpyrifos and diazinon interact with the estrogen receptors alpha and beta (ERα, ERβ) and/or the androgen receptor (AR) (Andersen et al. 2002; Chen et al. 2002; Kojima et al. 2004; Manabe et al. 2006; Orton et al. 2011; Shen et al. 2021). Chlorpyrifos also inhibits the expression of key sex steroid synthesizing enzymes, including aromatase (Usmani et al. 2003; Viswanath et al. 2010). Likewise, the pyrethroids deltamethrin, cypermethrin, and λ-cyhalothrin are known to exert estrogenic and anti-androgenic effects *in vitro* (Andersen et al. 2002; Chen et al. 2002; Fujino et al. 2019; Kjeldsen et al. 2013; Kojima et al. 2004; Orton et al. 2011; Sun et al. 2007). Dithiocarbamate fungicides such as mancozeb have also been shown to antagonize human AR activity *in vitro* (Archer and van Wyk 2015; Kjeldsen et al. 2013).

Increasing exposure to EDCs over the past few decades may be one the factors responsible for the consistent secular trend towards earlier puberty onset in girls observed in Western countries (Biro and Kiess 2016; Toppari and Juul 2010) and for a more recently described shift towards earlier male puberty onset (Brix et al. 2019; Herman-Giddens et al. 1997). However, few human studies have addressed the relationship between exposure to contemporary pesticides and puberty timing, with conflicting findings (Castiello and Freire 2021). Specifically, *in utero* exposure to the herbicide atrazine was associated with earlier age of menarche in mother-child pairs from urban and rural areas in the UK (Namulanda et al. 2017), and childhood exposure to OP pesticides was associated with delayed sexual development in urban and rural boys and girls in Belgium (Croes et al. 2015). Pyrethroid exposure in an urban setting was associated with pubertal delay in girls (Ye et al. 2017a) and pubertal acceleration in boys (Ye et al. 2017b) in a Chinese study, and the occupational exposure of pregnant Danish women to multiple pesticides was linked to

earlier breast development in female offspring (Wohlfahrt-Veje et al. 2012a) and lower testicular volume and penile length in the males (Wohlfahrt-Veje et al. 2012b). Other cross-sectional studies found no clear evidence of an association between residence in an agricultural area and pubertal development in South African boys (English et al. 2012) or breast development in Native American girls (Guillette et al. 2006).

Spain is the largest consumer of pesticides in the European Union (EU), using 74,000 tons of pesticides in 2019, including 34,000 tons of fungicides, the largest group (EUROSTAT 2022). With this background, the aim of this study was to investigate the association between concentrations of urinary metabolites of various non-persistent pesticides (OPs, pyrethroids, and dithiocarbamate fungicides) and pubertal development in girls and boys aged 7-11 years.

2. Material and methods

2.1. Study population

This cross-sectional study was conducted among children participating in the Environment and Childhood (INMA) multicenter birth cohort study, designed to investigate the effect of environmental exposures and diet during pregnancy on fetal and child development in different regions of Spain (Guxens et al. 2012). Five out of seven cohorts in the INMA study collected urine from the children at age 7-11 years. These five INMA cohorts enrolled 3,238 women during the first prenatal visit (10-13 weeks of gestation) in Asturias, Gipuzkoa (Basque Country), Sabadell (Catalonia), and Valencia (2003-2008) but at birth in Granada (Andalusia) (2000-2002). All cohorts included boys and girls except for Granada, which recruited boys alone, as the initial aim of this cohort was to investigate the influence of prenatal exposure to endocrine-disrupting chemicals on the risk of male urogenital malformations (Fernández et al. 2007). The Sabadell cohort in Northeastern Spain recruited mother-child pairs residing in the urban area of Sabadell, a medium-sized city, while the remaining cohorts recruited children from both urban and rural areas. INMA-Asturias and Gipuzkoa study areas, in Northern Spain, are characterized by the presence of urban centers with important industrial activity and small rural towns. The INMA-Granada study area, in Southeastern Spain, comprises the metropolitan area of Granada city and surrounding rural towns and villages. Finally, the Valencia-cohort is located in an area with intense agricultural activity surrounding the city of Valencia in Eastern Spain. Further details on the INMA study areas and recruitment strategies are described elsewhere (Guxens et al. 2012). A total of 1,976 out of 3,238 children (61%) born to women originally included in these cohorts underwent puberty development assessment at 7-11 years of age between 2010 and 2016

(Granada in 2010-2012, and Asturias, Gipuzkoa, Sabadell and Valencia in 2013-2016). Puberty assessment included physical examination of developmental stage, as described below (section 2.4), and hormonal measurements in Valencia and Granada. In this study, the association of pesticide exposure with pubertal stage was examined in 1,539 (606 girls and 933 boys) of the 1,976 children from the five cohorts for whom urine samples were available and were analyzed for metabolites of non-persistent pesticides (Figure 1).

2.2. Ethical aspects

The parents or guardians of all participants signed informed consent, and the research protocol was approved by the Ethics Committees of each region: Clinical Research Ethics Committee (CEIC) of the Principado de Asturias (ref. number 29/2003), CEIC Euskadi in Gipuzkoa (ref. number 11/2013), Biomedical Research Ethics Committee in Granada (ref. number 0509-N17), CEIC Parc de Salut MAR in Sabadell (ref. number 2005/2106/l), and CEIC DGSP-CSISP in Valencia (ref. number 20131007).

2.3. Measurement of urinary pesticide metabolites

A non-fasting spot urine sample was collected from each participant in Granada (5-8 p.m.) and Valencia (90% in the morning) on the day of the follow-up visit. Samples from Asturias were first morning voids, and samples from Sabadell and Gipuzkoa were pools of first morning and preceding bedtime voids. Urine samples (approx. 30 mL) were collected by study participants at their residences using sterile propylene containers and kept at 4 ºC until arrival at the hospital or follow-up site, where they were stored at -80 ºC. Pools of first morning and preceding bedtime voids were prepared by qualified research staff in Sabadell and Gipuzkoa following a standardized protocol. Samples were delivered to the ‘’UNETE Research Unit’’ of the Biomedical Research Center (CIBM), University of Granada (Spain), where pesticide measurements were performed. Urine samples were analyzed for concentrations of 3,5,6-trichloro-2-pyridinol (TCPy), a metabolite of chlorpyrifos and chlorpyrifos-methyl; 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), a metabolite of diazinon; diethyl thiophosphate (DETP) and diethyl dithiophosphate (DEDTP), two dialkyl phosphates (DAPs) that are metabolites of various OP insecticides; 3-phenoxybenzoic acid (3-PBA), a generic metabolite of pyrethroids; and ethylene thiourea (ETU), primary metabolite of ethylene-bis-dithiocarbamate (EBDC) fungicides such as mancozeb and maneb.

The metabolites/pesticides analyzed in this study were selected based on their ubiquity and toxicological/regulatory relevance. TCPy, 3-PBA, and DAP metabolites are widely used as urinary biomarkers of OP and pyrethroid

insecticides (Barr 2008; Yusà et al. 2015, 2022). Importantly, chlorpyrifos and pyrethroids are on the list of priority chemicals established by the European Human Biomonitoring Initiative (HBM4EU) (Ougier et al. 2021). Chlorpyrifos, one of the most widely used pesticides on European crops over the past few years, was banned in 2020 (Regulation (EU) 2020/17). Although diazinon has not been authorized in the EU since 2006, IMPy has been detected in urine samples collected several years after the prohibition of diazinon from Spanish children (Roca et al. 2014) and pregnant women (Bravo et al. 2020). Moreover, diazinon residues were detected in fruit, vegetable, and spices marketed in Spain in 2018 (AESAN 2018). DETP and DEDTP were selected as biomarkers of exposure to several common OP insecticides, including approved and non-approved insecticides such as chlorpyrifos, diazinon, ethion, parathion, phorate, and terbufos. Growing concerns about their potential adverse health effects of pyrethroids has led to the prohibition of some pyrethroids by the EU (PAN International 2021). Urinary ETU is considered a useful biomarker of human exposure to EBDC fungicides (Yusà et al. 2015), which are among the most frequently detected pesticides in food sold in Spain, including organic food (AESAN 2018). Mancozeb lost its approval in the EU in January 2021 (Regulation (EU) 2020/2087), but other EBDC fungicides such as ziram and metiram are still authorized. Notably, ETU is also used in the manufacture of neoprene, polyacrylate rubbers, plastic materials, and pharmaceutical compounds and in paper whitening, dry cleaning, and photography, although the main source of exposure is from agricultural fungicides (Mutic et al. 2017).

Ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) was used for the analysis of urinary pesticide metabolites. All metabolites were calibrated and extracted according to a previously described methodology (Freire et al. 2021; Suárez et al. 2021). Limits of detection (LD) were 0.039 µg/L for TCPy, 0.117 µg/L for IMPy and 3-PBA, 0.116 µg/L for DETP, 0.142 µg/L for DEDTP, 0.117 µg/L for 3-PBA, and 0.072 µg/L for ETU. In order to correct for urine dilution, urinary creatinine concentrations were measured in a Roche Cobas C-311 system using a commercial kit (CREJ2) based on the Jaffe method.

2.4. Puberty development assessment

Puberty development was assessed by clinical Tanner staging (Marshall and Tanner 1970) and/or the parent-reported Peterson's Pubertal Development Scale (PDS) (Petersen et al. 1988) at a mean age of 8.8 years (5^{th}-95^{th} percentile: 7.7-10.1 years). Puberty development was always assessed by individuals who were blinded to the results of the exposure assessment, eliminating the possibility of bias. Children in Asturias and Valencia (308 girls and 341 boys) were

evaluated for Tanner stages of genital development (boys), breast development (girls), and pubic hair development (boys and girls), while boys in Granada (n=279) were evaluated for genital development alone. A pediatrician or trained health care professional assessed genital (G) by visual inspection and palpation, pubic hair development (PH) by visual inspection, and breast development (B) by palpation. The development stage was classified on a scale ranging from 1 (pre-pubertal) to 5 (adult development). Puberty onset was defined as being in stage 2+ for genital development (G2+), pubic hair development (PH2+ or pubarche), or breast development (B2+ or thelarche).

The PDS was used to assess the children in Gipuzkoa and Sabadell (297 girls and 304 boys) and both Tanner staging and the PDS for those in Valencia (184 girls and 191 boys). The PDS is an interview-based continuous measure of pubertal development. Parents (in most cases mothers, 97%) in these 3 cohorts rated the pubertal status of their children using the PDS, which contains items on growth, body hair, and skin changes for all children, with sex-specific items on facial hair and voice change for males and on breast development and menarche for females (Petersen et al. 1988); there are four response options: "not yet started, barely started, definitely started, seems complete". The questionnaire does not contain any pictures or diagrams. The continuous score was transformed into a five-point ordinal scale (1-pre-puberty, 2-early puberty, 3-midpuberty, 4-late puberty, and 5-post-puberty) in accordance with Carskadon and Acebo (1993) and Shirtcliff et al. (2009). The PDS has proven to be a reliable and valid instrument to assess pubertal development in children (Carskadon and Acebo 1993; Koopman-Verhoeff et al. 2020), and both self-reported and parent-reported PDS have shown strong internal consistency and test-retest reliability (Koopman-Verhoeff et al. 2020). The PDS has been validated in previous studies, including the INMA-Valencia cohort, and results were in moderate-to-good agreement with the Tanner stages evaluated by physical examination (Koopman-Verhoeff et al. 2020). Although PDS categories cannot be directly translated into the 5-point Tanner scale, it can adequately differentiate between pre-, mid-, and post-pubertal stages (Koopman-Verhoeff et al. 2020). The algorithm of Carskadon and Acebo (1993) was used to calculate the pubertal development from three scale items: growth of body hair and the two sex-specific items. The algorithm of Shirtcliff et al. (2009) uses all five PDS indicators of sexual development, differentially gathering gonadal and adrenal signals of physical development. Breast development, menarche, and growth spurt are associated with gonadal hormonal signals in females, while testicular enlargement, growth spurt, voice deepening, and facial hair growth are associated with these signals in males. Pubic/body hair and skin changes are

associated with adrenal hormonal signals in both females and males (Shirtcliff et al. 2009). In this way, three distinct pubertal outcomes were derived from PDS results: overall puberty development, adrenal development (adrenarche), and gonadal development (gonadarche). As in the case of Tanner stages, puberty onset was defined as being in stage 2+ (early puberty stage or higher).

2.5. Covariates

Data on covariates were obtained from interviewer-based questionnaires administered to the parents by research staff during pregnancy and at the follow-up visit and from medical records. Maternal variables included age at delivery (years), schooling (up to primary; secondary; university), ethnicity (white; non-white), area of residence during pregnancy (urban; sub-urban; rural), smoking during pregnancy (no; yes), and weight gain during pregnancy (kg). Maternal ethnicity was not considered in analyses because only 2% of mothers were non-white. There were very few mothers that reported any use of pesticides or working in agricultural activities during pregnancy and this information was therefore not considered in this study, and maternal urinary pesticide metabolites (*i.e.*, OP insecticides) were only available for Valencia (Llop et al. 2017). Child-related variables included birth weight (g), gestational length (weeks), and age (years), urinary creatinine (mg/dL), height (cm), and weight status (underweight; normal weight; overweight; obese) at puberty assessment. Weight status was obtained by converting their BMI to a z-score for age and sex based on World Health Organization (WHO) reference curves for children (5-19 years) (WHO 2007) and then classifying the results as underweight (<-1 standard deviation [SD]), normal weight (±1 SD), overweight (>+1 SD, equivalent to BMI≥25 kg/m^2 at 19 years), and obese (>+2 SD; equivalent to BMI≥30 kg/m^2 at 19 years). Weight status was categorized into underweight/normal weight, overweight, or obese, because only 14 (0.9%) children were underweighted.

2.6. Statistical analysis

Urinary concentrations of pesticide metabolites were expressed as detection frequencies and 50th, 75th, 90th, and 95th percentiles. Descriptive statistics for pesticide metabolites with and without creatinine correction, participants' characteristics, and pubertal development were reported separately for the girls and boys. Spearman's correlation test was used to examine relationships between pesticide metabolite pairs. Mixed-effect logistic regression models were constructed to examine the association between pesticide exposure and puberty onset (stage 2+). The cohort (Asturias; Gipuzkoa; Granada; Sabadell; Valencia) was treated as a random variable (cluster variable) in models to account for heterogeneity among cohorts. Each urinary

pesticide metabolite was separately modeled with each outcome. IMPy and DETP were found above the LD in more than 60% of samples and were modeled as continuous variables. In this case, analytical results below the method LD were imputed with LD/√2, and concentrations were natural log transformed before analysis to obtain a normal distribution of residuals. ETU, detected in around 50% of samples, was classified as low (<LD), moderate (LD-75th percentile [P75]), or high (>75th percentile) exposure. IMPy and DETP and were also classified as low, moderate, or high, as for ETU, to assess possible non-monotonic relationships. Detection of TCPy and 3-PBA in <40% of samples only allowed them to be modeled in two broad categories (undetected/detected). DEDTP was detected in <5% of samples and was therefore not considered in analyses. The first model (basic model) included child age and urinary creatinine (log-transformed) as covariates. The second model (model 2) included child age, creatinine, maternal education, and child height, and model 3 (fully-adjusted model) also included child BMI at puberty assessment. The remaining covariates were not included in the models as they did not confound the association between pesticide exposure and puberty onset. Unadjusted urinary pesticide metabolite concentrations and urinary creatinine concentrations were considered as separate independent variables, reported to be a better approach for controlling measurement error bias due to variability in urine concentrations (Barr et al. 2005; O'Brien et al. 2016). Selection of confounders was based on biological considerations and previous studies on pesticide exposure and puberty timing (Wohlfahrt-Veje et al. 2012a; 2012b; Ye et al. 2017a, 2017b). Results are presented as odds ratios (ORs) with 95% confidence interval (CI) for being in pubertal stage 2+ per log-unit increase in urinary metabolite concentrations or for detected/moderate/high *versus* undetected/low concentrations.

Given that the BMI is a strong predictor of puberty onset (Loredana Marcovecchio and Chiarelli 2013; Reinehr and Roth 2019), the potential effect modification by child BMI was further examined through cross-product terms (metabolite x underweight or normal weight/overweight or obese) and stratification (underweight or normal weight *vs.* overweight or obese) of logistic models. In addition, the association between pesticide exposure and puberty status as an ordinal variable was examined by multinomial logistic regression in all girls and boys, grouping Tanner/PDS stages into 1, 2, or 3+, because very few girls or boys were in stage 4 or 5, as described below. Sensitivity analysis was further conducted by adjusting regression models for co-exposure, as follows: TCPy and IMPy models were simultaneously adjusted for TCPy, IMPy, 3-PBA, and ETU, as inclusion of DETP in the models may lead to over adjustment; and DETP, 3-PBA, and ETU models were

simultaneously adjusted for DETP, 3-PBA, and ETU. The significance level was set at 0.05. The statistical programs R v.4.1.0 package "nlme" (The R Project for Statistical Computing, https://www.r-project.org) and SPSS v.26 (IBM SPSS Statistics for Windows, Armonk, NY) were used for statistical analyses.

3. Results

3.1. General characteristics of study participants

No significant differences in general characteristics were found between participants included in the present study and those with data on puberty status at age 7-11 years but not on urinary pesticide biomarkers (n=437), except for a higher percentage of children from Gipuzkoa, Granada, and Valencia and a lower percentage of children from Asturias and Sabadell in the included *versus* non-included children (Supplementary material, Table S1). Children from Gipuzkoa and Valencia represented up to 24.2% and 24.4% of the participants in this study sample, respectively, followed by Asturias (18.4%), Granada (18.2%), and Sabadell (14.8%) (Table 1). The mean age of mothers at delivery was 32 years, 24% had primary schooling, 69% resided in urban areas during pregnancy, 27% reported smoking during pregnancy, 26% were overweight or obese before pregnancy, and they gained an average of 13.8 kg during pregnancy. The mean birth weight of children was 3,276 g and the mean gestational length 39.6 weeks. At puberty assessment, the mean height was 132 cm for girls and 134 cm for boys, and 41% of girls and 45% of boys were overweight or obese.

3.2. Puberty status of children

Among 308 girls with data on Tanner staging, 38.6% were in stage B2+ (6.5% in stage B3) and 23.8% in stage PH2+ (6.1% in stage PH3 or 4). Among 620 boys with data on genital development, 22.4% were in stage G2+ (1.6% in stage G3), and 6.5% of 341 boys with data on pubic hair growth were in stage PH2 (Table 2). Among 481 girls assessed with the PDS, 45.9% were classified in pubertal stage 2+ (19.7% in stage 3 or 4), 33.3% in adrenal development stage 2+ (10.5% in stage 3, 4 or 5), and 45.1% in gonadal development stage 2+ (9.5% in stage 3, 4, or 5) (Table 3). Among 495 boys assessed with the PDS, 26.3% were in pubertal stage 2+ (1.8% in stage 3), 13.1% in adrenal development stage 2+ (2.2% in stage 3 or 4), and 27.9% in gonadal development stage 2+ (5.6% in stage 3) (Table 3).

3.3. Urinary concentrations of pesticide metabolites

The most prevalent pesticide metabolite was IMPy, detected in 63.0% of urine samples from girls (median=0.277 µg/L) and 63.6% of those from boys (median=0.299 µg/L), followed by DETP in 60.6% (median=0.315 µg/L) and 65.4% (median=0.401 µg/L), ETU

in 53.5% (median=0.095 µg/L) and 50.0% (median=0.067 µg/L), TCPy in 34.8% (P75=0.085 µg/L) and 40.2% (median=0.096 µg/L), and 3-PBA in 39.6% (P75=0.288 µg/L) and 34.3% (median=0.184 µg/L), respectively (Table 4). Urinary concentrations of pesticide metabolites were higher in children from Valencia and lower in those from Sabadell and Granada. All metabolites except 3-PBA were detected in at least two-thirds of the children from the Valencia cohort (IMPy: 84%, ETU: 75%, DETP: 60%) (Table S2). In the total sample of girls and boys, all pesticide metabolite pairs except 3-PBA-ETU (rho=0.09, p=0.01) showed significant and weak or moderate correlations (rho ranging from 0.14 for 3-PBA-DETP to 0.49 for TCPy-DETP, p<0.001).

3.4. Association between pesticide exposure and pubertal development in girls

Results for the association between urinary pesticide metabolites and pubertal development in girls were similar between basic and fully-adjusted models, although ORs were generally higher in the latter (Table 5 and Table S3). In models that included all the girls, ETU and DETP were associated with significantly higher odds of having started puberty. Specifically, higher ETU concentrations were associated with higher odds of being in Tanner stage B2+ (fully-adjusted OR [95% CI]=4.27 [1.84-9.93] and 5.55 [2.83-12.91] for concentrations below and above the P75, respectively, *vs.* undetected concentrations) and PDS stage 2+ of overall puberty development (OR [95% CI]=1.71 [1.03-2.83] for concentrations>P75). The odds significantly increased for being in stage B2 *vs.* B1 and for being in PDS stage 3+ *vs.* 1 (Table S5). DETP concentrations>P75 were also associated with higher odds of PDS stage 2+ (OR [95% CI]=1.86 [1.07-3.24]) and increasing DETP was associated with slightly higher odds of being in stage B2+ and PDS stage 2+ of adrenal development (OR [95% CI]=1.04 [1.00-1.11] and 1.02 [1.00-1.07], respectively, per log-unit increase in concentration), particularly when comparing stage 3+ *vs.* 1 (Table S5). In addition, the presence of detectable concentrations of TCPy was modestly associated with higher odds of being in stage B2+ (OR [95% CI]=1.84 [0.97-3.52]).

Table 6 shows that the association between ETU and earlier breast development was stronger in girls with underweight/normal weight. In this way, the odds of being in stage B2+ was two-fold higher in girls with underweight/normal weight *versus* overweight/obesity (*i.e.*, ETU>P75: OR [95%CI]=10.08 [2.62-38.76] *vs.* 4.56 [1.10-18.92]). Although not significant, there was a marginal association between DETP and earlier breast development in girls with underweight/normal weight (*i.e.*, DETP>P75: OR=3.01 [0.85-10.68] *vs.* 1.24 [0.30-5.09] in

girls with overweight/obesity). In addition, the detection of TCPy was only modestly associated with higher odds of adrenarche in girls with underweight/normal weight alone (OR [95% CI]=1.50 [0.93-3.78]). However, interaction terms were not statistically significant, *i.e.*, $P_{interaction}$=0.75 (ETU), 0.70 (DETP), and 0.13 (TCPy).

Adjustment for co-exposure led to a slightly weaker but significant association between ETU and higher odds of being in stage B2+ (OR [95%CI]=4.08 [2.10-12.03] and 5.03 [2.39-12.59] for moderate and higher ETU concentrations, respectively), while associations of ETU and DETP with being in PDS stage 2+ did not remain significant (Table S7). Stratification of co-exposure models by weight status revealed a non-significant association between TCPy and higher odds of stage B2+ in girls with underweight/normal weight (OR [95%CI]=2.55 [0.90-7.24]). The remaining BMI-stratified associations were not substantially different than those from single exposure models (data not shown).

3.5. Association between pesticide exposure and pubertal development in boys

Results for boys were again similar between basic and fully-adjusted models, although some associations were strengthened by the inclusion of child BMI (Table 7 and Table S4). In fully-adjusted models that included all the boys, detected *vs.* undetected TCPy and 3-PBA were associated with significantly higher odds of being in Tanner stage G2+ (OR [95% CI]=1.97 [1.08-3.57] and 2.08 [1.15-3.81], respectively); these odds were higher although not significant when comparing stage G3 *vs.* G1 (Table S6). BMI-stratified models (Table 8) showed a stronger but non-significant association between TCPy and genital development in boys with normal weight ($P_{interaction}$=0.52), and a stronger association between 3-PBA and genital development that was significant in boys with overweight/obesity alone ($P_{interaction}$=0.03). A higher ETU (>P75) was associated with higher odds of genital development in boys with normal weight alone (OR [95%CI]=2.89 [1.08-7.74]) ($P_{interaction}$=0.07) and DETP with slightly lower odds of being in PDS stage 2+ in boys with overweight/obesity alone (OR [95%CI]=0.94 [0.89-0.99] per log-unit increase in concentration) ($P_{interaction}$=0.03).

Adjustment for co-exposure led to a stronger association between 3-PBA and higher odds of being in stage G2+ (OR [95%CI]=2.41 [1.26-4.62]) and a significant association between higher DETP and lower odds of being stage G2+ (OR [95%CI]=0.93 [0.88-0.99]) and adrenarche (OR [95%CI]=0.95 [0.91-1.00]) (Table S8). BMI-stratified co-exposure models revealed stronger associations between ETU and higher odds of being in stage G2+ in boys with underweight/normal weight (OR [95%CI]=4.11 [1.36-12.4] for higher ETU

concentrations), between 3-PBA and higher odds of being in stage G2+ in boys with overweight/obesity (OR [95%CI]=4.37 [1.60-11.96]), and between higher DETP and lower odds of adrenarche in boys with overweight/obesity (OR [95%CI]=0.93 [0.88-0.99]). The remaining results were essentially unchanged (data not shown).

4. Discussion

In this large sample of Spanish children, higher urinary concentrations of ETU and DETP, and possibly TCPy, were associated with earlier puberty development in girls, especially with earlier breast development in those with underweight/normal weight exposed to higher ETU. In boys, higher urinary TCPy and ETU concentrations were associated with earlier genital development in boys with underweight/normal weight, higher 3-PBA with earlier genital development in boys with overweight/obesity, and higher DETP with delayed overall puberty development and adrenarche in those with overweight/obesity. Most of these novel findings need to be verified in other population-based longitudinal studies; however, the data suggest an association of peri-pubertal exposure to ETU and certain insecticides (OPs, pyrethroids) with earlier puberty in girls and in boys, which may be modified by their BMI.

Exposure to mixtures of different pesticide residues is ubiquitous among the general population, and human biomonitoring studies have shown that children are exposed, mainly *via* their diet, at comparable levels throughout the world (Fernández et al. 2020; Li et al. 2019; Papadopoulou et al. 2019). However, limited biomonitoring studies are available on the pesticide exposure of Spanish children. The detection of TCPy in the present sample of children (35%) is similar to that in pregnant women from the INMA-Valencia cohort (detection frequency [DF], geometric mean [GM]=39%, 0.49 µg/L) (Llop et al. 2017) and lower than that reported for 5 to 12-year-old participants in the BIOVAL study in the same region (DF, median=74%, 1.13 µg/L and 86%, 3.40 µg/g, respectively) (Fernández et al. 2020; Roca et al. 2014). However, in the present study, TCPy was detected in the urine of up to 66% of the children from Valencia, an intense area of agricultural production including vegetables, fruits (*e.g.,* citrus), and rice. Detection of IMPy in two-thirds of the present samples indicates ongoing exposure to diazinon or its residue some years after its prohibition in the EU, most likely from food imported from non-EU countries (AESAN 2018). IMPy concentrations were similar to those reported for children in the BIOVAL study (DF=57%, median=5.16 µg/g) (Roca et al. 2014) but higher than those in pregnant women from the INMA-Valencia cohort (DF, GM=12%, 0.03 µg/L) (Llop et al. 2017). DETP detection was similar to that found in mothers from the INMA-Valencia cohort (DF=75%, GM=0.22 µg/L) and more frequent

than previously reported in children from Valencia (DF=21% and 36%, respectively) (Fernández et al. 2020; Roca et al. 2014) and from the Andalusian province of Almería (DF=18%) (González-Alzaga et al. 2020), whereas 3-PBA detection was less frequent than in children in the BIOVAL study (DF, median=79%, 1.63 µg/L) (Fernández et al. 2020).

Regarding ETU, urinary concentrations in the present children, particularly those from Valencia, are higher than concentrations in 6- to 18-year-old children participating in the U.S. 2003-2008 National Health and Nutrition Examination Survey (DF, GM=10.6%, 0.12 µg/L) (NHANES) (Stadler et al. 2022), within the range of urinary concentrations found in 3- to 10 year-old French children living near vineyards frequently treated with dithiocarbamates (median=0.43 µg/g, range=0.01-4.45 µg/g) (Raherison et al. 2019), but lower than those in 6- to 9-year-old children from agricultural communities in Costa Rica (GM=1.2 µg/L) (van Wendel de Joode et al. 2016). Overall, the extent to which children from the general population is exposed to these fungicides or their transformation products remains largely unknown (Stadler et al. 2022).

4.1. Fungicides: ETU

One of the most relevant study findings is the association between urinary ETU and earlier breast development in girls, given that girls with higher urinary ETU concentrations had 5-fold higher odds of being in Tanner stage B2+ (up to 10-fold higher odds in girls with underweight/normal weight) in comparison to those with undetected ETU. To our best knowledge, only one previous study in Denmark reported an association between exposure to non-persistent pesticides and earlier breast development, in a cohort of 83 girls whose mothers worked in greenhouses during pregnancy (Wohlfahrt-Veje et al. 2012a). The authors observed earlier breast development in 6- to 11-year-old girls prenatally exposed to multiple pesticides in comparison to non-exposed girls (mean breast development onset=8.9 *versus* 10.4 years, p=0.05). In the present study, the association between ETU and breast development was markedly stronger among girls with underweight/normal weight, likely because overweight may hamper the assessment of early breast stages (Wolff et al. 2014). On the other hand, estrogens synthesized by adipose tissue of girls with overweight/obesity (*e.g.*, in mammary fat) may contribute to breast development independently of the hypothalamic-pituitary-gonadal (HPG) axis. In this study, 21% of girls with underweight/normal weight were classified in stage B2+ *versus* 92% of those with overweight/obesity. In parallel, a higher ETU concentration was associated with increased odds of being in Tanner stage G2+ in boys with underweight/normal weight. In the

aforementioned Danish study, however, prenatal exposure to pesticides was related to lower testicular volume and penile length rather than increased genital size (*i.e.*, boys in the high exposure group had 24.7% smaller testes [95%CI=-62.2; -10.1] and 9.4% shorter penile length [95%CI=-16.8; -1.1] than the unexposed) (Wohlfahrt-Veje et al. 2012b). In contrast to the consistent evidence of a strong association between higher childhood BMI and earlier female puberty, data on the association between BMI and male puberty timing have been controversial (Busch et al. 2020). Hence, the effect modification of BMI on the association between ETU and genital development suggested by the present results is less clear. However, it may be explained by a greater predisposition to earlier puberty in boys with normal weight (Kleber et al. 2011; Lee et al. 2016), although there was a similar percentage of boys in stage G2+ in the underweight/normal weight (41%) and overweight/obesity (40%) groups.

ETU is an anti-thyroid compound and has been found to interfere with iodide uptake by inhibiting thyroid peroxidase activity (Hurley et al. 1998; Marinovich et al. 1997). In rodent studies, mancozeb was reported to reduce serum thyroxine (T4) levels and increase thyroid-stimulating hormone (TSH) production (Axelstad et al. 2011; Hurley et al. 1998; Kackar et al. 1997). In human studies, occupational exposure to mancozeb and other EBDC fungicides was associated with hypothyroid-like hormone imbalance (increased TSH and decreased T4) and other thyroid disorders in both men (Medda et al. 2017; Panganiban et al. 2004; Piccoli et al. 2016; Steenland et al. 1997) and women (Goldner et al. 2010). This is of interest because of the known cross-talk between thyroid hormones, estradiol, androgens, and gonadotropins (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) (Ren and Zhu 2022). Indeed, elevated TSH is one of the factors thought to contribute to central precocious puberty in girls (Jung et al. 2019). The specific mechanism underlying this relationship remains unclear, but thyroid hormones may affect the levels of gonadotropin-releasing hormone (GnRH) that are released by the hypothalamus (Ren and Zhu 2022) and that stimulate the pituitary secretion of LH and FSH, thereby activating the production of gonadal hormones and the progression of secondary sex characteristics.

Rodents exposed to either ETU or mancozeb have evidenced not only thyrotoxic but also reproductive effects, including disrupted estrus cycles, impaired embryo development, and altered reproductive hormone levels (Cecconi et al. 2007; Maranghi et al. 2013; Runkle et al. 2017). The mechanism(s) by which ETU, mancozeb, or other EBDC fungicides may specifically act on the reproductive system are poorly understood; however, the present results suggest that peri-pubertal exposure to ETU or its parent

compound(s) may induce the estrogen surge that triggers the initiation of puberty, particularly breast growth onset, either in a direct manner or by affecting mechanisms that directly or indirectly regulate this surge. This is the first report of an association between ETU and earlier puberty development and, given the cross-sectional design of the study, these findings should be interpreted with caution.

4.2. OP insecticides

The suggestive association between TCPy and higher odds of being in Tanner stage G2+ in boys is consistent with the estrogenic action exerted by chlorpyrifos *in vitro* (Andersen et al. 2002; Yu et al. 2015) but is not supported by experimental evidence on the anti-androgenic effects of chlorpyrifos and TCPy (Gao et al. 2021; Hazarika et al. 2021; Viswanath et al. 2010). However, after adjusting for co-exposure, TCPy was not associated with male genital development but was modestly associated with higher odds of being in Tanner stage B2+ in girls with underweight/normal weight, again suggesting an estrogenic effect. Other proposed mechanisms on how chlorpyrifos could accelerate puberty development are activation of the HPG axis through hypothalamic inflammation (Valsamakis et al. 2021) and immunotoxic effects (Bouman et al. 2005), as it has been shown to influence the activity of pro-inflammatory molecules (Camacho-Pérez et al. 2022) and interfere with the immune function (Lee and Choi 2020).

To the best of our knowledge, there are no published human data on TCPy/chlorpyrifos exposure and puberty development, and epidemiological studies in males have reported associations of urinary TCPy with reduced estradiol in adults (Meeker et al. 2008) and with reduced estradiol and FSH in adolescents (Suárez et al. 2021) rather than with increased levels. Animal studies have also found a reduction in estradiol, testosterone, LH, and FSH, and an induction of hypothyroidism after exposure to chlorpyrifos/chlorpyrifos-methyl in males and females (Abd-Elhakim et al. 2021; Jeong et al. 2006; Li et al. 2019; Peiris and Dhanushka 2017; Ventura et al. 2016), in disagreement with the present findings.

DETP is a metabolite of several OP insecticides, including chlorpyrifos and diazinon. In animal models, early-life exposure to some of these insecticides produced toxic effects on the HPG axis (Jayachandra and D'Souza 2014; Maitra and Mitra 2008). In partial agreement with the findings for TCPy, a higher DETP concentration was associated with earlier overall puberty development (higher odds of PDS stage 2+) in girls, but with delayed genital (lower odds of Tanner stage G2+) and adrenarche (lower odds of PDS adrenal stage 2+) in boys, although the former effect was not evident in co-exposure models. In partial

agreement with the associations observed for DETP in the present boys, a study in Belgium reported that the sum of dimethyl phosphate metabolites (∑DMPs) and diethyl phosphate metabolites (∑DEPs) were associated with lower odds of having completed genital development (OR [95%CI]=0.46 [0.22-0.96] and 0.53 [0.29-0.96], respectively) or having reached adult phase of estradiol and testosterone levels in 14-15-year-old adolescent males (Croes et al. 2015), suggesting an anti-androgenic action of OP exposure. However, ∑DEPs was associated with lower odds of complete breast development (OR [95%CI]=0.78 [0.61-1.00]) in Belgium adolescent females (Croes et al. 2015). Unlike observed for ETU, the association between DETP and delayed overall puberty development (lower odds of PDS stage 2+) was stronger in the boys with overweight/obesity. Overall, these results suggest that postnatal exposure to OP insecticides or metabolites may impact on pubertal development centrally by activating the HPG axis and thereby triggering puberty initiation or exerting an anti-androgenic action; however, the possible mechanism(s) underlying the associations observed with TCPy and DETP, and the role of BMI in these associations, remain to be elucidated.

4.3. Pyrethroids

3-PBA was only detected in one-third of the urine samples; nevertheless, boys with detected 3-PBA had increased odds of being in Tanner stage G2+, and this association was stronger in the boys with overweight/obesity. In the same line, a Chinese study found that urinary 3-PBA in boys aged 9-16 years (n=463), although at several-fold higher concentrations than in the present boys (P75: 2.98 *vs.* 0.288 ng/mL), was associated with earlier genital development (*i.e.*, the odds of being in stage G3 and G4 increased by 275 and 280%, respectively, per one-unit increase in log-transformed urinary 3-PBA) and higher FSH and LH levels (Ye et al. 2017b). However, Tanner stages were self-assessed in their study, and 3-PBA was associated with delayed breast development and pubic hair growth and with lower odds of having reached menarche in Chinese girls aged 9-17 years (Ye et al. 2017a). Observations in boys are supported by the finding of accelerated puberty onset in male mice after postnatal exposure to pyrethroids (Ye et al. 2017). In addition, two epidemiological studies found an association between urinary 3-PBA and elevated LH and FSH in men occupationally exposed (Han et al. 2008) and non-occupationally exposed (Meeker et al. 2009) to pyrethroids, consistent with an acceleration of puberty onset. With this background, although no direct evidence is available, it can be hypothesized that exposure to pyrethroids or their metabolites may lead to early puberty in boys through gonadotropin stimulation, possibly *via* oxidative stress and inflammation (Zhang et al. 2017). It remains

unclear whether this association is limited to boys with overweight/obesity.

4.4. Limitations and strengths

One of the major limitations of this study is its cross-sectional design, preventing the inference of causality. Moreover, the determination of non-persistent pesticide metabolites in spot urine samples or pools of two urine samples is limited to reflecting long-term exposure, because urinary metabolites have a short biological half-life. In fact, once in the human body, pesticides such as OPs and pyrethroids are typically metabolized and excreted in urine within 4-48 hours after exposure, depending on the compound (Egeghy et al. 2011), and several studies have indicated moderate temporal reliability for urinary metabolites. Thus, the intra-class correlation coefficient (ICC) for urinary DETP in 7-year-old European children was 0.37 for between-day variability and 0.35 for between-season variability (Casas et al. 2018). In pregnant Spanish women, the ICC for IMPy was in the range 0.40-0.50 (Bravo et al. 2020). A study of Costa Rican children aged 6-9 years exposed to agricultural pesticides found fair reliability for urinary ETU (ICC=0.67) and TCPy (ICC=0.52) but moderate reliability (ICC=0.32) for 3-PBA (van Wendel de Joode e al. 2016). However, in populations that are mainly exposed through their diet, it can be assumed that their exposure to pesticides is relatively continuous (Côté et al. 2014). In this regard, non-specific urinary metabolites such as 3-PBA or ETU may be valid biomarkers of chronic or sub-chronic exposure to dietary mixtures of pyrethroids or EBDC fungicide residues, while the specific metabolites (TCPy, IMPy) are dependent on recent exposure to the parent compound and may therefore show higher intra-individual variation. Another limitation is the variation in the timing of spot urine sample collection among cohorts in Granada (afternoon), Asturias (morning), and Valencia (morning), and it is not known whether concentrations differ between afternoon and morning urine samples, and this issue should be addressed in future studies by measuring repeated urine samples. The boys in the Granada cohort were only assessed for genital development using Tanner; hence, the results for genital development should be interpreted with greater caution. In addition, we cannot rule out that concentrations may have changed as a result of storage time, particularly for samples from Granada (collected in 2010-2012). However, any change in concentrations over time would produce an underestimation rather than overestimation of associations with pubertal development, given that misclassification is expected to be non-differential.

It is also possible that urinary 3-PBA concentrations were substantively underestimated, given that pyrethroid metabolites such as 3-PBA are largely present as phase II conjugates (glucuronide and/or

sulfate) in urine (up to 85%) (Baker et al. 2004), and this deconjugation step was omitted, although misclassification is again likely to be non-differential. The relatively low detection frequency of 3-PBA, TCPy, and ETU prevented assessment of the potential effect of the pesticide mixture, and a confounding effect of prenatal or postnatal exposure to other pesticides or EDCs cannot be ruled out. Moreover, diethyl phosphate (DEP) and DMP metabolites could not be measured due to the non-availability of reference standards, and measurement of total DAP concentration would have yielded information on exposure to a wider range of OP pesticides. Additionally, the use of two different instruments for the assessment of puberty development may represent a study limitation. However, Tanner and PDS outcomes were analyzed separately because these different measures of pubertal development are not entirely comparable as they yield different outcomes. The PDS assesses secondary sexual characteristics that are not captured by Tanner stages, including growth spurt, voice deepening, facial and body hair growth, and skin changes, providing an overall score for puberty development and additional outcomes related to the maturation of the HPG and hypothalamic-pituitary-adrenal (HPA) axes (gonadarche and adrenarche). Hence, Tanner and the PDS may be considered complementary instruments to assess pubertal development. Finally, the small number of boys who had reached pubarche or adrenarche limited the possibility of detecting associations with male adrenal development.

Study strengths include the contribution of data from a large sample of children on the association of ETU, TCPy, and DETP exposure with earlier puberty, adding to the scant information available on the relationship between pesticides and puberty timing. Furthermore, the sample derived from five different geographical locations and contained a sufficient proportion of children with overweight/obesity to permit evaluation of effect modification by BMI. It cannot be ruled out that some of the significant or suggestive results in the present study were due to chance, given the performance of multiple comparisons (5 exposure biomarkers x outcomes=25 comparisons in either girls or boys; 50 comparisons in BMI-stratified analysis). Nevertheless, these findings represent a potential cause of concern, due to the widespread exposure of children in the general population to pesticides and the possibility that altered pubertal timing may increase the risks of behavioral disorders during adolescence and of obesity, cardiovascular disease, and endocrine-related cancers in later life (Golub et al. 2008; Lakshman et al. 2008; 2009).

5. Conclusions

This study provides evidence that peri-pubertal exposure to ETU and certain

insecticides might be associated with pubertal outcomes, especially earlier breast development in girls and earlier genital development in boys. These results suggest that interference with the HPG axis by certain contemporary pesticides during childhood may potentially impact pubertal timing. To our best knowledge, this is the first study to report that exposure to ETU and TCPy, respectively, is associated with puberty timing in girls and boys, and it is the first to investigate effect modification by BMI on this association. Population-based longitudinal studies of large samples of children are needed to fully elucidate the role of exposure to pesticides in the population trend toward earlier puberty.

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Highlights

Higher urinary DETP and ETU were associated with earlier puberty development in girls.

Association between ETU and breast development was stronger in normal weight girls.

TCPy and 3-PBA were associated with earlier genital development in boys.

Higher ETU was associated with earlier genital development in normal weight boys.

Higher DETP was associated with delayed puberty development in overweight/obese boys.

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Table 1. Characteristics of 1,539 children in the INMA Project, Spain.

Characteristics	N (%) or mean ± SD		
	All	Girls (N=606)	Boys (N=933)
Cohort (age range)			
Asturias (7.7-9.6 yrs.)	284 (18.4)	125 (20.6)	159 (17.0)
Gipuzkoa (7.6-8.6 yrs.)	373 (24.2)	185 (30.5)	188 (20.1)
Granada (8.8-11.3 yrs.)	279 (18.2)	0 (0)	279 (29.9)
Sabadell (8.0-10.7 yrs.)	228 (14.8)	112 (18.5)	116 (12.4)
Valencia (8.2-9.8 yrs.)	375 (24.4)	184 (30.4)	191 (20.5)
Mother's age at delivery (years)	31.8 ± 4.3	32.1 ± 4.0	31.6 ± 4.4
Mother's educational attainment during pregnancy			
Up to primary	365 (23.7)	109 (18.0)	256 (27.4)
Secondary	626 (40.7)	254 (41.9)	372 (39.9)
University	548 (35.6)	243 (40.1)	305 (32.7)
Mother's ethnicity			
White	1509 (98.1)	598 (98.7)	911 (97.6)
Non-white	30 (1.9)	8 (1.3)	22 (2.4)
Mother's area of residence during pregnancy			
Urban	1059 (68.8)	474 (78.2)	569 (61.0)
Sub-urban	370 (24.0)	109 (18.0)	273 (29.2)
Rural	110 (7.1)	23 (3.8)	91 (9.7)
Mother's smoking during pregnancy			
Yes	410 (27.2)	183 (30.2)	227 (24.3)
No	1099 (72.8)	412 (68.0)	687 (73.6)
Maternal pre-pregnancy BMI			
Underweight/normal weight	1135 (73.7)	437 (72.1)	698 (74.8)
Overweight/obese	404 (26.3)	169 (27.9)	235 (25.2)
Weight gain during pregnancy (kg)	13.8 ± 5.1	13.5 ± 5.0	14.1 ± 5.2
Birth weight (g)	3,276 ± 469	3,195 ± 458	3,335 ± 468
Gestational length (weeks)	39.6 ± 1.6	39.6 ± 1.6	39.5 ± 1.5
Child urinary creatinine (mg/dL)	95.9 ± 68.1	86.1 ± 36.8	102.3 ± 81.7
Child age at puberty assessment (years)	8.76 ± 0.85	8.52 ± 0.72	8.92 ± 0.88
Child's height at puberty assessment (cm)	133.4 ± 7.3	131.86 ± 7.16	134.36 ± 7.20
Child's weight status at puberty assessment[a]			
Underweight or normal weight	868 (56.4)	356 (58.7)	512 (54.9)
Overweight	383 (24.9)	163 (26.9)	220 (23.6)
Obese	288 (18.7)	87 (14.4)	201 (21.5)

SD: standard deviation; BMI: Body mass index.

[a]Obtained by converting BMI to z-score for age and sex based on WHO reference curves for children (5-19 years).

Table 2. Puberty status of girls and boys based on clinical Tanner stage.

Tanner stage: AST+GRA+VAL[a]	Girls (N=308)		Boys (N=620)	
	mean age	N (%)	mean age	N (%)
Breast development (B)				
B1	8.6	189 (61.4)	–	–
B2	9.0	99 (32.1)	–	–
B3	9.0	20 (6.5)	–	–
Breast development onset (B2+)	8.7	119 (38.6)	–	–
AST	8.4	16 (13.0)	–	–
VAL	9.1	103 (56.3)	–	–
Genital development (G)				
G1	–	–	9.2	481 (77.6)
G2	–	–	9.4	129 (20.8)
G3	–	–	9.1	10 (1.6)
Genital development onset (G2+)	–	–	9.4	139 (22.4)
AST	–	–	8.3	2 (1.3)
GRA	–	–	9.9	57 (20.4)
VAL	–	–	9.1	80 (44.0)
Pubic hair growth (PH)				
PH1	8.7	235 (76.3)	8.7	598 (96.4)
PH2	8.9	54 (17.5)	9.1	22 (3.6)
PH3	9.0	17 (5.5)	–	0 (0)
PH4	8.8	2 (0.6)	–	0 (0)
Pubic hair growth onset (PH2+)[b]	8.5	73 (23.8)	8.9	22 (6.5)
AST	8.3	22 (17.6)	8.5	4 (2.5)
VAL	9.1	51 (28.0)	9.1	18 (9.9)

AST: Asturias; GRA: Granada; VAL: Valencia.

[a]VAL: 182 boys and 183 girls with data on Tanner stage.
[b]No data on pubic hair growth for boys in Granada (N=341 boys with data on pubic hair growth).

Table 3. Puberty status of girls and boys based on parent-reported PDS.

PDS: GIP+SAB+VAL	Girls (N=481)		Boys (N=495)	
	Mean age	N (%)	Mean age	N (%)
PDS stage: overall puberty				
1 (Pre-puberty)	8.4	260 (54.1)	8.5	365 (73.7)
2 (Early puberty)	8.8	126 (26.2)	8.6	121 (24.4)
3 (Midpuberty)	9.0	90 (18.7)	8.5	9 (1.8)
4 (Late puberty)	8.7	5 (1.0)	–	0 (0)
Puberty onset (PDS stage 2+)	8.9	221 (45.9)	8.7	130 (26.3)
GIP	7.7	47 (25.4)	7.7	45 (23.9)
SAB	9.3	63 (56.3)	9.2	32 (27.6)
VAL	9.1	111 (60.3)	9.1	53 (27.7)
PDS stage: adrenal development				
1 (Pre-puberty)	8.5	321 (66.7)	8.6	430 (86.9)
2 (Early puberty)	8.8	109 (22.7)	8.6	54 (10.9)
3 (Midpuberty)	8.6	44 (9.1)	8.4	9 (1.8)
4 (Late puberty)	9.2	6 (1.2)	8.9	2 (0.4)
5 (Post-puberty)	9.7	1 (0.2)	–	0 (0)
Adrenarche (PDS adrenal 2+)	8.7	160 (33.3)	8.5	65 (13.1)
GIP	7.7	42 (22.7)	7.4	21 (11.2)
SAB	9.3	50 (44.6)	9.1	14 (12.1)
VAL	9.1	68 (37.0)	9.0	30 (15.7)
PDS stage: gonadal development				
1 (Pre-puberty)	8.6	264 (54.9)	8.6	357 (72.1)
2 (Early puberty)	8.5	171 (35.5)	8.3	110 (22.2)
3 (Midpuberty)	8.9	43 (8.9)	8.6	28 (5.6)
4 (Late puberty)	9.3	1 (0.2)	–	0 (0)
5 (Post-puberty)	9.4	2 (0.4)	–	0 (0)
PDS gonadal 2+ (gonadarche)	8.7	217 (45.1)	8.7	138 (27.9)
GIP	7.7	83 (44.9)	7.7	77 (41.0)
SAB	9.4	48 (42.9)	9.3	20 (17.2)
VAL	9.1	86 (46.7)	9.0	41 (21.5)

GIP: Gipuzkoa; SAB: Sabadell; VAL: Valencia.

PDS: Pubertal Development Scale.

Table 4. Distribution of child urinary concentrations of pesticide metabolites.

Metabolites	%>LD	Percentiles							
		Unadjusted (µg/L)				Creatinine adjusted (µg/g)			
		50	75	90	95	50	75	90	95
Girls (N=606)									
TCPy	34.8	<LD	0.085	0.301	0.511	<LD	0.110	0.371	0.588
IMPy	63.0	0.277	1.079	2.742	3.763	0.354	1.380	3.029	4.096
DETP	60.6	0.315	2.633	9.901	20.706	0.439	3.284	12.498	24.632
3-PBA	39.6	<LD	0.288	0.935	2.047	<LD	0.378	1.328	3.019
ETU	53.5	0.095	0.367	0.964	1.981	0.114	0.501	1.469	2.559
Boys (N=933)									
TCPy	40.2	<LD	0.096	0.332	0.513	<LD	0.118	0.359	0.639
IMPy	63.6	0.299	1.118	2.417	4.003	0.363	1.282	2.761	4.195
DETP	65.4	0.401	2.877	8.042	13.986	0.519	2.907	9.099	17.582
3-PBA	34.3	<LD	0.184	0.760	1.470	<LD	0.251	0.819	1.699
ETU	50.0	0.067	0.286	0.798	1.212	0.081	0.349	1.054	1.778

LD: Limit of detection (TCPy: 0.039 µg/L; IMPy: 0.117 µg/L; DETP: 0.116 µg/L; 3-PBA: 0.117 µg/L; ETU: 0.072 µg/L)

Table 5. Associations between urinary pesticide metabolites and puberty development in girls.

Pesticide metabolites	Tanner stage 2+ (N=308)		PDS stage 2+ (N=481)		
	Breast development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.84 (0.97-3.52)	0.77 (0.42-1.45)	1.42 (0.83-2.23)	0.99 (0.71-1.87)	0.95 (0.62-1.57)
IMPy (log)	0.99 (0.92-1.06)	0.97 (0.92-1.04)	1.00 (0.94-1.04)	0.98 (0.94-1.03)	1.01 (0.96-1.06)
IMPy: >LD-P75 *vs.* <LD	1.17 (0.56-2.47)	0.77 (0.39-1.54)	0.91 (0.55-1.49)	0.74 (0.46-1.21)	0.77 (0.48-1.22)
IMPy: >P75 *vs.* <LD	1.70 (0.71-4.06)	0.69 (0.30-1.55)	1.36 (0.78-2.40)	0.94 (0.55-1.63)	1.25 (0.73-2.12)
DETP (log)	1.05 (1.00-1.11)	1.04 (0.98-1.09)	1.04 (1.00-1.11)*	1.02 (1.00-1.07)*	1.01 (0.98-1.04)
DETP: >LD-P75 *vs.* <LD	1.99 (0.97-4.12)	1.30 (0.68-2.51)	1.31 (0.80-2.14)	1.21 (0.75-1.95)	0.97 (0.61-1.52)
DETP: >P75 *vs.* <LD	1.88 (0.79-4.47)	1.12 (0.50-2.50)	1.86 (1.07-3.24)*	1.45 (0.85-2.47)	1.14 (0.68-1.91)
3-PBA: > *vs.* <LD	1.16 (0.62-2.15)	0.76 (0.43-1.36)	1.19 (0.72-1.76)	9.94 (0.64-1.55)	1.00 (0.67-1.55)
ETU: >LD-P75 *vs.* <LD	4.27 (1.84-9.93)**	1.16 (0.54-2.46)	1.47 (0.89-2.44)	1.24 (0.76-2.05)	1.18 (0.73-1.89)
ETU: >P75 *vs.* <LD	5.55 (2.83-12.91)**	1.33 (0.64-2.74)	1.71 (1.03-2.83)*	1.31 (0.80-2.13)	1.30 (0.81-2.09)

LD: Limit of detection. All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, and child weight status (normal weight, overweight, or obese) at 7-11 yrs. *p<0.05; **p<0.001

Table 6. Associations between urinary pesticide metabolites and puberty development in girls according to their weight status[a]

Pesticide metabolites	Normal weight					Overweight/obese			PI
	Tanner stage 2+ (N=176)		PDS stage 2+ (N=284)			Tanner stage 2+ (N=130)			
	Breast development n B2+=37	Pubic hair growth n PH2+=40	Overall puberty n stage 2+=96	Adrenarche n stage 2+=74	Gonadarche n stage 2+=97	Breast development n B2+=120	Pubic hair growth n PH2+=86	Overall puberty n stage 2+=125	
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	
TCPy: > *vs.* <LD	1.72 (0.71-4.24)	1.33 (0.55-3.20)	1.55 (0.89-3.73)	1.50 (0.93-3.78)	0.99 (0.65-2.29)	1.83 (0.61-5.45)	0.51 (0.20-1.27)	1.37 (0.54-2.38)	
IMPy (log)	1.00 (0.91-1.09)	1.03 (0.93-1.14)	1.01 (0.94-1.09)	1.02 (0.95-1.11)	1.00 (0.95-1.09)	0.98 (0.87-1.10)	0.91 (0.83-1.00)	1.00 (0.90-1.05)	
IMPy: >LD-P75 *vs.* <LD	0.96 (0.35-2.61)	0.94 (0.36-2.48)	0.89 (0.46-1.73)	0.91 (0.45; 1.85)	0.68 (0.36-1.27)	1.04 (0.29-3.68)	0.53 (0.19-1.48)	1.04 (0.49-2.19)	
IMPy: >P75 *vs.* <LD	1.71 (0.54-5.41)	1.31 (0.42-4.09)	1.54 (0.73-3.23)	1.39 (0.63-3.07)	1.06 (0.53-2.15)	2.16 (0.49-9.48)	0.37 (0.11-1.26)	1.31 (0.55-3.12)	
DETP (log)	1.08 (1.00-1.17)	1.04 (0.97-1.12)	1.03 (0.98-1.08)	1.04 (0.99-1.10)	1.00 (0.96-1.05)	1.04 (0.95-1.14)	1.04 (0.96-1.12)	1.05 (0.98-1.09)	
DETP: >LD-P75 *vs.* <LD	2.69 (0.94-7.64)	1.28 (0.49-3.33)	1.23 (0.64-2.36)	1.38 (0.68-2.76)	0.91 (0.50-1.67)	1.74 (0.52-5.85)	1.40 (0.56-3.53)	1.36 (0.63-2.92)	
DETP: >P75 *vs.* <LD	3.01 (0.85-10.68)	1.73 (0.54-5.56)	1.93 (0.92-4.03)	1.79 (0.81-3.92)	1.01 (0.51-2.03)	1.24 (0.30-5.09)	0.79 (0.26-2.45)	1.94 (0.85-4.45)	
3-PBA: > *vs.* <LD	0.75 (0.32-1.75)	0.67 (0.29-1.55)	1.05 (0.57-1.84)	1.08 (0.59-2.07)	0.85 (0.52-1.60)	1.26 (0.44-3.62)	0.93 (0.41-2.13)	1.49 (0.66-2.67)	
ETU: >LD-P75 *vs.* <LD	9.55 (2.27-40.08)**	1.51 (0.49-4.67)	1.34 (0.68-2.65)	1.66 (0.79-3.46)	1.14 (0.60-2.14)	3.13 (0.94-10.46)	0.88 (0.31-2.45)	1.56 (0.72-3.35)	
ETU: >P75 *vs.* <LD	10.08 (2.62-38.76)**	1.66 (0.60-4.61)	1.61 (0.84-3.07)	1.73 (0.87-3.47)	1.13 (0.61-2.12)	4.56 (1.10-18.92)*	1.04 (0.36-3.03)	1.91 (0.84-4.34)	

All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, cohort, maternal education, and child height.

[a]Weight status according to BMI z-score for age and sex.

*p<0.05; **p<0.001

Table 7. Associations between urinary pesticide metabolites and puberty development in boys.

Pesticide metabolites	Tanner stage 2+ (N=620)		PDS stage 2+ (N=495)		
	Genital development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.97 (1.08-3.57)*	0.77 (0.31-1.90)	0.93 (0.60-1.47)	0.98 (0.71-2.35)	1.39 (0.81-1.64)
IMPy (log)	1.07 (0.96-1.19)	0.97 (0.86-1.09)	0.98 (0.93-1.03)	0.99 (0.96-1.08)	1.01 (0.91-1.05)
IMPy: >LD-P75 *vs.* <LD	2.08 (0.94-4.71)	1.14 (0.35-3.75)	1.07 (0.66-1.75)	1.00 (0.52-1.92)	0.93 (0.56-1.53)
IMPy: >P75 *vs.* <LD	1.68 (0.70-4.13)	0.94 (0.25-3.52)	1.02 (0.59-1.76)	1.15 (0.57-2.34)	1.03 (0.60-1.78)
DETP (log)	0.97 (0.92-1.02)	0.96 (0.89-1.04)	0.98 (0.94-1.07)	0.96 (0.90-1.04)	1.00 (0.95-1.04)
DETP: >LD-P75 *vs.* <LD	0.58 (0.29-1.15)	0.62 (0.21-1.81)	0.88 (0.54-1.43)	0.61 (0.32-1.14)	1.32 (0.82-2.15)
DETP: >P75 *vs.* <LD	0.79 (0.37-1.69)	0.96 (0.31-2.97)	1.02 (0.61-1.72)	0.76 (0.39-1.49)	0.88 (0.51-1.52)
3-PBA: > *vs.* <LD	2.08 (1.15-3.81)*	1.22 (0.49-3.06)	0.79 (0.50-1.27)	1.01 (0.73-1.81)	1.12 (0.54-1.77)
ETU: >LD-P75 *vs.* <LD	1.58 (0.74-4.34)	0.74 (0.23-2.43)	0.79 (0.47-1.31)	0.67 (0.33-1.38)	0.81 (0.49-1.35)
ETU: >P75 *vs.* <LD	1.79 (0.89-3.53)	0.72 (0.25-2.11)	0.74 (0.44-1.22)	1.10 (0.59-2.07)	0.71 (0.42-1.20)

LD: Limit of detection. All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, and child weight status (normal weight, overweight, or obese) at 7-11 yrs.*p<0.05

Table 8. Associations between urinary pesticide metabolites and puberty development in boys according to their weight status[a]

Pesticide metabolites	Normal weight					Overweight/obese				
	Tanner stage 2+ (N=183)		PDS stage 2+ (N=282)			Tanner stage 2+ (N=158)		PDS stage 2+ (N=213)		
	Genital development n G2+=75 (41%)	Pubic hair growth n PH2+=10 (5%)	Overall puberty n stage 2+=64 (23%)	Adrenarche n stage 2+=29 (10%)	Gonadarche n stage 2+=72 (25%)	Genital development n G2+=64 (40%)	Pubic hair growth n PH2+=12 (7%)	Overall puberty n stage 2+=66 (31%)	Adrenarche n stage 2+=36 (17%)	Gonadarche n stage 2+=66 (31%)
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	2.18 (0.91-5.17)	0.70 (0.18-2.78)	0.94 (0.49-1.73)	1.06 (0.43-2.35)	1.26 (0.66-2.27)	1.56 (0.68-3.60)	0.68 (0.20-2.32)	0.87 (0.47-1.71)	1.08 (0.41-2.05)	1.43 (0.92-3.50)
IMPy (log)	1.03 (0.90-1.17)	0.92 (0.79-1.07)	0.96 (0.89-1.02)	0.98 (0.88-1.07)	1.00 (0.93-1.07)	1.10 (0.92-1.31)	1.04 (0.83-1.30)	1.01 (0.93-1.12)	0.99 (0.86-1.07)	1.02 (0.95-1.17)
IMPy: >LD-P75 *vs.* <LD	2.50 (0.79-7.95)	0.78 (0.14-4.28)	1.05 (0.54-2.04)	1.24 (0.48-3.25)	0.93 (0.47-1.83)	1.39 (0.44-4.35)	1.65 (0.29-9.22)	1.15 (0.55-2.39)	0.88 (0.36-2.17)	0.98 (0.47-2.06)
IMPy: >P75 *vs.* <LD	1.27 (0.34-4.73)	0.78 (0.12-5.06)	0.87 (0.41-1.87)	1.42 (0.50-4.00)	1.12 (0.53-2.35)	1.56 (0.47-5.14)	1.03 (0.15-7.11)	1.18 (0.53-2.63)	1.00 (0.37-2.65)	0.94 (0.42-2.10)
DETP (log)	0.96 (0.89-1.03)	0.98 (0.87-1.10)	1.02 (0.97-1.07)	0.97 (0.91-1.04)	1.00 (0.95-1.05)	0.98 (0.91-1.05)	0.94 (0.85-1.04)	0.94 (0.89-0.99)*	0.96 (0.90-1.01)	1.00 (0.93-1.06)
DETP: >LD-P75 *vs.* <LD	0.65 (0.16-1.21)	0.90 (0.18-5.54)	0.95 (0.47-1.91)	0.40 (0.14-1.13)	1.26 (0.64-2.47)	0.45 (0.16-1.21)	0.39 (0.09-1.78)	0.84 (0.43-1.67)	0.85 (0.37-1.97)	1.37 (0.68-2.76)
DETP: >P75 *vs.* <LD	0.42 (0.13-1.37)	0.97 (0.16-5.94)	1.72 (0.84-3.53)	1.10 (0.43-2.83)	1.07 (0.50-2.29)	1.28 (0.45-3.65)	0.94 (0.21-4.26)	0.57 (0.26-1.26)	0.62 (0.23-1.65)	0.71 (0.31-1.61)
3-PBA: > *vs.* <LD	1.22 (0.52-2.84)	0.81 (0.21-3.08)	0.65 (0.32-1.99)	0.71 (0.27-1.63)	1.16 (0.61-2.06)	3.64 (1.51-8.79)*	1.57 (0.44-5.55)	0.95 (0.49-1.92)	1.65 (0.67-3.55)	0.96 (0.52-2.18)
ETU: >LD-P75 *vs.* <LD	1.48 (0.49-4.53)	0.29 (0.03-2.79)	0.84 (0.40-1.75)	0.39 (0.11-1.39)	0.94 (0.46-1.95)	1.59 (0.55-4.56)	1.29 (0.28-5.89)	0.75 (0.37-1.55)	1.11 (0.44-2.80)	0.69 (0.33-1.42)
ETU: >P75 *vs.* <LD	2.89 (1.08-7.74)*	0.83 (0.19-3.67)	0.83 (0.42-1.67)	1.07 (0.43-2.68)	0.85 (0.42-1.75)	1.06 (0.38-2.95)	0.59 (0.12-2.87)	0.58 (0.27-1.25)	1.49 (0.60-3.69)	0.50 (0.22-1.10)

All models adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, cohort, maternal education, and child height.

[a]Weight status according to BMI z-score for age and sex.

*p<0.05

5.3 Resultado del objetivo 3:

ASSOCIATION OF PRENATAL PHTHALATE EXPOSURE WITH PUBERTAL DEVELOPMENT IN SPANISH BOYS AND GIRLS

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Resumen

Antecedentes: Los ftalatos son compuestos químicos de distribución ubicua en la población que poseen actividad anti-androgénica *in vitro*. La exposición a ftalatos podría tener un impacto en la cronología del desarrollo puberal.

Objetivo: Investigar la asociación de la exposición prenatal a ftalatos con el desarrollo puberal en niños y niñas.

Métodos: Un total de 788 parejas madre-hijo (409 niñas y 379 niños) participaron en el estudio. En muestras de orina de las madres recogidas durante el embarazo se analizaron las concentraciones de los metabolitos de 6 ftalatos (DEP, DiBP, DnBP, BBzP, DEHP y DiNP) y un plastificante no ftalato (DINCH®). A la edad de 7-10 años, se realizó la evaluación puberal de los niños y niñas mediante la escala de desarrollo puberal (PDS) cumplimentada por los padres. El análisis estadístico se efectuó utilizando regresión de Poisson con varianza robusta y se evaluó el papel modificador del IMC mediante análisis estratificado. El posible efecto de la co-exposición a los diferentes ftalatos se evaluó mediante regresión de suma de cuantiles ponderados (WQS).

Resultados: En niños, la exposición prenatal a DEHP se asoció con un mayor riesgo de haber iniciado la pubertad (riesgo relativo [RR]=1,32, IC95%=1,09-1,59 por cada aumento de una unidad logarítmica en las concentraciones de DEHP) y gonadarquia (RR=1,23, IC95%=1,00-1,50); en niñas, DEHP se asoció con mayor riesgo de adrenarquia (RR=1,25, IC95%=1,03-1,51). En niños, la exposición prenatal a DEP, DnBP y DEHP también se asoció con mayor riesgo de adrenarquia o gonadarquia (RRs=1,49-1,80) en aquellos con peso normal, y la exposición a BBzP y DINCH® con menor riesgo de adrenarquia (RR=0,49, IC95%=0,27-0,89 y RR=0,47, IC95%=0,24-0,90, respectivamente) en aquellos con sobrepeso/obesidad. En niñas, DiBP, DnBP y DINCH® se asociaron con un riesgo ligeramente mayor de gonadarquia (RR=1,14-1,19) en aquellas con

sobrepeso/obesidad. En los modelos WQS, no se observó asociación significativa entre la exposición combinada a ftalatos y el desarrollo puberal de niños y niñas.

Conclusión: La exposición prenatal a ciertos ftalatos se asoció con el desarrollo puberal a la edad de 7- 10 años, especialmente con una pubertad más temprana en niños con peso normal y niñas con sobrepeso/obesidad. Sin embargo, no se observó evidencia del efecto combinado de la mezcla de ftalatos.

Abstract

Background: Phthalates are widespread, anti-androgenic chemicals known to alter early development, with possible impact on puberty timing.

Aim: To investigate the association of prenatal phthalate exposure with pubertal development in boys and girls.

Methods: Urinary metabolites of six different phthalate diesters (DEP, DiBP, DnBP, BBzP, DEHP, and DiNP) and non-phthalate plasticizer DINCH® were quantified in two urine samples collected during pregnancy from mothers participating in the INMA Spanish cohort study. Pubertal assessment of their children at age 7-10 years (409 boys, 379 girls) was conducted using the parent-reported Pubertal Development Scale. Modified Poisson and Weighted Quantile Sum (WQS) regression was employed to examine associations between prenatal phthalates and risk of puberty onset, adrenarche, and gonadarche. Effect modification by child weight status was explored by stratified analysis.

Results: Prenatal exposure to DEHP was associated with higher risk of puberty onset (relative risk [RR]=1.32, 95% CI=1.09-1.59 per each log-unit increase in concentrations) and gonadarche (RR=1.23, 95% CI=1.00-1.50) in boys and higher risk of adrenarche (RR=1.25, 95% CI=1.03-1.51) in girls at age 7-10 years. In boys, prenatal exposure to DEP, DnBP, and DEHP was also associated with higher risk of adrenarche or gonadarche (RRs=1.49-1.80) in those with normal weight, and BBzP and DINCH® exposure with lower risk of adrenarche (RR=0.49, 95% CI=0.27-0.89 and RR=0.47, 95% CI=0.24-0.90, respectively) in those with overweight/obesity. In girls, DiBP, DnBP, and DINCH® were associated with slightly higher risk of gonadarche (RRs=1.14-1.19) in those with overweight/obesity. In the WQS model, the phthalate mixture was not associated with puberty in boys or girls.

Conclusion: Prenatal exposure to certain phthalates was associated with pubertal development at age 7-10 years, especially earlier puberty in boys with normal weight and girls with overweight/obesity. However, there was no evidence of effect of the phthalate mixture on advancing or delaying puberty in boys or girls.

Keywords

Phthalates; plasticizers; pregnancy; puberty; Pubertal Development Scale

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1. Introduction

Trend data suggest that age at puberty onset in girls has declined markedly during the past few decades and that pubertal timing may be altered in boys as well (Euling et al. 2008; Papadimitriou 2016). Hypothesized causes of these changes in pubertal timing include the increasing prevalence of childhood obesity and early-life exposure to endocrine disrupting chemicals (EDCs) ubiquitously present in food and the environment (Biro and Kiess 2016; Toppari and Juul 2010). EDCs may interfere with pubertal development by actions at different levels, including the neuroendocrine signals, the hypothalamic-pituitary axis, and peripheral target organs such as breast, hair follicles, and genitals (Parent et al. 2005). Children with early puberty are at higher risk for behavioral disorders and psychosocial difficulties during adolescence, early sexual debut, accelerated skeletal maturation and short adult height, and obesity, type 2 diabetes, cardiovascular disease, and endocrine-related cancers such as breast and testicular cancer in later life (Golub et al. 2008).

Phthalate diesters are a ubiquitous type of plasticizers used in the manufacture of a wide range of consumer products and as fragrance retainers (Hauser and Calafat 2005). They can be divided into low molecular weight (LMW) and high molecular weight (HMW) phthalates. HMW phthalates such as di-(2-ethylhexyl) phthalate (DEHP), butyl benzyl phthalate (BBzP), and di-iso-nonyl phthalate (DiNP) are commonly used in polyvinyl chloride (PVC) polymer and plastisol applications. They can be found in building materials, clothing, plastic toys, food packages, medical devices, among other consumer products (Wittassek et al. 2011). LMW phthalates such as di-ethyl phthalate (DEP), di-iso-butyl phthalate (DiBP), and di-n-butyl phthalate (DnBP) are often used as solvents in personal care products, and in adhesives, varnishes, and coatings (Wittassek et al. 2011). Restrictions on phthalates use have led to gradual replacement of these chemicals with several alternative substances such as di-iso-nonyl-cyclohexane-1,2-dicarboxylate (DINCH®), currently used in many consumer products (Tranfo et al. 2018). The widespread use of phthalates and non-phthalate plasticizers allows potentially high exposures to occur in the general population (Giovanoulis et al. 2018; Montazeri et al. 2019).

Phthalates are known EDCs that exert anti-androgenic activity *in vitro* and in animal studies by inhibition of steroidogenesis (Foster 2006; Hannas et al. 2011; Howdeshell et al. 2008; van den Driesche et al. 2011), although mechanisms of action of phthalates are complex and may also involve estrogen action and other mechanisms (Engel et al. 2018). There is a large body of data on the impact of human exposure to phthalates during early life and adulthood on reproductive development and function, including timing of pubertal

development (Basso et al. 2022; Mesquita et al. 2021; Radke et al. 2018). Thus, phthalate exposure has been associated with reduced anogenital distance, urogenital malformations, poorer sperm quality, and reduced testosterone levels in men (Mesquita et al. 2021; Radke et al. 2018); and with placental/umbilical dysfunction, adverse pregnancy outcomes (preterm delivery, low birth weight), ovarian insufficiency, endometriosis, hormone imbalance, and infertility in women (Basso et al. 2022; Mesquita et al. 2021). However, epidemiological studies on exposure to phthalates and pubertal development are still limited.

Though the mechanism of variations in pubertal timing caused in fetal life is not fully elucidated, the initiation and progression of puberty are under control of the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes, which are founded and matured during prenatal life (Kuiri-Hanninen et al. 2014). Thus, the prenatal environment may play a role in the timing of pubertal development. Animal studies have shown that *in utero* or early-life exposure to DEHP is associated with earlier ovarian development in female rats (Wang et al. 2016) and either earlier or later puberty in male rats (Andrade et al. 2006; Ge et al. 2007; Noriega et al. 2009). In humans, results of several cross-sectional studies examining childhood exposure to phthalates and puberty development have been largely inconsistent (Binder et al. 2018; Buttke et al. 2012; Frederiksen et al. 2012; Kasper-Sonnenberg et al. 2017; Mouritsen et al. 2013; Shi et al. 2015; Wolff et al. 2010; 2014; Zhang et al. 2015). Regarding *in utero* exposure, some studies found an association of certain phthalates with earlier puberty in girls (Cathey et al. 2020; Harley et al. 2019; Watkins et al. 2014; 2017a), while others showed an opposite or a null association (Berger et al. 2018; Berman et al. 2021a; Hart et al. 2014). In boys, one study reported earlier pubarche and gonadarche in relation to prenatal exposure to HMW phthalates (Berger et al. 2018) and two other studies found opposite or no association for different phthalates (Cathey et al. 2020; Harley et al. 2019).

The aim of this study is to investigate the association between prenatal exposure to several phthalates and pubertal development in boys and girls at 7-10 years of age. As some recent studies have demonstrated that the impact of phthalate exposure on pubertal development may be modified by child body mass index (BMI) (Berger et al. 2018; Binder et al. 2018; Shi et al. 2015), we further examined whether this association is modified by weight status.

2. Materials and Methods

2.1.Study population

Mother-child pairs included in the present study belong to the Environment and Childhood (INMA) Project, a multicenter

population-based birth cohort study designed to investigate the effect of environmental exposures and diet during pregnancy on fetal and child development in different geographic areas of Spain (http://www.proyectoinma.org). Recruitment and general characteristics of the INMA cohorts have been described in detail previously (Guxens et al. 2012). Assessment of maternal urinary phthalate biomarkers was performed in Gipuzkoa, Sabadell, and Valencia INMA cohorts, which were recruited during the first prenatal visit in the period 2003-2008. A total of 1298 children in these three cohorts underwent puberty development assessment at 7-10 years of age (range, Gipuzkoa: 7.6-8.5 years; Sabadell: 7.8-10.7 years; Valencia: 8.5-9.8 years). These constituted 57.9% of the babies born to women originally included in these cohorts (n=2241). The present study included 788 (409 boys, 379 girls) of these children for whom data were available on prenatal phthalate exposure and relevant covariates (Figure 1). No significant differences in characteristics of study participants were found between those included in the current study and those with data on puberty status at age 7-10 years but not on prenatal phthalate exposure or covariates (n=510), with the exception of a higher percentage of children from Sabadell and a lower percentage of children from Valencia in the included *versus* excluded children (Supplementary material, Table S1). Informed consent was obtained from all participants for each phase, and the research protocol was approved by the Ethic Committees of each region: CEIC Euskadi in Gipuzkoa (number 11/2013), CEIC Parc de Salut MAR in Sabadell (number 2005/2106/l), and CEIC DGSP-CSISP in Valencia (number 20131007).

2.2.Phthalate exposure assessment

Fasting spot urine samples were collected from mothers at 1st and 3rd (mean ± standard deviation= 33.1 ± 2.0 weeks) trimester of pregnancy. Phthalate metabolites were measured in the two samples collected during pregnancy for mothers in Sabadell and in pooled samples (1st and 3rd trimester) for mothers in Valencia and Gipuzkoa. For Sabadell, the average of the two urine pregnancy samples was used as biomarker of *in utero* phthalate exposure.

Urinary total concentrations (*i.e.* free and glucuronoconjugated species) of eight phthalate diester metabolites, corresponding to three LMW (<250 g/mol) phthalates (DEP, DiBP, DnBP) and two HMW (>250 g/mol) phthalates (BBzP and DEHP) (Table 1), were measured in all urine samples from the 788 mothers in the three cohorts (*i.e.*, total sample). Samples from Sabadell were determined by the Bioanalysis Research Group at the Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain) by ultra-performance liquid chromatography coupled to tandem mass spectrometry according to Valvi et al. (2015).

Samples from Valencia and Gipuzkoa were determined at the Norwegian Institute of Public Health (NIPH) (Oslo, Norway) by liquid chromatography coupled to mass spectrometry (Sabaredzovic et al. 2015). The limit of detection (LOD) ranged from 0.07 to 1.0 μg/L (Table 1). For comparability, we analyzed 10 samples with low to high MEP concentrations as reference selected from all Sabadell samples analyzed in Barcelona at the NIHP (Tamayo-Uria et al. 2019). NIPH was blinded to the concentrations of samples. Urinary concentrations of the phthalate metabolites determined in both laboratories were highly correlated (Spearman ranging from r=0.69 to 0.97). Due to the high correlations, the NIPH concentrations for subjects included in the comparison have been used. Maternal urine from Gipuzkoa and Valencia (*i.e.*, sub-sample of 460 pooled urines) was additionally analyzed for the concentrations of DiNP and DINCH® metabolites at the NIPH (Sabaredzovic et al. 2015), and their LODs were between 0.2 and 0.7 μg/L (Table 1). Urine creatinine was measured using the Jaffé method with Beckman Coulter© reactive in AU5400 (IZASA®) at the Echevarne Laboratory in Barcelona, Spain. The mean of maternal urinary creatinine between 1st and 3rd trimester was used to account for urine dilution of all samples.

2.3.Puberty assessment

Puberty assessment was conducted at a mean age of 8.7 years (range=7.6-10.7 years). In general, the normal age limit for the appearance of secondary sexual characteristics in Caucasian girls and boys from developing countries is 8 and 9 years, respectively. Parents (in most of the cases mothers, 97%) rated pubertal status of their children using the Peterson's Pubertal Development Scale (PDS), a commonly used measure developed as an alternative to physician rating measures (Petersen et al. 1988). The PDS may be a good alternative to physical examinations in epidemiological studies due to higher participation rates and lower costs but this instrument may not overcome the advantage of physical examinations (Carskadon and Acebo 1993). The PDS consists of 5 questions, three of which are common for both sexes and refer to growth spurt in height, appearance of pubic hair, and skin changes (pimples); and 2 specific questions on facial hair growth and deepening of the voice in boys, and breast development and menarche in girls. Answers are rated on a 4-point scale from 1 (no development initiated) to 4 (development seems complete). Following the criteria proposed by Carskadon and Acebo (1993) and Shirtcliff et al. (2009), the continuous score obtained from the PDS was converted to a 5-point ordinal scale, *i.e.*: 1-pre-puberty, 2-early puberty, 3-midpuberty, 4-late puberty, and 5-post-puberty. Carskadon and Acebo (1993) algorithm calculates the pubertal development

using 3 questions of this scale, including growth of body hair and the two questions specific for each sex. Shirtcliff et al. (2009) algorithm uses all the 5 indicators of sexual development of the PDS and differentially captures gonadal and adrenal signals of physical development. In girls, breast development, menarche, and growth spurt are associated with gonadal hormonal signals. In boys, testicular enlargement, growth spurt, deepening of voice, and facial hair growth are associated with gonadal hormones. For both sexes, pubic/body hair and skin changes are associated with adrenal hormones (Shirtcliff et al. 2009). In this study, three different outcomes were examined: overall pubertal development (based on Carskadon and Acebo algorithm); adrenal development or adrenarche, and gonadal development or gonadarche (based on Shirtcliff et al. algorithm), all categorized into stage 1 (pre-puberty) or stage 2+ (or having started puberty).

2.4.Covariates

Data on sociodemographic and lifestyle factors was obtained from interviewer-based questionnaires administered to the parents during the 1st and 3rd trimesters of pregnancy and at follow up visits. Maternal covariates included in this study were: age at delivery (years), educational attainment during pregnancy (up to primary/secondary/university schooling), parity at delivery (none/1/2 or more), place of residence during pregnancy (urban/rural), smoking during pregnancy (yes/no), passive smoking during pregnancy (yes/no), working during pregnancy (yes/no), and pre-pregnancy BMI. Pre-pregnancy BMI was obtained by dividing self-reported pre-pregnancy weight in kg by the square of the height measured at the 1st trimester (kg/m^2). Selected child covariates included: child age at puberty assessment (years), mother living with child's father (yes/no), and child weight status at 7-10 years of age (normal weight/overweight/obese). Weight status was obtained by converting child BMI to z-score for age and sex based on World Health Organization (WHO) reference curves for children (5-19 years) (WHO, 2007) and then grouping into normal weight (±1 standard deviation [SD]) and overweight/obese (>+1 SD, equivalent to BMI≥25 kg/m^2 at 19 years). Geographical site or cohort (Gipuzkoa/Sabadell/Valencia) was also included as a covariate. More than 98% of mothers were white and thus mother's ethnicity was not examined.

2.5. Statistical analysis

All phthalate diester metabolites were detected in more than 99% of the urine samples, while DINCH® metabolites were detected in 83-94% of the sub-sample of 460 pooled urines from Gipuzkoa and Valencia. Analytical results below the method LOD were substituted with the respective LOD value

divided by 2. To simplify the data analysis, we summed the concentrations of individual metabolite of DEHP, DiNP, and DINCH® instead of evaluating separately the concentrations of each metabolite measured. The sum was created based on molar concentrations by dividing each metabolite concentration by its molecular weight and then summing, *e.g.*, ∑DEHP= (MEHP/278.34) + (5OH-MEHP/294.35) + (5oxo-MEHP/292.33) + (5cx-MEPP/308.33) + (2cx-MMHP/338.31). The sum of concentrations (∑DEHPm) was expressed in µg/L by multiplying the molar sums with the molecular weight of their respective parent compound. For univariate analyses, we calculated geometric means (GM) and selected percentiles of urinary concentrations of phthalate biomarkers, which were left skewed and therefore natural-log transformed (ln-transformed) prior to analysis to approximate a normal distribution. We reported descriptive statistics for phthalate biomarkers without creatinine correction, participants' characteristics, and pubertal development (stage 2+) for boys and girls separately. Puberty status and phthalate biomarkers by cohort is also reported. Spearman correlations were calculated to evaluate the relationship between phthalate biomarkers.

The association between individual phthalate biomarkers and puberty onset (being in stage 2+) was assessed by estimating relative risk (RR) using Poisson regression with robust error variance (Zou 2004). Each urinary phthalate metabolite or grouping was modeled separately with each outcome (*i.e.*, puberty, adrenarche, and gonadarche), separately for boys and girls (sex-stratified models). Crude and adjusted Poisson regression models were employed using ln-transformed phthalate biomarkers. Adjusted models included the following covariates as independent variables: 1) basic model: maternal mean urinary creatinine (ln-transformed), cohort, and child age at puberty assessment (included *a priori*); 2) fully-adjusted model: additionally adjusted for mother's age, education, pre-pregnancy BMI, parity, and smoking during pregnancy. To control for urine dilution variability and reduce potential error bias, urinary creatinine was included in models as a separate covariate as recommended in previous studies (Barr et al. 2005; O'Brien et al. 2016). The remaining covariates were chosen based on prior literature on prenatal phthalate exposure and puberty timing (Berger et al. 2018; Ferguson et al. 2014; Harley et al. 2019) (*i.e.*, child age, mother's education, and pre-pregnancy BMI) and the 10% change-in-estimate criterion (*i.e.*, mother's age, parity, and smoking during pregnancy). Phthalate biomarkers determined in the total sample of boys and girls were subsequently categorized into quartiles using the first quartile as the referent category, and p-values for trend of increasing risk across quartiles were calculated using a likelihood

ratio test. Results are presented as the risk of puberty onset (stage 2+) with 95% confidence interval (CI) per each unit of log-transformed urinary metabolite concentrations increase or for 2nd, 3rd, or 4th *versus* 1st quartile of concentrations.

As BMI is a strong predictor of puberty onset (Marcovecchio and Chiarelli 2013; Reinehr and Roth 2019), we examined the potential effect modification by child weight status through stratification (normal weight *vs.* overweight/obese) of single exposure models and interaction models with cross-product terms for metabolites x weight status. Child weight status was not included as a covariate in the models because it may be on the causal pathway (Berman et al. 2021b; Harley et al. 2017), but we further conducted sensitivity analysis including weight status (normal weight *vs.* overweight/obese) in the models to explore whether results changed substantially from those of the main analyses, possibly reflecting mediation.

Because simultaneous exposure to multiple EDCs may be harmful even when individual exposures are below observable effect levels (Kortenkamp 2014), the association of the phthalate mixture with pubertal development was examined with Weighted Quantile Sum (WQS) Regression (Carrico et al. 2015), using the same covariates as those used in logistic regression, including sensitivity analysis adjusting for weight status. WQS index is regressed from multivariate model which constructs the unidirectional weighted index from quantiled chemical exposure variables, thus reducing potential multicollinearity and dimensionality while providing an overall mixture effect estimate (Tanner et al. 2020). Weights are expressed as percentages that sum to one and indicate the relative strength of each compound within the mixture. Phthalate biomarkers available for all study participants (*i.e.,* DEP, DiBP, DnBP, BBzP, and DEHP), previously ln-transformed, were binned as quartiles. Based on the expectation that *in utero* exposure to anti-androgenic or estrogenic chemicals would be related to delayed puberty in boys and earlier puberty in girls, WQS index was first constructed using weights as positive when analyzing the mixture effect among girls and as negative when analyzing the effect among boys. However, results from epidemiological studies have been inconsistent, showing phthalate exposure associated with either earlier or later puberty in boys and girls. Therefore, index was further constructed using weights as positive in boys and as negative in girls. The chemical of concern threshold was set to a weight of 20%, a value consistent with equal weighting (100%/5 phthalate biomarkers). Finally, to evaluate the stability and generalizability of our results, repeated holdout validation was performed. This method combines cross-validation and bootstrap resampling by splitting data into 40-60% training test sets and repeating WQS

regression 100 times as previously performed in other environmental epidemiological studies (Galbán-Velázquez et al. 2021; Tanner et al. 2020). We expressed estimates as the risk of being in stage 2+ per inter-quartile-range (IQR) increase in the WQS index with 95% CI. The significance level was set at 0.05. Data analyses were conducted in R v. 4.1.2 (R Core Team, 2018) using the glm2, lmtest, and gWQS (Renzetti et al. 2018) packages and SPSS v.26 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp).

3. Results

More than 50% of the mothers in this sample had secondary or university education, were primiparous, resided in urban areas, did not smoke but were passive smokers during pregnancy, worked throughout the pregnancy, and were overweight or obese before pregnancy. At age 7-10 years, children were predominantly normal-weight and lived with their mother and father. Among boys, 26.4% had started puberty, 24.4% were in adrenal stage 2+, and 10.8% in gonadal stage 2+. Among girls, 43.8% had started puberty, 31.9% were in adrenal stage 2+, and 40.4% in gonadal stage 2+ (Table 2). Only 1.2% and 5.6% of boys were in stages 3-4 of adrenal and gonadal development, respectively, *versus* 8.5% and 10.6% of girls (Table 2). Pubertal status differed by cohort, with older cohorts (Sabadell and Valencia) having a greater proportion of children in stage 2+, particularly girls; however, the proportion of children having reached gonadarche was greater in Gipuzkoa (Table S2).

Regarding phthalate biomarkers, MEP had by far the highest urinary concentration (50^{th} percentile [P50]=185.42 µg/L), followed by ∑DEHPm (P50=98.56 µg/L), MiBP (P50=26.40 µg/L), MnBP (P50=24.25 µg/L), ∑DiNPm (P50=19.91 µg/L), and MBzP (P50=10.14 µg/L). Median concentration for the sum of DINCH® metabolites was 1.27 µg/L (Table 3). Descriptive analysis by cohort showed that mothers in Gipuzkoa had lower urinary MEP, MnBP, DEHP, and DiNP concentration, while mothers in Sabadell had higher MEP and MBzP (Table S2). Moderate positive correlations (Spearman coefficient, r=0.40-0.59, p<0.001) were observed between phthalate biomarkers, while ∑DINCHm was negatively correlated with MEP (r=-0.12, p<0.05) and MnBP (r=-0.16, p<0.05), and positively only with MiBP (r=0.26, p<0.001) (Table S3).

Basic and fully-adjusted models for the association between single phthalate biomarkers and pubertal development showed similar results, but the fully-adjusted model slightly attenuated or strengthen some RRs (Table 4). In sex-stratified models, DEHP exposure was associated with higher risk of having started puberty both in boys and girls. Specifically, higher maternal urinary ∑DEHPm concentrations were associated with higher risk of puberty onset (RR=1.32, 95%

CI=1.09; 1.59 per each unit of log-transformed urinary phthalate biomarker increase) and gonadarche (RR=1.23, 95% CI=1.00-1.50) in boys and higher risk of adrenarche (RR=1.25, 95% CI=1.03-1.51) in girls. After stratification of the phthalate biomarkers into quartiles (Table S4), boys in the 4th *versus* 1st quartile of ∑DEHPm showed a higher risk of puberty onset (RR=1.94, 95% CI=1.05-3.59), but a trend toward higher risk across ∑DEHPm quartiles was not observed. Girls in the 3rd and 4th quartile of ∑DEHPm were at higher risk of adrenarche (RR=1.59, 95% CI=1.00-2.52 and RR=1.72, 95% CI=1.03-2.86, p-trend=0.06). Associations of MiBP and MnBP with higher risk of gonadarche in girls were not evident in the analysis based on quartiles, while some further associations were suggested in boys, including higher risk of adrenarche in relation to increasing MEP (p-trend=0.19) and MiBP (p-trend=0.01) and lower risk of adrenarche in relation to increasing MBzP (RR=0.34, 95% CI=0.14-0.83 for boys in the 4th *vs.* 1st quartile, p-trend=0.02).

Table 5 shows that associations with earlier puberty in boys were observed among those with normal weight. Thus, ∑DEHPm and MnBP as well as MEP were significantly associated with higher risk of gonadarche (∑DEHPm) and adrenarche (MnBP and MEP) (RRs=1.49-1.80) only in normal-weight boys. Conversely, MBzP and ∑DINCHm were associated with lower risk of adrenarche (RR=0.49, 95% CI=0.27-0.89 and RR=0.47, 95% CI=0.24-0.90, respectively) only in boys with overweight/obesity. In girls, MiBP, MnBP, and ∑DINCHm were associated with slightly higher risk of gonadarche (RRs=1.14-1.19) in those with overweight/obesity, whereas the association between ∑DEHPm and adrenarche no longer remained significant after stratification. Interaction terms were significant for MEP and adrenarche in boys (*Pinteraction*=0.03), ∑DEHPm and gonadarche in boys (*Pinteraction*=0.008), and for MnBP and adrenarche in girls (*Pinteraction*=0.03). Inclusion of child BMI as a covariate in the main models led to a significant association between MEP and lower risk of puberty (RR=0.88, 95% CI=0.80-0.97) in girls, while associations with ∑DEHPm (Table 4) remained unchanged in boys and girls (Table S5).

The mixture effect estimates of the WQS models were not statistically significant and RRs were mostly close to 1 either in models using weights as negative in boys and as positive in girls (Fig. 2a) or using weight as positive in boys and as negative in girls (Fig. 2b). Sensitivity analysis including weight status in the WQS models showed similar results (data not shown).

4. Discussion

In this study, higher prenatal concentrations of urinary metabolites of certain phthalates, including DiBP, DnBP and DEHP, were associated with a slight increase

in the risk of puberty onset at age 7-10 years, especially in boys with normal weight and girls with overweight/obesity. There were some exceptions: prenatal BBzP and DINCH® were associated with lower risk of adrenarche in boys with overweight/obesity and DEP with lower risk of puberty in all girls. Associations between DEHP and earlier puberty in boys and girls, and between DEP and delayed puberty in girls were independent of child weight status. However, results of the mixture effect model did not provide clear evidence that combined exposure to phthalates is associated with puberty development on boys or girls at this age.

The observed association between maternal ∑DEHPm and higher risk of overall puberty onset and gonadarche in boys is not consistent with exposure to anti-androgenic or estrogenic compounds but is supported by results from the CHAMACOS (California) and ELEMENT (Mexico) cohorts showing associations between prenatal DEHP exposure and earlier gonadarche and pubarche (Berger et al. 2018) and increased testicular volume in boys (Ferguson et al. 2014). As in our study, these associations were independent of child BMI, suggesting that they are not mediated by child weight status. However, in contrast to our study, associations in the CHAMACOS cohort were seen only in boys with overweight/obesity (Berger et al. 2018). In the present study, there were more boys with normal weight than overweight/obesity (229 *vs.* 180), which may increase power to detect effects. However, most of the RRs were above 1 in boys with normal weight and RRs were generally below 1 in those with overweight/obesity, suggesting that difference in results between groups is not explained by difference in sample size. Scientific literature shows that the association between BMI and puberty in boys is less clear than in girls (Burt Solorzano and McCartney 2010), and whether the effect of prenatal phthalate exposure on pubertal development is stronger in boys with normal weight remains to be studied further.

Associations of MEP and MnBP with higher risk of adrenarche in boys are not supported by previous findings of delayed pubarche or lack of association with puberty development in relation to prenatal DEP and DnBP exposure (Cathey et al. 2020; Ferguson et al. 2014; Harley et al. 2019). Our results indicate a possible mediation effect of BMI with regard to these two phthalates, which is consistent with the previous observation of an association between maternal urinary MEP and MnBP and risk of obesity in boys (Harley et al. 2017). Among boys with overweight/obesity, MBzP and ∑DINCHm were associated with delayed adrenarche, which may be consistent with an anti-androgenic action *in utero* (Andrade et al. 2006). These associations were not seen in models adjusted for BMI, again implying the possibility of a mediation effect. Previous cohort studies partially support our finding

regarding MBzP: in models adjusted for BMI, prenatal MBzP was associated with delayed pubarche in Mexican boys (Ferguson et al. 2014; Watkins et al. 2017b) and California boys (Berger et al. 2018), but in the latter study associations were observed only in boys with normal weight. However, further studies are needed to rule out that some of our findings could be chance findings.

In general, associations seen in girls were weaker than in boys. Nonetheless, our results suggest an increase in the risk of adrenarche with increasing prenatal ∑DEHPm which is consistent with the expectation that pubertal development in girls would be accelerated by estrogenic or anti-androgenic chemicals. Our finding is supported by data from the Mexican cohort showing an association between prenatal DEHP (specifically MEHP) and earlier pubarche in girls (Watkins et al. 2014; 2017a). Maternal concentrations of DEHP metabolites in the Mexican study were in the range of ours but MEHP was also associated with later thelarche (Watkins et al. 2014; 2017a). By contrast, maternal urinary DEHP was associated with later thelarche and menarche in California girls (Berger et al. 2018). Our result is also supported by animal studies showing that early-life exposure to DEHP leads to earlier puberty in female rats (Losa-Ward et al. 2012; Ma et al. 2006; Wang et al. 2016); however, that DEHP exposure is positively associated with adrenarche but not gonadarche in girls remains unclear. As observed in boys, prenatal DEHP exposure was associated with earlier adrenarche in girls regardless of their BMI, which is not in agreement with previous data demonstrating a link between prenatal DEHP and risk of obesity in girls (Harley et al. 2017).

To our best knowledge, this is the first study to report an association of prenatal exposure to DiBP, DnBP, and DINCH® with puberty in girls. Thus, maternal urinary or serum MiBP and MnBP were not associated with thelarche, menarche, or pubarche timing among girls (Harley et al. 2019; Hart et al. 2014; Watkins et al. 2014; 2017a), while no previous study examined the impact of DINCH® exposure on pubertal development. DnBP and its metabolite MnBP have demonstrated to exert anti-androgenic effects by altering steroid hormone biosynthesis *via* inhibition of steroidogenic acute regulatory (StAR) protein expression (Wang et al. 2007) and, in line with our findings, a study of female rats found developmental DnBP exposure to be associated with earlier pubertal onset (Hu et al. 2013). For its part, DiBP exerted anti-androgenic and adverse reproductive effects in various animal studies (Yost et al. 2019), and the non-phthalate plasticizer DINCH® has been recently shown to interfere with the human estrogen and androgen receptors (Engel et al. 2018). In the present study, MiBP and MnBP were associated with higher risk of gonadarche only in girls with overweight/obesity and this association

appeared to be mediated, at least in part, by BMI. Several phthalates, including the metabolites of DnBP, have been shown to promote adipogenesis (Desvergne et al. 2009), supporting a possible mediation by weight status suggested in this study. However, prenatal MnBP was not associated with BMI and body fat in girls in the CHAMACOS cohort (Harley et al. 2017). On the other hand, it has been hypothesized that phthalate exposure effects may be more apparent in girls with overweight/obesity, as overweight may compromise assessment of pubertal stage, particularly early breast stages (Wolff et al. 2014). Indeed, the only previous study that examined interaction between BMI and prenatal phthalates found that MBzP and ∑DEHP were associated with later puberty only in normal-weight girls (Berger et al. 2018), whereas a cross-sectional study found an association between DEHP exposure and earlier breast development in Chinese girls, particularly in those with higher body fat (Shi et al. 2015). A likely explanation to the positive association between MiBP and MnBP and gonadarche seen only in the group of girls with overweight/obesity is that prenatal exposure to DiBP and DnBP may lead to both childhood obesity and earlier puberty. Alternative explanations such as sample size and other statistical issues are unlike given that there are fewer overweight than normal-weight girls in our sample and general characteristics of our children do not differ between weight strata (data not shown).

Unlike ∑DEHP and MnBP, MEP was associated with reduced risk of puberty in girls, and this association was independent of their weight status. Conversely, in other cohorts, maternal urinary MEP was associated with earlier menarche (Watkins et al. 2014; 2017a) and pubarche in girls (Harley et al. 2019). Studies on childhood exposure found associations with earlier thelarche and pubarche (Frederiksen et al. 2012; Wolff et al. 2014), and only one cross-sectional study in Germany using the PDS and adjusting their models for BMI found similar findings to ours (Kasper-Sonnenberg et al. 2017). There is the possibility that the result for MEP in this study is due to chance, as it differs from results for other phthalates and analysis based on quartiles of exposure did not suggest any trend towards later puberty with increasing MEP.

Overall, previous epidemiological studies on prenatal phthalate exposure and puberty timing in boys or girls have been largely inconsistent. Such inconsistencies may be attributed to several factors, including timing of exposure (*i.e.*, exposure measure in prenatal studies may reflect a more susceptible window of exposure), child age, exposure profile, other factors related to puberty timing, such as ethnicity, epigenetics, nutrition, physical activity, and psychological factors (Biro et al. 2006), and other prenatal and/or exposures early in life. It should be also borne

in mind that phthalates and their monoester metabolites can act through complex mechanisms, which include activation or inactivation of the steroid hormone receptors (*i.e.*, estrogen receptors ERα and ERβ, and the androgen receptor) and non-androgen receptors such as the peroxisome proliferator-activated receptors PPARα and PPARγ (Benjamin et al. 2017; Engel et al. 2017; Hurst and Waxman 2003). Together with the fact that several other chemicals may target similar pathways (Søeborg et al. 2012), this makes interpretation of effects in humans very difficult. In this study, the phthalate mixture was not associated with pubertal development in boys or girls, suggesting that other factors in childhood could be more important than prenatal exposures.

Additionally, the variety of outcome measures may also explain the differences in the results of epidemiological studies. In this study, puberty assessment was performed using the PDS instead of the Tanner scale, considered the "gold standard" for assessing pubertal status. PDS is not a complete estimation of Tanner stages, and no direct questions about pubic hair are included. Moreover, the PDS is limited especially because premature adrenarche by parents often is mistaken as signs of true puberty (Koopman-Verhoeff et al. 2020). However, the PDS has been validated in previous studies, including the INMA-Valencia cohort, and their results were in moderate to good agreement with the physical examination according to Tanner stages (Beneito et al. 2017; Bond et al. 2006; Koopman-Verhoeff et al. 2020). Koopman-Verhoeff et al. (2020) showed that both self-reported and parent-reported PDS have strong internal consistency and test-retest reliability and indicated that, although the PDS categories do not provide a one-to-one translation to the 5-point Tanner scale, it may adequately differentiate between pre-, mid-, and post-pubertal categories. There may be still some error in the pubertal stage assessment, but errors in outcome are likely to be non-differential.

There are several other limitations to the current study. Phthalates are quickly metabolized and two urinary measurements during pregnancy may not accurately reflect usual exposure. In addition, phthalate exposure pattern may vary by trimester of pregnancy (Li et al. 2019). Nonetheless, urinary metabolites of LMW phthalates (*e.g.,* DEP) have shown high reliability in pregnant women from the INMA study, indicating that the use of personal care products by these women is less variable throughout the pregnancy, while reliability of HMW phthalates present in PVC products (*e.g.*, DEHP) was low, indicating changes in diet or other habits (Casas et al. 2018). On the other hand, estimates obtained from regression models are potentially subject to selection bias, as the proportion of children from Sabadell was higher compared to the original cohort and exposure and outcome

measures varied by cohort (Table S2). However, loss to follow up or missing phthalate data is unlikely related to the exposure or outcome. Our study may be also limited by the age range, particularly in boys, as we could not assess the entire pubertal period. In Europe, the mean age at breast development onset (stage B2) in girls is 10.5 years and the mean age at genital development onset in boys (stage G2) is 11 years (Brix et al. 2019; Marco-Hernández et al. 2008). However, this study was focused on puberty onset and there was a relatively high proportion of children (*i.e.*, 11-44%) in stage 2+ based on the PDS. Furthermore, trimester-specific associations could not be assessed due to limited number of mothers with exposure data in the 1st and 3rd trimesters of pregnancy. Information on the nature and magnitude of postnatal exposure was not available, which may add to prenatal exposure, and we cannot exclude or confirm a confounding effect by other co-exposures which is a major challenge for future epidemiological studies. Additional limitations are that data on DiNP and DINCH® exposure were only available for 236 boys and 224 girls, and other common phthalates, including di-n-octyl phthalate (DnOP) and di-iso-decyl phthalate (DiDP), were not measured.

Strengths of this study include a longitudinal design, a relatively large sample size, inclusion of three different geographical locations, and performance of mixture effect analysis. Further investigation is needed to estimate single chemical effect estimates, examine interactions between chemicals, and identify relevant biological pathways.

5. Conclusions

In conclusion, we found that prenatal exposure to certain phthalates, including DiBP, DnBP and DEHP, was associated with pubertal development at age 7-10 years, especially earlier puberty in boys with normal weight and girls with overweight/obesity. The clinical relevance of these results are uncertain, but they represent a potential cause of concern, due to widespread exposure to phthalates in the general population and the possibility that even subtle alterations of pubertal timing may increase the risk of adverse health effects during adolescence and later in life (Golub et al. 2008). Our results suggest that childhood obesity may be an effect modifier in the association between prenatal phthalate exposure and puberty development. However, there was no clear evidence of effect of the phthalate mixture on advancing or delaying puberty in boys or girls, suggesting that other factors in childhood could be more important than prenatal exposure. Given the inconsistency of existing data, further research should be conducted in different cultural settings.

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Highlights

MEP, MnBP, and ∑DEHP were associated with earlier puberty in normal weight boys.

∑DEHP was associated with earlier adrenarche in girls regardless of their BMI.

MBzP and ∑DINCH were associated with delayed adrenarche in overweight/obese boys.

MiBP, MnBP, and ∑DINCH were associated with earlier gonadarche in overweight girls.

Child obesity modifies the association of prenatal phthalate exposure with puberty.

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Table 1. Phthalate diesters, phthalate substitute DINCH®, and their respective metabolites

Grouping	Parent compound	Abbreviation	Human urinary metabolite	Abbreviation	LOD (ng/mL)	Molecular weight (g/mol)
LMW phthalates	Di-ethyl phthalate	DEP	Mono-ethyl phthalate	MEP	0.2-1.0	194.19
	Di-iso-butyl phthalate	DiBP	Mono-iso-butyl phthalate	MiBP	0.2-0.5	222.24
	Di-n-butyl phthalate	DnBP	Mono-n-butyl phthalate	MnBP	0.2-1.0	222.24
HMW phthalates	Butylbenzyl phthalate	BBzP	Mono-benzyl phthalate	MBzP	0.07-0.5	256.25
	Di-(2-ethyl-hexyl) phthalate	DEHP	Mono-(2-ethyl-hexyl) phthalate	MEHP	0.2-1.0	278.34
			Mono-(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP	0.2-0.5	294.35
			Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP	0.2-0.5	292.33
			Mono-(2-ethyl-5-carboxypentyl) phthalate	MECPP	0.7-1.0	308.33
	Di-iso-nonyl phthalate	DiNP	Mono-hydroxy-iso-nonyl phthalate	OH-MiNP	0.2	308.37
			Mono-oxo-iso-nonyl phthalate	oxo-MiNP	0.2	306.36
			Mono-carboxy-iso-octyl phthalate	cx-MiNP	0.7	322.35
Non-phthalate plasticizers	Di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate	DINCH®	Cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester	OH-MINCH	0.2	318.39
			Cyclohexane-1,2-dicarboxylate-mono-(oxo-isononyl) ester	oxo-MINCH	0.2	312.40

LMW: Low molecular weight (<250 g/mol); HMW: High molecular weight (>250 g/mol).

Table 2. Characteristics of 788 mother-child pairs in the INMA Project, Spain.

Characteristics	n (%) or mean ± SD		
	All	Boys (N=409)	Girls (N=379)
Cohort (age range)			
Gipuzkoa (7.6-8.5 years)	231 (29.3)	117 (28.6)	114 (30.1)
Sabadell (7.8-10.7 years)	328 (41.6)	173 (42.3)	155 (40.9)
Valencia (8.5-9.8 years)	229 (29.1)	119 (29.1)	110 (29.0)
Mother's age at delivery (years)	32.2 ± 3.7	32.30 ± 3.70	32.16 ± 3.71
Mother's educational attainment during pregnancy			
Up to primary	170 (21.6)	91 (22.2)	79 (20.8)
Secondary	306 (38.8)	158 (38.6)	148 (39.1)
University	312 (39.6)	160 (39.1)	152 (40.1)
Parity at delivery			
None	451 (57.2)	231 (56.5)	220 (58.0)
1	291 (36.9)	155 (37.9)	136 (35.9)
2 or more	46 (5.8)	23 (5.6)	23 (6.1)
Mother's urban residence during pregnancy=yes	636 (80.7)	329 (80.4)	307 (81.0)
Mother's smoking during pregnancy=yes	218 (27.7)	109 (26.6)	109 (28.7)
Mother's passive smoking during pregnancy=yes	494 (62.7)	258 (63.9)	236 (62.9)
Mother's working during pregnancy=yes	705 (89.5)	353 (86.3)	352 (92.9)
Maternal pre-pregnancy BMI (kg/m^2)	23.5 ± 4.3	23.2 ± 4.1	23.3 ± 4.1
<18.5	38 (4.8)	22 (5.4)	16 (4.2)
18.5-24.9	546 (69.3)	277 (67.7)	269 (71.0)
>24.9	204 (25.9)	110 (26.9)	94 (24.8)
Maternal urinary creatinine (g/L)	0.93 ± 0.36	0.94 ± 0.35	0.91 ± 0.36
Child age at puberty assessment (years)	8.70 ± 0.74	8.71 ± 0.73	8.70 ± 0.75
Mother lives with child's father at 7-10 years of age=yes	781 (99.1)	405 (99.0)	376 (99.2)
Child weight status at 7-10 years of age[a]			
Normal weight	456 (57.9)	229 (56.0)	227 (59.9)
Overweight/obese	332 (42.1)	180 (44.0)	152 (40.1)
Pubertal development at 7-10 years of age[b]			
Overall puberty onset (PDS 2+)	274 (34.8)	108 (26.4)	166 (43.8)
Stage 1	514 (65.2)	301 (73.6)	213 (56.2)
Stage 2	195 (24.8)	103 (25.2)	92 (24.2)
Stage 3	78 (9.9)	5 (1.2)	73 (19.3)
Stage 4	1 (0.1)	0 (0)	1 (0.3)
Adrenarche (PDS adrenal 2+)	165 (20.9)	44 (10.8)	121 (31.9)
Stage 1	623 (79.1)	365 (89.2)	258 (68.1)
Stage 2	128 (16.2)	39 (9.5)	89 (23.5)
Stage 3	32 (4.1)	4 (1.0)	28 (7.4)
Stage 4	5 (0.6)	1 (0.2)	4 (1.1)
Gonadarche (PDS gonadal 2+)	253 (32.1)	100 (24.4)	153 (40.4)
Stage 1	535 (67.9)	309 (75.6)	226 (59.6)
Stage 2	190 (24.1)	77 (18.8)	113 (29.8)
Stage 3	63 (8.0)	23 (5.6)	40 (10.6)
Stage 4	0 (0)	0 (0)	0 (0)

[a]Obtained by converting BMI to z-score for age and sex based on WHO reference curves for children (5-19 years): normal weight (±1 standard deviation), overweight/obese (>+1 standard deviation, equivalent to BMI≥25 kg/m^2 at 19 years)

[b]Based on the Petersen's Pubertal Development Scale (PDS): overall pubertal development (based on Carskadon and Acebo algorithm); adrenal and gonadal development (based on Shirtcliff et al. algorithm)

Table 3. Distribution of maternal urinary concentrations (μg/L) of phthalate metabolites (pooled samples or average of two urine samples collected during pregnancy)

Phthalate metabolites	Boys								Girls							
	%>LOD	GM	Percentiles						%>LOD	GM	Percentiles					
			10	25	50	75	90	95			10	25	50	75	90	95
Total sample[a]					N=409								N=379			
MEP	100	198.24	50.47	92.43	187.59	413.64	920.79	1269.82	100	193.52	45.16	82.17	183.26	433.98	921.59	1456.75
MiBP	100	27.55	9.76	16.87	27.23	43.27	69.89	97.66	100	26.44	9.90	16.72	25.57	43.36	64.59	86.55
MnBP	99.5	25.47	7.88	12.82	23.64	39.71	96.78	205.09	99.1	26.12	6.96	13.12	24.86	43.61	98.09	251.18
MBzP	99.7	10.29	2.54	4.81	10.68	22.06	30.09	57.77	99.7	10.12	2.41	4.39	9.61	22.15	41.21	62.20
MEHP	100	7.29	2.43	4.05	7.37	13.14	20.98	26.10	100	6.61	2.02	3.98	6.80	11.51	21.60	28.93
MEHHP	100	21.77	7.72	11.64	21.34	37.90	59.58	73.90	100	20.32	6.36	11.84	19.94	35.32	59.50	79.55
MEOHP	100	14.86	5.24	8.45	14.43	25.37	37.95	48.04	100	13.88	4.41	8.25	13.88	23.90	39.05	53.25
MECPP	100	33.12	13.85	20.48	30.86	48.82	77.83	121.90	99.7	31.09	11.91	18.53	29.83	51.05	84.19	108.59
∑DEHPm	–	103.24	40.62	59.81	100.78	164.82	244.52	355.07	–	96.50	34.02	57.82	96.34	160.62	261.13	355.97
Sub-sample[b]					N=236								N=224			
OH-MiNP	99.2	3.28	1.15	1.74	3.00	5.33	9.13	16.47	100	3.20	1.21	1.81	2.87	5.36	9.15	13.68
oxo-MiNP	100	3.27	1.09	1.55	3.19	5.48	9.68	16.35	100	3.16	1.13	1.78	2.80	4.93	9.91	13.84
cx-MiNP	100	9.87	5.35	6.41	8.79	12.87	18.98	28.79	100	9.30	5.24	6.41	8.39	12.06	18.59	22.55
∑DiNPm	–	22.47	10.06	12.90	20.32	32.72	47.78	70.00	–	21.44	10.36	13.92	19.50	28.15	47.92	68.02
OH-MINCH	93.6	0.68	0.29	0.35	0.51	0.98	3.41	8.42	93.3	0.65	0.24	0.36	0.49	0.82	3.35	7.89
oxo-MINCH	85.2	0.53	<LOD	0.25	0.46	1.02	2.07	4.81	83.5	0.49	<LOD	0.25	0.42	0.86	2.13	4.70
∑DINCHm	–	1.70	0.56	0.83	1.31	2.57	7.09	16.30	–	1.62	0.55	0.81	1.24	2.27	6.64	16.05

LOD: Limit of detection; GM: Geometric mean.

[a]Total sample: Gipuzkoa, Sabadell and Valencia cohorts (N=788); [b]Sub-sample: Gipuzkoa and Valencia cohorts (N=460).

Table 4. Association between prenatal phthalate exposure and puberty onset in boys (N=409) and girls (N=379) at age 7-10 years.

Phthalate biomarkers	Pubertal stage 2+[a]	Crude model				Basic model[b]				Fully-adjusted model[c]			
		Boys		Girls		Boys		Girls		Boys		Girls	
		RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
MEP	Overall	1.07	0.93-1.23	1.00	0.91-1.10	1.12	0.96-1.31	0.93	0.84-1.04	1.11	0.95-1.31	0.91	0.82-1.01
	Adrenal	1.08	0.83-1.42	1.02	0.90-1.15	1.20	0.90-1.63	0.93	0.81-1.07	1.29	0.95-1.73	0.91	0.79-1.05
	Gonadal	0.91	0.79-1.06	0.96	0.86-1.07	1.07	0.92-1.26	0.98	0.88-1.10	1.02	0.86-1.20	0.95	0.77-1.15
MiBP	Overall	0.90	0.74-1.11	1.00	0.86-1.16	0.97	0.76-1.23	1.00	0.84-1.19	0.96	0.76-1.21	0.99	0.83-1.18
	Adrenal	1.16	0.78-1.70	1.01	0.82-1.23	1.35	0.94-1.95	1.01	0.79-1.29	1.37	0.96-1.95	0.99	0.79-1.25
	Gonadal	1.00	0.81-1.23	1.10	0.95-1.29	1.01	0.79-1.28	1.13	0.96-1.33	0.98	0.78-1.23	1.14	0.98-1.33
MnBP	Overall	1.07	0.93-1.24	1.13	1.04-1.23*	1.04	0.88-1.24	0.99	0.90-1.10	1.04	0.87-1.23	0.97	0.88-1.07
	Adrenal	1.01	0.78-1.32	1.03	0.91-1.17	0.99	0.71-1.36	0.92	0.79-1.07	1.00	0.73-1.35	0.90	0.77-1.05
	Gonadal	0.91	0.77-1.09	1.10	1.00-1.20*	1.01	0.83-1.22	1.14	1.00-1.24*	0.99	0.82-1.21	1.10	0.99-1.22
MBzP	Overall	1.02	0.88-1.17	1.10	0.99-1.21	1.10	0.89-1.35	0.96	0.85-1.09	1.08	0.88-1.33	0.95	0.84-1.07
	Adrenal	0.85	0.64-1.15	1.14	1.00-1.30*	0.87	0.60-1.26	1.00	0.86-1.17	0.89	0.61-1.29	0.99	0.85-1.15
	Gonadal	0.86	0.73-1.00*	0.97	0.87-1.09	1.10	0.90-1.35	1.03	0.89-1.18)	1.12	0.92-1.36	1.02	0.89-1.18
∑DEHPm	Overall	1.19	0.99-1.43	1.22	1.06-1.39*	1.30	1.06-1.58*	1.10	0.93-1.30	1.32	1.09-1.59*	1.11	0.95-1.30
	Adrenal	0.93	0.69-1.26	1.26	1.06-1.49*	0.96	0.68-1.37	1.23	1.01-1.50*	1.09	0.75-1.59	1.25	1.03-1.51*
	Gonadal	1.00	0.79-1.26	0.98	0.84-1.14	1.21	0.98-1.48	0.98	0.81-1.18	1.23	1.00-1.50*	0.97	0.81-1.17
Sub-sample[d] (236 boys, 224 girls)		Boys		Girls		Boys		Girls		Boys		Girls	
		RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI	RR	95%CI
∑DiNPm	Overall	1.22	0.96-1.55	1.14	0.92-1.42	1.24	0.95-1.61	0.92	0.72-1.17	1.26	0.96-1.64	0.92	0.74-1.14
	Adrenal	0.71	0.41-1.23	0.98	0.72-1.35	0.71	0.37-1.37	0.87	0.61-1.24	0.77	0.41-1.42	0.86	0.61-1.21
	Gonadal	0.89	0.68-1.18	1.24	1.02-1.50*	0.89	0.65-1.20	1.21	0.97-1.51	0.95	0.70-1.28	1.20	0.98-1.48
∑DINCHm	Overall	0.82	0.65-1.02	0.82	0.70-0.96*	0.91	0.71-1.17	1.00	0.83-1.21	0.94	0.73-1.21	1.00	0.84-1.19
	Adrenal	0.68	0.75-0.97*	0.80	0.66-0.98*	0.66	0.41-1.05	0.86	0.68-1.08	0.68	0.44-1.06	0.88	0.70-1.12
	Gonadal	1.16	0.99-1.35	1.08	0.97-1.21	1.05	0.88-1.26	1.09	0.95-1.25	1.09	0.89-1.32	1.08	0.95-1.24

[a]Based on Petersen's Pubertal Development Scale (PDS): overall pubertal development (based on Carskadon and Acebo algorithm); and adrenal and gonadal development (based on Shirtcliff et al. algorithm)

[b]Adjusted for mother's urinary creatinine (log-transformed), cohort, and child age at puberty assessment.

[c]Additionally adjusted for mother's age, education, pre-pregnancy BMI, parity, and smoking during pregnancy.

[d]Sub-sample: Gipuzkoa and Valencia cohorts.

RR: Relative risk per each log-unit increase in urinary phthalate metabolite concentration.

*p<0.05

Table 5. Adjusted associations between prenatal phthalate exposure and puberty onset in boys and girls according to child weight status[a]

Phthalate biomarkers	Pubertal stage stage 2+	Normal weight						Overweight/obese					
		Boys (N=229)			Girls (N=227)			Boys (N=180)			Girls (N=152)		
		n stage 2+	RR	95%CI	n stage 2+	RR	95%CI	n stage 2+	RR	95%CI	n stage 2+	RR	95%CI
MEP	**Overall**	56	1.21	0.96-1.54	67	0.82	0.66-1.02	52	1.05	0.83-1.33	99	0.94	0.85-1.04
	Adrenal	23	1.80	1.29-2.51*	54	0.91	0.70-1.17	21	0.93	0.56-1.55	67	0.91	0.77-1.07
	Gonadal	53	1.06	0.83-1.35	64	0.85	0.70-1.03	47	0.97	0.77-1.23	89	1.01	0.90-1.14
MiBP	**Overall**	56	0.89	0.61-1.28	67	0.84	0.60-1.18	52	1.00	0.73-1.37	99	1.09	0.93-1.27
	Adrenal	23	1.48	0.94-2.34	54	0.94	0.66-1.33	21	1.34	0.77-2.31	67	1.00	0.76-1.32
	Gonadal	53	1.01	0.72-1.44	64	1.05	0.79-1.40	47	0.96	0.72-1.29	89	1.19	1.02-1.38*
MnBP	**Overall**	56	1.20	0.92-1.55	67	0.92	0.76-1.12	52	0.93	0.72-1.20	99	1.00	0.92-1.09
	Adrenal	23	1.53	1.07-2.18*	54	0.95	0.76-1.19	21	0.68	0.46-1.01	67	0.85	0.70-1.04
	Gonadal	53	1.20	0.91-1.60	64	1.05	0.86-1.29	47	0.93	0.71-1.22	89	1.14	1.03-1.26*
MBzP	**Overall**	56	1.06	0.80-1.42	67	0.86	0.69-1.08	52	1.03	0.76-1.39	99	0.97	0.87-1.09
	Adrenal	23	1.41	0.96-2.07	54	0.98	0.79-1.21	21	0.49	0.27-0.89*	67	1.00	0.83-1.19
	Gonadal	53	1.21	0.93-1.58	64	0.96	0.74-1.23	47	0.91	0.69-1.21	89	1.01	0.88-1.17
∑DEHPm	**Overall**	56	1.46	1.13-1.89*	67	1.09	0.81-1.47	52	1.16	0.83-1.62	99	1.10	0.91-1.32
	Adrenal	23	1.34	0.86-2.08	54	1.23	0.92-1.64	21	0.85	0.40-1.82	67	1.18	0.92-1.52
	Gonadal	53	1.49	1.12-1.97*	64	0.86	0.61-1.21	47	0.85	0.60-1.22	89	1.01	0.82-1.25
Sub-sample[b]		**Boys (N=132)**			**Girls (N=127)**			**Boys (N=103)**			**Girls (N=98)**		
		n stage 2+	RR	95%CI	n stage 2+	RR	95%CI	n stage 2+	RR	95%CI	n stage 2+	RR	95%CI
∑DiNPm	**Overall**	33	1.11	0.74-1.68	31	1.01	0.67-1.51	33	1.15	0.82-1.52	60	0.89	0.70-1.14
	Adrenal	15	0.82	0.46-1.46	26	0.84	0.51-1.38	13	0.81	0.29-2.24	35	0.86	0.56-1.32
	Gonadal	38	0.87	0.54-1.42	43	1.34	0.95-1.89	35	0.94	0.63-1.42	59	1.11	0.85-1.44
∑DINCHm	**Overall**	33	0.70	0.49-1.01	31	1.06	0.78-1.43	33	1.11	0.81-1.52	60	0.91	0.74-1.12
	Adrenal	15	0.80	0.44-1.46	26	0.84	0.57-1.23	13	0.47	0.24-0.90*	35	0.84	0.62-1.13
	Gonadal	38	1.12	0.84-1.49	43	0.97	0.79-1.19	35	0.96	0.72-1.27	59	1.16	1.01-1.35*

All models adjusted for urinary creatinine (log-transformed), child age, cohort, and mother's age, education, pre-pregnancy BMI, parity, and smoking during pregnancy.

[a]Weight status according to BMI z-score for age and sex: normal weight (±1 standard deviation); overweight/obese (>+1 standard deviation, equivalent to BMI≥25 kg/m^2 at 19 years)

[b]Sub-sample: Gipuzkoa and Valencia cohorts.

RR: Relative risk per each log-unit increase in urinary phthalate metabolite concentration.

*p<0.05

Fig. 11: *Flow-chart of the study population.*

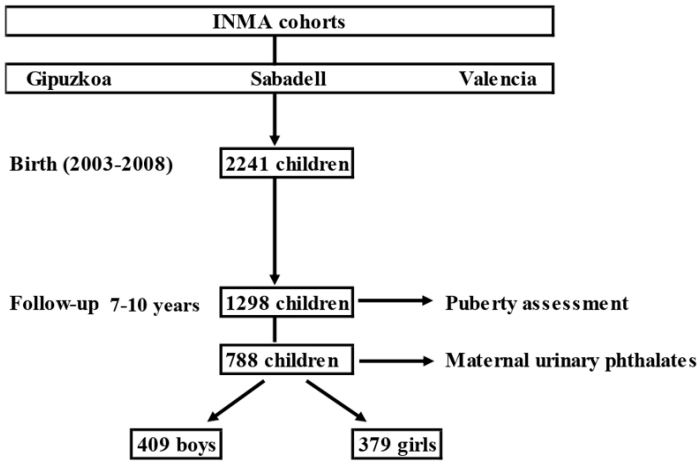


Fig. 12a: *WQS index constructed using weights as negative when analyzing the effect among boys and as positive among girls.*

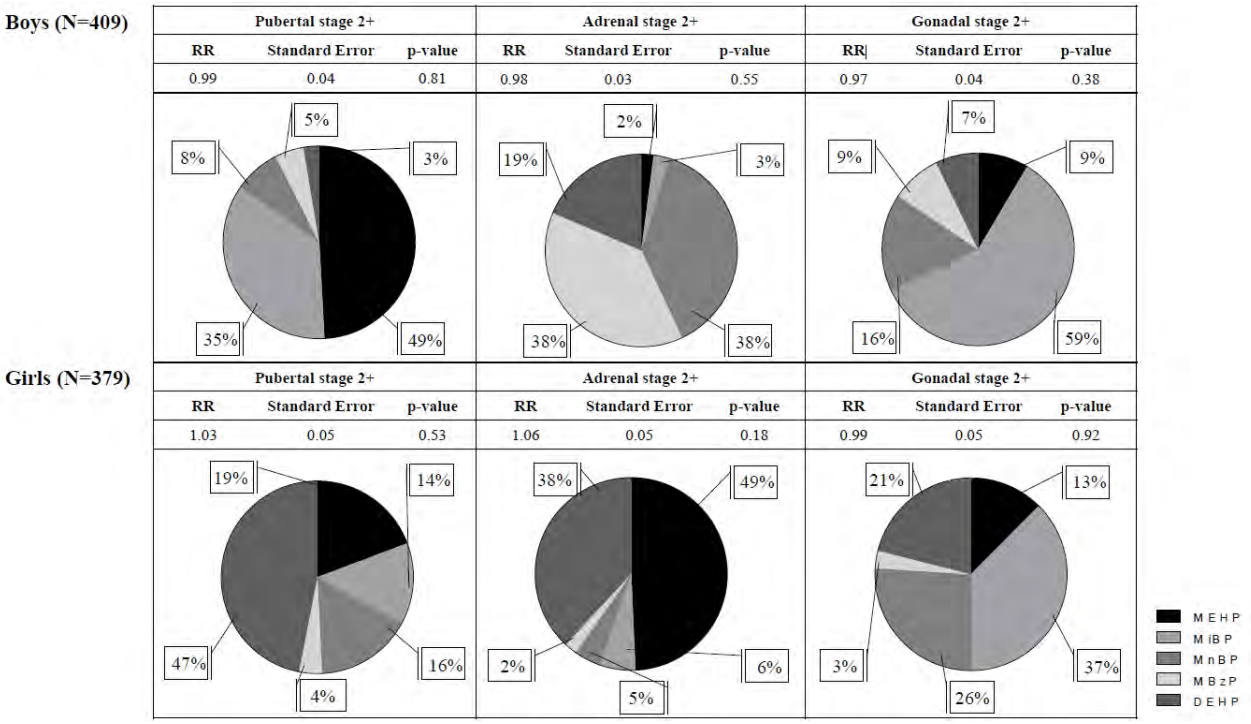
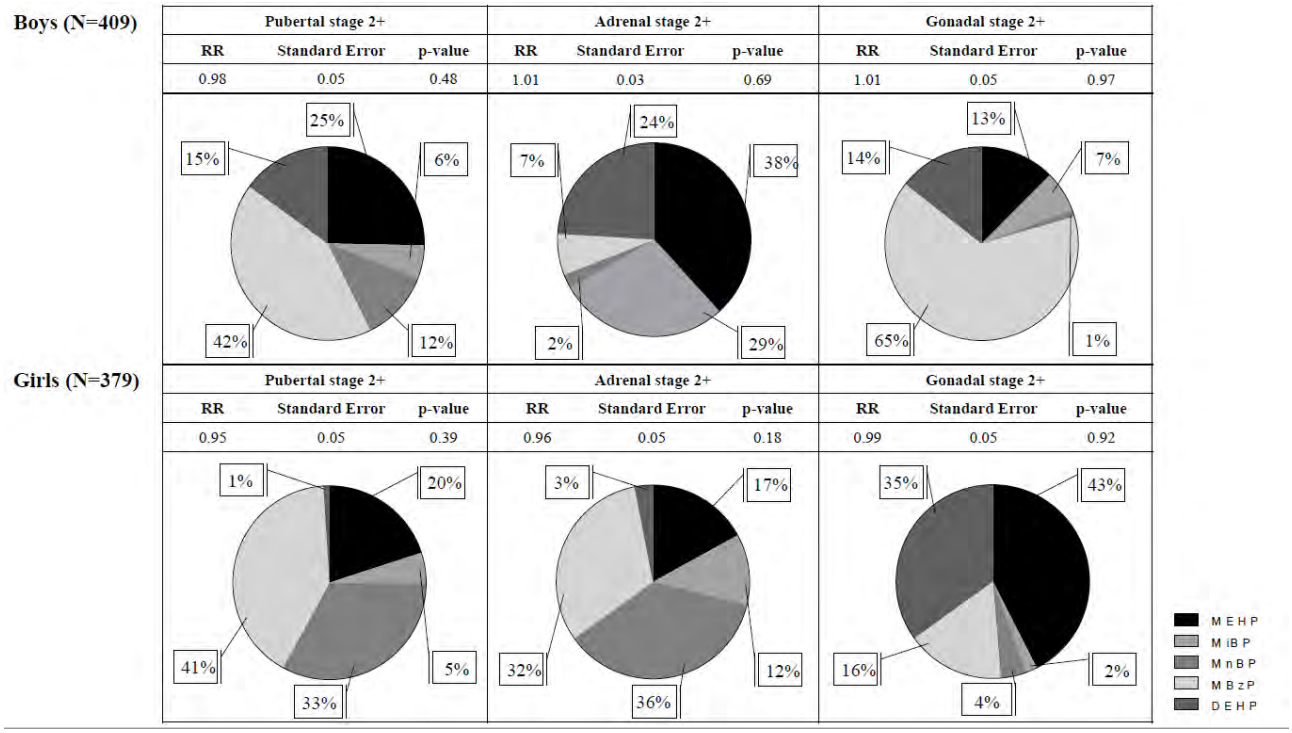


Fig. 2b: *WQS index constructed using weights as positive when analyzing the effect among boys and as negative among girls.*



5.4 Resultado del objetivo 4:

EXPOSURE TO NON-PERSISTENT PESTICIDES AND SEXUAL MATURATION OF SPANISH ADOLESCENT MALES

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En revisión (enviado el 13 de septiembre de 2022)
Chemosphere, Factor de impacto: 8,943, 1er Cuartil

Resumen

Antecedentes: Diversos pesticidas no persistentes son posibles disruptores endocrinos y pueden interferir en la maduración sexual.

Objetivo: Examinar la asociación entre biomarcadores urinarios de pesticidas no persistentes y la maduración sexual en varones adolescentes del Proyecto Infancia y Medio Ambiente (INMA).

Métodos: Se midieron los metabolitos de varios pesticidas en muestras de orina de 201 varones de 14 a 17 años de edad de las cohortes INMA de Granada y Menorca, entre ellos: 3,5,6-tricloro-2-piridinol (TCPy), metabolito de clorpirifós; 2-isopropil-4-metil-6-hidroxipirimidina, metabolito de diazinón; malatión diácido (MDA), metabolito de malatión; tiofosfato de dietilo (DETP) y ditiofosfato de dietilo, metabolitos no específicos de insecticidas organofosforados; ácido 3-fenoxibenzoico (3-PBA) y ácido dimetilciclopropano carboxílico, metabolitos de piretroides; 1-naftol (1-N), metabolito de carbaril; y etilentiourea, metabolito de fungicidas ditiocarbamatos. La maduración sexual se evaluó mediante la escala de Tanner y el volumen testicular (VT) valorado por un pediatra, y mediante la escala PDS auto-cumplimentada por los adolescentes. Se empleó regresión logística multivariante para examinar la asociación entre cada metabolito de pesticidas y la probabilidad de estar en estadío 5 de Tanner de desarrollo genital (G5) y púbico (P5), en estadío ≥4 del desarrollo puberal general, gonadal y adrenal según la PDS y de tener un VT ≥25 mL.

Resultados: Concentraciones de DETP >percentil 75 (P75) se asociaron con menor probabilidad de estar en estadiío G5 (OR=0,27; IC95%=0,10-0,70), la detección de TCPy con menor probabilidad de estar en estadío gonadal ≥4 (OR=0,50; 95%IC=0,26-0,96), y

concentraciones intermedias de MDA (<P75) con menor probabilidad de estar en estadío de desarrollo adrenal ≥4 (OR=0,32; 95%IC=0,11-0,94). En cambio, la detección de 1-N se asoció con mayor probabilidad de estar en estadío adrenal ≥4 (OR=2,61; IC95%=1,30-5,24) y con menor probabilidad de presentar VT ≥25 mL (OR=0,42; IC95 %=0,19-0,90). **Conclusión:** La exposición a ciertos pesticidas podría estar asociada con el estado de maduración sexual en varones adolescentes.

Abstract

Background: Several non-persistent pesticides are endocrine disrupting chemicals and may impact on sexual maturation.

Objective: To examine the association between urinary biomarkers of non-persistent pesticides and sexual maturation in adolescent males in the Environment and Childhood (INMA) Project.

Methods: The metabolites of several pesticides were measured in spot urine samples collected from 201 boys aged 14-17 years, including: 3,5,6-trichloro-2-pyridinol (TCPy), metabolite of chlorpyrifos; 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPy), metabolite of diazinon; malathion diacid (MDA), metabolite of malathion; diethyl thiophosphate (DETP) and diethyl dithiophosphate, non-specific metabolites of organophosphates; 3-phenoxybenzoic acid (3-PBA) and dimethyl cyclopropane carboxylic acid, metabolites of pyrethroids; 1-naphthol (1-NPL), metabolite of carbaryl; and ethylene thiourea (ETU), metabolite of dithiocarbamate fungicides. Sexual maturation was assessed using Tanner stages, self-reported Pubertal Development Scale, and testicular volume (TV). Multivariate logistic regression was employed to examine associations between urinary pesticide metabolites and the odds of being in Tanner stage 5 of genital development (G5) or pubic hair growth (PH5); stage ≥4 of overall pubertal development, gonadarche, and adrenarche; or having mature TV (≥25 mL).

Results: DETP concentrations>75th percentile (P75) were associated with lower odds of being in stage G5 (OR=0.27; 95% CI=0.10-0.70), detectable TCPy with lower odds of gonadal stage≥4 (OR=0.50; 95% CI=0.26-0.96), and intermediate detectable MDA concentrations (<P75) with lower odds of adrenal stage≥4 (OR=0.32; 95% CI=0.11-0.94). Conversely, detectable 1-NPL was associated with higher odds of adrenal stage≥4 (OR=2.61; 95% CI=1.30-5.24) but lower odds of mature TV (OR=0.42; 95% CI=0.19-0.90).

Conclusion: Exposure to certain pesticides may be associated with sexual maturity status in adolescent males.

Keywords: non-persistent pesticides; endocrine disruption; sexual maturation; gonadarche; adrenarche

1. Introduction

Pesticides are widely used in the food production chain and in urban and domestic settings. In the general population, diet is the main source of exposure to contemporary pesticides (Barr et al., 2010; Fernández et al., 2020), which are rapidly metabolized and mainly excreted through urine (Egeghy et al., 2011). Organophosphate (OP) and carbamate insecticides are used on crops, although some have been banned in the European Union (EU); for instance, the OP chlorpyrifos have not been approved since 2020 due to its possible adverse health effects (Dalsager et al., 2019; EU Pesticides Database (v.2.2) Active Substance, n.d.; Guo et al., 2019). Synthetic pyrethroids are relatively newer, becoming more available in industrial and domestic products (Tang et al., 2018). Among fungicides, ethylene-bis-dithiocarbamates (EBDC) such as maneb and mancozeb were banned in the EU in 2017 and 2021, respectively (EU Pesticides Database (v.2.2) Active Substance, n.d.). Human biomonitoring studies have shown comparable levels of dietary exposure to pesticides among adults and children worldwide (Holme et al., 2016; Li et al., 2019; Papadopoulou et al., 2019).

Numerous non-persistent pesticides have been found to exert endocrine-disrupting activity and interfere with the mechanisms that regulate the initiation and progression of puberty and sexual maturation by interacting with the receptors or metabolism of steroid hormones (Kojima et al., 2004; Orton et al., 2011). Thus, several OP insecticides (Archer and van Wyk, 2015; Kjeldsen et al., 2013; Kojima et al., 2004; Manabe et al., 2006; Yu et al., 2015), pyrethroids (Kjeldsen et al., 2013; Kojima et al., 2004; Manabe et al., 2006), carbamates (Kojima et al., 2004; Tange et al., 2016), and EBDC fungicides (Archer and van Wyk, 2015; Kjeldsen et al., 2013) bind to the estrogen receptors (ERα and/or -β) and/or the androgen receptor (AR) in vitro, and they can interfere with the metabolism of steroid hormones (Andersen et al., 2002). Nevertheless, few epidemiological studies have assessed their potential impact on pubertal development, with inconsistent results (Castiello and Freire, 2021). Thus, urinary concentrations of dialkyl phosphate (DAP) metabolites of OPs were associated with delayed genital development and lower testosterone levels in 14-15-year-old boys in Belgium (Croes et al., 2015), whereas

urinary 3-PBA, metabolite of pyrethroids, were associated with earlier genital development in 9-16-year-old boys in China (Ye, Pan, et al., 2017a). In a Danish cohort, boys born to mothers who worked in greenhouses during pregnancy had smaller testicular size and shorter penis length at the age of 6-11 years compared to non-exposed boys (Wohlfahrt-Veje et al., 2012).

Within the framework of the INMA (Environment and Childhood) Project in Spain, out group recently reported that exposure to OP insecticides, pyrethroids, and EBDC fungicides was associated with earlier puberty development in a large sample of 7-11-year-old girls and boys (Castiello et al., 2023). We also previously reported that urinary metabolites of chlorpyrifos, diazinon, and carbaryl were associated with altered serum levels of sex hormones in 134 adolescent males from the INMA-Granada cohort at the age of 15-17 years (Freire et al., 2021; Suárez et al., 2021). The objective of the present study of a larger sample of late adolescent males from the INMA Project was to evaluate the association of urinary metabolites of OPs, pyrethroid, and carbamate insecticides and EBDC fungicides with their sexual maturation status.

2. Material and methods

2.1 Study population

The INMA Project is a multicenter population-based mother-child cohort study that investigates the effect of environmental exposures and diet during pregnancy on fetal and child development in different geographic areas of Spain (http://www.proyectoinma.org).

Recruitment and general characteristics of the INMA cohorts are described elsewhere (Guxens et al., 2012). All cohorts included boys and girls except for Granada, which recruited only boys (Fernandez et al., 2007). Measurement of urinary metabolites of pesticides in adolescents (14-17 years) was performed in participants from urban and rural areas in Menorca (Eastern Spain) and Granada (Southern Spain) cohorts. The INMA-Granada cohort was established in 2000-2002 by recruiting 668 mother-son pairs at delivery. 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=300, 44.9%). 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). In Menorca, 482 pregnant women were recruited during pregnancy in 1997-1998. Mother-child pairs underwent several follow-ups from birth to the child age of 7-8 years (participation rate of 97-100%). At 9-10 years, 88% of the children included in the original INMA-Menorca cohort participated in a follow up, and at 14-16 years, 72% of children (n=345) participated in a new follow up. Of these 345 children, 139 males underwent pubertal assessment. Therefore, at 14-17 years of age, 290 boys in Menorca and Granada underwent assessment of pubertal status, of which 201 (69.3%) had their urine analysed for urinary pesticide metabolites. Study participants in the INMA-Granada cohort were more likely to reside in an urban area and less likely to have a stable partner that those initially recruited in the cohort, but no substantial differences were observed in the general characteristics of participants between the current and the previous follow up (Supplementary material, Table S1). An informed consent form was signed by the parents of all participants before gathering personal information and biological samples. The research protocol, including urine collection, was approved by the Biomedical Research Ethics Committee of Granada and the Balearic Islands.

2.2 Urinary pesticide metabolites

A first morning spot urine sample was collected from each study participant the same day of the clinical examination, which was kept at -80ºC until analysis. Urine samples were analysed for the following metabolites: diethyl-thiophosphate (DETP) and diethyl-dithiophosphate (DEDTP), non-specific metabolites of OP insecticides; 3,5,6-trichlo-2-pyridinol (TCPy), specific metabolite of chlorpyrifos and chlorpyrifos-methyl; 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), specific metabolite of the OP diazinon; 3-phenoxybenzoic acid (3-PBA), generic metabolite of pyrethroids; 1-naphtol (1-NPL), metabolite of the carbamate insecticide carbaryl; and ethylene thiourea (ETU), major metabolite of EBDC fungicides. It was not possible to measure DEP or DMP metabolites because reference standards were not available. Malathion dicarboxylic acid (MDA), a specific metabolite of malathion, and 2,2-dimethylcyclopropane carboxylic acid (DCCA) (sum of *cis*- and *trans*-isomers), a metabolite of *cis*- and *trans*-isomers of the pyrethroids permethrin, cypermethrin, and cyfluthrin, were

measured in the urine of a sub-sample of 161 boys.

Ultra-high-performance liquid chromatography coupled to mass spectrometry was employed to analyse DETP, DEDTP, TCPy, IMPy, 3-PBA, 1-NPL, and ETU, and liquid chromatography coupled to mass spectrometry was used to analyse the acid metabolites MDA and DCCA, as previously described (Freire et al., 2021; Rodríguez-Carrillo et al., 2022; Suárez et al., 2021). All metabolites were calibrated and extracted according to Suárez et al. (2021). Flow rates for chromatographic separation of MDA and DCCA were set at 0.3 mL/min. Limits of detection (LOD) and quantification (LOQ), retention times, analytical parameters of calibration curves, mean accuracy, selected reaction monitoring (SRM), and relative standard deviation (RSD) values are reported in Supplementary Material (Table S2). Urine dilution was considered by using a commercial kit (CREJ2) to measure urine creatinine concentrations in a Roche Cobas C-311 system, following the Jaffe method.

2.3 Assessment of sexual maturity

Tanner stages of genital development (G) and pubic hair growth (PH) were assessed in 175 and 181 boys, respectively, and Petersen's Pubertal Development Scale (PDS) score (Petersen et al., 1988) was obtained in 197 boys. The Tanner scale for boys classifies G and PH in one of five stages, ranging from prepubertal/absence of development to adult stage/complete maturation (Marshall and Tanner, 1970). Tanner stages were assessed by a paediatric endocrinologist in the Granada cohort and were self-rated in the Menorca cohort. Testicular volume (TV) was measured in 139 boys from Granada by comparison with the Prader orchidometer, a chain of 12 numbered beads of increasing size from 1 to 25 mL (Prader, 1966). When the size differed between testicles, the larger volume was recorded. All TV measurements were performed in duplicate, considering the arithmetic mean of the two measurements if they differed. Both self-reported Tanner and PDS were answered by study participants the same day of the clinical visit and urine collection.

The PDS contains five items: three for both males and females (growth spurt in height, pubic hair, and skin changes/pimples) and two solely for males (facial hair and voice deepening) (Petersen et al., 1988). Responses are on a four-point scale

from 1 (development not commenced) to 4 (complete development). The continuous PDS score was transformed into five ordinal stages using the algorithm of Petersen et al. (Petersen et al. (1988) and Carskadon and Acebo (1993) as follows: 1-prepubertal, 2-early pubertal, 3-midpubertal, 4-late pubertal, and 5-postpubertal. The PDS has proven to be a reliable and valid instrument to assess pubertal development in children (Carskadon and Acebo, 1993; Koopman-Verhoeff et al., 2020), and both self-reported and parent-reported PDS have shown strong internal consistency and test-retest reliability (Koopman-Verhoeff et al., 2020). The PDS score was also categorized according to adrenal and gonadal development scales following the algorithm of Shirtcliff et al., (2009) using all five PDS indicators and differentially gathering gonadal and adrenal signs of physical development. In males, growth spurt, voice deepening, and facial hair growth are associated with gonadal development and pubic/body hair and skin changes with adrenal development (Shirtcliff et al., 2009).

2.4 Covariates

Information on potential confounders was obtained from follow-up questionnaires administered to the adolescents and parents and included: cohort (Granada/Menorca), age (years), body mass index (BMI) z-score, weight status (normal weight/overweight/obese), passive smoking, area of residence (urban/sub-urban/rural), maternal age (years), maternal education (up to primary/secondary/university), and maternal stable partner (yes/no). The weight and height of boys were measured using standardized procedures. The BMI was calculated and converted to a z-score for age and sex based on World Health Organization reference curves for children (5-19 years) (WHO, 2007), classifying the boys as normal weight (±1 standard deviation [SD]) or overweight/obese (>+1 SD). Covariates also included the timing of urine sampling (spring/summer/fall/winter), given its potential influence on dietary patterns and pesticide exposure (Pontual et al., 2021).

2.5 Statistical analysis

Urinary concentrations of pesticide metabolites were expressed as detection frequencies and as 25th, 50th, 75th, and 95th percentiles. DEDTP and 3-PBA were excluded from association analysis because they were only detected in 1 sample and 35 samples, respectively. Pesticide metabolites

detected in <50% of urine samples (TCPy and 1-NPL) were converted to dichotomous variables (detected/undetected) before regression analysis, and the concentration of metabolites detected in ≥50% of samples was categorized as low (<LOD), moderate (LOD-75th percentile), or high (>75th percentile). Tanner stage and TV were categorized as reaching sexual maturity (Tanner G=5, Tanner PH=5, TV≥25) or not. PDS stages were categorized as late/postpuberty (stage≥4) or pre/early/midpuberty (stage <4) for overall pubertal, adrenal, and gonadal development. Data were missing on passive smoking and marital status for nine participants, respectively, and on BMI for one. Missing data on covariates were imputed by using the mode for categorical covariates (passive smoking and marital status) and the median for BMI.

The association between pesticide exposure and the odds of sexual maturity was assessed using logistic regression, modelling each pesticide metabolite (independent variable) separately with each outcome (dependent variable). Adjusted models included the following covariates as independent variables: 1) model 1 (basic model): child age, cohort, and urinary creatinine (ln-transformed) (included *a priori*). Unadjusted urinary pesticide metabolites and urinary creatinine concentrations were considered as separate independent variables, considered a better approach for controlling measurement error bias caused by variability in urine concentrations (Barr et al., 2005; O'Brien et al., 2016); 2) model 2: additionally adjusted for maternal education as a proxy of socioeconomic status; and model 3 (fully-adjusted model): additionally adjusted for child BMI z-score. BMI was introduced in a third step to examine whether results changed from those of model 2, possibly reflecting mediation, since pesticide exposure could have an impact on obesity (Pinos et al. 2021), which is strongly associated with pubertal development (Reinehr and Roth 2019). The remaining covariates were tested for potential confounding following the 10% change-in-estimate criterion, but none of them confounded the association between exposures and outcomes.

Since Tanner stages were self-reported in the Menorca cohort, a sensitivity analysis was performed by excluding boys in this cohort from the fully-adjusted model with Tanner outcomes. In addition, given that

individuals are typically exposed to multiple pesticides simultaneously, further analyses were performed in the total sample of boys to assess potential confounding by co-exposure by adjusting regression models simultaneously for all pesticide metabolites (except for DETP to avoid overestimating exposure as they are unspecific metabolites that share parent compounds with other metabolites included in the model). Results are presented as odds ratios (ORs) with 95% confidence interval (CI) for reaching sexual maturity with detected versus undetected concentrations, moderate/high versus low concentrations, or with each log-unit increase in urinary metabolite concentrations. The significance level was set at $p<0.05$. IBM SPSS Statistics v.26 was used for data analyses.

3. Results

Study participants had a mean age of 16.2 years (range=14.3-17.9), one-quarter were overweight/obese, one-third were passive smokers, and approximately half of them resided in urban areas. Their mothers had a mean age of 46.3 years; and more than three-quarters had a stable partner and low/middle education, respectively (Table 1).

Table 2 and Table S3 exhibits the distribution of urinary pesticide metabolite concentrations. The metabolite detected in the highest proportion of samples was MDA (87.6%, median=0.31 μg/L), followed by DCCA (68.3%, median=1.35 μg/L), IMPy (67.2%, median=0.27 μg/L), ETU (63.2%, median=0.15 μg/L), DETP (52.2%, median=0.25 μg/L), TCPy (31.3%, 75th percentile=0.04 μg/L), 1-NPL (29.4%, 75th percentile=0.20 μg/L), and 3-PBA (17.4%, 95th percentile=0.31 μg/L) (Table S3).

The sexual maturity status of participants is reported in Table 3 and Table S4. Regarding Tanner stage, 38.9% and 55.2% of boys had completed genital and pubic hair development, respectively. Regarding the PDS, 50.0% of boys were in stage ≥4 for gonadarche and 46.7% for adrenarche. Among 139 boys with available data on TV, 46.8% had a TV≥25mL (Table 3).

Results of logistic regression analysis are exhibited in Tables 4 and 5. In general, estimates obtained from the three models were similar; however, inclusion of BMI z-score in the model led to a slightly stronger association in some cases. In the fully-adjusted model, higher concentrations of DETP (>75th percentile) were associated with lower

odds of being in Tanner stage G5 (OR=0.27; 95% CI=0.10-0.71) (Table 4), while detectable concentrations of TCPy were associated with lower odds of being in gonadal stage≥4 (OR=0.51; 95% CI=0.27-0.99) (Table 5). In addition, intermediate (LOD-75th percentile) but not higher concentrations of MDA were associated with lower odds of being in adrenal stage≥4 (OR=0.30; 95% CI=0.10-0.90) (Table 4) and marginally associated with lower odds of mature TV (OR=0.36, 95% CI=0.12-1.09) (Table 4). Conversely, intermediate ETU concentrations were marginally associated with higher odds of mature TV (fully-adjusted model: OR=2.34; 95% CI=0.95-5.81) (Table 4), and detectable 1-NPL was significantly associated with higher odds of adrenal stage≥4 (fully-adjusted model: OR=2.67; 95% CI=1.32-5.40) (Table 5) but lower odds of mature TV (fully-adjusted model: OR=0.41; 95% CI=0.19-0.89) (Table 4).

The association between DETP and genital development remained unchanged after exclusion of boys from Menorca (Table S5), and results of regression models adjusted for co-exposure were not substantially different from the results of single exposure models (Table S6).

4. Discussion

In this sample of adolescent males from the general population, urinary metabolites of OP insecticides and 1-NPL were associated with delayed sexual maturation, especially delayed genital development. Regarding the adrenal axis, malathion exposure was associated with delayed and 1-NPL with accelerated adrenarche. These associations seem to be independent of socioeconomic status, BMI, and pesticide co-exposure.

Despite increasing restrictions on their utilization, OP insecticides remain widely used in agriculture (Hernández et al., 2019) and were detected in 31-87% of the present urine samples (collected in 2012-2019). MDA was the most prevalent, with concentrations that were higher than those reported for children aged 5-12 years from Valencia, Eastern Spain (detection frequency [DF]=87% and P95=5.42 μg/L in the present study *vs.* DF=7% and P95=1.39 μg/L in Valencia) (Fernández S. et al., 2020b), reflecting generalized exposure to malathion, currently authorized in the EU. Surprisingly, IMPy was detected in the urine of 67% of participants, despite the EU prohibition of its parent compound, diazinon, since 2006 (The Commission of the European Communities, 2007), although IMPy

concentrations were lower than those found in 2010 in 6-11-year-old children from Valencia (median=0.24 *vs*. 5.16 µg/g creatinine) (Roca et al., 2014), but slightly higher than those found in 5-12-year-old children from Valencia in 2016 (P95=4.28 *vs*. 0.75 µg/L) (Fernández S. et al., 2020b). However, TCPy was detected in only 31% of the boys, with concentrations that were lower than those described by Roca et al. (2014) for Valencian children (P75=0.03 µg/g in the present study *vs.* P50=3.40 µg/g), although chlorpyrifos was approved in the EU during the urine sample collection period (The European Commission, 2020). The association between exposure to OP insecticides and delayed genital/gonadal development is consistent with their anti-androgenic action and with the finding in Belgian boys aged 14-15 years of an association between urinary concentrations of DAPs and delayed genital development (Croes et al., 2015). Additionally, a Danish cohort study observed that the sons of mothers occupationally exposed during pregnancy to multiple pesticides, including OPs, had smaller testicular size and shorter penis length at the age of 6-11 years compared with sons of non-exposed mothers (Wohlfahrt-Veje et al., 2012). These observations are supported by experimental findings that exposure to chlorpyrifos, diazinon, and malathion induces smaller testicular size and structural abnormalities in adolescent (Jayachandra and D'Souza, 2014; Slimen et al., 2014) and adult (Farag et al., 2010; Joshi et al., 2007) mice. This is biologically plausible because various OP insecticides exert anti-androgen activity by binding to ERα, ERβ, or AR and by interfering with the expression of genes involved in the metabolism of steroid hormones (Kojima et al., 2004; Manabe et al., 2006). In the present study, urinary MDA seemed to be associated with smaller TV and delayed adrenarche, consistent with an animal study in which malathion exposure was found to downregulate gonadal development by targeting the expression pattern of transcription factors, activin A, orphan nuclear or sex steroid receptors, and steroidogenic enzymes (Prathibha et al., 2014). Interestingly, in our study conducted in children from the INMA cohorts at the age of 7-11 years, urinary DETP was associated with delayed puberty onset in boys with overweight or obesity, which is in line with the present results, whereas TCPy was associated with earlier genital growth onset (Castiello et al., 2023), suggesting that the effect of pesticide exposure on

pubertal development may depend on the age-time window.

The frequency of urinary 3-PBA detection was substantially lower in these boys than recorded in other biomonitoring studies. In addition, urinary 3-PBA concentrations in the current study were much lower (P95=0.16 μg/g) than those from larger samples of children from Japan (P50=1.40 μg/g and P95=13.09 μg/g) (Osaka et al., 2016), the United States (P50=2.50 μg/g and P95=13.09 μg/g) (Naeher et al., 2010), and China (P50 = 1.42 μg/g and P95=12.53 μg/g) (Ye et al., 2017a, 2017b). In Valencia, Spain, 3-PBA was detected in 23% and 79% of urine samples from children, respectively, and concentrations were higher than in the present study (P95=12.33 μg/g and P95 =11.57 μg/L, respectively), while detection of DCCA was higher (68% *vs.* 23%) but its concentrations were lower (6.74 μg/L *vs.* 46.65 μg/L) than those reported for children from Valencia (Fernández et al., 2020b). Concentrations of DCCA (metabolite of pyrethroid insecticides permethrin, cypermethrin, and cyfluthrin) were not associated with sexual maturity status in this study, and this metabolite was not analysed in children from the INMA cohorts at peripubertal age (Castiello et al., 2023). Various *in vitro* bioassays have documented the interaction of certain pyrethroids with estrogen and androgen receptors (Du et al., 2010; Kojima et al., 2004; Tange et al., 2014; Zhang et al., 2008). However, animal studies have reported contrasting results for the impact on sexual maturation according to the compound in question. For instance, exposure to cypermethrin accelerated puberty in mice by inducing the expression of StAr and Cyp11A1 genes related to testosterone production in Leydig cells and by inducing the synthesis of gonadotrophins in pituitary cells (Ye et al., 2017), whereas prenatal and postnatal exposure to bifenthrin was found to reduce testosterone production by downregulating gene expression in Leydig cells (Jin et al., 2013).

To our best knowledge, this is the first study to assess the association between 1-NPL and pubertal development in children. 1-NPL is the hydrolysis product not only of the insecticide carbaryl but also of naphthalene, a polycyclic aromatic hydrocarbon. Thereby, exposure misclassification can be caused by differences in exposure source (Meeker et al., 2007). No published study could be traced on the association of 1-NPL with sexual maturity in adolescents. A study of adult males by Meeker et al.

(2006) found an association between urinary 1-NPL and lower testosterone levels, in agreement with the association of 1-NPL with delayed genital development in the present study and with lower testosterone and FSH levels in a previous investigation (Freire et al., 2021). A study in adult male rats found that exposure to carbaryl was associated with reduced testicular size, decreased testosterone levels, and increased gonadotrophin levels (Fattahi et al., 2012). Although the mechanisms underlying these relationships are poorly understood, possible explanations include its estrogenic and anti-androgenic effects (Andersen et al., 2002; Klotz et al., 1997; Tange et al., 2016) and GnRH neuronal damage (Smulders et al., 2003) demonstrated in experimental studies. Conversely, the present observation of an association between 1-NPL and accelerated adrenal development is in agreement with our previous finding of an association between urinary 1-NPL and higher serum DHEAS concentrations (Freire et al., 2021), a marker of adrenal development.

Dithiocarbamates are the most widely used fungicides in the EU, where Spain is one of the largest consumers of agricultural fungicides (Eurostat, n.d.). Exposure to mancozeb has been associated with adverse reproductive effects in males (Runkle et al., 2017), although no published study could be found that addressed the impact of childhood ETU exposure on sexual maturation. ETU was detected in 63% of the present urine samples, with concentrations similar to those in French children aged 3 to 10 years living near vineyards frequently treated with dithiocarbamates (Raherison et al., 2019) but higher than those observed in occupationally exposed pregnant women in California (Castorina et al., 2010) and male farmers in North Carolina (Arcury et al., 2017). In this study, ETU was slightly associated with a larger TV but with no evidence of a linear increasing trend. EBDC fungicides have demonstrated anti-androgenic effects *in vitro* (Manabe et al., 2006; Yu et al., 2015) and a toxic effect on testicles and sperm quality in animal models (Kackar et al., 1997; Khan and Sinha, 1996), and they might therefore be expected to delay sexual maturation in adolescents. However, childhood exposure to ETU was associated with earlier puberty development boys and girls from the INMA Project at the age of 7-11 years, particularly earlier breast development in girls and earlier genital development in boys (Castiello et al., 2023). In this

line, prenatal exposure of rats to mancozeb was found to increase circulating levels of kisspeptin, a hypothalamic mediator of puberty onset (Overgaard et al., 2013). ETU is a known antithyroid compound (Hurley et al., 1998; Marinovich et al., 1997) that reduces thyroxine levels and increases the production of thyroid-stimulating hormone (TSH) in animal studies (Axelstad et al., 2011; Kackar et al., 1997; Medda et al., 2017; Panganiban et al., 2004; Piccoli et al., 2016). This is of interest due to the known interaction between the thyroid and gonadal axis (Ren and Zhu, 2022), and elevated TSH levels have been proposed as a contributing factor in central precocious puberty in girls (Jung et al., 2019). Nevertheless, the association observed in the present study was weak and could be a chance finding.

The sample size was relatively small, offering relatively low statistical power, and the cross-sectional design of the study prevents confirmation of a causal relationship between pesticide exposure and sexual maturation. Additionally, the quantification of non-persistent pesticide metabolites in spot urine samples indicates recent exposure because their biological half-life is short (4-48 hours) (Egeghy et al., 2011), and several studies have shown moderate temporal reliability for urinary pesticide metabolites. For instance, the intra-class correlation coefficient (ICC) for urinary DETP in 7-year-old European children was 0.37 for between-day variability and 0.35 for between-season variability (Casas et al. 2018). The ICC for IMPy was in the range 0.40-0.50 in pregnant Spanish women (Bravo et al. 2020), while in Costa Rican children (6-9 years) residing in an agricultural area urinary ETU and TCPy showed fair reliability (ICC=0.67 and 0.52, respectively) (van Wendel de Joode e al. 2016). Nevertheless, given that the diet is the main source of pesticide exposure in the general population, the exposure may be more or less continuous, with children receiving low daily doses of pesticides with their food; hence, metabolites of the pesticides can be expected to maintain stable levels in serum and target tissues (Côté et al., 2014; Wielgomas, 2013). Thus, a recent study in non-occupationally exposed adults found good repeatability (ICC>0.75) for urinary TCPy and pyrethroid metabolites 3-PBA and DCCA over 1-year period, concluding that one 24-h urine sample may be considered sufficient to characterize long-term exposure to non-specific pyrethroid metabolites (Klimowska et al. 2020). Future studies should be

developed to maximize reproducibility and achieve good characterization of the temporal variability. The detection frequency of some of the analyzed metabolites was low, reducing the sensitivity to detect effects and hampering assessment of the combined effect of mixtures of pesticides (Keil et al., 2020). In this regard, it is very likely that urinary 3-PBA concentrations were substantively underestimated, given that pyrethroid metabolites such as 3-PBA are largely present as phase II conjugates (glucuronide and/or sulphate) in urine (up to 85%) (Baker et al. 2004), and this deconjugation step was omitted. In addition, information on urinary concentrations of methyl phosphate metabolites would have provided a broader picture of the effect of OP insecticide exposure. It is also not possible to rule out the potential confounding effect of other EDCs. The strength of this study is that it is the first to examine the association between biomarkers of exposure to non-persistent pesticides and human male sexual maturation, contributing to scant knowledge on this relationship in this age window (14 to 17 years). The inclusion of boys from two different geographical areas is an additional strength. However, the study involved multiple comparisons, and it cannot be ruled out that some of the significant results may be due to chance.

5. Conclusions

These findings contribute to increasing evidence that exposure to non-persistent pesticides may be related to sexual maturation, suggesting a delaying effect for OP and carbamate insecticides and an accelerating effect for EBDC fungicides. Prospective studies with larger sample sizes and improved exposure assessment are warranted to verify these results and assess the impact of exposure to mixtures of pesticides.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Table 1. Characteristics of study participants (N=201)

Characteristic	N (%) or mean ± SD	Granada (N=150)	Menorca (N=51)
Cohort			
Granada (age range=15.9-17.9 years)	150 (74.6)	–	–
Menorca (age range=14.3-14.8 years)	51 (25.4)	–	–
Age (years)	16.2 ± 0.9	16.6 ± 0.4	14.6 ± 0.1
Season of follow up visit			
Spring	69 (34.3)	34 (22.7)	35 (68.6)
Summer	35 (17.4)	21 (14.0)	14 (27.5)
Autumn	64 (31.8)	63 (42.0)	1 (2.0)
Winter	33 (16.4)	32 (21.3)	1 (2.0)
BMI (kg/m^2)	22.75 ± 4.66	23.23 ± 4.84	21.34 ± 3.79
BMI z-score (kg/m^2)	0.48 ± 1.24	0.48 ± 1.29	0.47 ± 1.04
Overweight/obese (BMI≥25 kg/m^2)	50 (25.0)	43 (28.9)	7 (13.7)
Passive smoking	64 (33.3)	58 (38.7)	6 (11.8)
Area of residence			
Urban	105 (52.2)	105 (70.0)	0 (0)
Sub-urban/rural	96 (47.8)	45 (30.0)	51 (100)
Urinary creatinine (mg/dL)	184.7 ± 58.3	184.1 ± 57.5	186.3 ± 60.9
Maternal age	46.3 ± 4.7	46.9 ± 4.7	44.4 ± 4.1
Maternal education			
Primary	93 (47.9)	68 (45.3)	25 (49.0)
Secundary	59 (30.4)	49 (32.7)	18 (35.3)
Univeristy	41 (21.1)	33 (22.0)	8 (15.7)
Maternal stable partner (yes)	181 (90.0)	10 (7.1)	10 (19.6)

SD: Standard deviation; BMI: Body mass index.

Table 2. Urinary concentrations (μg/L) of pesticide metabolites in first morning voids.

Metabolites	LOD	% >LOD	Percentiles				Max
			25	50	75	95	
OP insecticides							
TCPy	0.039	31.3	<LOD	<LOD	0.04	0.18	1.21
IMPy	0.117	67.2	<LOD	0.27	0.76	4.28	
MDA (N=161)	0.052	87.6	0.15	0.31	0.55	1.12	1.83
DETP	0.116	52.2	<LOD	<LOD	0.74	5.42	54.95
Pyrethroids							
3-PBA	0.117	17.4	<LOD	<LOD	<LOD	0.31	0.72
DCCA (cis+trans) (N=161)	0.172	68.3	<LOD	1.35	3.56	6.74	8.95
Carbamates							
1-NPL	0.156	29.4	<LOD	<LOD	0.20	0.82	2.88
Dithiocarbamate fungicides							
ETU	0.072	63.2	<LOD	0.15	0.60	2.55	19.37

LOD: Limit of detection

Table 3. Sexual maturity status of study participants.

Outcome	N	n (%)
Genital Tanner stage	175	
G=3		11 (6.3)
G=4		96 (54.9)
G=5		68 (38.9)
Pubic hair Tanner stage	181	
PH=2		3 (1.7)
PH=3		20 (11)
PH=4		58 (32)
PH=5		100 (55.2)
Overall pubertal development*	197	
Pre + early + mid-pubertal		135 (68.5)
Late + post-puberty (PDS≥4)		62 (31.5)
Gonadal development**	196	
Pre + early + mid-pubertal		98 (50.0)
Late + post-puberty (PDS≥4)		98 (50.0)
Adrenal development**	197	
Pre + early + mid-pubertal		105 (53.3)
Late + post-puberty (PDS≥4)		92 (46.7)
TV≥25 mL	139	65 (46.8)

*Based on PDS-Carskadon and Acebo algorithm;

**Based on PDS-Shirtcliff et al. algorithm

Table 4. Association between urinary pesticide metabolites, Tanner stage, and testicular volume (TV).

Outcomes	Pesticide metabolites	OR (95% CI)	OR (95% CI)	OR (95% CI)
Tanner G=5 (N=175)[a]	**TCPy: ≥ *vs.* <LOD**	1.57 (0.81-3.04)	1.52 (0.78-2.97)	1.39 (0.70-2.75)
	IMPy (ref: <LOD)			
	LOD-P75	1.33 (0.63-2.79)	1.43 (0.67-3.06)	1.50 (0.69-3.25
	>P75	1.08 (0.44-2.64)	1.12 (0.45-2.74)	1.16 (0.46-2.91)
	p-Trend	0.91	0.97	0.97
	MDA (ref: <LOD)			
	LOD-P75	1.00 (0.34-2.93)	0.97 (0.33-2.87)	0.93 (0.31-2.80)
	>P75	0.67 (0.19-2.34)	0.66 (0.19-2.31)	0.64 (0.15-2.28)
	p-Trend	0.39	0.32	0.35
	DETP (ref: <LOD)			
	LOD-P75	0.77 (0.35-1.68)	0.76 (0.34-1.65)	0.91 (0.40-2.05)
	>P75	0.28 (0.11-0.70)*	0.25 (0.10-0.65)*	0.27 (0.10-0.71)*
	p-Trend	0.006	0.004	0.005
	DCCA (ref: <LOD)			
	LOD-P75	0.83 (0.38-1.82)	1.79 (0.79-4.04)	1.67 (0.73-3.85)
	>P75	0.78 (0.32-1.86)	0.91 (0.34-2.48)	0.76 (0.27-2.14)
	p-Trend	0.58	0.61	0.41
	ETU (ref: <LOD)			
	LOD-P75	0.84 (0.38-1.82)	0.80 (0.36-1.75)	0.80 (0.36-1.78)
	>P75	0.78 (0.32-1.86)	0.75 (0.31-1.80)	0.81 (0.33-1.99)
	p-Trend	0.65	0.62	0.74
	1-NPL: ≥ vs. <LOD	1.00 (0.49-2.03)	0.97 (0.48-1.99)	0.89 (0.43-1.86)
Tanner PH=5 (N=181)[b]	**TCPy: ≥ *vs.* <LOD**	1.09 (0.54-2.21)	1.12 (0.55-2.29)	1.04 (0.50-2.18)
	IMPy (ref: <LOD)			
	LOD-P75	0.49 (0.21-1.12)	0.46 (0.20-1.07)	0.51 (0.22-1.22)
	>P75	0.48 (0.18-1.25)	0.47 (0.18-1.23)	0.55 (0.50-1.46)
	p-Trend	0.77	0.32	0.40
	MDA (ref: <LOD)			
	LOD-P75	1.37 (0.46-4.05)	1.45 (0.48-4.35)	1.33 (0.42-4.23)
	>P75	0.94 (0.27-3.24)	0.97 (0.28-3.37)	0.94 (0.25-3.53)
	p-Trend	0.63	0.63	0.61
	DETP (ref: <LOD)			
	LOD-P75	0.78 (0.34-1.77)	0.78 (0.34-1.77)	1.00 (0.42-2.36)
	>P75	0.64 (0.27-1.54)	0.65 (0.27-1.58)	0.79 (0.31-1.97)
	p-Trend	0.37	0.39	0.50
	DCCA (ref: <LOD)			
	LOD-P75	1. 78(0.79-4.04)	1.76 (0.77-4.01)	1.64 (0.70-3.78)
	>P75	2.14 (0.77-5.97)	2.11 (0.74-5.95)	1.81 (0.60-5.43)
	p-Trend	0.18	0.20	0.34

	ETU (ref: <LOD)			
	LOD-P75	0.52 (0.22-1.22)	0.52 (0.22-1.24)	0.54 (0.22-1.32)
	>P75	0.56 (0.21-1.47)	0.57 (0.22-1.49)	0.68 (0.25-1.85)
	p-Trend	0.52	0.53	0.41
	1-NPL: ≥ vs. <LOD	1.49 (0.70-3.19)	1.52 (0.71-3.26)	1.49 (0.68-3.28)
	TCPy: ≥ *vs.* <LOD	1.12 (0.54-2.32)	1.05 (0.05-2.20)	1.03 (0.49-2.16)
	IMPy (ref: <LOD)			
	LOD-P75	1.58 (0.68-3.66)	1.76 (0.74-4.17)	1.73 (0.72-4.13)
	>P75	1.02 (0.38-2.69)	1.03 (0.38-2.78)	1.03 (0.38-2.77)
	p-Trend	0.62	0.58	0.59
	MDA (ref: <LOD)			
	LOD-P75	0.39 (0.13-1.16)	0.36 (0.12-1.09)	0.36 (0.12-1.09)
	>P75	0.92 (0.27-3.18)	0.89 (0.26-3.10)	0.89 (0.26-3.13)
	p-Trend	0.37	0.37	0.38
	DETP (ref: <LOD)			
TV≥25 mL (N=139)	LOD-P75	1.32 (0.56-3.10)	1.34 (0.57-3.18)	1.41 (0.58-3.41)
	>P75	0.93 (0.36-2.34)	0.89 (0.35-2.30)	0.92 (0.36-2.40)
	p-Trend	0.68	0.61	0.63
	DCCA (ref: <LOD)			
	LOD-P75	0.97 (0.44-2.15)	1.04 (0.46-2.33)	1.00 (0.44-2.27)
	>P75	0.86 (0.33-2.25)	0.97 (0.36-2.58)	0.92 (0.34-2.49)
	p-Trend	0.75	0.93	0.81
	ETU (ref: <LOD)			
	LOD-P75	2.47 (1.01-6.02)*	2.37 (0.96-5.84)	2.34 (0.95-5.81)
	>P75	1.66 (0.63-4.34)	1.63 (0.62-4.30)	1.66 (0.62-4.40)
	p-Trend	0.92	0.91	0.89
	1-NPL: ≥ vs. <LOD	0.45 (0.21-0.95)*	0.42 (0.20-0.91)*	0.41 (0.19-0.89)*

LOD: Limit of detection; P75: 75th percentile.
Model 1: adjusted for cohort, age, and urinary creatinine (mg/dL)
Model 2: adjusted for cohort, age, urinary creatinine, and maternal education
Model 3: adjusted for cohort, age, urinary creatinine, maternal education, and child BMI z-score (continuous)
[a]N=147 for MDA and DCCA
[b]N=149 for MDA and DCCA
*p-value<0.05

Table 5. Association between urinary pesticide metabolites and pubertal development score (PDS)

Outcomes	Pesticide metabolites	OR (95% CI)	OR (95% CI)	OR (95% CI)
Overall puberty PDS≥4 (N=197)*	**TCPy: ≥ *vs.* <LOD**	0.64 (0.32-1.29)	0.63 (0.31-1.29)	0.61 (0.29-1.26)
	IMPy (ref: <LOD)			
	LOD-P75	1.15 (0.54-2.44)	1.22 (0.57-2.62)	1.36 (0.62-3.02)
	>P75	0.61 (0.25-1.53)	0.63 (0.25-1.60)	0.66 (0.26-1.70)
	p-Trend	0.71	0.20	0.20
	MDA (ref: <LOD)			
	LOD-P75	2.28 (0.68-7.56)	2.12 (0.63-7.12)	1.94 (0.56-6.71)
	>P75	2.62 (0.67-10.13)	2.52 (0.65-9.82)	2.60 (0.65-10.38)
	p-Trend	0.27	0.27	0.27
	DETP (ref: <LOD)			
	LOD-P75	1.20 (0.55-2.63)	1.16 (0.52-2.55)	1.40 (0.61-3.19)
	>P75	0.60 (0.25-1.43)	0.58 (0.24-1.40)	0.67 (0.27-1.63)
	p-Trend	0.16	0.15	0.19
	DCCA (ref: <LOD)			
	LOD-P75	1.15 (0.51-2.61)	1.20 (0.52-2.76)	1.12 (0.51-2.83)
	>P75	0.85 (0.32-2.25)	0.91 (0.33-2.46)	0.73 (0.25-2.10)
	p-Trend	0.66	0.75	0.43
	ETU (ref: <LOD)			
	LOD-P75	1.59 (0.73-3.47)	1.17 (0.56-2.44)	1.71 (0.75-3.91)
	>P75	1.56 (0.64-3.78)	0.87 (0.10-7.59)	1.92 (0.76-4.88)
	p-Trend	0.53	0.53	0.41
	1-NPL: ≥ vs. <LOD	1.84 (0.91-3.71)	1.82 (0.90-3.69)	1.93 (0.94-3.99)
Adrenal development PDS≥4 (N=197)*	**TCPy: ≥ *vs.* <LOD**	0.84 (0.45-1.57)	0.86 (0.46-1.62)	0.81 (0.43-1.54)
	IMPy (ref: <LOD)			
	LOD-P75	1.02 (0.39-1.94)	1.02 (0.51-2.06)	1.17 (0.57-2.39)
	>P75	0.87 (0.39-1.94)	0.90 (0.40-2.03)	0.96 (0.42-2.18)
	p-Trend	0.68	0.75	0.74
	MDA (ref: <LOD)			
	LOD-P75	0.35 (0.12-0.99)	0.32 (0.11-0.94)*	0.30 (0.10-0.90)*
	>P75	0.41 (0.12-1.40)	0.38 (0.11-1.33)	0.38 (0.11-1.32)
	p-Trend	0.42	0.39	0.37
	DETP (ref: <LOD)			
	LOD-P75	0.55 (0.26-1.22)	0.51 (0.24-1.09)	0.53 (0.25-1.16)
	>P75	0.57 (0.26-1.22)	0.55 (0.26-1.20)	0.57 (0.26-1.24)
	p-Trend	0.27	0.28	0.28
	DCCA (ref: <LOD)			
	LOD-P75	0.76 (0.35-1.65)	0.74 (0.34-1.63)	0.73 (0.33-1.63)
	>P75	0.49 (0.19-1.22)	0.48 (0.19-1.24)	0.49 (0.19-1.28)
	p-Trend	0.13	0.13	0.14

	ETU (ref: <LOD)			
	LOD-P75	0.94 (0.47-1.91)	0.95 (0.46-1.95)	1.03 (0.50-2.14)
	>P75	1.73 (0.78-3.86)	1.79 (0.79-4.03)	2.02 (0.88-4.68)
	p-Trend	0.11	0.10	0.09
	1-NPL: ≥ vs. <LOD	2.65 (1.33-5.26)*	2.67 (1.33-5.35)*	2.67 (1.32-5.40)*
Gonadal development PDS≥4 (N=196)*	**TCPy: ≥ *vs.* <LOD**	0.54 (0.29-1.02)	0.54 (0.28-1.02)	0.51 (0.27-0.99)*
	IMPy (ref: <LOD)			
	LOD-P75	1.20 (0.61-2.38)	1.25 (0.62-2.50)	1.36 (0.67-2.77)
	>P75	0.69 (0.31-1.55)	0.72 (0.31-1.62)	0.76 (0.33-1.73)
	p-Trend	0.22	0.25	0.27
	MDA (ref: <LOD)			
	LOD-P75	1.90 (0.71-5.12)	1.77 (0.65-4.82)	1.69 (0.62-4.62)
	>P75	2.13 (0.66-6.87)	2.05(0.63-6.66)	2.07 (0.64-6.72)
	p-Trend	0.31	0.32	0.32
	DETP (ref: <LOD)			
	LOD-P75	1.09 (0.53-2.56)	1.03 (0.50-2.16)	1.18 (0.55-2.52)
	>P75	0.80 (0.38-1.70)	0.77 (0.36-1.66)	0.85 (0.39-1.85)
	p-Trend	0.48	0.46	0.55
	DCCA (ref: <LOD)			
	LOD-P75	1.40 (0.66-3.01)	1.49 (0.68-3.24)	1.47 (0.67-3.23)
	>P75	1.22 (0.50-2.97)	1.33 (0.53-3.32)	1.20 (0.47-3.08)
	p-Trend	0.49	0.46	0.36
	ETU (ref: <LOD)			
	LOD-P75	0.88 (0.44-1.79)	0.86 80.42-1.78)	0.90 (0.43-1.86)
	>P75	1.22 (0.55-2.71)	1.23 (0.55-2.77)	1.41 (0.61-3.22)
	p-Trend	0.78	0.66	0.92
	1-NPL: ≥ vs. <LOD	1.08 (0.55-2.11)	1.06 (0.54-2.09)	1.01 (0.54-2.15)

LOD: Limit of detection; P75: 75th percentile.

Model 1: adjusted for age, cohort, and urinary creatinine (mg/dL)

Model 2: adjusted for age, cohort, urinary creatinine, and maternal education.

Model 3: adjusted for age, cohort, urinary creatinine, maternal education, and child BMI z-score (continuous)

[a]N=157 for MDA and DCCA

*p-value<0.05

5.5 Resultados del objetivo 5, artículo 1:

ORGANOPHOSPHATE PESTICIDE EXPOSURE, HORMONE LEVELS, AND INTERACTION WITH PON1 POLYMORPHISMS IN MALE ADOLESCENTS

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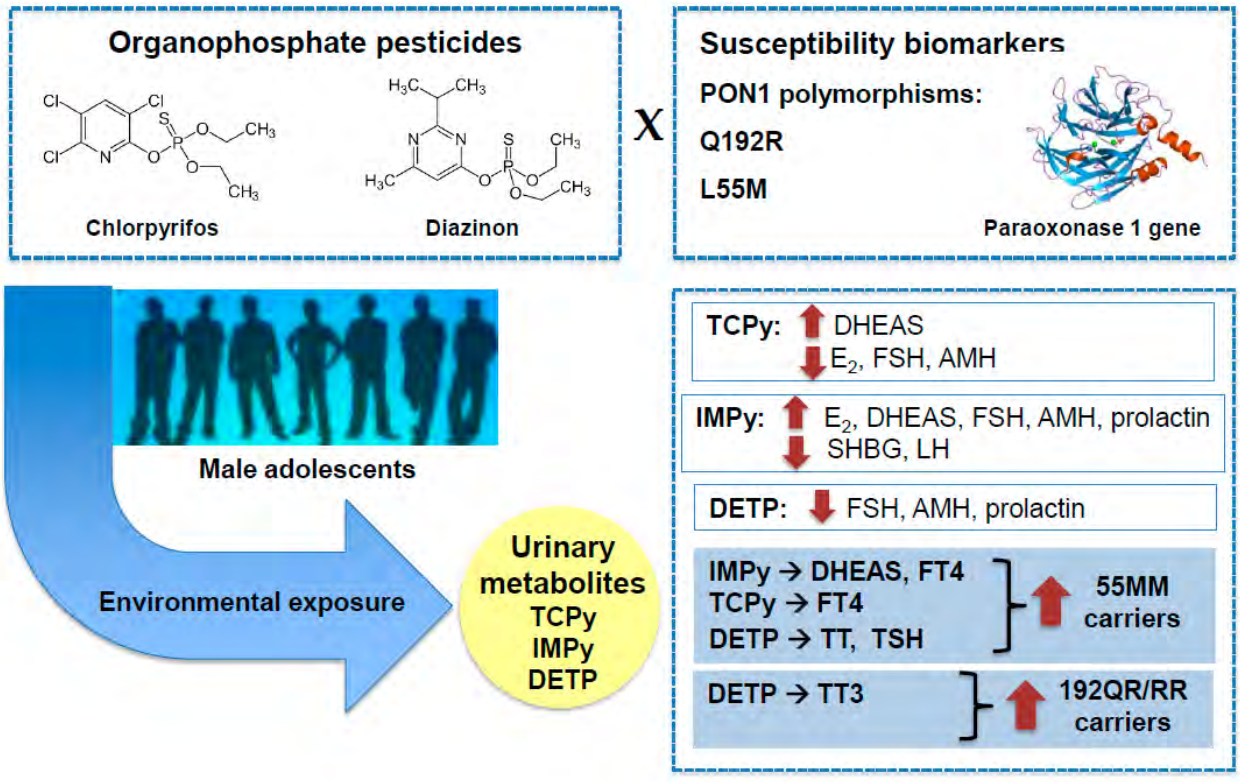
Resumen Gráfico



Resumen

Objetivo: Examinar la asociación entre los metabolitos urinarios de pesticidas organofosforados (OP) y las concentraciones séricas de hormonas tiroideas y reproductivas en adolescentes varones y evaluar el efecto potencial de la interacción entre la exposición a pesticidas OPs y polimorfismos en el gen de la paraoxonasa 1 (PON1) sobre los niveles hormonales.

Métodos: Los sujetos del estudio (N=117) eran varones de 16 a 17 años de edad de la cohorte INMA-Granada en España. Se recogió una muestra de orina de cada participante y se midieron las concentraciones de creatinina y de 3,5,6-tricloro-2-piridinol (TCPy), metabolito de clorpirifós/clorpirifós-metilo, 2-isopropil-6-metil-4-pirimidinol (IMPy), metabolito de diazinón y dietiltiofosfato (DETP) y dietilditiofosfato (DEDTP), metabolitos no específicos de pesticidas OPs. En una muestra de sangre venosa se cuantificaron las concentraciones séricas de las principales hormonas sexuales (testosterona total [TT], estradiol [E2], sulfato de dehidroepiandrosterona [DHEA-S], globulina transportadora de hormonas sexuales [SHBG], hormona luteinizante [LH], hormona folículo estimulante [FSH], hormona antimülleriana [AMH], factor de crecimiento insulínico tipo 1 [IGF-1] y prolactina), y de hormonas tiroideas (tiroxina libre [FT4], triiodotironina total [TT3] y hormona estimulante del tiroides [TSH]). Se analizaron los polimorfismos PON1 Q192R y L55M en muestra de sangre extraída durante la misma visita clínica.

Resultados: Los modelos de regresión lineal múltiple mostraron que la detección de TCPy estaba asociada con un aumento de DHEA-S y disminución de los niveles de E_2, FSH y AMH; la detección de IMPy con aumento de E_2, DHEA-S, FSH, AMH y prolactina y disminución de SHBG y LH; y la detección de DETP con aumento marginalmente significativo de TT y TT3 y disminución de FSH, AMH y prolactina. La magnitud del efecto de IMPy y DETP sobre los niveles de DHEA-S y TT, respectivamente, fue mayor en los sujetos que portaban el genotipo PON1 55MM, mientras que el efecto de TCPy, IMPy y DETP sobre los niveles de hormona tiroidea fue mayor en los portadores del genotipo PON1 192QR/RR o 55MM.

Conclusiones: En varones adolescentes, la exposición no ocupacional a pesticidas OPs se asoció con cambios en los niveles de hormonas sexuales y tiroideas, y la magnitud de algunas asociaciones fue mayor en adolescentes genéticamente más susceptibles a la exposición a pesticidas OP que portan el genotipo PON1 55MM.

Abstract

Objective: To examine the association between urinary metabolites of organophosphate (OP) pesticides and serum concentrations of thyroid and reproductive hormones in male adolescents and to assess the potential effect of interactions between OP pesticides and paraoxonase 1 (PON1) polymorphisms on hormone levels.

Methods: Study subjects (N=117) were male 16- to 17-year-olds from the Environment and Childhood (INMA)-Granada cohort in Spain. Concentrations of 3,5,6-trichloro-2-pyridinol (TCPy), a metabolite of chlorpyrifos/chlorpyrifos-methyl, 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), a metabolite of diazinon, and diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP), non-specific metabolites of OP pesticides, were measured in a spot urine sample from each subject and adjusted for creatinine. Levels of reproductive hormones (total testosterone [TT], estradiol [E_2], dehydroepiandrosterone sulfate [DHEAS], sex hormone binding globulin [SHBG], luteinizing hormone [LH], follicle stimulating hormone [FSH], anti-Müllerian hormone [AMH], insulin growth factor 1 [IGF-1], and prolactin), thyroid hormones (free thyroxine [FT4], total triiodothyronine [TT3], and thyroid stimulating hormone [TSH]), and PON1 Q192R and L55M polymorphisms were determined in blood drawn during the same clinical visit.

Results: Multiple linear regression models showed that detectable levels of TCPy were associated with an increase in DHEAS and decreases in E_2, FSH, and AMH; detectable IMPy with increases in E_2, DHEAS, FSH, AMH, and prolactin and decreases in SHBG and LH; and detectable DETP with marginally-significant increases in TT and TT3 and decreases in FSH, AMH, and prolactin. The effect of IMPy and DETP on DHEAS and TT levels, respectively, was higher in subjects that carried the PON1 55MM genotype, while the effect of TCPy, IMPy, and DETP on thyroid hormone levels was higher in PON1 192QR/RR or 55MM genotype carriers.

Conclusions: In male adolescents, non-occupational exposure to OP pesticides was associated with several changes in reproductive and thyroid hormone levels, and the magnitude of some associations was greater in adolescents genetically more susceptible to OP pesticide exposure who carry the PON1 55MM genotype.

Keywords

Chlorpyrifos; diazinon; paraoxonase 1; reproductive hormones; thyroid hormones; male hormones

1. Introduction

Organophosphate (OP) pesticides are a class of insecticides widely used throughout the world. While most residential uses of OP pesticides have been banned in many countries, their use is still permitted in food crops for pest control. Dietary intake is the main source of OP pesticide exposure for adults and children from the general population (Hyland et al., 2019; Lu et al., 2006). OP pesticides do not accumulate in the human body and more than 75% of their content is metabolized to dialkylphosphates (DAPs) and then excreted in the urine within 6 to 48 h after exposure (Barr and Angerer, 2006). Currently, the most sensitive and reliable biomarkers of OP exposure are urinary levels of DAPs or specific metabolites of OP pesticides such as chlorpyrifos, malathion, and diazinon.

Several OP pesticides can exert estrogenic and/or anti-androgenic activity and disturb sex steroid-synthesizing enzymes, including CYP19 (aromatase) and CYP17 (Andersen et al., 2002; Kojima et al., 2004; Orton et al., 2011). For example, chlorpyrifos, a broad-spectrum OP insecticide, has shown estrogenic activity (Andersen et al., 2002; Orton et al., 2011) and the capacity to strongly inhibit CYP1A2 and CYP3A4 (Usmani et al., 2003, 2006), the main P450 enzymes that metabolize estradiol, estrone, and testosterone (T) in the liver. Other OPs such as dichlorvos, pirimiphos-methyl, azinphos-methyl, and fenitrothion exhibit anti-androgenic activity *in vitro* (Andersen et al., 2002; Orton et al., 2011). Animal studies have demonstrated that OP pesticides may also affect the hypothalamus and the pituitary, resulting in thyroid disruption (Campos and Freire, 2016; Leemans et al., 2019). For instance, depletion of thyroid hormone (thyroxine [T4] and triiodothyronine [T3]) levels has been observed in mice and fish, respectively, after exposure to chlorpyrifos at low (De Angelis et al., 2009) and high (Besson et al., 2020) doses.

In humans, various studies have suggested that exposure to OP pesticides alters circulating levels of male reproductive hormones (Aguilar-Garduño et al., 2013; Blanco-Muñoz et al., 2010; Meeker et al., 2006a, 2008; Melgarejo et al., 2015; Omoike et al., 2015; Panuwet et al., 2018; Recio et al., 2005; Yucra et al., 2006), but their results have been inconsistent. Thus, urinary levels of DAPs or chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCPy) were associated with decreased T levels in agricultural workers (Aguilar-Garduño et al., 2013; Yucra et al., 2006) and non-occupationally exposed men

(Meeker et al., 2006a; Omoike et al., 2015), whereas positive or null associations were found between OP exposure and male T in other studies (Blanco-Muñoz et al., 2010; Melgarejo et al., 2015; Panuwet et al., 2018; Recio et al., 2005). Associations of OP urinary biomarkers with luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, inhibin B, and estradiol have also been inconsistent across studies (Aguilar-Garduño et al., 2013; Blanco-Muñoz et al., 2010; Meeker et al., 2006a, 2008; Melgarejo et al., 2015; Recio et al., 2005; Yucra et al., 2006). Regarding thyroid function, some authors found associations between OP exposure and changes in serum thyroid stimulating hormone (TSH) and thyroid hormone levels among male agricultural workers (Lacasaña et al., 2010a; Toft et al., 2006) and adults from the general population (Fortenberry et al., 2012; Jain, 2017; Meeker et al., 2006b). Results of occupational studies are in line with experimental findings that OP pesticides exposure causes hypothyroid-like effects, *i.e.* increased TSH with either a decrease, increase, or no change in T4 and/or T3 levels (Campos and Freire, 2016).

Polymorphisms in genes encoding enzymes involved in xenobiotic metabolism may contribute to inter-individual variance in susceptibility to the toxicity of environmental chemicals. The esterase paraoxonase-1 (PON1) is a key enzyme in the hydrolysis of the active metabolites (oxons) of a number of OP pesticides, such as chlorpyrifos and diazinon (Costa et al., 2013). Two common polymorphisms in the PON1 gene coding sequence at positions 192 and 55 have been related to differences in PON1 activity. The Q192R polymorphism (a Gln(Q)/Arg(R) substitution) confers differential catalytic activity on some OP substrates, while the L55M polymorphism (a Met(M)/Leu(L) substitution) has been associated with low serum PON1 levels (Costa et al., 2013). A study in Mexican male floriculture workers examined the interaction between OP pesticide exposure and PON1 polymorphisms on thyroid hormone levels (Lacasaña et al., 2010b). The results showed that 192RR genotype carriers had a larger increase in TSH and total T3 values associated with higher urinary levels of DAP metabolites in comparison to carriers of 192QQ and QR genotypes.

With this background, the objective of this study was to examine the association between urinary metabolites of OP pesticide exposure and serum concentrations of

reproductive and thyroid hormones in 16-17-year-old male adolescents, as well as the possible interaction between OP pesticide exposure and PON1 polymorphisms on hormone levels.

2. Material and methods

2.1. Study population

Subjects were recruited from the Environment and Childhood (INMA) Project, a multicenter population-based birth cohort study that aims to investigate the effect of environmental exposures and diet during pregnancy on fetal and child development in different parts of Spain (Guxens et al., 2012). Participants were male adolescents from the INMA-Granada cohort (baseline *n*=668) in Andalusia, Southern Spain, who underwent clinical follow up visit at the age 15-17 years (Castiello et al., 2020). Briefly, all boys who participated at 4-5 and/or 9-11 years follow up (*n*=220 and 300, respectively) were asked to participate in a new follow-up visit in 2017-2019. Among those approached, a total of 155 (approximately 51%) accepted participation, providing a urine specimen and undergoing physical examination at the San Cecilio University Hospital (HUSC) of Granada, and 135 (87%) of them also provided a blood sample. Sociodemographic data were obtained from questionnaires administered to parents during the clinical visit. The present study was conducted in 117 of these adolescents with available data on urinary biomarkers of OP pesticides, PON1 polymorphisms, serum hormones, and covariates. There were no significant differences between participants in the current study (*n*=117) and those with data on urinary OP pesticide metabolite levels but not on levels of serum hormones, PON1 polymorphisms, or covariates (*n*=38), with the exception of a lower percentage with family income <25000 euros (34 *vs.* 50%) in the included *versus* excluded adolescents (Supplementary material, Table S1). The parents of all participants signed informed consent, and the study protocol was approved by the Biomedical Research Ethics Committee of Granada.

2.2. OP metabolites in urine

A single spot urine sample was collected from each participant from the first morning void on the day of their hospital visit and was stored at -80 ºC until analysis. Urine samples were analyzed for concentrations of TCPy and 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), which are specific metabolites of chlorpyrifos/chlorpyrifos-methyl and diazinon, respectively, and for concentrations of two non-specific

diethylphosphate metabolites, *i.e.* diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP). DETP and DEDTP are major metabolites of several OPs, including chlorpyrifos and diazinon. It was not possible to measure DEP or DMP metabolites because reference standards were not available.

Analysis of OP urinary metabolites was performed by ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS), using an UHPLC Ultimate 3000 (Thermo Fischer) and a Q Exactive Focus mass spectrometer (Thermo Fischer) at the laboratory of the "UNETE research unit" of the Biomedical Research Center (CIBM), University of Granada. All reagents were analytical grade unless otherwise specified. IMPy, TCPy, DETP, and DEDTP were purchased from Sigma-Aldrich. Solvents for extraction procedures (*i.e.*, methanol and acetonitrile), ammonium hydroxide, and formic acid were from Sigma-Aldrich, which also supplied liquid chromatography-mass spectrometry (LC-MS)-grade acetonitrile, water, and ammonium bicarbonate. OASIS MAX cartridges were supplied by Waters. Water (18.2 MΩ cm) was purified using an in-house Milli-q system (Millipore). For chemical analyses, stock standard solutions (1000 mg/L) of each compound were prepared in acetonitrile and stored at 4 ºC. The solutions remained stable for at least four months. Working standards were prepared immediately before use by dilution with pure acetonitrile.

Internal standard (IS) and standard solutions were added to 1 mL of urine (for calibration samples), followed by the addition of 2 mL of 1% aqueous ammonium hydroxide solution to the sample, which was then kept at room temperature for 20 min to reach stabilization. The cleanup and preconcentration step was done by solid-phase extraction as follows: the sorbent was activated with 3 mL of methanol, 3 mL of deionized water, and 2 mL of 1% aqueous ammonium hydroxide solution (v/v); the sample extract was loaded onto the sorbent bed, and the cartridge was rinsed with 2 mL of 1% formic acid in deionized water and then vacuum dried. Analytes extracted from the sorbent were eluted in a new tube with the addition of 4 mL of 1% formic acid in methanol/1% formic acid in acetonitrile 25/75 (v/v). The cleaned extract was concentrated by dryness under a stream of nitrogen. Next, 200 µL of 10 mM (pH 9.5) ammonium bicarbonate/acetonitrile, 20/80 (v/v), was added to the preconcentrated extract, which was then mixed and transferred to

an insert chromatography vial for injection into the UHPLC-MS/MS system.

Compounds were chromatographically separated using a LUNA 3 µm NH2 100 Å (100 x 2 mm) from Phenomenex. The gradient mobile phase consisted of 10 mM ammonium bicarbonate (solvent A) and acetonitrile (solvent B). Gradient conditions were as follows: 0.0-5.5 min, 80% solvent B; 5.5-7.5 min, 10% solvent B; 7.5-12.5 min, 80% solvent B. The flow rate was 0.2 mL/min, and the injection volume was 25 µL. The column temperature was maintained at 25ºC. The MS was operated in negative ESI mode using optimized selected reaction monitoring (SRM). Multiple samples were fortified at different concentration levels to establish the accuracy and precision of the method. The recovery of spiked urine samples was determined, and the precision of the extraction method was calculated from the percentage relative standard deviation (%RSD) within and between batches. Data on SRM transitions, retention times, analytical parameters of the calibration curves, mean accuracy, and RSD values are reported in Supplementary material (Table S2). Limits of detection (LOD) were 0.039 µg/L for TCPy, 0.117 µg/L for IMPy, 0.116 µg/L for DETP, and 0.142 µg/L for DEDTP. Detectable OP pesticide metabolite levels were adjusted by urinary creatinine concentrations to account for urine dilution, expressing creatinine-adjusted concentrations as nanograms per gram (ng/g) of creatinine. Urinary creatinine was analyzed in a Roche Cobas C-311 system using a commercial kit (CREJ2) based on the Jaffé method.

2.3. DNA extraction and genotyping

DNA was extracted from whole blood with Maxwell® RSC equipment, quantified by PicoGreen assay, and normalized to 50 ng/µL. Genome-wide DNA genotyping was performed by Infinium technology from Illumina with the Global Screening Array (GSA) v2.0+MD at the Human Genotyping Laboratory of the Spanish National Cancer Research Centre, a member of CeGen-PRB3-Carlos III Health Institute (ISCIII), Madrid, following Illumina's recommendations. Each plate contained 2 HapMap control samples duplicates. Genotype calling was performed with the GenomeStudio v2.0 software (Illumina). Next, additional genetic variants were imputed with the Michigan Server and the HRC reference panel, from which we retrieved genotypes of the following single nucleotide

polymorphisms (SNPs) in PON1 gene: rs662_C (Q192R) and rs854560_T (L55M). We checked the minor allele frequency (MAF) and the deviation from the Hardy Weinberg equilibrium. The analysis performed showed no significant linkage disequilibrium between the polymorphisms (D'=0.94, R^2=0.20).

2.4. Serum hormones

A non-fasting venous blood sample was drawn between 4 and 8 pm on the same day as the collection of the urine sample. Blood samples were centrifuged, and the resulting serum was stored at −80 °C until analysis. Reproductive and thyroid hormone levels in serum samples were analyzed by electrochemiluminescence immunoassay using a Roche® kit (Elecsys System, Roche Diagnostics) at the laboratory of the Scientific and Technological Platform of ibs.GRANADA. The reproductive hormones included total testosterone (TT), 17β-estradiol (E_2), dehydroepiandrostenedione (DHEA), sex-hormone binding globulin (SHBG), LH, FSH, anti-Müllerian hormone (AMH), insulin-like growth factor-1 (IGF-1), and prolactin. Thyroid hormones included free T4 (FT4), total T3 (TT3), and TSH. Regarding quality control, repeatability and intermediate precision ranges for all hormones are shown in Supplementary material (Table S3). Information on assay sensitivities is also reported in Supplementary material.

2.5. Statistical analysis

Descriptive statistics were calculated for the general characteristics of participants, the distributions of urinary OP pesticide metabolite concentrations (both unadjusted and creatinine-adjusted levels) and serum hormones, and the allelic frequencies of PON1 polymorphisms. Percentiles were used to describe the distribution of data on OP pesticide metabolites and hormones. Hormone levels below the LOD were assigned a value of half the LOD; however, no imputation method was used for OP metabolites because >20% of the samples had levels below the LOD of the method for each metabolite.

Multivariable linear regression was used to explore associations between individual OP metabolites and serum levels of reproductive and thyroid hormones. Hormone levels were natural logarithm transformed before regression analysis to reduce skewness. Urinary biomarkers of OP pesticide exposure were modeled as dichotomous variables with undetected concentrations as the

reference category. Based on biological considerations and following previous studies (Meeker et al., 2006a, 2006b, 2008; Melgarejo et al., 2015), all models were adjusted for age (16 or 17 years), body mass index (BMI; continuous, kg/m^2), time of day when blood was collected (continuous), and timing of blood/urine sampling by season (spring, summer, fall, or winter). Following the 10% change-in-estimate criterion, area of residence (urban or sub-urban/rural), annual household income (<25000, 25000-35000, or >35000 euros), and body fat mass (continuous, %) were also considered for inclusion in the models; however, none of these variables appeared to confound the associations between urinary OP metabolites and hormones. The regression coefficients were back-transformed to facilitate interpretation of the results, which were expressed as the percentage variation in hormone levels between the detection and non-detection of exposure. Interaction terms and stratified analysis were used to explore the potential for interaction between OP metabolite and possession of either the susceptibility allele (*i.e.* 192QR+RR or 55LM+MM genotype) or the homozygous recessive genotype (*i.e.* 192RR or 55MM). Stratified analysis was performed when a statistically significant (p-value<0.05) interaction was observed, with the exception of the 192RR genotype, which was present in only a limited number of participants. R version 3.4.3 (SAS Institute Inc., Cary, NC, USA) was used for data analyses.

3. Results

General characteristics of the study participants are summarized in Table 1. Most of the adolescents were 16 years old, had their biological samples collected in the fall or winter, resided in urban areas, and had an annual household income of ≥25000 euros. The mean BMI of participants was 23 kg/m^2, and the mean body fat mass was 13.4%. IMPy was detected in 76% of urine samples, with a median concentration of 0.254 µg/L (218.4 ng/g creatinine), and TCPy in 34% of samples. DETP was detected in 56% of samples (median of 0.251 µg/L or 111.8 ng/g creatinine) and DEDTP in only one participant (Table 2). Overall, reproductive hormone levels did not reveal clinical signs of endocrine disturbance (Table 3). However, it should be noted that 14% of participants had low testosterone levels (<6.20 nmol/L). Regarding thyroid hormones, two subjects had slightly elevated TSH (with normal FT4) and two had low FT4 (with normal TSH). Genotype frequencies were 56.4% (QQ), 36.8%

(QR), and 6.8% (RR) for the Q192R polymorphism, and 38.5% (LL), 44.4% (LM), and 17.1% (MM) for the L55M polymorphism (Table 3).

Results of linear regression analysis between urinary concentrations of OP pesticide metabolites and hormone levels are exhibited in Table 4. Detected concentrations of urinary IMPy were significantly associated with increases in E_2, DHEAS, FSH, AMH, and prolactin levels and with decreases in SHBG and LH; TCPy was associated with an increase in DHEAS and decreases in E_2, FSH, and AMH; and DETP was associated with marginally-significant increases in TT and TT3 and with significant decreases in FSH, AMH, and prolactin.

Significant interactions were found between IMPy and Q192R polymorphism on levels of TT ($P_{interaction}$=0.05 for 192RR genotype) and DHEAS ($P_{interaction}$=0.02 for 192R genotype), and between IMPy and 55MM genotype on levels of DHEAS ($P_{interaction}$=0.04), SHBG ($P_{interaction}$=0.05), and FT4 ($P_{interaction}$=0.05). For TCPy, significant interactions were found with 55MM genotype on SHBG ($P_{interaction}$=0.01) and FT4 ($P_{interaction}$=0.02) and with 192RR genotype on IGF-1 ($P_{interaction}$=0.01). Finally, there were significant interactions between DETP and 55MM genotype on TT ($P_{interaction}$=0.03), E_2 ($P_{interaction}$=0.01), and prolactin ($P_{interaction}$=0.03).

According to the stratified analysis, participants carrying 55MM genotype showed higher increases in DHEAS (95%, 95%CI=31; 191%) and TT (91%, 95%CI=6; 145%) levels for detected *versus* undetected IMPy and DETP, respectively, compared with those carrying 55LL or LM genotype (7%, 95%CI=-12; 32% and 8%, 95%CI=-12; 31%, respectively) (Table 5). Regarding thyroid hormones, carriers of 192QR or RR genotype showed a higher increase in TT3 levels (11%, 95%CI=3; 21%) for detected DETP in comparison to carriers of QQ genotype (2%, 95%CI=-6; 10%), while those carrying 55MM genotype had higher increases in FT4 (25%, 95%CI=-2; 58%) and TSH levels (66%, 95%CI=7; 158%) for detected TCPy and DETP, respectively, in comparison to carriers of 55LL or LM genotype (2%, 95%CI=-4; 7% and 0%, 95%CI=-17; 21%, respectively) (Table 5).

4. Discussion

To our knowledge, this is the first study to examine the association between OP pesticide exposure biomarkers and hormone levels and the

interaction with PON1 polymorphisms in non-occupationally exposed subjects. Results obtained suggest that: 1) exposure of male adolescents to OP pesticides may lead to changes in their reproductive and thyroid hormone levels, and some of these changes could result from inhibition of steroidogenesis and pituitary hormones secretion or from interference with deiodination; 2) the effect of IMPy and DETP on DHEAS and TT levels, respectively, is higher among adolescents who are genetically more susceptible to OP pesticides due to possession of PON1 55MM genotype; and 3) IMPy, TCPy, and DETP showed an apparent effect on thyroid hormone levels in adolescents carrying PON1 192QR/RR or 55MM genotype.

OP pesticides are among the most frequently employed insecticides in agriculture. In Spain, they represent around one-third of the total amount of insecticides, acaricides, and nematicides used for plant protection (Hernández et al., 2019). In the European Union (EU), chlorpyrifos was commonly used to control insects in numerous crops for many years, but its commercialization and use was banned in January 31 2020 (Commission Implementing Regulation (EU) 2020/17). Diazinon has not been approved for use in EU countries since 2006. However, IMPy was among the most frequently detected pesticide metabolites in the urine of 6-11-year-old Spanish children a few years later, with a detection rate of 57% (median=5.16 ng/g), while TCPy and DETP were detected in 86% (median=3.40 ng/g) and 36% of the children, respectively (Roca et al., 2014). In Almería, an area of intensive horticulture activity adjacent to the province of Granada, DETP was detected in urine from 21.8% of children aged 3-11 years (Hernández et al., 2019). In comparison to the above findings, boys from the INMA-Granada cohort had higher levels of IMPy and DETP and lower levels of TCPy. In general, urinary levels of TCPy in the present study are several times lower than those reported for non-occupationally exposed men in other countries (Fortenberry et al., 2012; Meeker et al., 2006a, 2006b, 2008; Omoike et al., 2015). Low concentrations of TCPy might be due to a reduction in exposure to chlorpyrifos over recent years, whereas the relatively high detection rate of IMPy in the urine of participants was less expected and suggests ongoing dietary exposure to diazinon. In fact, data obtained from the Spanish control program in 2018 on pesticide residues in food revealed the detection of diazinon in fruit, vegetables, and spices and reported the wide presence of chlorpyrifos in fruit,

vegetables, spices, cereals, meat, beans, seeds, and processed food (AESAN, 2018).

4.1. Reproductive hormones

Evidence linking OP pesticide exposure to altered male reproductive hormones has been somewhat contradictory. However, some results in the present study are in partial agreement with previous reports, as discussed below. In line with our findings, urinary TCPy (median=3.16 *vs* <0.04 μg/L in this study) was inversely associated with E_2 levels in adult men recruited at a fertility clinic (Meeker et al., 2008). In addition, several animal studies have shown a decrease in serum E_2 levels after exposure to chlorpyrifos or chlorpyrifos-methyl (Jeong et al., 2006; Manjunatha and Philip, 2016; Ventura et al., 2016), and one observed that chlorpyrifos reduces the expression of co-repressors of estrogen receptor (ER) activity, resulting in lower E_2 levels (Ventura et al., 2016). Another possible mechanism for the inverse association between TCPy and E_2 may be interference with aromatization (the process that converts steroid precursors such as DHEAS and androgens to E_2) by TCPy, chlorpyrifos, or chlorpyrifos-methyl. In men, approximately half of circulating E_2 and testosterone is produced by peripheral conversion of DHEA. Hence, inhibition of aromatase, responsible for DHEA conversion to testosterone, or 3β-hydroxysteroid dehydrogenase (3β-HSD), responsible for testosterone conversion to E_2, could lead to reduced E_2 and increased DHEAS levels, which is consistent with our findings. This is biologically plausible, because chlorpyrifos has been shown to exert anti-androgenic activity by reducing the expression of key steroidogenic enzymes, including aromatase, 3β-HSD, and 17β-HSD *in vitro* (Viswanath et al., 2010). In addition, rat studies demonstrated that chlorpyrifos induces oxidative stress in the pituitary gland, inhibiting the secretion of LH and FSH (Joshi et al., 2007; Shittu et al., 2012). If the site of chlorpyrifos action was the pituitary gland, an inverse association of TCPy with AMH might be expected to accompany the inverse association with FSH observed in this study, given that AMH is considered a marker of *FSH* action on Sertoli cells and may therefore predict FSH levels. However, Meeker et al. (2006a) found no association of urinary TCPy (mean=2.41 μg/L) with FSH or LH. At any rate, these associations are novel and should be interpreted with caution, because a clear relationship between exposure to chlorpyrifos and male reproductive

hormones has not previously been reported, and this is the first study to examine these associations in adolescents.

Total DAPs levels have been associated with increased FSH and prolactin (Aguilar-Garduño et al., 2013) and decreased LH (Blanco-Muñoz et al., 2010; Recio et al., 2005) in agricultural workers with higher urinary DETP levels than those of the present participants. We found similar results for IMPy, which was additionally associated with increases in E_2, DHEAS, and AMH, and a decrease in SHBG. The parent compound of IMPy diazinon has been shown to induce oxidative damage and inhibit TT synthesis in mice testes (Sarabia et al., 2009). Furthermore, diazinon inhibited steroidogenic acute regulatory (*StAR*) protein expression in rats (Siavashpour et al., 2018). Other animal studies have also demonstrated that diazinon disrupts the pituitary-gonadal axis, increasing prolactin and FSH levels and decreasing LH and E_2 levels (ElMazoudy and Attia, 2012; Maxwell and Dutra, 2005). Hence, with the exception of the positive association with E_2, our findings are supported by experimental evidence and suggest that diazinon may alter the hypothalamic-pituitary axis, affecting the secretion of pituitary hormones (LH, FSH, prolactin) and possibly interfering in steroidogenesis.

Regarding DETP, the observed association with increased TT is in line with findings in agricultural workers of positive associations with total DAPs (Blanco-Muñoz et al., 2010), diethylphosphate (DEP), and DEDTP (Panuwet et al., 2018), but not with findings of an inverse association between DEP and TT in non-occupationally exposed men with DETP levels comparable to those of our population (Omoike et al., 2015). As observed for TCPy, DETP was also associated with reductions in FSH, AMH, and prolactin in the present study, possibly due to the inhibition of pituitary hormone secretion by parent compounds of DETP, which include chlorpyrifos and diazinon; however, other OP pesticides are also metabolized to DETP, hampering the interpretation of results.

We found that the effect of IMPy and DETP on levels of DHEAS and TT, respectively, was higher for PON1 55MM genotype carriers, which may be explained by the fact that the 55L allele results in higher PON1 mRNA and serum levels and therefore higher activity in comparison to the 55M allele (Leviev et al., 1997). To our best knowledge, no study has examined the interaction between OP pesticide

exposure and PON1 polymorphisms on reproductive hormones.

4.2. Thyroid hormones

Few epidemiological studies have examined the impact of OP exposure on thyroid hormone levels, and scant data are available on children and adolescents (Suhartono et al., 2018). Our observation of a positive association between TCPy at low levels and FT4 is in disagreement with a previous report of an inverse association between TCPy and FT4 in adult men (Meeker et al., 2006b) but is in agreement with National Health and Nutrition Examination Survey (NHANES) findings of a positive association between TCPy and TT4 in males aged 12-18 years (Fortenberry et al., 2012). A possible mechanism underlying the suggestive associations of TCPy and IMPy with elevated FT4 may be the inhibition by chlorpyrifos or diazinon of extrathyroidal enzymes such as type II 5'-deiodinase, involved in the conversion of T4 to active T3. Inhibition of deiodination might increase serum levels of T4; however, no study has demonstrated the inhibition of deiodinase activity by chlorpyrifos or diazinon.

The positive association of DETP with TSH is consistent with previous observations in occupationally and non-occupationally exposed men, suggesting that OP exposure may induce hormonal changes compatible with hypothyroidism (Lacasaña et al., 2010a; Meeker et al., 2006b; Toft et al., 2006). Possible mechanisms underlying the hypothyroid-like effect of OP compounds include: alteration of T4 production/secretion by the thyroid gland; induction of uridine diphospho-glucuronosyltransferase (UDPGT) and sulfotransferase (SULT), enzymes involved in the hepatic metabolism of thyroid hormones; and stimulation of extrathyroidal conversion of T4 to T3 (De Angelis et al., 2009; Ghisari and Bonefeld-Jorgensen, 2005; Jeong et al., 2006). Stimulation of deiodination is the most likely mechanism, because it is compatible with the observed increase in TT3 associated with DETP.

As found for IMPy and DETP in relation to DHEAS and TT, the association of TCPy and DETP with thyroid hormones was more pronounced for genotype 55MM carriers. By contrast, the study of Mexican floricultural workers by Lacasaña et al. (2010b) observed no interaction between urinary DAPs and L55M polymorphism but found an interaction between total DAPs and dimethylphosphate metabolites and Q192R polymorphism on TSH and TT3, in partial agreement

with the present finding of an interaction between DETP and Q192R polymorphism on increased TT3. Q192R polymorphism exerts a substrate-dependent effect on enzymatic activity. In this way, paraoxon and chlorpyrifos oxon (active metabolites of parathion and chlorpyrifos) are hydrolyzed faster by the R isoform, while diazoxon (the active metabolite of diazinon) is hydrolyzed faster by the Q isoform (Mackness et al., 1998). Our finding may therefore suggest that 192R allele carriers are more susceptible to the effect on TT3 of yet-to-be-identified parent compound(s) of DETP present in the urine.

4.3. Limitations and strengths

This study has some limitations, including its cross-sectional design, which impedes the establishment of causal relationships and does not elucidate whether the associations between OP pesticide metabolites and serum hormone levels are transient or permanent. In addition, the sample size was limited and may have provided inadequate statistical power, particularly for the interaction analysis with the homozygous recessive genotype and the analysis stratified by PON1 polymorphisms. A further limitation is that urinary OP pesticide metabolite levels typically reflect recent exposure, due to their short biological half-lives, and the measurement of single urine samples may increase the risk of exposure misclassification. Repeated urine samples should be gathered in future studies to improve the accuracy of exposure assessment. Our study was also limited by the lack of data on total DAP concentrations, an overall biomarker of OP exposure, hampering comparison with the results of previous epidemiological studies. Another potential limitation of the study is the lack of information on the iodine status of study participants, which may have a major effect on susceptibility to the effects of thyroid-disrupting pesticides (Medda et al., 2017). Finally, the performance of multiple analyses means that some of the significant associations observed may be due to the chance. Nevertheless, many of the statistically significant results were supported by existing epidemiological and experimental evidence, as discussed above for the most relevant associations. Regarding the experimental data, although animal studies offer valuable insight into possible modes of action, account should be taken of possible differences between species in the response to endocrine disrupting

chemicals. In this way, extrapolation of exposure-response data from animals is limited by the differences between animals and humans. For instance, humans and rats may differ in their sensitivity to thyroid hormone deficiency (Zoeller, 2004), while certain endocrine disruptors may alter the expression of estrogen and thyroid receptors in a species-specific manner (Jocsak et al., 2019).

Strengths of this study include: the assessment of two specific OP pesticide metabolites (IMPy and TCPy); the examination of numerous hormonal parameters, including hormones not previously studied in relation to pesticide exposure; and the use biomarkers of susceptibility to OP pesticides. The results can be considered of public health importance, given that: 1) a large proportion of the general population is exposed to low levels of OP pesticides, and 2) adolescents may be especially vulnerable to changes in reproductive hormone levels, which may indicate altered reproductive function.

5. Conclusions

This study of male adolescents from the general population observed several biologically plausible associations between exposure to low doses of OP pesticides and altered hormone levels and provides evidence that PON1 polymorphisms, particularly L55M polymorphism, may confer differential susceptibility to OP pesticide toxicity. However, longitudinal studies in larger samples are warranted to elucidate whether non-occupational exposure to OP pesticides can cause permanent changes in hormone levels and to explore the influence of genetic susceptibility to OP toxicity on the risk of adverse health effects.

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COMPETING FINANCIAL INTEREST DECLARATION

The authors declare no actual or potential competing financial conflict of interests.

Highlights

1. OP pesticides are potential endocrine disruptors that may affect hormone levels.
2. We assessed the link between OP urinary metabolites and hormones in boys.
3. We also examined the interaction between OP metabolites and PON1 polymorphisms.
4. OP exposure was associated with altered reproductive and thyroid hormone levels.
5. Some associations were greater in subjects carrying the PON1 55MM genotype.

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Table 1. Characteristics of study participants (N=117)

Variables	n (%) or AM ± SD
Age (years)	
16	92 (78.6)
17	25 (21.4)
Season of blood sampling	
Spring	23 (19.7)
Summer	15 (12.8)
Fall	54 (46.2)
Winter	25 (21.4)
Area of residence	
Urban	82 (70.1)
Sub-urban/rural	35 (29.9)
Family income (euros)	
<25000	40 (34.2)
25000-35000	55 (47.0)
>35000	22 (18.8)
BMI (kg/m^2)	23.05 ± 4.96
Body fat mass (%)	13.4 ± 7.2
Urinary creatinine (mg/dL)	187 ± 58.6

AM: Arithmetic mean; SD: Standard deviation; BMI: Body mass index.

Table 2. Urinary concentrations of OP insecticide metabolites in first morning voids.

Metabolites	DF (%)	Unadjusted (µg/L)				Creatinine-adjusted (ng/g)			
		Median	Percentiles		Max.	Median	Percentiles		Max.
			25th	75th			25th	75th	
TCPy	34.2	<0.039	<0.039	0.084	1.213	<20.85	<20.85	42.07	448.6
IMPy	76.9	0.254	0.083	0.810	27.11	218.4	102.2	409.5	17,647
DETP	56.4	0.251	<0.116	0.623	40.35	111.8	<62.06	272.1	26,555
DEDTP	0.9	<0.142	<0.142	<0.142	0.308	<75.93	<75.93	<75.93	311.3

DF: Detection frequency.

Table 3. Serum concentrations of hormones and frequencies of PON1 genotypes.

Hormones (normal range*)	**Median**	**5^{th}-95^{th} percentiles**
TT (6.20-30.60 nmol/L)	10.69	4.33-21.47
E_2 (94.8-223 pmol/L)	25.51	9.17-94.41
DHEA-S (2,435-17,072 nmol/L)	9,518	4,172-16,729
SHBG (13.6-86.0 nmol/L)	32.95	16.01-72.01
LH (0.50-5.00 mU/mL)	4.25	1.64-12.98
FSH (0.80-4.40 mU/mL)	3.58	1.46-57.17
Prolactin (86-324 μU/mL)	233.2	125.7-438.3
AMH (23-128 pmol/L)	54.30	24.39-274.9
IGF-1 (221-577 ng/mL)	331.5	206.3-468.8
FT4 (12-22 pmol/L)	16.09	13.07-19.58
TT3 (1.3-3.1 nmol/L)	2.15	1.59-2.70
TSH (0.27-4.20 mU/mL)	1.89	0.81-3.94
PON1 polymorphisms	***n***	**%**
Q192R		
QQ (homozygous dominant)	66	56.4
QR (heterozygous)	43	36.8
RR (homozygous recessive)	8	6.8
QR + RR	51	43.6
L55M		
LL (homozygous dominant)	45	38.5
LM (heterozygous)	52	44.4
MM (homozygous recessive)	20	17.1
LM + MM	72	61.5

*For reproductive hormones: normal values for males aged 12-19 years in Tanner stage G4 or G5.

Table 4. Crude and adjusted associations between urinary OP pesticide metabolites and serum hormone levels.

Hormones	IMPy				TCPy				DETP			
	Crude model		Adjusted model*		Crude model		Adjusted model*		Crude model		Adjusted model*	
	% var.	*95%CI*	% var.	*95%CI*	% var.	*95%CI*	% var.	*95%CI*	% var.	*95%CI*	% var.	*95%CI*
TT	-12	-29; +10	-13	-30; +8	-14	-29; +4	-13	-29; +5	**+22**	**+1; +46**	**+16**	**-2; +41**
E_2	**+35**	**-5; +91**	**+46**	**+5; +105**	**-32**	**-50; -8**	**-36**	**-53; -14**	-22	-52; +5	-12	-35; +17
DHEAS	+16	-3; +38	**+20**	**+1; +44**	**+23**	**+6; +45**	**+23**	**+5; +45**	-2	-16; +14	0	-16; +16
SHBG	-11	-25; +7	**-16**	**-28; -2**	-10	-23; +6	-4	-17; +10	+14	-1; +33	+2	-10; +17
LH	**-30**	**-45; -12**	**-27**	**-42; -9**	+13	-9; +40	+12	-10; +38	+1	-18; +23	+10	-10; +35
FSH	**+74**	**+3; +194**	**+77**	**+12; +180**	**-48**	**-67; -17**	**-38**	**-59; -6**	**-52**	**-69; -26**	**-55**	**-69; -34**
AMH	**+42**	**+2; +97**	**+39**	**+3; +87**	**-25**	**-44; +1**	**-17**	**-37; +9**	**-28**	**-46; -5**	**-28**	**-44; -7**
IGF-1	+1	-9; +13	0	-11; +11	+7	-3; +18	+6	-4; +18	0	-10; +9	-2	-10; +8
Prolactin	**+30**	**+12; +51**	**+27**	**+9; +48**	-7	-20; +6	-10	-22; +4	**-18**	**-28; -7**	**-17**	**-28; -6**
FT4	+1	-5; +7	+2	-5; +8	+4	-2; +9	+4	-2; +10	+2	-4; +7	+2	-3; +8
TT3	-4	-10; +2	-3	-10; +3	0	-6; +6	-1	-7; +5	**+5**	**0; +11**	**+5**	**0; +11**
TSH	-10	-27; +10	-9	-27; +12	+2	-15; +22	0	-20; +20	+7	-10; +28	+8	-10; +29

*Adjusted for age (16 or 17 yrs), BMI (continuous), timing of blood/urine samples by season (spring, summer, fall, or winter), and time of day (continuous). % var: percentage variation in hormone level for detected *versus* undetected urinary OP metabolite.
Significant or marginally significant associations in bold ($p<0.10$).

Table 5. Association between urinary OP pesticide metabolites and hormone levels stratified by PON1 genotype[†]

	TT		E_2		DHEAS		SHBG		Prolactin		FT4		TT3		TSH	
	% var.	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***	**% var.**	***95%CI***
IMPy																
192QQ					**+53**	**+19; +95**										
192QR+RR					-5	-29; +29										
55LL+LM					+7	-12; +32	**-21**	**-34; -7**			-2	-8; +4				
55MM					**+95**	**+31; +191**	+17	-19; +68			**+27**	**-1; +64**				
TCPy																
55LL+LM							-10	-24; +5			+2	-4; +7				
55MM							+22	-11; +70			**+25**	**-2; +58**				
DETP																
192QQ													+2	-6; +10		
192QR+RR													**+11**	**+3; +21**		
55LL+LM	+7	-12; +31	-24	-45; +5					**-22**	**-32; -11**					0	-17; +21
55MM	**+91**	**+6; +145**	+176	-33; +374					+23	-17; +85					**+66**	**+7; +158**

[†]Only associations for which the interaction term was significant are shown; % var: percentage variation in hormone level for detected *versus* undetected urinary OP metabolite.

%var: percentage variation in hormone level.

Significant or marginally significant associations in bold ($p<0.10$).

5.6 Resultados del objetivo 5, artículo 2:

URINARY METABOLITES OF NON-PERSISTENT PESTICIDES AND SERUM HORMONES IN SPANISH ADOLESCENT MALES

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Resumen

Objetivo: Evaluar la relación entre las concentraciones urinarias de etilentiourea (ETU), el principal metabolito de los fungicidas bisditiocarbamatos de etileno; ácido 3-fenoxibenzoico (3-PBA), un metabolito común de muchos piretroides, y 1-naftol (1N), un metabolito del carbamato insecticida carbarilo, con las concentraciones hormonales en varones adolescentes y examinar las interacciones entre los metabolitos de pesticidas y polimorfismos en genes implicados en el metabolismo de xenobióticos, incluidos CYP2C19 y CYP2D6, en relación con las concentraciones de hormonas.

Métodos: Se realizó un estudio transversal en 134 varones de la cohorte INMA-Granada. Se recogieron muestras de orina y suero de los participantes durante la misma visita clínica a la edad de 15-17 años. Se cuantificaron las concentraciones urinarias de ETU, 3-PBA y 1N y los niveles séricos de las principales hormonas reproductivas (testosterona, 17β-estradiol [E2], sulfato de dehidroepiandrosterona [DHEA-S], globulina transportadora de hormonas sexuales [SHBG], hormona luteinizante [LH], hormona estimulante del folículo [FSH], hormona antimülleriana [AMH] y prolactina), y tiroideas (tiroxina libre [FT4], triiodotironina [TT3] y hormona estimulante de la tiroides [TSH]). Se midieron también las concentraciones de factor de crecimiento insulínico tipo 1 (IGF-1), hormona adrenocorticotrópica (ACTH) y cortisol y se analizaron los polimorfismos genéticos CYP2C19 G681A y CYP2D6 G1846A en una submuestra de 117 participantes. El análisis estadístico se realizó mediante regresión lineal múltiple y análisis estratificado.

Resultados: Se detectó ETU en la orina de 74,6% de los participantes, 1N en el 38,1% y 3-PBA en el 19,4%. Se observaron asociaciones positivas entre la detección de 3-PBA y TT3 y entre la detección de 1N y DHEA-S. Se encontraron asociaciones marginalmente significativas entre 1N y disminución de las concentraciones de E2 y FSH.
La magnitud de las asociaciones observadas para 1N y DHEAS eran mayores en los portadores de los polimorfismos con pérdida de función en los genes CYP2C19 y CYP2D6 (portadores de genotipo GA y AA). Además, se observó una asociación positiva entre las concentraciones de ETU y de cortisol solo en los portadores de los genotipos 1846GA y AA del gen CYP2D6.
Conclusiones: Las asociaciones observadas entre metabolitos urinarios de pesticidas y los niveles de hormonas tiroideas y reproductivas deben verificarse en estudios con un mayor tamaño muestral. Son necesarios, además, estudios que investiguen las interacciones gen-ambiente para identificar individuos más susceptibles a los efectos adversos de los pesticidas.

Abstract

Objective: To assess the relationship of urinary concentrations of ethylenethiourea (ETU), the main degradation product of ethylene bis-dithiocarbamate fungicides, 3-phenoxybenzoic acid (3-PBA), a common metabolite of many pyrethroids, and 1-naphthol (1N), a metabolite of the carbamate insecticide carbaryl, with hormone concentrations in adolescent males; and to examine interactions between pesticide metabolites and polymorphisms in xenobiotic metabolizing enzymes, including CYP2C19 and CYP2D6, in relation to hormone concentrations.
Methods: A cross-sectional study was conducted in 134 males from the Spanish Environment and Childhood (INMA)-Granada cohort. Urine and serum samples were collected from participants during the same clinical visit at the age of 15-17 years. First morning urine void was analyzed for concentrations of ETU, 3-PBA, and 1N. Serum was analyzed for concentrations of reproductive hormones (testosterone, 17β-estradiol [E2], dehydroepiandrosterone sulfate [DHEAS], sex hormone binding globulin [SHBG], luteinizing hormone [LH], follicle stimulating hormone [FSH], anti-Müllerian hormone [AMH], and prolactin), thyroid hormones (free thyroxine [FT4], total triiodothyronine [TT3], and thyroid stimulating hormone [TSH]), insulin growth factor 1 (IGF-1), adrenocorticotropic hormone (ACTH), and cortisol. CYP2C19 G681A and CYP2D6

G1846A polymorphisms were determined in blood from 117 participants. Multiple linear regression, interaction terms, and stratified analyses were performed.

Results: Urinary ETU was detected in 74.6% of participants, 1N in 38.1%, and 3-PBA in 19.4%. Positive associations between detectable 3-PBA and TT3 and between detectable 1N and DHEAS were found, and marginally-significant associations of 1N with reduced E2 and FSH were observed. Poor CYP2C19 and CYP2D6 metabolizers (GA and AA genotype carriers) showed a greater increase in DHEAS for detected versus undetected 1N compared with GG genotype carriers. Poor CYP2D6 metabolizers (1846GA and AA genotypes) evidenced increased cortisol for detected versus undetected ETU.

Conclusions: The associations observed between urinary pesticide metabolites and altered thyroid and reproductive hormones are novel and should be verified in studies with larger sample size. Further research on gene-environment interactions is warranted to establish individual susceptibility to pesticides and the risk of adverse health effects.

Keywords:

Non-persistent pesticides; pyrethroids; fungicides; reproductive hormones; thyroid hormones; gene-environment interaction

1. INTRODUCTION

Most pesticides used today are considered non-persistent compounds because they readily break down in the environment and do not accumulate in the body throughout life. They are widely used in agricultural land, public indoor spaces, and residential buildings, although pesticide residues in food are the main exposure source for the general population (Curl et al., 2019; Hyland et al., 2019). Concerns have been raised by the classification of numerous non-persistent pesticides as confirmed or suspected endocrine-disrupting (ED) chemicals. It is estimated that at least 10% of approved pesticides in the European Union (EU) possess ED properties (McKinlay et al., 2008; PAN Europe, 2015). Experimental studies have reported that many current-use pesticides (or their metabolites) may interfere with the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid axes (Leemans et al., 2019; Orton et al., 2011). However, the effects of current human exposure levels on reproductive and thyroid function are poorly understood. Spain is the largest consumer of pesticides in the EU, especially fungicides, purchasing 71,987 tons of pesticides in 2017 (EUROSTAT, 2019). Pesticide residues are also more frequently detected in fruit and vegetables from Spain than from most other European countries (EFSA, 2019). Dithiocarbamates are broad-spectrum fungicides used in a wide range of food crops (López-Fernández et al., 2012), and the ethylene bis-dithiocarbamates (EBDCs) mancozeb, maneb, and thiram are metabolized to ethylene thiourea (ETU). ETU can interfere with thyroid hormone biosynthesis by inhibiting thyroid peroxidase activity and is therefore considered an ED compound (Marinovich et al., 1997). In addition, some EBDC formulations contain ETU. In experimental studies, exposure of rodents to EBDCs produced a decrease in T4 and an increase in thyroid-stimulating hormone (TSH) (Axelstad et al., 2011; Kackar et al., 1997). In human studies, urine and blood ETU levels in workers exposed to EBDC fungicides were associated with an increase in TSH and iodine excretion (Medda et al., 2017; Panganiban et al., 2004; Steenland et al., 1997). Moreover, mancozeb demonstrated anti-androgenic activity *in vitro* (Kjeldsen et al., 2013), but no association between urinary ETU and testosterone levels was observed in a small sample of male farmers (Panuwet et al., 2018).

Pyrethroids are a large group of synthetic insecticides commonly used in agriculture, gardens, and homes and

have become even more widespread and available to consumers since the application of restrictions on organophosphate use (Horton et al., 2011; Meeker et al., 2009). Many pyrethroids possess ED properties, having the potential to interact with the hypothalamic-pituitary-gonadal axis (Ye and Liu, 2019) and to disturb thyroid homeostasis (Leemans et al., 2019). In this regard, *in vitro* studies demonstrated that the pyrethroids deltamethrin, cypermethrin, and λ-cyhalothrin elicit estrogenic and anti-androgenic activity (Andersen et al., 2002; Kjeldsen et al., 2013; Kojima et al., 2004) and that several pyrethroids exert antagonistic action on the thyroid receptor (Du et al., 2010). In humans, urinary levels of 3-phenoxybenzoic acid (3-PBA), a major metabolite of pyrethroids, including cyhalothrin, cypermethrin, deltamethrin, fenvalerate, permethrin, and tetramethrin, were associated with increased levels of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in men attending a fertility clinic (Han et al., 2008; Meeker et al., 2009) and in 9- to 16-year-old boys (Ye et al., 2017). Research in adult males also found associations of 3-PBA with increased 17β-estradiol (E2) (Han et al., 2008) and of the pyrethroid metabolites *cis*-/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane) carboxylic acid with reduced E2, testosterone, and inhibin B (Meeker et al., 2009). However, three studies found no association between urinary 3-PBA and male reproductive hormones (Panuwet et al., 2018; Radwan et al., 2014; Yoshinaga et al., 2014). In relation to thyroid hormones varied results have been published from epidemiological studies in men from the general population, with some authors reporting an inverse association of urinary 3- PBA with total thyroxine (TT4) and triiodothyronine (TT3) (Hwang et al., 2020) but others finding no association with thyroid disruption (Jain, 2016; Meeker et al., 2009).

Carbamate insecticides were heavily used in agriculture and also sold as household insecticides in the past but they are no longer in use in the EU due to their toxicity. *In vitro* studies indicated that carbaryl, a broad-spectrum carbamate insecticide, may act as an endocrine modulator in mammalian cells (Klotz et al., 1997) and as a thyroid antagonist by interfering with the thyroid hormone receptor-signaling pathway (Sun et al., 2008). Although carbaryl has not been approved for use in EU countries since 2007, it is still detected in foods marketed in the EU and grown in the EU or other countries (EFSA, 2019).

To our best knowledge, only two epidemiological studies have examined the link between carbamate exposure and hormone levels among non-occupationally exposed men. One of these found an association of urinary levels of 1- naphthol (1N), the primary metabolite of carbaryl, with lower testosterone, E2, and LH levels (Meeker et al., 2006a), whereas the other study found no association between 1N and thyroid hormones (Meeker et al., 2006b). Cytochrome (CYP) enzymes are one of the main enzyme systems in xenobiotic metabolism, playing a key role in the biotransformation of numerous environmental contaminants. Polymorphisms in the genes that code for these enzymes may alter levels of toxic chemical intermediates and thereby influence the risk of adverse health effects.

The CYPs CYP2C19 and CYP2D6 are involved in the metabolism of at least 15% and 4% of pesticides, respectively (Abass et al., 2012). Pesticides reported to be metabolized at least in part by human hepatic CYP2C19 or CYP2D6 include carbaryl and other carbamates (Tang et al., 2002), while CYP2C19 is involved in the metabolism of various pyrethroids such as *cis*-permethrin, cypermethrin, deltamethrin, esfenvalerate, and λ-cyhalothrin (Godin et al., 2007; Scollon et al., 2009). The common loss-of-function CYP2C19 G681A and CYP2D6 G1846A variants are among the most widely investigated single nucleotide polymorphisms (SNPs) in relation to pesticide metabolism (Gómez-Martín et al., 2015). Despite the wide-scale use of pesticides and the consequent human exposure, little is known about the health effects on children and adolescents, and there has been no study on the interaction with variants in CYP genes. The European Human Biomonitoring Initiative (HBM4EU) project has identified several non-persistent pesticide-related effect biomarkers and their mechanistic pathways within the adverse outcome pathway (AOP) framework, including circulating hormonal (estrogen and thyroid hormone) levels. With this background, the aim of this study was to assess the relationship of urinary concentrations of ETU and 3-PBA, exposure biomarkers of EBDC fungicides and numerous pyrethroids, respectively, and of 1N, exposure biomarker of the carbamate insecticide carbaryl, with serum levels of reproductive and thyroid hormones in adolescent males. A further objective was to examine the role of interactions with CYP2C9 G681 and CYP2D6 G1846A polymorphisms.

2. MATERIALS AND METHODS

2.1. Study population

A cross-sectional study was conducted in males aged 16 to 17 years from the Environment and Childhood (INMA)-Granada mother-son cohort in Southern Spain, who participated in a clinical follow-up visit in 2017-2019 (Castiello et al., 2020). The INMA Project is a multicenter population-based birth cohort study to investigate the effect of environmental exposures and diet during pregnancy, infancy, and adolescence on fetal and child development in different parts of Spain (Guxens et al., 2012). The baseline INMA-Granada cohort comprised 668 mother-son pairs, of whom a random sample of boys was asked to participate in follow-up visits at the ages of 4-5 years (N=220) and 9-11 years (N=300). All boys who participated at 4-5 and/or 9-11 years were asked to take part in a new follow-up visit. A total of 155 of these boys accepted participation, but only 135 provided both urine and blood samples. The present study was conducted in 134 of these boys who had available data on their current urinary pesticide metabolites, serum hormones, and covariates. All participants were in Tanner stage 4 or 5 (almost/fully developed genitalia). Interaction between pesticide metabolites and CYP polymorphisms on hormone levels was examined in a subsample of 117 participants with available genetic data. Further details on study participation have been reported elsewhere (Castiello et al., 2020; Suárez et al., 2021). An informed consent form was signed by the parents of all participants before collecting personal information and biological samples. The research protocol, including urine and blood collection, was approved by the Biomedical Research Ethics Committee of Granada.

2.2. Biological sample analysis

Participants self-collected a first-morning-void urine sample on the day of their clinical visit (between 4 and 8 p.m.), when a non-fasting venous blood sample was also drawn. Serum was separated from blood within 4 hours of collection. Urine, serum, and whole blood samples were stored at -80 ºC until analysis.

2.2.1. Pesticide metabolites in urine

Analysis of urinary ETU, 3-PBA, and 1N levels was performed by ultra-highperformance liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS), using an UHPLC Ultimate 3000 (Thermo Fischer) and Q Exactive

Focus mass spectrometer (Thermo Fischer) at the "UNETE research unit" of the Biomedical Research Center (CIBM), University of Granada. Detailed information on the analytical method and quality control procedures were recently published (Suárez et al., 2021). Supplementary material (Table 1) provides information on selected reaction monitoring (SRM) transitions, retention time, analytical parameters of calibration curves, mean accuracy, and relative standard deviation (RSD) for each metabolite. The standard curve for each metabolite is depicted in Supplementary Material (Figure 1). Limits of detection (LODs) were 0.072 µg/L for ETU, 0.117 µg/L for 3-PBA, and 0.156 µg/L for 1N. Urinary creatinine concentrations (mg/dL) were measured according to the Jaffé method with a Roche Cobas C-311 analyzer (Hitachi, Japan). Creatinine concentrations were used to standardize detectable pesticide metabolite levels based on urinary dilution, expressing metabolite levels as both volume-based concentrations (µg/L) and creatinine-adjusted concentrations (µg/g creatinine).

2.2.2. Serum hormones

Serum samples were analyzed for concentrations of: sex steroids (total testosterone, E2, and dehydroepiandrostenedione sulfate [DHEAS]), pituitary gonadotropins (LH and FSH), and other non-steroidal sex hormones (sex hormone binding globulin [SHBG], anti-Müllerian hormone [AMH], and prolactin); insulin-like growth factor-1 (IGF-1); other hormones of the hypothalamic-pituitary-adrenal axis, including adrenocorticotropic hormone (ACTH) and cortisol; and thyroid hormones (free T4 [FT4], TT3, and TSH). Hormone measurements were performed by electrochemiluminescence immunoassay using a Roche® kit (Elecsys System, Roche Diagnostics) at the *Instituto de Investigación Biosanitaria de Granada* (ibs.GRANADA). LODs of serum hormones and the repeatability and intermediate precision ranges were previously reported (Suárez et al., 2021). Reference ranges of hormone concentrations for males in Tanner stage 4 or 5 are reported in Table 3.

2.2.3. DNA extraction and genotyping

DNA was extracted from whole blood with Maxwell® RSC equipment, quantified by PicoGreen assay, and normalized to 50 ng/µL. Genome-wide DNA genotyping was performed by Infinium technology from Illumina with

the Global Screening Array (GSA) v2.0+MD at the Human Genotyping Laboratory of the Spanish National Cancer Research Centre (Carlos III Health Institute, Madrid), following Illumina's recommendations. Each plate contained 2 HapMap duplicate control samples. Genotype calling was performed with GenomeStudio v2.0 software (Illumina). Next, additional genetic variants were imputed with the Michigan Server and HRC reference panel, from which genotypes of the G681A SNP in CYP2C19 gene (rs4244285) and the G1846A SNP in CYP2D6 gene (rs3892097) were retrieved. These SNPs potentially encode CYP2C19*2 and CYP2D6*4 variants, respectively, which are the most frequent causes of metabolic inactivation of CYP2C19 and CYP2D6. The minor allele frequency (MAF) and deviation from the Hardy Weinberg equilibrium were calculated. No significant linkage disequilibrium was found between the polymorphisms (D'=0.49, $\chi 2$=1.17, p-value=0.28).

2.3. Statistical analysis

Detection frequencies of 3-PBA and 1N were lower than 40%; therefore, no imputation was made for urinary values below LODs for these metabolites. Concentrations of 3-PBA and 1N were converted to dichotomous variables (detected or undetected) before regression analysis. ETU was detected in more than 70% of urine samples and was therefore modeled as both a continuous and dichotomous variable (detected/undetected). Urine samples with ETU concentrations below the LOD were assigned a value of LOD/√2. Spearman's correlation analysis was performed to assess relationships between creatinine-adjusted concentrations of the three metabolites.

Linear regression analysis was conducted to explore the relationship between urinary pesticide metabolites and hormonal parameters. Each pesticide metabolite (independent variable) was modeled separately with the levels of each hormone (dependent variables). Hormone and ETU concentrations were log-transformed to reduce skewness. Urinary pesticide metabolite concentrations and urinary creatinine concentrations were considered as separate independent variables in accordance with previous studies (Barr et al., 2005). First, crude regression models that included pesticide metabolite concentrations and urinary creatinine were constructed. Based on biological considerations and previous reports (Meeker et al., 2006a, 2006b, 2008), models were further adjusted for

age (16 or 17 years), body mass index (BMI; continuous, kg/m2), time of day when blood was drawn (continuous), and timing of blood/urine sampling by season (spring, summer, fall, or winter). Area of residence (urban or sub-urban/rural), annual household income (<25000, 25000-35000, or >35000 euros), and body fat mass (continuous, %) were also considered for inclusion in the models, but none of these variables confounded associations between urinary pesticide metabolites and hormones. Study covariates are described in detail elsewhere (Castiello et al., 2020). Residuals of all linear regression models were checked for normality. Regression coefficients were back-transformed to facilitate interpretation of the results, which were expressed as the percentage variation in hormone concentration for detected *versus* undetected pesticide metabolite or, in the case of continuous ETU, as the percentage variation in hormone concentration for each doubling in urinary concentration. Because subjects are typically exposed to multiple pesticides at the same time, sensitivity analyses were performed to assess potential confounding by coexposure to pesticides by adjusting regression models simultaneously for the three pesticide metabolites. Following WHO recommendations, sensitivity analysis was also performed by excluding highly dilute or concentrated urine samples (creatinine <30 or >300 mg/dL) (WHO, 1996). Finally, sensitivity analyses were performed by excluding boys taking medications (in the past 12 months) that affect thyroid and/or sex hormone levels and those with history of physician-diagnosed thyroid disease or any endocrine related disorder.

The possible interaction of pesticide exposures with CYP2C19 G681A and CYP2D6 G1846A polymorphisms was explored by interaction terms (detected pesticide metabolite*CYP polymorphism) and stratification of the adjusted regression models by absence and presence of the null allele, *i.e.* GG genotype carriers (wild type homozygotes) and GA or AA genotype carriers (susceptibility genotype). The significance level was set at $p<0.05$. R version 3.4.3 (SAS Institute Inc., Cary, NC, USA) was used for data analyses.

3. RESULTS

No significant differences were found in general characteristics between study participants with all available data (n=134) and those with data on urinary pesticide metabolites but no data on serum hormones or covariates (n=21), except for a higher household income in

the included *versus* excluded adolescents (Supplementary material, Table 2). Most of the 134 adolescents with available data on urinary pesticide metabolites and serum hormone concentrations were aged 16 years, attended the followup visit in fall or winter, resided in urban areas, and came from middle-income families (Table 1). The mean BMI and body fat mass of participants were 23.1 kg/m2 and 13.4%, respectively (28.4% were overweight or obese). The mean urinary creatinine concentration was 186 mg/dL.

Table 2 exhibits the distribution of pesticide metabolite urinary concentrations. The highest detection frequency was for ETU (74.6%, median=0.514 µg/g creatinine), followed by 1N (38.1%, 75th percentile=0.173 µg/g) and 3-PBA (19.4%, 95th percentile=0.178 µg/g). 1N concentrations were positively and significantly correlated with 3-PBA (correlation coefficient, r=0.25, p-value=0.004), 3-PBA was marginally correlated with ETU (r=0.15, p-value=0.08), while 1N and ETU were not correlated (r=0.12, p-value=0.17). At least one pesticide metabolite was detected in the urine of 62 participants (46%), with detectable urine concentrations of two metabolites in 44 (33%) and three metabolites in 9 (7%). Overall, hormone concentrations were within the reference range for this age group (Table 3). However, it should be noted that 13% of the participants had testosterone concentrations below the reference range (<6.20 nmol/L) for this age group, 14% had prolactin concentrations above the normal range (>342 µU/mL), and almost 30% had low estradiol concentrations (<26.80 pmol/L).

Regarding thyroid hormones, two subjects had slightly elevated TSH (with normal FT4)

and two had low FT4 (with normal TSH). Genotypic frequencies of CYP polymorphisms in the subsample of participants with available genetic data were 82.1% (GG) and 17.9% (GA+AA) for the CYP2C19*2 variant and 65.8% (GG) and 34.2% (GA+AA) for the CYP2D6*4 variant (Table 3). Only six participants had the GA/AA genotype for both variants.

Multivariate linear regression analysis revealed significant positive associations between 3-PBA and TT3 concentrations with 8% (95%CI=2; 16, p-value=0.01) higher TT3 among those with detectable 3PBA. Further, detectable concentrations of 1N was associated with 18% (95%CI=2; 39, p-value=0.03) higher DHEAS, and there was indication, although the association did

not reach statistical significance, that 1N was associated with reduced E2 (-24%, 95%CI=-44; 3, p-value=0.08) and FSH (-31%, 95%CI=-54; 3, p-value=0.07) (Table 4). No significant association was observed for ETU. Residuals of all models were normally distributed (data not shown). Simultaneous adjustment for the three pesticide metabolites did not significantly affect the results (Supplementary material, Table 3). Likewise, results did not appreciably differ when participants with urinary creatinine>300 mg/dL (*n*=5) were excluded (data not shown); no urine sample had a creatinine level below 30 mg/dL. Results of the sensitivity analysis that excluded boys taking medications that affect hormone levels (N=3) and those with a history of hypothyroidism (N=2), hyperthyroidism (N=3) or other endocrine disorder (gynecomastia, N=1) revealed a slightly stronger association between 3-PBA and TT3 (10%, 95%CI=2; 17, p-value=0.01), while the remaining associations did not substantially change. Statistically significant interactions were found between ETU and CYP2C19 G681A polymorphism on testosterone, DHEAS, FSH, and TSH level; and between 1N and CYP2D6 G1846A polymorphism on ACTH level (Supplementary material, Table 4). In the stratified analyses, significant increases in DHEAS, LH, and FSH were observed for detected *versus* undetected ETU among poor CYP2C19 metabolizers alone (Table 5a) and a significant increase in cortisol for detected *versus* undetected ETU among poor CYP2D6 metabolizers alone (Table 5b). Poor CYP2C19 metabolizers also showed significant increases in FSH and prolactin for detected *versus* undetected 3-PBA; and significant higher increases in DHEAS, prolactin, and IGF-1 for detected *versus* undetected 1N in comparison to GG genotype carriers (Table 5a). Likewise, poor CYP2D6 metabolizers had a higher but not significant increase in DHEAS concentration for detected *versus* undetected 1N in comparison to GG genotype carriers (Table 5b).

4. DISCUSSION

This study investigated the association of pesticide exposures (as measured by concentrations of urinary pesticide metabolites) with serum hormone concentrations in Spanish adolescent males. Despite the small sample size, our results suggest that 3- PBA, an exposure biomarker of numerous pyrethroids, is associated with elevated serum TT3 and that 1N, an exposure biomarker of carbaryl, is associated with elevated DHEAS and a reduction in E2 and FSH.

These associations were independent of pesticide co-exposure. Results of the interaction analyses should be interpreted with considerable caution given the limited number of subjects, particularly in the CYP2C19 polymorphism-stratified analyses. However, the data suggest that the effect of 1N on DHEAS was higher among subjects who carry the CYP2C19*2 or CYP2D6*4 variant allele, while it appears that ETU is associated with increased cortisol in carriers of the CYP2D6*4 variant allele. We highlight the low testosterone level observed in 13% of these healthy adolescents, which may reflect the age-independent secular decline in testosterone observed in some countries (Andersson et al., 2007). A relationship has been suggested between this phenomenon and a higher BMI and greater environmental exposures in adolescents and young adult men (Patel et al., 2020).

4.1. Fungicides: ETU

ETU is a non-specific exposure biomarker of several EBDC fungicides, including mancozeb, and this metabolite showed the highest urinary concentrations in the study participants. Strikingly, they are within the range of urinary ETU concentrations measured in urine from 3- to 10-year-old French children living in an area of vineyards frequently treated with dithiocarbamates (Raherison et al., 2019) and higher than those in pregnant women from an agricultural community in California (Castorina et al., 2010). Fungicides represent around half of the total amount of pesticides used in Spain (37,982 tons in 2017) (EUROSTAT, 2019), and dithiocarbamates are among the most frequently detected ED pesticides in food sold in this country, including organic food (AESAN, 2018).

ETU is a known antithyroid compound (Axelstad et al., 2011; Marinovich et al., 1997) and type 2B carcinogen (Hurley et al., 1998). Exposure to mancozeb was found to cause hypothyroid-like effects in rodents (Axelstad et al., 2011; Cecconi et al., 2007), while exposure to EBDCs was associated with increased TSH and thyroid gland disorders in agricultural workers (Medda et al., 2017; Panganiban et al., 2004; Steenland et al., 1997). A review described mancozeb as a reproductive and developmental toxicant in mammals and suggested that chronic mancozeb exposure might lead to male infertility (Runkle et al., 2017). In addition, the EBDC thiram has been shown to irreversibly inhibit 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts cortisol into inactive cortisone (Atanasov et al.,

2003), and this mechanism may possibly underlie the putative association of ETU with increased cortisol in poor CYP2D6 metabolizers. The lack of an association between ETU and thyroid hormones in the present study may be attributable the lower ETU levels in comparison to previous studies and the small sample size, which limited the statistical power to detect subtle effects; however, the present ETU levels are comparable to those reported in small studies of EBDC applicators experiencing toxic thyroid effects (Panganiban et al., 2004; Steenland et al., 1997). Differences in exposure pattern and route and previous thyroid status may also account for discrepancies with previous reports.

4.2. Pyrethroids: 3-PBA

Urinary 3-PBA levels represent exposure to most pyrethroids and were several folds lower than those reported for 6-11-year-old children recruited in 2010 from Valencia, Eastern Spain (mean=4.76 μg/g) (Roca et al., 2014) and for children and adolescents in multiple countries in the 1990s and 2000s, including China and the USA (Egeghy et al., 2011; Ye et al., 2017b). Levels in the present participants are also lower than those reported in studies of adult males, which found an inverse association of 3-PBA with TT4 and TT3 (Hwang et al., 2019) or no association between 3-PBA and thyroid hormones (Jain, 2016; Meeker et al., 2009). Studies in pregnant women with higher urinary 3-PBA levels than in the present adolescents suggested that pyrethroid exposure has hypothyroid-like effects (Chevrier et al., 2019; Hu et al., 2019) or no effect on thyroid hormone status (Zhang et al., 2013).

To our knowledge, this is the first study to describe an association between urinary 3-PBA levels and TT3 in adolescents exposed to low levels of pyrethroids. Pyrethroid residues are frequently detected in fruit and vegetables marketed in Spain (AESAN, 2018), and several pyrethroids proved able to suppress the transactivity of the thyroid hormone receptor (TR) in a reporter gene assay (Du et al., 2010). Interestingly, 3-PBA has also shown an ability to interact with the TR and may therefore have biological relevance (Du et al., 2010). It might be hypothesized that the association of 3-PBA with TT3 elevation in the present study may result from stimulation of type I iodothyronine 5-monodeiodinase activity by 3-PBA or any of its parent compounds, which may increase conversion of T4 to T3, as suggested by findings in mice exposed to fenvalerate (Maiti et al., 1995; Maiti and Kar, 1997). If so, pyrethroids (or 3-PBA)

may also exert thyroid-disrupting effects beyond the TR and thyroid gland. However, we cannot rule out the possibility that the association observed in our study is due to chance, and further research is warranted to verify this finding.

4.3. Carbamates: 1N

Urinary 1N is the primary biomarker of human exposure to the insecticide carbaryl and accounts for more than 85% of all carbaryl metabolites in urine (Maroni et al., 2000). However, 1N is a major urinary metabolite of both carbaryl and naphthalene and exposure misclassification due to differences in exposure source can therefore be expected (Meeker et al., 2007). Nevertheless, the present findings are generally consistent with the reported association between urinary 1N and decreased E2 and LH levels (Meeker et al., 2006a) but not with that between 1N and thyroid hormones (Meeker et al., 2006b), both observed in male adults with substantially higher urinary 1N levels than these adolescent males. In the former study (Meeker et al. 2006a), an inverse association was also found between 1N and testosterone, whereas 1N was positively associated with DHEAS in our global sample and inversely associated with ACTH in poor CYP2D6 metabolizers.

Although experimental studies on the hormonal activity of carbaryl are limited, available data suggest it may act as an endocrine disruptor. Carbaryl weakly activated estrogen- and progesterone-responsive reporter genes in breast MCF-7 and endometrial cancer cells (Klotz et al., 1997), and low doses of carbaryl decreased levels of gonadotropin-releasing hormone (GnRH) and gonadotropic hormones in fish (Ghosh et al., 1990). More recently, both carbaryl and its metabolite 1N exhibited moderate estrogenic activity and potent anti-androgenic activity in a reporter gene assay (Tange et al., 2016). The mechanisms of action underlying the associations observed in our study remain elusive; however, the possible inverse association of 1N with FSH may be explained by a decrease in levels of GnRH, responsible for releasing FSH and LH from the hypothalamus, due to inhibition of the neurotransmitter norepinephrine (Cooper et al., 1999). Nevertheless, an inverse association between 1N and LH might be expected to accompany the inverse association with FSH observed in the present study, but this was not the case. The mechanisms underlying the association of 1N with E2, DHEAS, and ACTH are

less apparent. DHEAS is a precursor sex steroid hormone mainly produced in the adrenal cortex under the influence of ACTH. In males, approximately half of circulating E2 and testosterone is produced by the peripheral conversion of DHEA. Hence, the present findings could be explained by the capacity of carbaryl to inhibit the steroidogenic enzyme aromatase, responsible for DHEA conversion to testosterone; however, there are no data to support the hypothesis that carbaryl interferes with aromatase.

4.4. Interaction with CYP polymorphisms

The genotypic frequencies of the CYP variants observed are in agreement with those of polymorphisms detected in pesticide-metabolizing genes among children living near intensive agriculture areas in Southern Spain (25.8% for genotype CYP2C19 681GA or AA and 32.2% for genotype CYP2D6 1846GA or AA) (Gómez-Martín et al., 2015). Around 2-5% of Caucasians are poor metabolizers of substrates catalyzed by CYP2C19 through possession of the 681AA genotype (Goldstein et al., 1997), while 5- 14% of Caucasians lack CYP2D6 activity, most frequently due to their possession of the CYP2D6 1846AA genotype (Bozina et al., 2009). There is limited information on the effect of interaction between pesticide exposure and polymorphisms in genes encoding CYP enzymes. Koutros et al. (2011) evaluated the interaction between pesticide use and 1,913 SNPs in candidate genes involved in xenobiotic metabolism in relation to prostate cancer risk. They found no interaction between pesticide use and CYP2C19 SNPs; however, they observed an association between certain CYP2C19 SNPs and prostate cancer risk. They proposed that polymorphisms in genes coding for pesticide-metabolizing enzymes may alter levels of toxic pesticide intermediates and thereby influence the risk of prostate cancer (Koutros et al., 2011). In this study, poor CYP2C19 and CYP2D6 metabolizers showed a greater increase in DHEAS for detected *versus* undetected 1N, while poor CYP2D6 metabolizers evidenced increased cortisol for detected *versus* undetected ETU. However, there was no strong evidence of a positive interaction of CYP2C19*2 and CYP2D6*4 variants with pesticide exposure, although these results should be interpreted with caution because of the limited number of participants in the stratified sample.

4.5. Limitations and strengths

Major limitations of this study include its modest sample size, which may have provided inadequate statistical power. The small number of participants reduced the capacity of the study to detect possible subtle changes in hormone concentrations, particularly in stratified analyses. In addition, its cross-sectional design prevented the identification of causal relationships between urinary pesticide metabolites and hormones and the classification of the associations observed as transient or sustained. Nevertheless, continued low-dose exposure to ED pesticides may have significant consequences for public health, given that alterations of male reproductive hormone levels may lead, for instance, to a decline in semen quality. In fact, urinary 3-PBA and 1N levels have been associated with lower sperm motility and higher sperm DNA damage (Meeker et al., 2004a, 2004b). The urinary metabolites measured in this study derive from pesticides (*i.e.,* pyrethroids, carbamates, and dithiocarbamates) that are metabolized rapidly and eliminated in the urine within 24 hours. Hence, urinary concentrations indicate recent exposure, and the measurement of single urine samples may increase the risk of exposure misclassification. On the other hand, low daily doses of non-persistent pesticides such as pyrethroids are known to achieve approximately steady-state levels in internal tissues. Moreover, it should be considered that the limit of detection of 3-PBA (0.117 µg/g) was relatively high in this study, hampering the identification of possible significant effects of low 3-PBA levels on hormone concentrations. Another limitation is that the performance of multiple analyses mean that the significant associations observe may be due to chance. Nevertheless, some of the statistically significant results are supported by existing epidemiological and experimental evidence, as discussed above. It should also be noted that other CYP enzymes involved in pesticide metabolism were not considered in the study. This is the case of the CYP3AP1-44G/A variant, another common polymorphism of the CYP genes involved in pesticide metabolism (Gómez-Martín et al., 2015), which could not be imputed by genome-wide DNA genotyping. Finally, there was no assessment of exposure to other ED pesticides (*e.g.,* azole fungicides, herbicides) or ED chemicals used in daily life (*e.g.* bisphenol A, phthalates), which may have resulted in biased effect estimates. Given that humans are exposed to multiple chemicals at any given time, extreme caution is required

in the interpretation of associations with single chemicals. Strengths of this study include its assessment of numerous hormone levels as combined effect biomarkers and its analysis of gene-environment interactions with two relevant pesticide metabolizing gene variants.

5. CONCLUSIONS

In this study of male adolescents, urinary levels of pyrethroids and carbaryl exposure biomarkers were related to certain changes in hormone levels, observing associations between 3-PBA and increased TT3 and between 1N and increased DHEAS. The effect of 1N on DHEAS appeared to be greater in carriers of the CYP2C19*2 or CYP2D6*4 variant allele. These novel associations need to be verified in prospective studies with larger sample sizes. Future studies should assess the effects of mixtures (pesticides and other endocrine disruptors) and gene-environment interactions in order to characterize individual susceptibility to pesticides and the risk of adverse health effects. Finally, it should be borne in mind that data on circulating hormone levels may not be sufficiently sensitive to detect small changes produced by newer pesticides. Hence, there is a need for novel specific biomarkers that are more sensitive to variations in the hypothalamic-pituitary-thyroid, gonadal, and -adrenal axes.

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AUTHOR CONTRIBUTIONS

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COMPETING FINANCIAL INTEREST DECLARATION

The authors declare no actual or potential competing financial conflict of interests.

Highlights

1. Urinary ETU was detected in 75% of boys, followed by 1N (38%) and 3-PBA (19%).

2. Urinary 3-PBA and 1N were associated with TT3 and DHEAS elevation, respectively.

3. Urinary 1N was marginally associated with reduced E2 and FSH.

4. Poor metabolizers of CYP2C19 and CYP2D6 had higher DHEAS increase in relation to 1N.

5. Further studies should examine endocrine effects of pesticide mixtures exposure.

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Table 1. Characteristics of study participants (N=134)

Variables	n (%) or Mean ± SD
Age (years)	
16	107 (79.9)
17	27 (20.1)
Season of blood sampling	
Spring	29 (21.6)
Summer	16 (11.9)
Fall	60 (44.8)
Winter	29 (21.6)
Area of residence	
Urban	96 (71.6)
Sub-urban/rural	38 (28.4)
Family income (€)	
Low (<25000)	48 (35.8)
Middle (25000-35000)	60 (44.8)
High (>35000)	26 (19.4)
Body mass index (kg/m^2)	23.09 ± 4.73
Body fat mass (%)	13.4 ± 6.8
Urinary creatinine (mg/dL)	186 ± 56.7

SD: Standard deviation.

Table 2. Concentrations of non-persistent pesticide metabolites in urine among 134 males at age 16-17 years.

Metabolites	DF (%)	Unadjusted (µg/L)			Creatinine-adjusted (µg/g)		
		Percentiles			Percentiles		
		50th	75th	95th	50th	75th	95th
ETU	74.6	0.288	0.876	2.547	0.141	0.514	1.587
3-PBA	19.4	<0.117	<0.117	0.253	<0.063	<0.063	0.178
1N	38.1	<0.156	0.341	0.818	<0.084	0.173	0.616

DF: Detection frequency.

Table 3. Serum hormone concentrations and genotypic frequencies of CYP polymorphisms.

Hormones (normal range*) (N=134)	**Median**	**5th-95th percentiles**
Testosterone (6.20-30.60 nmol/L)	10.95	4.40-22.10
E_2 (26.80-80.02 pmol/L)	27.44	9.18-92.66
DHEAS (2,435-17,072 nmol/L)	9.596	4.138-16.698
SHBG (13.6-86.0 nmol/L)	32.59	15.99-68.35
LH (0.50-5.00 mU/mL)	4.30	1.71-11.32
FSH (0.80-4.40 mU/mL)	3.57	1.45-57.89
AMH (7.85-338 pmol/L)	51.91	24.36-267.21
Prolactin (86-342 µU/mL)	234.81	133.24-437.10
IGF-1 (221-577 ng/mL)	332.83	214.62-468.80
ACTH (1.32-16.72 pmol/L**)	3.26	1.323-7.050
Cortisol (68.2-327 nmol/L**)	146.10	69.47-307.91
FT4 (12-22 pmol/L)	16.20	13.11-19.60
TT3 (1.3-3.1 nmol/L)	2.14	1.62-2.71
TSH (0.27-4.20 mU/mL)	1.82	0.81-3.88
CYP polymorphisms (N=117)	***n***	**%**
CYP2C19 G681A (CYP2C19*2)		
GG (normal)	96	82.1
GA + AA (poor metabolizers)	21	17.9
CYP2D6 G1846A (CYP2D6*4)		
GG (normal)	77	65.8
GA + AA (poor metabolizers)	40	34.2

*Laboratory reference range. For reproductive hormones: normal values for males in Tanner stage 4 or 5
**Reference range in the afternoon (16-20h)

Table 4. Percentage variation in hormone concentrations associated with urinary levels of pesticide metabolites (N=134).

Hormones	Crude models[a]								Adjusted models[b]							
	ETU				3-PBA		1N		ETU				3-PBA		1N	
	Detected *vs.* undetected		Continuous		Detected vs. undetected		Detected *vs.* undetected		Detected *vs.* undetected		Continuous		Detected *vs.* undetected		Detected *vs.* undetected	
	% var	*95%CI*	*% var*[c]	*95%CI*	*% var*	*95%CI*	*% var*	*95%CI*	*%var*	*95%CI*	*% var*[c]	*95%CI*	*% var*	*95%CI*	*% var*	*95%CI*
Testosterone	-6	-23; 15	0	-3; 5	11	-11; 38	2	-15; 22	-2	-19; 20	0	-4; 4	10	-10; 38	-4	-21; 15
E_2	-11	-36; 22	-1	-8; 6	-14	-40; 22	-27	-45; -1**	-13	-36; 20	-2	-8; 5	-13	-39; 24	-24	-44; 3*
DHEAS	-13	-26; 3	-3	-5; 2	-4	-20; 16	23	6; 43**	-10	-22; 6	-2	-5; 2	-2	-18; 17	18	2; 39**
SHBG	9	-7; 28	3	-1; 6	13	-5; 34	-2	-16; 13	4	-10; 20	1	-2; 4	9	-6; 27	0	-12; 15
LH	4	-16; 29	-1	-5; 4	-3	-23; 21	15	-5; 40	5	-14; 30	-1	-5; 3	6	-16; 34	13	-8; 36
FSH	27	-21; 99	6	-3; 18	-15	-49; 44	-44	-63; -14**	-4	-38; 43	3	-5; 12	-19	-49; 29	-31	-54; 3*
AMH	5	-22; 42	1	-5; 7	-0	-28; 38	-25	-43; -2**	-12	-34; 13	-2	-7; 4	-6	-30; 27	-17	-36; 8
Prolactin	12	-3; 28	1	-2; 4	12	-3; 31	-7	-17; 6	9	-5; 25	1	-1; 3	8	-7; 27	-1	-13; 13
IGF-1	-0	-10; 10	1	-1; 3	-3	-12; 8	-1	-10; 8	-1	-10; 9	6	-2; 3	-5	-15; 6	2	-7; 13
ACTH	4	-16; 30	1	-3; 6	12	-12; 42	0	-18; 22	5	-17; 30	2	-2; 7	8	-15; 38	-1	-19; 22
Cortisol	13	-5; 36	-1	-5; 3	18	-2; 44*	5	-10; 24	14	-5; 36	-1	-4; 3	16	-5; 43	6	-11; 26
FT4	-2	-8; 3	0	-2; 1	4	-2; 10	2	-3; 7	-2	-8; 4	-1	-2; 1	5	-2; 11	2	-3; 8
TT3	3	-3; 9	0	-1; 2	7	0; 14**	5	-0; 10*	4	-2; 10	0	-1; 2	8	2; 16**	5	-1; 10
TSH	-2	-18; 16	1	-3; 5	2	-16; 24	5	-11; 23	1	-16; 20	1	-2; 5	2	-17; 23	5	-11; 25

[a]Adjusted only for urinary creatinine (continuous); [b]Adjusted for urinary creatinine (continuous), age (16 or 17 yrs), BMI (continuous), timing of blood/urine samples by season (spring, summer, fall, or winter), and time of day (continuous).

%var: percentage variation in hormone concentration for detected *versus* undetected pesticide metabolite;

[c]Percentage variation in hormone concentration per each doubling increment in ETU level.

**p<0.05; *p<0.10

Table 5a. Percentage variation in hormone concentrations associated with urinary pesticide metabolites stratified by CYP2C19 G681A (a) and CYP2D6 G1846A (b) genotype among 117 males.

Hormones	ETU			3-PBA			1N		
	n>LOD[a]	*%var*	*95%CI*	*n>LOD*[†]	*%var*	*95%CI*	*n>LOD*[a]	*%var*	*95%CI*
Testosterone									
GG	74	8	–16; 38	17	10	–17; 46	32	–9	–27; 15
GA+AA	13	–28	–54; 5*	3	–22	–56; 39	11	34	–21; 125
E_2									
GG	74	–12	–40; 31	17	–11	–42; 39	32	–27	–49; 4*
GA+AA	13	–35	–71; 42	3	–27	–74; 112	11	14	–58; 213
DHEAS									
GG	74	–17	–33; 2*	17	–2	–23; 23	32	21	0; 46**
GA+AA	13	57	5; 134**	3	–20	–56; 45	11	70	13; 153**
SHBG									
GG	74	6	–13; 27	17	17	–4; 43	32	–4	–19; 13
GA+AA	13	–7	–41; 49	3	8	–41; 99	11	46	–13; 143
LH									
GG	74	6	–20; 39	17	1	–26; 36	32	6	–17; 36
GA+AA	13	58	13; 125**	3	25	–25; 107	11	–3	–41; 58
FSH									
GG	74	–25	–56; 31	17	–30	–61; 27	32	–35	–61; 7*
GA+AA	13	118	0; 381**	3	225	18; 802**	11	–10	–69; 156
AMH									
GG	74	–23	–46; 8	17	–5	–36; 42	32	–23	–44; 7
GA+AA	13	6	–31; 65	3	32	–23; 129	11	–0	–41; 70
Prolactin									
GG	74	2	–15; 22	17	–1	–19; 21	32	–7	–22; 9
GA+AA	13	20	–13; 65	3	57	4; 136**	11	39	0; 93**
IGF-1									
GG	74	–2	–16; 10	17	–8	–20; 5	32	4	–7; 16
GA+AA	13	–4	–26; 25	3	–20	–41; 9	11	35	5; 73**
ACTH									
GG	74	1	–26; 38	17	3	–27; 43	32	–4	–28; 27
GA+AA	13	–12	–44; 43	3	–16	–55; 54	11	–10	–49; 62
Cortisol									
GG	74	16	–8; 45	17	20	–7; 54	32	5	–15; 28
GA+AA	13	–20	–55; 45	3	10	–49; 141	11	13	–46; 132
FT4									
GG	74	–3	–10; 4	17	5	–3; 14	32	1	–5; 8
GA+AA	13	7	–12; 28	3	2	–18; 31	11	9	–12; 39
TT3									
GG	74	3	–4; 12	17	9	1; 18**	32	7	0; 14*
GA+AA	13	4	–16; 28	3	–11	–30; 21	11	16	–5; 43
TSH									
GG	74	14	–10; 43	17	14	–12; 48	32	–3	–22; 20
GA+AA	13	–36	–2; 14	3	–1	–57; 129	11	–6	–56; 105

b)	ETU			3PBA			1N		
Hormones	***n>LOD†***	***%var***	***95%CI***	***n>LOD†***	***%var***	***95%CI***	***n>LOD†***	***%var***	***95%CI***
Testosterone									
GG	58	–4	–27; 27	13	–7	–33; 28	27	–24	–42; 2*
GA+AA	29	–4	–35; 40	7	15	–25; 77	16	13	–21; 61
E_2									
GG	58	–9	–40; 38	13	–30	–57; 13	27	–17	–44; 22
GA+AA	29	–17	–59; 65	7	27	–41; 174	16	–30	–63; 32
DHEAS									
GG	58	–6	–24; 18	13	15	–11; 49	27	21	–2; 52*
GA+AA	29	–20	–40; 15	7	–30	–53; 3*	16	35	–3; 89*
SHBG									
GG	58	–6	–22; 13	13	5	–18; 30	27	–9	–24; 8
GA+AA	29	–3	–30; 34	7	23	–13; 76	16	10	–18; 50
LH									
GG	58	8	–18; 43	13	–7	–33; 29	27	26	–4; 69*
GA+AA	29	16	–26; 82	7	15	–31; 88	16	–34	–56; 0*
FSH									
GG	58	–17	–51; 41	13	–28	–62; 34	27	–20	–52; 32
GA+AA	29	–21	–69; 98	7	43	–48; 301	16	10	–54; 163
AMH									
GG	58	–21	–47; 16	13	–9	–43; 45	27	–18	–44; 18
GA+AA	29	–8	–41; 44	7	42	–12; 128	16	10	–27; 68
Prolactin									
GG	58	8	–10; 29	13	10	–10; 36	27	3	–14; 22
GA+AA	29	17	–16; 64	7	5	–27; 53	16	0	–27; 27
IGF-1									
GG	58	–4	–16; 9	13	–8	–21; 8	27	12	–2; 28*
GA+AA	29	3	–15; 25	7	–8	–25; 14	16	–1	–18; 20
ACTH									
GG	58	8	–23; 52	13	8	–27; 61	27	26	–10; 80
GA+AA	29	–21	–45; 13	7	–8	–39; 39	16	–27	–48; 1*
Cortisol									
GG	58	3	–20; 33	13	31	–3; 76*	27	10	–13; 42
GA+AA	29	40	2; 91**	7	–8	–36; 34	16	–5	–31; 30
FT4									
GG	58	0	–8; 9	13	6	–4; 18	27	–2	–10; 6
GA+AA	29	–4	–12; 5	7	–4	–13; 6	16	6	–2; 15
TT3									
GG	58	3	–6; 12	13	5	–5; 16	27	8	–1; 18*
GA+AA	29	3	–8; 14	7	6	–6; 19	16	2	–8; 12
TSH									
GG	58	–6	–26; 21	13	18	–11; 59	27	–4	–24; 22
GA+AA	29	–16	–44; 24	7	–16	–46; 30	16	20	–17; 74

All models are adjusted for urinary creatinine (continuous), age (16 or 17 yrs), BMI (continuous), timing of blood/ urine samples by season (spring, summer, fall, or winter), and time of day (continuous). %var: percentage variation in hormone concentration for detected *vs.*undetected urinary pesticide metabolite.; [a]Number of subjects with detectable concentrations of urinary pesticide metabolites; **p<0.05; *p<0.10

6. DISCUSIÓN

La literatura científica más reciente sugiere que la exposición a contaminantes ambientales con actividad hormonal, incluidos los pesticidas no persistentes, que ocurre en periodos de especial vulnerabilidad como la etapa perinatal y la infancia, puede afectar al inicio y la progresión de la pubertad. Los resultados de esta Tesis Doctoral indican que niños y niñas de diferentes regiones de España están expuestos a diferentes tipos de pesticidas no persistentes y de ftalatos en medida variable, que la exposición a insecticidas OPs, fungicidas EBDC y ftalatos se asocia con un adelanto en el desarrollo puberal y que la magnitud de algunas de estas asociaciones se ve modificada por el IMC. Además, se describen variaciones en los niveles de hormonas sexuales en adolescentes expuestos a diferentes clases de pesticidas, especialmente OPs, y la fuerza de las asociaciones parece variar según la susceptibilidad genética individual. La identificación de la exposición a algunos pesticidas y ftalatos como factor asociado al adelanto de la pubertad se suma al resto de los determinantes ambientales que influyen en la variabilidad interindividual de la pubertad y que de forma conjunta pueden estar relacionados con la tendencia hacia un desarrollo más precoz de la pubertad, observado en las décadas más recientes tanto en niñas como en niños.

Los resultados de esta Tesis Doctoral sirven de fundamento a los argumentos que pediatras, ginecólogos/as y matronas puedan ofrecer como recomendaciones encaminadas a reducir la presencia de sustancias con actividad hormonal en la alimentación y en cualquier exposición que ocurra en el día a día en las embarazadas y en los niños/as desde las primeras etapas de su desarrollo. Esto se alinea con la tendencia de la comunidad médica que trata de enfocar la atención a la salud integral del niño y del adolescente desde una mirada global, que inevitablemente debe tener en consideración el entorno en el que el individuo nace y se desarrolla. Así, está emergiendo la necesidad de investigar las vertiginosas transformaciones medioambientales a las que asistimos en las décadas más recientes y sus repercusiones en la salud humana, con especial atención a las mujeres y hombres en edad fértil y a los niños/as y adolescentes por su mayor vulnerabilidad a las influencias ambientales.

En el ámbito científico y académico, existe una creciente demanda por parte de la clínica para la consideración rigurosa del papel de la disrupción endocrina en relación con la pubertad normal y sus variantes (193,247,248). Más concretamente, se han identificado diferentes aspectos especialmente críticos a priorizar en el planteamiento de iniciativas de investigación (193), entre los que destaca la evaluación, mediante una metodología

reproducible, de exposiciones ambientalmente relevantes teniendo en cuenta las ventanas críticas de especial vulnerabilidad a los efectos de los DEs. En los estudios realizados en el contexto de esta Tesis Doctoral, la caracterización de la exposición se ha realizado a través de la cuantificación en orina de biomarcadores de exposición validados, lo que permite la comparación de los niveles de exposición con otros estudios de biomonitorización humana realizados en diferentes áreas geográficas (120,122,140,142,249–253). Los resultados obtenidos proporcionan evidencias de que la exposición es ubicua y afecta a la población pediátrica. Cabe destacar que algunos de los pesticidas y ftalatos analizados en esta Tesis entran dentro del listado de sustancias químicas establecidas como prioritarias en el marco de la Iniciativa Europea de Biomonitorización Humana (HBM4EU, *European Human Biomonitoring Initiative*) (254,255). La iniciativa HMB4EU nació en 2016 con el objetivo de establecer una red europea para generar conocimiento sobre la exposición humana a contaminantes químicos y sus posibles impactos en la salud, habiéndose identificado 18 sustancias consideradas prioritarias para los programas de biomonitorización humana, entre las que destacan los pesticidas modernos, incluidos el insecticida organofosforado clorpirifós y los piretroides, los ftalatos y el Hexamoll® DINCH (256).

Por otro lado, en el abordaje de la pubertad como posible blanco para la disrupción endocrina, es preciso considerar el conjunto de marcadores puberales que puedan reflejar el impacto sobre los factores que orquestan el desarrollo puberal (193). En este sentido, en esta Tesis Doctoral se han utilizado escalas clínicas estandarizadas en distintos rangos de edad con la finalidad de describir, por un lado, la aparición de los primeros signos de desarrollo en niños/as en edad pre/peri-puberal (7-11 años) y, por el otro, el grado de maduración sexual en adolescentes de 14 a 17 años. Actualmente, la escala de Tanner es considerada el "patrón oro" para la evaluación clínica del desarrollo puberal, mientras que la escala PDS ofrece información valiosa sobre el grado de desarrollo de caracteres sexuales secundarios no considerados en la clasificación de Tanner. Así, mediante la evaluación conjunta de diversos hitos puberales, hemos podido observar asociaciones entre la exposición a fungicidas EBCD en niñas y adelanto del desarrollo mamario y entre la exposición a insecticidas OPs y adelanto global de la aparición de los caracteres sexuales secundarios incluida la adrenarquia. En varones, se ha observado una asociación entre la exposición a OPs, piretroides y fungicidas y adelanto en el desarrollo genital, mientras que, en una etapa más tardía de la pubertad, la exposición OPs parece asociarse con retraso en la maduración sexual global y la exposición a fungicidas con un mayor

tamaño testicular. Estos resultados deben ser corroborados por otros estudios, pero sugieren que los pesticidas podrían presentar diferentes espectros de acción directa o indirecta sobre los mecanismos que regulan la aparición y el desarrollo de los diferentes caracteres sexuales. Además, en una pequeña muestra de adolescentes varones, se ha explorado la relación entre la exposición a pesticidas no persistentes y los niveles de hormonas sexuales involucradas en los ejes gonadal y adrenal, y los resultados obtenidos sugieren que la exposición a ciertos pesticidas, especialmente insecticidas OPs, podría interferir en la esteroidogénesis gonadal y adrenal y en la secreción de hormonas hipofisarias, con efecto promotor e inhibitorio según el pesticida analizado.

Una limitación importante de los resultados que se presentan es el diseño transversal de la mayoría de los estudios, que impide establecer un nexo de casualidad entre la exposición y las manifestaciones puberales. Además, por la naturaleza no persistente de los compuestos analizados, la determinación de los metabolitos de pesticidas y ftalatos en una única muestra de orina puede llevar a error de clasificación de la exposición. De hecho, al tratarse de compuestos con vida media biológica relativamente corta (de aproximadamente 4 a 48 horas), uno de los mayores desafíos de los estudios epidemiológicos que evalúan compuestos no persistentes es mejorar la caracterización de la exposición mediante la recogida de muestras de orina repetidas que permitan reducir el posible error de clasificación. Además, la frecuencia de detección para algunos metabolitos ha sido relativamente baja, probablemente en consecuencia de limitaciones metodológicas, de modo que, en el planteamiento de futuras investigaciones, sería deseable mejorar los métodos analíticos para obtener una mayor sensibilidad que permita cuantificar concentraciones bajas de los metabolitos urinarios, y así poder tratar estos biomarcadores de forma continua y analizar su efecto combinado. Entre las fortalezas, cabe destacar el amplio tamaño muestral de los estudios sobre desarrollo puberal y su carácter multicéntrico; además, el diseño longitudinal del estudio sobre ftalatos, con el embarazo como ventana de especial susceptibilidad, contribuye a ampliar el conjunto de evidencias todavía limitadas sobre el impacto de la exposición intrauterina a ftalatos sobre el desarrollo puberal. Así, a la luz de los resultados obtenidos, se han planteado nuevos objetivos en el marco del Proyecto INMA, entre ellos el estudio de la relación entre la exposición infantil a pesticidas/exposición prenatal a ftalatos y otros DEs y la edad de la menarquia en niñas y relación entre la exposición prenatal a pesticidas y el desarrollo puberal en niños y niñas.

No obstante, considerando el escenario real de exposición a DEs en la población general, es poco probable que se obtenga una evidencia completa sobre el impacto de la exposición combinada a estos compuestos durante las ventanas críticas del desarrollo sobre el desarrollo puberal. Por tanto, las evidencias disponibles, a las que se suman los resultados de esta Tesis Doctoral, ofrecen indicios de que la exposición a insecticidas, OPs y piretroides y a fungicidas EBDC podría afectar negativamente a la salud reproductiva de niños y niñas y, por lo tanto, siguiendo el principio de precaución, deben instaurarse acciones preventivas para evitar o limitar el daño potencial derivado de la exposición a estos tóxicos (257).

La cadena de producción alimentaria convencional es altamente dependiente del uso intensivo de productos químicos, entre los cuales se encuentran numerosos pesticidas con capacidad de actuar como DEs. Utilizando los datos oficiales del Programa de Control de Residuos de Plaguicidas recopilados por la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN), un informe de "Ecologistas en Acción" publicado en noviembre 2022 describe los residuos de pesticidas encontrados en alimentos comercializados en España y muestra que la exposición de la población a estas sustancias a través de la alimentación es preocupante (258). Según el informe, España sigue siendo líder europeo en ventas de pesticidas, con 75.775 toneladas empleadas en 2020. No obstante, el número de muestras de alimentos analizados para la detección de residuos de pesticidas en nuestro país es muy inferior a la media de la UE, con 3,26 muestras de alimentos por cada 100.000 habitantes frente a una media de 17,25 muestras en la UE, por lo que la validez de estos datos está cuestionada. El informe describe que el 35% de las muestras analizadas presentaba residuos de 125 tipos diferentes de pesticidas, 51 reconocidos DEs, de los cuales más de la mitad (51%) son pesticidas cuyo uso no está actualmente autorizado en la UE. Además, el informe muestra que solo el 1% de los pesticidas detectados supera los límites legales establecidos, lo cual pone de manifiesto que la legislación actual, que fija unos límites máximos de residuos y permite solo cantidades inferiores a éstos, es insuficiente para controlar el problema de los compuestos con actividad hormonal, debido a que incluso la exposición a pequeñas dosis es capaz de alterar el equilibrio del sistema hormonal sin que exista una relación dosis-respuesta lineal, lo cual impide establecer un nivel umbral de no efecto.

En este contexto, los resultados de esta Tesis Doctoral muestran niveles de exposición en los niños/as españoles que varían, aproximadamente, entre un 20% hasta un máximo de 87% en las frecuencias de detección para los pesticidas analizados, siendo

los pesticidas más frecuentemente detectados los insecticidas OPs y piretroides y los fungicidas EBDC, ampliamente utilizados en la industria agroalimentaria. Además, uno de los pesticidas más frecuentemente detectado, en 63-77% de los niños/as, fue el diazinón, cuyo uso está prohibido en la UE desde el año 2006, es decir, varios años antes de la recogida de las muestras de orina analizadas en nuestro estudio, lo cual pone de manifiesto un control ineficiente de las autoridades sobre la aplicación de las medidas legislativas, ya de por sí deficientes para evitar los daños a la salud. Por otro lado, los ftalatos están sujetos actualmente a una creciente presión regulatoria en la UE para reducir la exposición a aquellos considerados peligrosos para la salud. Por ejemplo, la UE no permite el uso de DEHP, BBzP, DiBP y DnBP sin autorizaciones específicas, estando prohibidos en juguetes y artículos de puericultura; aquellos considerados tóxicos para la reproducción no pueden usarse en cosméticos y se están determinando las concentraciones máximas permitidas de ftalatos como DEHP, BBzP y DnBP en materiales en contacto con los alimentos. No obstante, la magnitud de la exposición a este tipo de componentes plásticos, que se refleja en los resultados de esta Tesis Doctoral con frecuencias de detección próximas al 100% en las mujeres embarazadas de diferentes cohortes españolas, requiere medidas mucho más estrictas y controles más diligentes por parte de las autoridades como la *European Food Safety Authority* (EFSA) y la *European Chemical Agency* (ECHA).

Por lo tanto, nuestros resultados respaldan la creciente preocupación sobre los modelos de alimentación y consumo convencionales, claramente insostenibles ambientalmente y perjudiciales para la salud, especialmente la de los más pequeños. En este sentido, los pediatras y los profesionales sanitarios que acompañamos las familias en etapas tan importantes como la infancia, el embarazo y, en general, la edad fértil, estamos asistiendo a una creciente demanda de orientaciones precisas para prevenir o contrarrestar los riesgos derivados de las exposiciones ambientales.

El adelanto puberal en las niñas representa una causa común de preocupación de las familias y uno de los principales motivos de consulta en endocrinología infantil, aunque en la mayoría de los casos se trata de una condición benigna que no repercute en el desarrollo global y que no requiere tratamiento (259). La tendencia observada hacia edades cada vez más tempranas de aparición de la menarquia y de la telarquia ha generado un intenso debate sobre la posibilidad de revisar los límites de edad clásicos de la pubertad normal; sin embargo, aún no disponemos de evidencias suficientemente rigurosas para redefinir los límites hasta ahora considerados válidos. Además, la posibilidad de reducir

los límites inferiores de la pubertad normal puede conllevar el riesgo de no evaluar a niños y niñas con patologías orgánicas subyacentes a las manifestaciones puberales. Por lo tanto, parece seguir siendo válido evaluar a toda niña menor de 8 años y a todo niño menor de 9 años con sospecha de pubertad precoz y adaptar las decisiones diagnósticas y terapéuticas en cada caso considerando la edad, las perspectivas de crecimiento, el resultado de pruebas diagnósticas y, finalmente, la aceptación de eventuales tratamientos a largo plazo. En este contexto, y a la luz de lo expuesto en esta Tesis Doctoral, el diagnóstico medioambiental debería entrar en el abordaje de la patología puberal, en conjunto con las otras consideraciones diagnósticas. Así, consideramos fundamental la búsqueda, mediante una anamnesis dirigida, de indicios que orienten al pediatra sobre la contribución, desde la etapa perinatal hasta la pubertad, de factores ambientales en la etiología de anomalías puberales. El patrón de la dieta habitual, con descripción de la presencia de productos de origen vegetal, su origen y tratamiento previo al consumo, el tipo de envasado de los alimentos y los materiales domésticos empleados para su procesamiento, las características del entorno del hogar desde el embarazo hasta la actualidad, la cercanía del hogar o el colegio del niño a actividades industriales o de producción agrícola, el trabajo de los padres y la posibilidad de exposición ocupacional a sustancias químicas, la presencia de mascotas y el uso de pesticidas en interiores y exteriores entrarían dentro de las preguntas orientadas a la caracterización de una posible exposición de los niños a DEs y otros contaminantes ambientales. De hecho, estas son algunas de las preguntas recogidas en la "Hoja Verde de Anamnesis" propuesta como instrumento para la incorporación de la evaluación ambiental en la historia clínica del niño en las Unidades de Salud Medioambiental Pediátrica (PEHSUs, del inglés: *Pediatric Environmental Health Specialty Units*) (260), que tienen como objetivo vigilar y reconocer los riesgos medioambientales relacionados con la salud infantil y proporcionar información y educación y que actualmente están presentes en España en las regiones de Valencia, Murcia y Cataluña.

Entre las medidas orientadas a disminuir el impacto de los pesticidas sobre la salud, la promoción de una alimentación ecológica, de temporada y de cercanía, es primordial debido a que la dieta es la principal fuente de exposición a pesticidas y otros DEs. La comunidad médica y en especial los pediatras, ginecólogos/as y matronas deben subsanar activamente la actual falta de información sobre las sustancias tóxicas que pueden contender los productos alimenticios y fomentar una alimentación ecológica, sostenible y saludable, mediante recomendaciones como consumir fruta y verdura fresca a diario,

elegir alimentos de temporada, producidos localmente y sin el uso de pesticidas o fertilizantes químicos y, en todos los casos, lavar y pelar la fruta y la verdura antes del uso, evitar el agua embotellada y otros envases de plástico, reducir el consumo de cosméticos innecesarios y preferir materiales de origen natural siempre que sea posible.

El control legislativo actual sobre la cadena de producción alimentaria convencional es ineficiente para proteger la salud de la población frente a los compuestos con capacidad de alterar el sistema hormonal. Es necesario que las administraciones locales y los gobiernos apuesten a una producción alimentaria libre de tóxicos, incrementando las restricciones en el uso de sustancias potencialmente dañinas y fomentando los sistemas de producción alimentaria sostenible y saludable. La estrategia "De la granja a la mesa" del "Pacto Verde" (*Green Deal*) de la UE para incrementar la sostenibilidad y seguridad alimentaria propone, entre otros objetivos, reducir al 50% el uso de pesticidas antes del 2030. Este objetivo ambicioso parece estar lejos de ser alcanzado. No obstante, consideramos que un cambio de paradigma hacia una producción sostenible y libre de tóxicos es posible mediante el fomento de la agroecología como instrumento para transformar el sistema agrario actual y restituir la seguridad al consumidor. Sin embargo, es probable que esta transformación sea lenta, por lo tanto, la prevención mediante un consumo crítico por parte de las familias es no solo una forma de protegerse de los riesgos derivados la alimentación convencional si no también una herramienta para estimular y exigir un cambio en el escenario de producción y consumo actuales.

7. CONCLUSIONES

La revisión de la literatura científica más actual junto al análisis de los resultados de la presente Tesis Doctoral nos permite enunciar las siguientes conclusiones:

1. Los niños y niñas en edad peripuberal de diferentes regiones geográficas de España están expuestos a pesticidas no persistentes en medida variable según el compuesto. Según los resultados del estudio realizado en el marco del Proyecto INMA, la exposición a ETU (metabolito de fungicidas) y a ciertos insecticidas (OPs y piretroides) en edad peripuberal podría estar asociada con un desarrollo mamario más temprano en las niñas y un desarrollo genital más temprano en los niños, siendo el estado nutricional un factor que pueda modificar estas asociaciones. Estos resultados sugieren que ciertos pesticidas modernos podrían interferir con el eje HPG durante la infancia, afectando el momento de inicio de la pubertad. La exposición a concentraciones ambientalmente relevantes de ciertos pesticidas no persistentes podría ser uno de los factores implicados en el descenso progresivo en la edad de inicio de la pubertad, registrado en las últimas décadas, especialmente en niñas.

2. La exposición a ciertos ftalatos, incluidos DEHP y DnBP, durante el periodo intrauterino puede repercutir sobre la programación fetal del sistema reproductivo y asociarse con un inicio más temprano de la pubertad en niños y niñas. Nuestros resultados amplían el conocimiento actual sobre la relación entre la exposición prenatal a ftalatos y desarrollo puberal y sugieren que la obesidad infantil puede modificador dicha asociación.

3. La exposición a pesticidas no persistentes en la adolescencia podría estar relacionada con alteraciones en el grado de maduración sexual en varones, observándose un efecto retardador para los insecticidas OPs y carbamatos y un efecto acelerador para los fungicidas EBDC en niños del Proyecto INMA evaluados a la edad de 14-17 años.

4. La evaluación de la posible asociación entre la exposición a insecticidas OPs, piretroides, carbamatos y fungicidas y los niveles de hormonas reproductivas en adolescentes varones de la cohorte INMA-Granada ha puesto de manifiesto diversas asociaciones biológicamente plausibles entre la exposición a dosis bajas de pesticidas y alteraciones a diferentes niveles de los ejes hormonales HPG y HPA, que podrían explicar

posibles alteraciones en el desarrollo puberal y en la salud reproductiva masculina relacionadas con estas exposiciones.

5. La presencia de variantes genéticas relacionadas con la capacidad metabólica frente a xenobióticos puede conferir una mayor susceptibilidad a los potenciales efectos adversos en salud de los pesticidas no persistentes. En particular el polimorfismo L55M del gen PON1 y las variantes CYP2C19*2 o CYP2D6*4 pueden conferir una menor capacidad metabólica frente a los pesticidas OPs y carbamatos, respectivamente.

6. La mayoría de los estudios epidemiológicos que han investigado la relación entre exposición a pesticidas no persistentes y desarrollo puberal describe que la exposición a pesticidas no persistentes durante períodos críticos de desarrollo y crecimiento puede alterar el inicio y la progresión normal de la pubertad. Sin embargo, la calidad de la evidencia proporcionada es inadecuada para establecer una relación unívoca, y es probable que estos efectos dependan del tipo de pesticida, la co-exposición a múltiples compuestos y el momento de la exposición, entre otros factores.

7. Mientras que nuevos estudios clínicos y epidemiológicos incorporan diseños más adecuados para la caracterización de la exposición y la evaluación de las consecuencias a largo plazo, es urgente implementar medidas precautorias para anticiparse al daño causado por tales exposiciones. Los sanitarios deben conocer los riesgos asociados y dar recomendaciones pertinentes a las gestantes y madres.

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9. ANEXOS

9.1 ANEXO I: Hoja de información a las familias y consentimiento informado

INMA: Infancia y Medioambiente
HOJA DE INFORMACIÓN A LAS FAMILIAS

Apreciados padres,

En primer lugar, queremos agradecer nuevamente vuestra participación en el estudio, en el que vosotros y vuestros hijos lleváis más de 15 años colaborando. El estudio que iniciamos en el año 2000 en Granada forma parte del proyecto de investigación INMA (Infancia y Medio Ambiente) (www.proyectoinma.org), en el que, como sabéis, participan más de 3.500 niños y niñas de distintas áreas geográficas de España, y a los que se está siguiendo de manera prospectiva, desde la gestación hasta la adolescencia.

Esta hoja de información proporciona detalles de la nueva etapa en el seguimiento del estudio INMA-Granada. Por favor, tomaos el tiempo necesario para leer cuidadosamente la siguiente información.

¿Por qué hacemos el Proyecto INMA?

Como es bien sabido, la salud depende del medioambiente en el que se desarrolla la actividad de nuestros hijos. Por ejemplo, la exposición a algunos compuestos químicos durante el embarazo y las primeras etapas de la vida se relaciona con el adecuado desarrollo del sistema nervioso, inmunológico y reproductivo. Los estudios de cohortes de nacimiento, como el estudio INMA, proporcionan una gran cantidad de información sobre estas exposiciones ambientales y su papel en la salud. El conocimiento generado permite el desarrollo de estrategias de prevención para crear ambientes más sanos y seguros para las generaciones presentes y futuras.

Este año comenzamos un nuevo seguimiento de los chicos participantes en el estudio INMA-Granada, que ya han alcanzado los 15-17 años. Ahora es el turno de evaluar la dieta, la actividad física, la pubertad, el estilo de vida, así como el desarrollo neuropsicológico, la salud cardiovascular y respiratoria, y el peso de los chicos.

¿Qué es lo que comprende este nuevo seguimiento?

En este nuevo seguimiento, a los chicos se les realizará un examen médico y completarán un cuestionario. Las madres y los padres completarán también un cuestionario. La visita tendrá una duración de alrededor de una hora y 30 minutos.

Al tratarse de menores, necesitamos la autorización y colaboración de los padres, ya que el estudio implica un examen médico y la toma de muestras biológicas que serán realizados por profesionales sanitarios.

TOMA DE MUESTRAS BIOLÓGICAS AL NIÑO

- Recogida de muestra de **sangre** (20 mL) para medición de hormonas y bioquímica sanguínea (colesterol y triglicéridos, glucosa…..), y para determinar la presencia de determinadas variaciones de los genes relacionadas con la toxicidad de algunos contaminantes ambientales.

- Recogida de muestra de **orina** (mínimo 20 mL) y **pelo** (50 mg) para medir la concentración de residuos de compuestos químicos-contaminantes ambientales.

CUESTIONARIOS Y ENCUESTAS A LOS PADRES Y AL NIÑO

Mediante entrevistas los padres y los niños responderán a varios cuestionarios relacionados con la salud general de su hijo, ocupación de los padres, lugar de residencia del niño, exposiciones ambientales, dieta, ejercicio físico, entorno familiar, amigos, hábitos de sueño y conducta del niño, entre otros.

EXAMEN FISICO

Se realizará un examen físico de los chicos incluyendo antropometría completa (medición de peso, talla, circunferencia abdominal y de cadera, y composición corporal mediante bioimpedancia), evaluación del desarrollo puberal, prueba de función respiratoria y medición de la presión arterial.

EVALUACIÓN NEUROPSICOLÓGICA: Se realizará una evaluación de la función ejecutiva, atención y memoria de trabajo mediante pruebas sencillas por ordenador.

¿Cuánto tiempo permaneceré en el estudio?

Le recordamos que la participación en el estudio es voluntaria y que ustedes pueden dejar de participar en cualquier momento. No obstante, le queremos insistir en que su participación es sumamente valiosa, solo así podremos lograr un mejor futuro para los niños. Si decidiera retirarse del estudio, comuníquelo a los investigadores responsables.

¿Recibiré algún beneficio por participar en el estudio?

Su participación en el estudio es altruista y por lo tanto no conlleva ningún beneficio económico. Sin embargo, la principal ventaja es que su hijo tendrá una revisión médica completa y estará ayudando a poder entender mejor los factores que influyen en la salud de las actuales y futuras generaciones de niños, reduciendo la exposición a contaminantes ambientales y sus efectos sobre la salud.

¿Cuáles son los costos?

Su participación en este estudio no representa ningún coste para usted.

¿La participación en el estudio conlleva algún riesgo para mi hijo?

Las pruebas que se realizarán a su hijo (evaluación del neurodesarrollo, antropometría, medición de presión arterial) no entrañan ningún riesgo para su salud.

La muestra de sangre se obtendrá como cualquier análisis de sangre convencional. Bajo circunstancias normales los riesgos incluyen una ligera molestia o hematoma en el punto de la punción. La recogida de orina y de mucosa bucal son procedimientos sencillos que no conllevan ningún riesgo para su hijo.

¿Se mantendrá confidencial la información?

Toda la información, tanto los datos de índole personal como los de carácter clínico, será recogida y tratada, y estará protegida de acuerdo a la Ley Orgánica 15/1999 de Protección de Datos de Carácter Personal (LOPD 15/1999 de 13 de diciembre), Ley 1/1996 de Protección Jurídica del Menor, y la Ley 41/2002 reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica.

De acuerdo a la LOPD 15/1999, los datos personales que se le requieren son los necesarios para cubrir los objetivos del estudio. En ninguno de los informes del estudio aparecerá su nombre ni el de su hijo, y su identidad no será revelada a persona alguna salvo para cumplir con los fines del estudio, y en el caso de urgencia médica o requerimiento legal. Cualquier información de carácter personal que pueda ser identificable será conservada y procesada por medios informáticos en condiciones de seguridad.

El acceso a dicha información quedará restringido al personal autorizado que estará obligado a mantener la confidencialidad de la información. Los resultados del estudio podrán ser comunicados a las autoridades sanitarias y, eventualmente, a la comunidad científica a través de congresos y/o publicaciones.

Los datos serán utilizados para los fines específicos de este estudio y en todo caso si fuese necesario podrán ser también utilizados con otros fines de tipo docente o carácter científico.

De acuerdo con la ley vigente, tiene usted derecho al acceso de sus datos personales; asimismo, y si está justificado, tiene derecho a su rectificación y cancelación. Si así lo desea, deberá solicitarlo al médico que le atiende en este estudio.

¿Cómo serán identificadas y conservadas las muestras?

Las muestras biológicas no estarán asociadas a su identidad, se les adjudicará un código al que sólo accederán los investigadores del proyecto. Las muestras serán procesadas en la Plataforma de Servicios Científico-Técnicos del Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), donde trabajan los investigadores del proyecto. Una vez analizadas, las muestras serán almacenadas por tiempo indefinido en el Biobanco del Sistema Sanitario Público de Andalucía (SSPA), en Granada, reuniendo los requisitos de la Ley 14/2007 de Investigación Biomédica. En cualquier caso se respetará su voluntad de aceptar o rechazar su almacenamiento indefinido, manifestada en la hoja de consentimiento informado.

¿Me comunicarán los resultados de las pruebas?

Los resultados del examen clínico, de la evaluación neuropsicológica, y de los análisis clínicos y de genes que se efectúen le serán entregados personalmente. En el caso de que en la investigación se obtengan resultados con un posible impacto sobre la

salud de su hijo y hubiera medidas preventivas o algún tipo de actuación médica, recibirá dicha información de forma personalizada y el equipo médico se pondrá a su disposición para orientar cualquier actuación necesaria.

¿Cuáles son los derechos como participante?

La participación en el estudio es voluntaria. Puede optar por no participar o abandonar el estudio en cualquier momento. La retirada del estudio no supondrá ninguna sanción o pérdida de beneficios médicos o derechos legales para ustedes.
Igualmente, si en algún momento desea retirar las muestras suyas o de su hijo o la información recogida en el proyecto, podrá hacerlo, sin tener que justificar su decisión. Se interrumpirá el tratamiento de los datos, pero los efectos de la revocación no podrán afectar a investigaciones ya realizadas.

¿A quién debo llamar si tengo una pregunta o un problema?

Para preguntar acerca de cualquier cuestión relacionada con el estudio, comuníquese con los investigadores del equipo INMA de Granada en el número de teléfono 958241000 ext. 20366 (Dra. Carmen Freire, correo electrónico: cfreire@ugr.es), 958241000 ext. 20367 (Dra. Marieta Fernández, correo electrónico: marieta@ugr.es), ó 958246179 (Dr. Nicolás Olea, correo electrónico: nolea@ugr.es).

¿Dónde puedo obtener más información?

Podrá encontrar información sobre INMA en: http://www.proyectoinma.org

Gracias por leer esta hoja de información y esperamos que desee formar parte de este nuevo seguimiento.

Este nuevo seguimiento de INMA-Granada ha sido aprobado por el Comité de Ética de la Investigación de Granada (CEI-Granada), con fecha 28/03/2017.

Usted recibirá una copia de esta hoja de información. Puede también pedir una copia del protocolo (plan completo del estudio) directamente telefoneando al 958241000, extensión 20366.

Código de identificación IDNUM: .....

HOJA DE CONSENTIMIENTO INFORMADO

Consentimiento por escrito del participante – Ejemplar para INMA

Investigador principal: Dr. Nicolás Olea Serrano.

Nombre del estudio: "INMA (Infancia y Medio Ambiente): Exposiciones pre- y postnatales a contaminantes ambientales, dieta, crecimiento fetal y desarrollo neuro-inmuno-endocrino"

Institución responsable: Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA)-FIBAO

Persona que proporciona la información y la hoja de consentimiento:

Yo D/Dª..... (nombre y apellidos)

Declaramos que:

- Hemos leído la hoja de información sobre el Proyecto INMA que se nos ha entregado.
- Hemos tenido oportunidad de hacer preguntas sobre el Proyecto INMA.
- Hemos recibido suficiente información sobre el Proyecto INMA.
- Hemos hablado con....., quien nos ha aclarado las dudas.
- Comprendemos que nuestra participación es voluntaria.
- Comprendemos que podemos retirarnos del estudio en cualquier momento y sin necesidad de justificación y sin que ello repercuta en la atención médica o derechos legales de nuestro hijo.
- Comprendemos que el Proyecto INMA está diseñado para incrementar los conocimientos médicos.
- Comprendemos que la información que proporcionamos será tratada con estricta confidencialidad y que solo yo, si los pido, y los responsables del estudio los conocerán.
- Somos conscientes de que el estudio ha sido aprobado por el Comité de Ética de la Investigación de Granada.
- Damos el consentimiento para que se utilicen las muestras solo para los fines descritos en el Proyecto INMA.
- Prestamos libremente nuestra conformidad para participar en el Proyecto INMA.

Damos nuestro consentimiento para que se realice:

- La recogida de orina: ( ) SI ( ) NO
- La extracción de sangre: ( ) SI ( ) NO
- La recogida de muestra de pelo: ( ) SI ( ) NO

Nombre de la madre, padre o tutor	y	Nombre del adolescente
Sr/a _____		Sr/a _____
Firma		Firma
DNI:		DNI.....

Lugar y fecha:.....

5

Código de identificación IDNUM: .....

HOJA DE CONSENTIMIENTO INFORMADO

Consentimiento por escrito del participante – Ejemplar para la familia

Investigador principal: Dr. Nicolás Olea Serrano.

Nombre del estudio: "INMA (Infancia y Medio Ambiente): Exposiciones pre- y postnatales a contaminantes ambientales, dieta, crecimiento fetal y desarrollo neuro-inmuno-endocrino"

Institución responsable: Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA)-FIBAO

Persona que proporciona la información y la hoja de consentimiento:

Yo D/Dª..... (nombre y apellidos)

Declaramos que:

- Hemos leído la hoja de información sobre el Proyecto INMA que se nos ha entregado.
- Hemos tenido oportunidad de hacer preguntas sobre el Proyecto INMA.
- Hemos recibido suficiente información sobre el Proyecto INMA.
- Hemos hablado con....., quien nos ha aclarado las dudas.
- Comprendemos que nuestra participación es voluntaria.
- Comprendemos que podemos retirarnos del estudio en cualquier momento y sin necesidad de justificación y sin que ello repercuta en la atención médica o derechos legales de nuestro hijo.
- Comprendemos que el Proyecto INMA está diseñado para incrementar los conocimientos médicos.
- Comprendemos que la información que proporcionamos será tratada con estricta confidencialidad y que solo yo, si los pido, y los responsables del estudio los conocerán.
- Somos conscientes de que el estudio ha sido aprobado por el Comité de Ética de la Investigación de Granada.
- Damos el consentimiento para que se utilicen las muestras solo para los fines descritos en el Proyecto INMA.
- Prestamos libremente nuestra conformidad para participar en el Proyecto INMA.

Damos nuestro consentimiento para que se realice:

- La recogida de orina: ( ) SI ( ) NO
- La extracción de sangre: ( ) SI ( ) NO
- La recogida de muestra de pelo: ( ) SI ( ) NO

Nombre de la madre, padre o tutor: y Nombre del adolescente:

Sr/a _____ _____

Firma Firma

DNI: ..... DNI.....

Lugar y fecha:.....

6

9.2 ANEXO II: Dictamen favorable del Comité de Ética de la Investigación de la provincia de granada para el proyecto: "Exposición a pesticidas no persistentes, desarrollo puberal y susceptibilidad genética en niños y adolescentes: Proyecto INMA-Ado-Pub-Pest."

DICTAMEN ÚNICO EN LA COMUNIDAD AUTÓNOMA DE ANDALUCÍA

D/Dª: Juan Morales Arcas como secretario/a del CEI de Granada

CERTIFICA

Que este Comité ha evaluado la propuesta de (No hay promotor/a asociado/a) para realizar el estudio de investigación titulado:

TÍTULO DEL ESTUDIO:	Exposición a pesticidas no persistentes disruptores endocrinos, tiempo de la pubertad y susceptibilidad genética en niños y adolescentes: Proyecto INMA-Ado-PubPest. , (Exposición a pesticidas y pubertad.)
Protocolo, Versión:	1
HIP, Versión:	1
CI, Versión:	1

Y que considera que:

Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y se ajusta a los principios éticos aplicables a este tipo de estudios.

La capacidad del/de la investigador/a y los medios disponibles son apropiados para llevar a cabo el estudio.

Están justificados los riesgos y molestias previsibles para los participantes.

Que los aspectos económicos involucrados en el proyecto, no interfieren con respecto a los postulados éticos.

Y que este Comité considera, que dicho estudio puede ser realizado en los Centros de la Comunidad Autónoma de Andalucía que se relacionan, para lo cual corresponde a la Dirección del Centro correspondiente determinar si la capacidad y los medios disponibles son apropiados para llevar a cabo el estudio.

Lo que firmo en GRANADA a 11/01/2018

D/Dª. Juan Morales Arcas, como Secretario/a del CEI de Granada

CERTIFICA

Que este Comité ha ponderado y evaluado en sesión celebrada el 22/12/2017 y recogida en acta 11/2017 la propuesta del/de la Promotor/a (No hay promotor/a asociado/a), para realizar el estudio de investigación titulado:

TÍTULO DEL ESTUDIO:	Exposición a pesticidas no persistentes disruptores endocrinos, tiempo de la pubertad y susceptibilidad genética en niños y adolescentes: Proyecto INMA-Ado-PubPest. ,(Exposición a pesticidas y pubertad.)
Protocolo, Versión:	1
HIP, Versión:	1
CI, Versión:	1

Que a dicha sesión asistieron los siguientes integrantes del Comité:

Presidente/a
D/Dª. Fidel Fernández Quesada

Vicepresidente/a
D/Dª. Francisco Manuel Luque Martínez

Secretario/a
D/Dª. Juan Morales Arcas

Vocales
D/Dª. JOSÉ LUIS MARTÍN RODRÍGUEZ
D/Dª. Luis Javier Martínez González
D/Dª. José Antonio López Escámez
D/Dª. Juan Mozas Moreno
D/Dª. ANTONIO MORALES ROMERO
D/Dª. JUAN DIAZ GARCIA
D/Dª. Juana María de Haro Castellano
D/Dª. CRISTINA LUCIA DAVILA FAJARDO
D/Dª. Juan Ramón Delgado Pérez
D/Dª. José Dario Sánchez López
D/Dª. José Uberos Fernández
D/Dª. MARIA ESPERANZA DEL POZO GAVILAN
D/Dª. MAXIMILIANO OCETE ESPINOLA
D/Dª. Joaquina Martínez Galán
D/Dª. AURORA BUENO CAVANILLAS
D/Dª. Paloma Muñoz de Rueda
D/Dª. Manuel Gálvez Ibáñez
D/Dª. Esther Espínola García
D/Dª. MIGUEL LÓPEZ GUADALUPE
D/Dª. JUAN ROMERO COTELO
D/Dª. José Luis Martín Ruiz

Que dicho Comité, está constituido y actua de acuerdo con la normativa vigente y las directrices de la Conferencia Internacional de Buena Práctica Clínica.

Lo que firmo en GRANADA a 11/01/2018

9.3 ANEXO III: Cuestionario INMA para la evaluación del desarrollo puberal

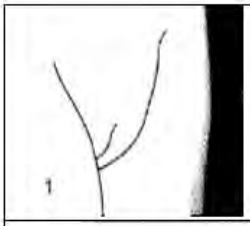
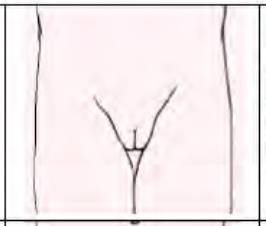
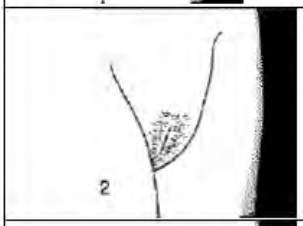
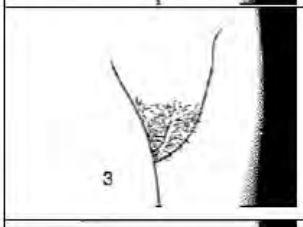
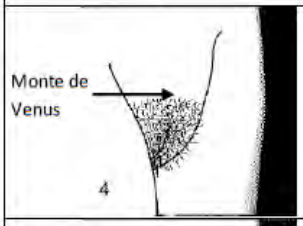
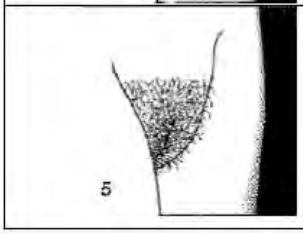
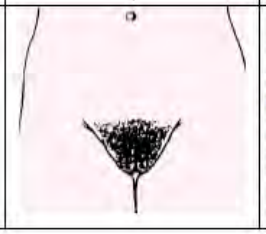
FICHA 2a

Estadíos de Tanner

Niñas (A rellenar por la niña)

Infancia y Medio Ambiente

DESARROLLO DEL VELLO PUBIANO FECHA: __/__/__

		**P1** **Prepuberal** Ligera vellosidad infantil. Similar a la del abdomen
		**P2** Vello escaso, lacio y ligeramente pigmentado. Generalmente a lo largo de los labios.
		**P3** Vello rizado y oscuro, aunque escasamente desarrollado. Sobre los labios, se extiende escasamente sobre el monte de Venus.
		**P4** Vello púbico de tipo adulto. El vello cubre el Monte de Venus pero no la cara interna de los muslos
		**P5** Vello tipo adulto en tipo y cantidad. Distribución clásica femenina en forma de triángulo (también cara interna de los muslos). El 10% puede extenderse fuera del triángulo pubiano (estadio 6)

FICHA 2b

Estadíos de Tanner

Niñas (A rellenar por la niña)

Infancia y Medio Ambiente

DESARROLLO DE LA MAMA

		**M1** **Prepuberal** Mama infantil. Sólo el pezón está ligeramente sobreelevado
		**M2** Brote mamario. Las areolas y los pezones sobresalen como un cono. Indica la existencia de tejido glandular subyacente. Aumento del tamaño de la areola.
		**M3** Crecimiento con elevación de la mama y la areola en el mismo plano. El tejido de la mama crea un pequeño cono.
		**M4** Mayor crecimiento. La areola y el pezón se distinguen como una segunda elevación, por encima del contorno de la mama.
		**M5** Desarrollo mamario total. La areola se encuentra a nivel de la piel de la mama, sólo sobresale el pezón. (Nota: en algunos casos, la mujer adulta puede mantenerse en el estadio 4)

FICHA 2a

Estadíos de Tanner

Niños (A rellenar por el niño)

Infancia y Medio Ambiente

DESARROLLO DEL VELLO PUBIANO FECHA: __/__/__

		**P1** **Prepuberal** Ligera vellosidad infantil. Similar a la del abdomen
		**P2** Vello escaso, lacio y ligeramente pigmentado. Generalmente arraigado al pene.
		**P3** Vello rizado y oscuro (claramente pigmentado), aunque escasamente desarrollado. Arraigado al pene.
		**P4** Vello púbico de tipo adulto, pero no con respecto a la distribución. El vello crece hacia los pliegues inguinales, pero no en la cara interna de los muslos.
		**P5** Vello tipo adulto en tipo y cantidad. Distribución horizontal en forma de triángulo (también cara interna de los muslos). El 80% puede extenderse fuera del triángulo pubiano extendiéndose hacia la línea alba (estadio 6).

FICHA 2b

Estadíos de Tanner

Niños (A rellenar por el niño)

Infancia y Medio Ambiente

DESARROLLO DE LOS GENITALES

		**G1** ☐ **Prepuberal** Pene, escroto y testículos infantiles, más o menos del mismo tamaño y forma que en la infancia.
		**G2** ☐ Agrandamiento del escroto y testículos. La piel del escroto se vuelve más roja, delgada y arrugada. El pene no tiene agrandamiento o es insignificante.
		**G3** ☐ Agrandamiento del pene, principalmente en longitud. Continúa el desarrollo testicular y escrotal.
		**G4** ☐ Aumento del tamaño del pene, con crecimiento del diámetro y desarrollo del glande. Continúa el crecimiento testicular y escrotal. La piel del escroto se oscurece.
		**G5** ☐ Genitales de tipo y tamaño adulto.

Infancia y Medio Ambiente

ESCALA DE DESARROLLO PUBERAL (PDS)

Instrucciones: *Los chicos y las chicas pasan por varias fases de desarrollo a diferentes edades. Algunos chico/as empiezan antes y otros más tarde. En las siguientes preguntas, marca por favor, la opción que mejor te describa.*

CHICOS Y CHICAS						
1.¿Dirías que tu crecimiento en estatura ha comenzado (has empezado a dar el estirón)? *(estirón = crecimiento repentino, mayor crecimiento de lo habitual)*	☐ No	☐ Sí, un poco	☐ Sí, sin duda	☐ Desarrollo completado	☐ No sabe/No contesta	
2. En relación al crecimiento del vello corporal: ¿Dirías que el vello corporal te ha empezado a crecer? *(vello corporal = axila o vello púbico)*	☐ No	☐ Sí, un poco	☐ Sí, bastante	☐ Desarrollo completado	☐ No sabe/No contesta	
3. ¿Has notado algún cambio en tu piel, especialmente granos (acné)?	☐ No	☐ Sí, un poco	☐ Sí, bastante			
4. ¿Crees que tu desarrollo ha empezado más temprano o más tarde que la mayoría de los otros chicos/as de tu edad?	☐ Mucho más temprano	☐ Un poco antes	☐ Sobre la misma edad	☐ Un poco más tarde	☐ Mucho más tarde	☐ No sabe/No contesta
CHICOS						
5. ¿Has notado una profundización (tono de voz más grave) en tu voz?	☐ No	☐ Sí, un poco	☐ Sí, bastante	☐ Desarrollo completado	☐ No sabe/No contesta	
6. ¿Te ha empezado a crecer vello en la cara?	☐ No	☐ Sí, un poco	☐ Sí, bastante	☐ Desarrollo completado	☐ No sabe/No contesta	
CHICAS						
7. ¿Te han empezado a crecer los pechos?	☐ No	☐ Sí, un poco	☐ Sí, sin duda	☐ Desarrollo completado	☐ No sabe/No contesta	
1. ¿Has empezado a menstruar (te ha venido la regla)?	☐ No	☐ Sí	☐ No sabe/No contesta			
8.a ¿Cuál fue la fecha de tu primera menstruación?	Día _____	Mes _____	Año _____			
8.b Si no sabes la fecha, ¿qué edad tenías?	_____ años					
8.c ¿Cuándo tuviste la última menstruación (indicar el primer día)?	Día _____	Mes _____				

8.d Duración de tu menstruación	_____dias					
8.e ¿Tienes sangrado entre periodos?	☐ No	☐ Sí				
8.f ¿Tu menstruación es cada 28-30 días (regular) o son tus ciclos irregulares?	☐ Regular	☐ Irregular				
8.g ¿Tienes dolor durante tus periodos menstruales?	☐ No	☐ Sí				

9.4 ANEXO IV: Material suplementario del artículo: "Childhood exposure to non-persistent pesticides and pubertal development in spanish girls and boys: evidence from the INMA (environment and childhood) cohort"

Table S1. General characteristics of study participants and of subjects not included in the present study.

Characteristics	Children included N=1,539	Children not included N=437[a]
Cohort (%)		
Asturias	18.4	27.2*
Gipuzkoa	24.2	4.6
Granada	18.1	4.2
Sabadell	14.8	52.8
Valencia	24.4	11.1
Mother's age at delivery (mean, years)	31.8	31.2
Mother's educational attainment during pregnancy (%)		
Up to primary	23.7	24.6
Secondary	40.7	38.9
University	35.6	36.5
Mother's ethnicity (%)		
White	98.1	98.2
Non-white	1.9	1.8
Mother's area of residence during pregnancy (%)		
Urban	68.8	65.4
Sub-urban	24.0	26.3
Rural	7.1	8.3
Mother's smoking during pregnancy (%)		
Yes	27.2	26.5
No	72.8	73.5
Maternal pre-pregnancy BMI (%)		
Underweight/normal weight	73.7	75.1
Overweight/obese	26.3	24.9
Weight gain during pregnancy (mean, kg)	13.1	14.5
Birth weight (mean, g)	3,276	3,210
Gestational length (mean, weeks)	39.6	39.0
Child's height at puberty assessment (mean, cm)	133.4	132.9
Child's weight status at puberty assessment (%)		
Underweight/normal weight	56.4	52.5
Overweight	24.9	25.1
Obese	18.7	20.4

[a]Subjects with data on pubertal development at age 7-11 years but not on urinary concentrations of pesticides.

*$p<0.05$ (χ^2 for difference between included and not included children)

Table S2. Detection frequency and median concentration (µg/L) of urinary pesticide metabolites by cohort.

Metabolites	Asturias (2013-2015)	Gipuzkoa (2014-2016)	Granada (2010-2012)	Sabadell (2013-2015)	Valencia (2013-2014)
TCPy					
%>LD	26.1	31.4	45.7	15.8	65.9
Median	0.004	0.010	0.015	0.000	0.117
IMPy					
%>LD	43.3	74.5	62.8	35.1	84.0
Median	0.082	0.419	0.242	0.015	0.620
DETP					
%>LD	60.6	65.4	48.6	49.1	70.1
Median	0.277	0.447	0.092	0.016	0.762
3-PBA					
%>LD	43.3	37.3	21.0	16.2	44.0
Median	0.064	0.048	0.012	0.000	0.071
ETU					
%>LD	33.8	48.8	17.1	36.8	75.5
Median	0.031	0.067	0.001	0.026	0.313

LD: Limit of detection.

Table S3. Model 1 (basic) and 2 for the association between urinary pesticide metabolites and puberty development in girls.

Pesticide metabolites	Model 1					Model 2				
	Tanner stage 2+ (N=308)		PDS stage 2+ (N=481)			Tanner stage 2+ (N=308)		PDS stage 2+ (N=481)		
	Breast development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche	Breast development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.50 (0.86-2.59)	0.79 (0.44-1.42)	1.39 (0.88-2.18)	1.23 (0.77-1.96)	1.06 (0.69-1.64)	1.59 (0.89-2.85)	0.78 (0.42-1.45)	1.27 (0.80-2.03)	1.12 (0.70-1.81)	0.95 (0.60-1.50)
IMPy (log)	1.01 (0.95-1.07)	0.98 (0.93-1.04)	0.97 (0.93-1.02)	0.97 (0.93-1.02)	1.00 (0.95-1.04)	1.00 (0.94-1.07)	0.98 (0.92-1.04)	0.98 (0.93-1.03)	0.98 (0.93-1.03)	1.01 (0.96-1.06)
IMPy: >LD-P75 *vs.* <LD	1.38 (0.73-2.62)	0.94 (0.40-1.79)	0.85 (0.52-1.38)	0.86 (0.52-1.41)	0.85 (0.54-1.35)	1.16 (0.59-2.28)	0.77 (0.39-1.54)	0.88 (0.55-1.42)	0.75 (0.46-1.21)	0.76 (0.48-1.21)
IMPy: >P75 *vs.* <LD	1.39 (0.67-2.85)	0.72 (0.33-1.53)	0.99 (0.57-1.73)	0.96 (0.55-1.68)	1.11 (0.66-1.87)	1.48 (0.69-3.21)	0.69 (0.31-1.55)	1.21 (0.71-2.06)	0.92 (0.53-1.57)	1.17 (0.70-1.97)
DETP (log)	1.03 (0.98-1.08)	1.03 (0.98-1.08)	1.02 (0.99-1.05)	1.02 (0.99-1.06)	1.00 (0.97-1.03)	1.04 (0.99-1.09)	1.04 (0.98-1.09)	1.03 (0.99-1.06)	1.03 (1.00-1.07)	1.01 (0.98-1.04)
DETP: >LD-P75 *vs.* <LD	1.28 (0.70-2.37)	1.29 (0.69-2.42)	0.88 (0.56-1.40)	1.07 (0.67-1.72)	0.76 (0.49-1.17)	1.28 (0.68-2.41)	1.30 (0.68-2.50)	1.12 (0.71-1.79)	1.14 (0.71-1.83)	0.90 (0.57-1.40)
DETP: >P75 *vs.* <LD	1.16 (0.56-2.38)	1.00 (0.46-2.17)	1.28 (0.77-2.14)	1.33 (0.79-2.24)	0.92 (0.57-1.49)	1.37 (0.64-2.93)	1.12 (0.50-2.49)	1.68 (0.99-2.83)	1.42 (0.84-2.40)	1.10 (0.67-1.81)
3-PBA: > *vs.* <LD	0.93 (0.55-1.58)	0.74 (0.43-1.30)	0.97 (0.65-1.46)	0.91 (0.60-1.38)	0.92 (0.62-1.35)	1.06 (0.61-1.86)	0.76 (0.43-1.36)	1.08 (0.71-1.66)	0.98 (0.64-1.51)	1.01 (0.67-1.52)
ETU: >LD-P75 *vs.* <LD	3.26 (1.61-6.59)**	1.16 (0.56-2.42)	1.13 (0.71-1.82)	1.15 (0.70-1.87)	0.99 (0.63-1.54)	3.90 (1.84-8.29)**	1.16 (0.55-2.45)	1.40 (0.86-2.26)	1.24 (0.76-2.03)	1.17 (0.73-1.85)
ETU: >P75 *vs.* <LD	3.31 (1.68-6.53)**	1.43 (0.71-2.88)	1.30 (0.78-2.16)	1.40 (0.83-2.35)	1.21 (0.75-1.97)	3.60 (1.74-7.42)*	1.33 (0.64-2.74)	1.54 (0.95-2.49)	1.28 (0.79-2.07)	1.24 (0.78-1.98)

LD: Limit of detection

Model 1: Adjusted for cohort (random effect), urinary creatinine (log-transformed), and child age. Model 2: Additionally adjusted for maternal education and child height.

*p<0.05; **p<0.001

Table S4. Model 1 (basic) and 2 for the association between urinary pesticide metabolites and puberty development in boys.

Pesticide metabolites	Model 1					Model 2				
	Tanner stage 2+ (N=620)		PDS stage 2+ (N=495)			Tanner stage 2+ (N=620)		PDS stage 2+ (N=495)		
	Genital development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche	Genital development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.88 (1.06-3.33)*	0.79 (0.33-1.93)	0.95 (0.61-1.48)	0.96 (0.54-1.70)	1.43 (0.93-2.21)	1.82 (1.02-3.27)*	0.79 (0.32-1.93)	0.94 (0.60-1.47)	0.95 (0.53-1.69)	1.47 (0.94-2.30)
IMPy (log)	1.09 (0.98-1.20)	0.98 (0.87-1.10)	0.99 (0.94-1.04)	0.99 (0.92-1.06)	1.03 (0.98-1.09)	1.06 (0.96-1.18)	0.97 (0.86-1.10)	0.98 (0.93-1.03)	0.98 (0.91-1.05)	1.02 (0.97-1.08)
IMPy: >LD-P75 *vs.* <LD	2.02 (0.94-4.35)	1.19 (0.37-3.80)	1.18 (0.70-1.98)	1.02 (0.51-2.03)	1.05 (0.63-1.76)	1.94 (0.88-4.28)	1.18 (0.37-3.83)	1.08 (0.66-1.76)	1.06 (0.56-2.02)	0.91 (0.56-1.50)
IMPy: >P75 *vs.* <LD	1.75 (0.75-4.05)	0.99 (0.27-3.62)	1.15 (0.64-2.06)	1.17 (0.55-2.51)	1.20 (0.68-2.12)	1.56 (0.66-3.71)	0.97 (0.26-3.59)	1.03 (0.60-1.78)	1.23 (0.61-2.47)	1.01 (0.59-1.74)
DETP (log)	0.97 (0.92-1.02)	0.96 (0.89-1.03)	0.98 (0.95-1.01)	0.96 (0.92-1.00)	1.00 (0.97-1.04)	0.97 (0.92-1.02)	0.96 (0.89-1.04)	0.98 (0.94-1.01)	0.96 (0.92-1.00)	1.00 (0.97-1.04)
DETP: >LD-P75 *vs.* <LD	0.61 (0.32-1.18)	0.63 (0.22-1.84)	0.93 (0.58-1.50)	0.64 (0.34-1.19)	1.38 (0.86-2.21)	0.58 (0.30-1.14)	0.62 (0.21-1.81)	0.88 (0.55-1.43)	0.63 (0.34-1.18)	1.30 (0.81-2.10)
DETP: >P75 *vs.* <LD	0.79 (0.38-1.65)	0.96 (0.31-2.96)	1.10 (0.66-1.84)	0.81 (0.42-1.57)	0.95 (0.56-1.61)	0.78 (0.37-1.67)	0.95 (0.31-2.96)	1.02 (0.60-1.71)	0.79 (0.40-1.53)	0.85 (0.49-1.47)
3-PBA: > *vs.* <LD	2.14 (1.20-3.79)*	1.17 (0.48-2.87)	0.78 (0.49-1.23)	1.01 (0.57-1.80)	1.14 (0.74-1.77)	2.15 (1.19-3.88)*	1.20 (0.48-2.98)	0.77 (0.49-1.23)	0.99 (0.55-1.78)	1.08 (0.69-1.69)
ETU: >LD-P75 *vs.* <LD	1.44 (0.69-3.00)	0.78 (0.24-2.50)	0.84 (0.50-1.39)	0.73 (0.36-1.47)	0.85 (0.52-1.40)	1.48 (0.70-3.11)	0.76 (0.24-2.48)	0.81 (0.49-1.35)	0.73 (0.36-1.48)	0.82 (0.50-1.36)
ETU: >P75 *vs.* <LD	1.69 (0.87-3.28)	0.77 (0.27-2.22)	0.75 (0.45-1.26)	1.09 (0.58-2.07)	0.71 (0.42-1.19)	1.65 (0.84-3.25)	0.74 (0.25-2.14)	0.75 (0.45-1.23)	1.18 (0.64-2.20)	0.70 (0.41-1.17)

LD: Limit of detection

Model 1: Adjusted for cohort (random effect), urinary creatinine (log-transformed), and child age. Model 2: Additionally adjusted for maternal education and child height.

*p<0.05

Table S5. Multinomial regression models for the associations between urinary pesticide metabolites and puberty status in girls.

Pesticide metabolites	Tanner (N=308)				PDS (N=481)					
	Breast development (ref.: B1)		Pubic hair growth (ref.: PH1)		Overall puberty (ref.: stage 1)		Adrenarche (ref: stage 1)		Gonadarche (ref: stage 1)	
	B2 (N=99)	B3+ (N=20)	PH2 (N=54)	PH3+ (N=19)	Stage 2 (N=126)	Stage 3+ (N=95)	Stage 2 (N=109)	Stage 3+ (N=51)	Stage 2 (N=171)	Stage 3+ (N=46)
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.78 (0.93-3.42)	2.73 (0.72-10.4)	0.75 (0.38-1.48)	0.88 (0.29-2.69)	1.44 (0.89-2.31)	1.41 (0.81-2.46)	0.93 (0.58-1.49)	1.14 (0.60-2.16)	0.93 (0.61-1.42)	1.17 (0.57-2.41)
IMPy (log)	1.00 (0.93-1.07)	0.96 (0.83-1.10)	0.98 (0.90-1.05)	0.97 (0.86-1.09)	0.98 (0.93-1.04)	1.03 (0.97-1.10)	0.97 (0.92-1.02)	1.00 (0.93-1.08)	1.01 (0.96-1.06)	1.01 (0.94-1.09)
DETP (log)	1.05 (0.99-1.11)	1.09 (0.98-1.23)	1.02 (0.96-1.08)	1.10 (0.98-1.24)	1.03 (0.99-1.06)	1.06 (1.01-1.11)*	1.01 (0.97-1.05)	1.07 (1.01-1.14)*	1.01 (0.97-1.04)	1.00 (0.95-1.06)
3-PBA: > *vs.* <LD	1.21 (0.65-2.26)	0.75 (0.21-2.62)	0.71 (0.37-1.35)	0.91 (0.31-2.65)	1.30 (0.81-2.09)	1.01 (0.57-1.78)	0.82 (0.51-1.33)	1.28 (0.68-2.41)	1.00 (0.66-1.52)	0.97 (0.46-2.04)
ETU: >LD-P75 *vs.* <LD	4.30 (1.81-10.25)**	4.89 (0.98-24.23)	1.28 (0.55-2.96)	0.84 (0.21-3.27)	1.32 (0.75-2.32)	1.81 (0.91-3.58)	1.14 (0.65-1.98)	1.60 (0.72-3.54)	1.03 (0.63-1.69)	2.64 (1.06-6.54)*
ETU: >P75 *vs.* <LD	5.74 (2.42-13.60)**	4.58 (0.92-22.81)	1.66 (0.74-3.74)	0.70 (0.19-2.57)	1.54 (0.88-2.70)	2.13 (1.10-4.13)*	1.11 (0.64-1.92)	1.97 (0.92-4.21)	1.24 (0.76-2.02)	2.12 (0.87-5.19)

LD: Limit of detection.

All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, child weight status (normal weight, overweight, or obese) at 7-11 yrs.

*p<0.05; **p<0.001

Table S6. Multinomial regression models for the associations between urinary pesticide metabolites and puberty status in boys.

Pesticide metabolites	Tanner stage 2+ (N=620)		PDS stage 2+ (N=495)					
	Genital development (ref: G1)		Overall puberty		Adrenarche		Gonadarche	
	G2 (N=129)	G3+ (N=10)	Stage 2 (N=121)	Stage 3+ (N=9)	Stage 2 (N=54)	Stage 3+ (N=11)	Stage 2 (N=121)	Stage 3+ (N=9)
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.73 (0.93-3.22)	7.81 (0.87-70.24)	0.92 (0.60-1.40)	1.12 (0.29-4.39)	1.01 (0.56-1.82)	0.83 (0.23-2.93)	1.49 (0.94-2.38)	1.12 (0.51-2.50)
IMPy (log)	1.08 (0.96-1.22)	0.98 (0.78-1.23)	0.98 (0.93-1.03)	0.95 (0.82-1.11)	1.02 (0.94-1.10)	0.90 (0.80-1.01)	1.01 (0.95-1.07)	1.02 (0.92-1.13)
DETP (log)	0.97 (0.92-1.02)	0.97 (0.85-1.10)	0.97 (0.94-1.01)	1.03 (0.90-1.17)	0.95 (0.91-0.99)*	0.99 (0.89-1.10)	1.01 (0.97-1.05)	0.97 (0.91-1.03
3-PBA: > *vs.* <LD	1.90 (1.02-3.54)*	4.51 (0.83-24.52)	0.78 (0.49-1.24)	1.00 (0.23-4.33)	1.06 (0.56-1.98)	0.81 (0.20-3.24)	1.37 (0.85-2.22)	0.46 (0.16-1.26)
ETU: >LD-P75 *vs.* <LD	1.98 (0.93-4.21)	0.36 (0.04-3.55)	0.76 (0.46-1.25)	1.43 (0.33-6.25)	0.61 (0.29-1.31)	0.98 (0.26-3.70)	0.84 (0.43-1.63)	3.28 (0.97-11.10)
ETU: >P75 *vs.* <LD	1.80 (0.85-3.83)	1.26 (0.24-6.43)	0.74 (0.42-1.28)	0.43 (0.04-4.18)	1.43 (0.71-2.90)	0.34 (0.04-2.95)	0.85 (0.48-1.47)	0.38 (0.12-1.22)

LD: Limit of detection.

All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, child weight tatus (normal weight, overweight, or obese) at 7-11 yrs.

*p<0.05; **p<0.001

Table S7. Associations between pesticide co-exposure and puberty development in girls.

Pesticide metabolites	Tanner stage 2+ (N=308)		PDS stage 2+ (N=481)		
	Breast development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.71 (0.84-3.48)	0.82 (0.42-1.59)	1.33 (0.79-2.23)	1.12 (0.67-1.88)	0.93 (0.57-1.51)
IMPy (log)	0.97 (0.90-1.04)	0.98 (0.92-1.04)	0.98 (0.93-1.03)	0.97 (0.92-1.02)	1.01 (0.96-1.06)
IMPy: >LD-P75 *vs.* <LD	0.92 (0.41-2.06)	0.80 (0.39-1.63)	0.73 (0.42-1.27)	0.74 (0.44-1.27)	0.76 (0.45-1.27)
IMPy: >P75 *vs.* <LD	1.13 (0.42-3.00)	0.73 (0.31-1.72)	1.06 (0.57-1.98)	0.94 (0.51-1.73)	1.24 (0.69-2.24
DETP (log)	1.02 (0.96-1.09)	1.04 (0.99-1.10)	1.02 (0.99-1.06)	1.02 (0.99-1.06)	1.00 (0.97-1.04)
DETP: >LD-P75 *vs.* <LD	1.48 (0.67-3.25)	1.34 (0.67-2.68)	1.14 (0.67-1.94)	1.26 (0.75-2.12)	0.92 (0.57-1.50)
DETP: >P75 *vs.* <LD	1.43 (0.55-3.72)	1.19 (0.50-2.82)	1.59 (0.87-2.89)	1.49 (0.83-2.67)	1.06 (0.61-1.85)
3-PBA: > *vs.* <LD	1.07 (0.55-2.08)	0.68 (0.37-1.24)	1.31 (0.77-2.26)	0.91 (0.58-1.42)	0.98 (0.64-1.49)
ETU: >LD-P75 *vs.* <LD	4.08 (2.10-12.03)*	1.02 (0.47-2.22)	1.31 (0.77-2.26)	1.27 (0.74-2.17)	1.22 (0.74-2.03)
ETU: >P75 *vs.* <LD	5.03 (2.39-12.59)**	1.16 (0.54-2.49)	1.47 (0.82-2.65)	1.48 (0.82-2.65)	1.43 (0.82-2.48)

LD: Limit of detection.

All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, child weight status (normal weight, overweight, or obese) at 7-11 yrs., and for all pesticide metabolites simultaneously:

TCPy model: adjusted for IMPy (log-transformed), 3-PBA (detected/undetected), and ETU (low/moderate/high).

IMPy model: adjusted for TCPy (detected/undetected), 3-PBA (detected/undetected), and ETU (low/moderate/high).

DETP model: adjusted for 3-PBA (detected/undetected) and ETU (low/moderate/high).

3-PBA model: adjusted for DETP (log-transformed) and ETU (low/moderate/high).

ETU model: adjusted for DETP (log-transformed) and 3-PBA (detected/undetected).

*p<0.05; **p<0.001

Table S8. Associations between pesticide co-exposure and puberty development in boys.

Pesticide metabolites	Tanner stage 2+ (N=620)		PDS stage 2+ (N=495)		
	Genital development	Pubic hair growth	Overall puberty	Adrenarche	Gonadarche
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
TCPy: > *vs.* <LD	1.36 (0.68-2.72)	0.72 (0.25-2.05)	1.06 (0.67-1.69)	1.04 (0.56-1.94)	1.61 (0.99-2.60)
IMPy (log)	1.05 (0.95-1.16)	0.97 (0.85-1.10)	0.98 (0.93-1.04)	0.99 (0.92-1.06)	1.01 (0.95-1.08)
IMPy: >LD-P75 *vs.* <LD	1.84 (0.80-4.22)	1.20 (0.35-4.06)	1.15 (0.68-1.93)	1.06 (0.52-2.13)	0.91 (0.52-1.59)
IMPy: >P75 *vs.* <LD	1.21 (0.45-3.23)	1.03 (0.23-4.51)	1.12 (0.61-2.06)	1.24 (0.56-2.74)	0.97 (0.52-1.82)
DETP (log)	0.93 (0.88-0.99)*	0.96 (0.88-1.04)	0.98 (0.95-1.02)	0.95 (0.91-1.00)*	1.01 (0.97-1.05)
DETP: >LD-P75 *vs.* <LD	0.42 (0.20-0.87)*	0.62 (0.20-1.87)	0.98 (0.59-1.61)	0.59 (0.31-1.15)	1.39 (0.84-2.30)
DETP: >P75 *vs.* <LD	0.43 (0.18-1.01)	0.93 (0.27-3.25)	1.24 (0.70-2.20)	0.72 (0.34-1.50)	0.93 (0.51-1.68)
3-PBA: > *vs.* <LD	2.41 (1.26-4.62)*	1.48 (0.55-3.96)	0.86 (0.54-1.39)	1.10 (0.60-2.04)	1.18 (0.74-1.89)
ETU: >LD-P75 *vs.* <LD	1.98 (0.88-4.45)	0.84 (0.24-2.86)	0.84 (0.49-1.43)	0.80 (0.38-1.67)	0.79 (0.46-1.33)
ETU: >P75 *vs.* <LD	1.93 (0.90-4.10)	0.78 (0.25-2.43)	0.81 (0.48-1.39)	1.35 (0.68-2.65)	0.67 (0.38-1.18)

LD: Limit of detection.

All models are adjusted for cohort (random effect), urinary creatinine (log-transformed), child age, maternal education, child height, child weight status (normal weight, overweight, or obese) at 7-11 yrs., and for all pesticide metabolites simultaneously:

TCPy model: adjusted for IMPy (log-transformed), 3-PBA (detected/undetected), and ETU (low/moderate/high).

IMPy model: adjusted for TCPy (detected/undetected), 3-PBA (detected/undetected), and ETU (low/moderate/high).

DETP model: adjusted for 3-PBA (detected/undetected) and ETU (low/moderate/high).

3-PBA model: adjusted for DETP (log-transformed) and ETU (low/moderate/high).

ETU model: adjusted for DETP (log-transformed) and 3-PBA (detected/undetected).

*p<0.05; **p<0.001

9.5 ANEXO V: Material suplementario del artículo: "Association of prenatal phthalate exposure with pubertal development in spanish boys and girls"

Table S1. Characteristics of participants in the original cohort, of study participants, and of subjects not included in the current analysis.

Characteristics	Mothers in the original cohort n=2241	Mother-child pairs included n=788[a]	Mother-child pairs not included n=510[b]
Cohort (%)			
Gipuzkoa	28.5	29.3	32.7*
Sabadell	34.6	41.6	27.1
Valencia	36.9	29.1	40.2
Mother's age at delivery (mean, years)	31.6	32.2	31.7
Mother's educational attainment during pregnancy (%)			
Up to primary	26.4	21.6	18.6
Secondary	40.9	38.8	44.6
University	32.6	39.6	36.8
Parity at delivery (%)			
None	55.1	57.2	56.6
1	37.4	36.9	37.8
2 or more	7.4	5.8	5.6
Mother's urban residence during pregnancy=yes (%)	79.6	80.7	81.0
Mother's smoking during pregnancy=yes (%)	32.2	27.7	28.8
Mother's passive smoking during pregnancy=yes (%)	65.7	62.7	63.0
Mother's working during pregnancy=yes (%)	85.6	89.5	89.2
Maternal pre-pregnancy BMI (%)			
<18.5 kg/m^2	4.7	4.8	3.3
18.5-24.9 kg/m^2	69.3	69.3	73.0
>24.9 kg/m^2	26.0	25.9	23.6
Child age at puberty assessment (mean, years)	–	8.70	8.90
Mother lives with child's father at 8-10 years=yes (%)	–	99.1	99.7
Child weight status at 8-10 years of age (%)			
Normal weight	–	57.9	58.1
Overweight	–	23.7	24.3
Obese	–	18.4	17.6

[a] Subjects included in the current analysis.

[b] Subjects with data on puberty status at age 7-10 years but not on maternal urinary concentrations of phthalates.

*p<0.05 ($\chi 2$ for difference between children included and not included).

Table S2. Pubertal status and maternal urinary phthalates by cohort.

	Gipuzkoa (7.6-8.5 yrs)		Sabadell (7.8-10.7 yrs)		Valencia (8.5-9.8 yrs)	
	Boys (N=117)	Girls (N=114)	Boys (N=173)	Girls (N=155)	Boys (N=119)	Girls (N=110)
Puberty onset (stage 2+), %						
Overall	22.2	23.7	23.7	48.4	34.3	58.1
Adrenal	9.4	21.0	8.7	38.7	15.1	33.6
Gonadal	39.3	45.6	15.0	32.9	23.5	45.4
Phthalate biomarkers, median (µg/L)						
MEP	94.53	96.23	334.31	321.05	171.25	119.11
MiBP	24.71	24.57	28.63	28.90	28.23	24.02
MnBP	11.93	10.60	27.51	29.70	33.07	32.78
MBzP	3.92	4.13	20.61	21.99	9.46	7.94
∑DEHPm	57.39	57.30	118.20	114.25	131.10	102.61
∑DiNPm	16.80	2.00	–	–	25.24	23.90
∑DINCHm	15.61	1.89	–	–	1.02	0.94

Table S3. Spearman correlation between phthalate biomarkers (raw variables)

		MEP	MiBP	MnBP	MBzP	∑DEHPm	∑DiNPm	∑DINCHm
Total sample (788 mothers)	**MEP**		0.24**	0.32**	0.42**	0.35**	0.22**	–0.12*
	MiBP	0.24**		0.49**	0.43**	0.49**	0.43**	0.26**
	MnBP	0.32**	0.49**		0.61**	0.55**	0.45**	–0.16*
	MBzP	0.42**	0.43**	0.61**		0.56**	0.57**	0.02
	∑DEHPm	0.35**	0.49**	0.55**	0.56**		0.60**	0.01
Sub-sample (460 mothers)	**∑DiNPm**	0.22**	0.43**	0.45**	0.57**	0.60**		0.05
	∑DINCHm	–0.12*	0.26**	–0.16*	0.02	0.01	0.05	

*p<0.05; **p<0.001

Table S3. Association between prenatal phthalate exposure based on quartiles and puberty onset in boys (N=409) and girls (N=379) at age 7-10 years.

Phthalate biomarkers	Pubertal stage 2+[a]	Quartiles	Fully-adjusted model[b]					
			Boys			Girls		
			n stage 2+	RR	95% CI	n stage 2+	RR	95% CI
MEP	**Overall**	Q1	20	1	–	45	1	–
		Q2	32	1.40	0.84-2.35	37	0.72	0.52-0.99*
		Q3	30	1.28	0.75-2.17	38	0.76	0.54-1.07
		Q4	26	1.33	0.77-2.29	46	0.78	0.56-1.08
		p-trend		0.88			0.69	
	Adrenal	Q1	9	1	–	32	1	–
		Q2	11	1.28	0.54-2.99	22	0.63	0.39-1.00
		Q3	8	0.89	0.32-2.43	32	0.88	0.57-1.37
		Q4	16	2.28	1.00-5.21*	35	0.81	0.52-1.25
		p-trend		0.19			0.39	
	Gonadal	Q1	25	1	–	45	1	–
		Q2	32	1.28	0.81-2.04	36	0.86	0.62-1.20
		Q3	22	0.98	0.56-1.71	32	0.87	0.60-1.26
		Q4	21	1.02	0.60-1.75	40	0.93	0.65-1.32
		p-trend		0.54			0.83	
MiBP	**Overall**	Q1	27	1	–	43	1	–
		Q2	29	1.28	0.83-1.98	42	0.83	0.59-1.16
		Q3	27	1.11	0.66-1.85	42	0.92	0.65-1.29
		Q4	25	1.05	0.62-1.85	39	0.84	0.58-1.20
		p-trend		0.67			0.69	
	Adrenal	Q1	18	1	–	34	1	–
		Q2	8	0.65	0.28-1.53	28	0.66	0.43-1.03
		Q3	5	0.45	0.17-1.20	30	0.78	0.51-1.20
		Q4	18	1.85	0.89-3.56	29	0.75	0.48-1.19
		p-trend		0.01			0.34	
	Gonadal	Q1	23	1	–	38	1	–
		Q2	29	1.40	0.86-2.29	31	0.77	0.51-1.15
		Q3	26	1.05	0.63-1.76	42	1.25	0.87-1.79
		Q4	22	0.91	0.51-1.64	42	1.17	0.81-1.69
		p-trend		0.30			0.18	
MnBP	**Overall**	Q1	25	1	–	36	1	–
		Q2	26	1.21	0.71-2.05	39	0.77	0.53-1.11
		Q3	31	1.28	0.72-1.17	34	0.59	0.39-0.88*
		Q4	26	2.09	0.59-2.00	57	0.81	0.56-1.17
		p-trend		0.70			0.14	
	Adrenal	Q1	11	1	–	27	1	–
		Q2	9	1.23	0.52-2.92	31	0.88	0.55-1.44
		Q3	13	1.36	0.58-3.18	26	0.66	0.39-1.11
		Q4	11	1.27	0.48-3.35	37	0.79	0.48-1.30
		p-trend		0.81			0.76	

	Gonadal	Q1	31	1	–	35	1	–
		Q2	24	1.02	0.63-1.64	42	1.26	0.86-1.84
		Q3	23	1.05	0.62-1.79	30	0.95	0.60-1.51
		Q4	22	1.05	0.61-1.82	46	1.31	0.84-2.03
		p-trend		0.98			0.58	
MBzP	**Overall**	Q1	23	1	–	42	1	–
		Q2	25	1.03	0.59-1.79	41	0.85	0.62-1.16
		Q3	36	1.38	0.78-2.44	32	0.64	0.43-0.94*
		Q4	23	1.16	0.60-2.24	51	0.83	0.58-1.19
		p-trend		0.61			0.27	
	Adrenal	Q1	18	1	–	31	1	–
		Q2	6	0.25	0.09-0.65*	27	0.80	0.51-1.24
		Q3	12	0.37	0.17-0.83*	24	0.67	0.41-1.12
		Q4	8	0.34	0.14-0.83*	39	0.82	0.51-1.32
		p-trend		0.02			0.43	
	Gonadal	Q1	29	1	–	43	1	–
		Q2	24	1.30	0.79-2.13	46	1.13	0.82-1.55
		Q3	27	1.91	0.90-2.63	25	0.75	0.47-1.18
		Q4	19	1.95	0.84-2.70	39	1.08	0.70-1.66
		p-trend		0.52			0.24	
∑DEHPm	**Overall**	Q1	19	1	–	28	1	–
		Q2	30	1.61	0.94-2.75	36	1.07	0.71-1.60
		Q3	26	1.60	0.87-2.97	55	1.43	0.99-2.06
		Q4	33	1.94	1.05-3.59*	47	1.40	0.93-2.11
		p-trend		0.87			0.36	
	Adrenal	Q1	9	1	–	20	1	–
		Q2	13	1.37	0.58-2.05	26	1.23	0.74-2.05
		Q3	12	1.47	0.53-4.08	41	1.59	1.00-2.52*
		Q4	10	1.33	0.44-4.08	24	1.72	1.03-2.86*
		p-trend		0.79			0.06	
	Gonadal	Q1	25	1	–	35	1	–
		Q2	27	1.34	0.81-1.65	38	1.14	0.79-1.65
		Q3	20	1.22	0.67-2.15	46	1.34	0.92-1.96
		Q4	28	1.69	0.99-2.88	34	1.04	0.68-1.60
		p-trend		0.81			0.40	

[a]Pubertal development based on Petersen's Pubertal Development Scale (PDS): Overall pubertal development (based

on Carskadon and Acebo algorithm); and adrenal and gonadal development (based on Shirtcliff et al. algorithm)

[b]Adjusted for mother's urinary creatinine, cohort, and child age at puberty assessment, and mother's age education, pre-pregnancy BMI, parity, and smoking during pregnancy.

*p<0.05

Table S4. Sensitivity analysis including child weight status as a covariate in the main models.

Phthalate biomarkers	Pubertal stage 2+	Fully-adjusted model[a]			
		Boys (N=409)		Girls (N=379)	
		OR	95%CI	OR	95%CI
MEP	**Overall**	1.17	0.92-1.49	0.76	0.60-0.96*
	Adrenal	1.35	0.94-1.92	0.82	0.65-1.03
	Gonadal	1.04	0.80-1.35	0.92	0.74-1.13
MiBP	**Overall**	0.95	0.68-1.34	1.00	0.70-1.42
	Adrenal	1.45	0.92-2.28	1.00	0.70-1.43
	Gonadal	1.01	0.70-1.46	1.32	0.94-1.84
MnBP	**Overall**	1.06	0.81-1.38	0.93	0.72-1.21
	Adrenal	0.99	0.69-1.42	0.80	0.62-1.04
	Gonadal	1.00	0.75-1.35	1.23	0.97-1.56
MBzP	**Overall**	1.12	0.82-1.52	0.79	0.58-1.08
	Adrenal	0.87	0.55-1.37	0.90	0.67-1.21
	Gonadal	1.18	0.85-1.65	0.99	0.75-1.30
∑DEHPm	**Overall**	1.54	1.08-2.18*	1.10	0.76-1.60
	Adrenal	1.10	0.63-1.90	1.28	0.89-1.84
	Gonadal	1.47	0.99-2.15	0.91	0.64-1.30
Sub-sample (N=460)		**Boys (N=236)**		**Girls (N=224)**	
		OR	**95%CI**	**OR**	**95%CI**
∑DiNPm	**Overall**	1.41	0.91-2.19	0.77	0.44-1.36
	Adrenal	0.66	0.29-1.48	0.76	0.44-1.33
	Gonadal	0.90	0.56-1.45	1.58	0.94-2.65
∑DINCHm	**Overall**	0.91	0.63-1.31	0.96	0.67-1.37
	Adrenal	0.63	0.33-1.20	0.82	0.56-1.20
	Gonadal	1.15	0.83-1.59	1.14	0.85-1.52

[a]Adjusted for mother's urinary creatinine, cohort, child age, mother's age, education, pre-pregnancy BMI, parity, smoking during pregnancy, and child weight status (normal weight *vs.* overweight/obese).
*$p<0.05$

Table S5. Sensitivity analysis including child weight status as a covariate in the main models.

Phthalate biomarkers	Pubertal stage 2+	Fully-adjusted model[a]			
		Boys (N=409)		Girls (N=379)	
		RR	95%CI	RR	95%CI
MEP	Overall	1.11	0.95-1.31	0.88	0.80-0.97*
	Adrenal	1.29	0.95-1.73	0.91	0.79-1.05
	Gonadal	1.01	0.86-1.20	0.96	0.86-1.06
MiBP	Overall	0.96	0.76-1.21	0.99	0.83-1.16
	Adrenal	1.37	0.96-1.95	0.99	0.79-1.25
	Gonadal	0.98	0.78-1.23	1.14	0.98-1.31
MnBP	Overall	1.04	0.87-1.23	0.96	0.87-1.05
	Adrenal	0.99	0.73-1.35	0.90	0.77-1.05
	Gonadal	1.00	0.82-1.21	1.10	0.99-1.21
MBzP	Overall	1.08	0.88-1.33	0.91	0.82-1.02
	Adrenal	0.89	0.61-1.29	0.98	0.85-1.15
	Gonadal	1.12	0.92-1.36	0.99	0.87-1.13
∑DEHPm	Overall	1.32	1.09-1.60*	1.09	0.93-1.27
	Adrenal	1.09	0.75-1.59	1.25	1.03-1.51*
	Gonadal	1.23	1.00-1.51*	0.97	0.81-1.15
Sub-sample[b] (N=460)		Boys (N=236)		Girls (N=224)	
		RR	95%CI	RR	95%CI
∑DiNPm	Overall	1.25	0.95-1.63	0.89	0.71-1.11
	Adrenal	0.77	0.41-1.42	0.86	0.61-1.21
	Gonadal	0.94	0.69-1.28	1.19	0.95-1.48
∑DINCHm	Overall	0.94	0.73-1.20	0.98	0.82-1.16
	Adrenal	0.68	0.44-1.06	0.88	0.70-1.12
	Gonadal	1.09	0.89-1.32	1.07	0.94-1.21

[a]Adjusted for mother's urinary creatinine, cohort, child age, mother's age, education, pre-pregnancy BMI, parity, and smoking during pregnancy, and child weight status (normal weight *vs.* overweight/obese).

[b]Sub-sample: Gipuzkoa and Valencia cohorts.

RR: Relative risk per each log-unit increase in urinary phthalate metabolite concentration.

*p<0.05

9.6 ANEXO VI: Material suplementario del artículo: **"Exposure to non-persistent pesticides and sexual maturation of spanish adolescent males"**

Table S1. General characteristics of study participants in the INMA-Granada cohort at 15-17, 9-11, and 4-5 years-old follow up, and baseline.

Characteristics		15-17 yrs (n=151)	9-11 yrs (n=300)	4-5 yrs (n=220)	Baseline (n=668)
Area of residence, %	Urban	70.2*	70.5	64.3	62.5
	Suburban/rural	29.8	29.5	35.7	37.5
Maternal age at delivery, mean (years)		30.3	32.1	29.8	31.3
Maternal education, %	Primary	47.9	46.0	14.3*	53.1
	Secondary	30.4	32.0	68.6	30.8
	University	21.1	22.0	17.1	16.1
Marital status, %	Stable partner	90.0*	92.3	98.1	97.1
	No stable partner	10.0	7.7	1.9	2.9

*p<0.05

Table S2. Distribution of LOD-LOQ values of pesticide metabolites.

Pesticides	TCPy	IMPy	MDA	DETP	DEDTP	3-PBA	DCCA	1-NPL	ETU
LOD (µg/L)	0.039	0.117	0.052	0.116	0.142	0.117	0.055	0.156	0.072
LOQ (µg/L)	0.13	0.391	0.172	0.387	0.474	0.389	0.184	0.527	0.241
Q-SRM	197.91	151.09	273	169.01	184.98	213.05	113	143.04	103.03
Rt (min)	3.21	1.74	6.38	3.14	2.29	3.36	2.61	1.45	1.73
a	-0.76826	-0.80231	32,986	-122,351	-153,302	-0.58052	47,924	-106,224	-243,736
b	0.91352	0.60619	0.9203	0.84444	0.84401	0.83928	12,798	0.8809	0.97101
R^2	0.997	0.994	0.996	0.996	0.996	0.994	0.999	0.997	0.999
Mean accuracy (%)	88.29	92.77	98.64	101.98	99.46	93.58	90.49	101.68	94.64
% RSD	13.03	10.99	11.51	14.74	8.58	14.19	10.47	9.11	13.28

Q-SRM: Selected reaction monitoring; Rt: Retention time; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

Table S3. Urinary concentrations (µg/L) of pesticide metabolites by cohort.

Metabolites	LOD	GRANADA (N=150)						MENORCA (N=51)					
		% >LOD	P25	P50	P75	P95	Max	% >LOD	P25	P50	P75	P95	Max
OP insecticides													
TCPy	0.039	32.0	<LOD	<LOD	0.04	0.13	1.21	29.4	<LOD	<LOD	0.04	0.52	1.09
IMPy	0.117	74.7	<LOD	0.33	0.81	4.68	27.11	47.1	<LOD	<LOD	0.58	3.25	5.42
MDA*	0.052	86.7	0.14	0.30	0.50	1.00	1.50	100	0.81	1.08	1.55	1.77	1.83
DETP	0.116	53.3	<LOD	<LOD	0.69	3.89	40.35	47.1	<LOD	<LOD	0.91	33.37	54.95
Pyrethroids													
3-PBA	0.117	19.3	<LOD	<LOD	<LOD	0.31	0.72	11.8	<LOD	<LOD	<LOD	0.28	0.45
DCCA (cis+trans)*	0.172	66.0	<LOD	1.06	3.43	6.12	8.26	100	3.31	5.55	6.74	7.41	8.95
Carbamates													
1-NPL	0.156	38.0	<LOD	<LOD	0.26	1.01	2.88	3.9	<LOD	<LOD	<LOD	<LOD	0.82
Dithiocarbamate fungicides													
ETU	0.072	74.0	<LOD	0.26	0.70	2.55	19.37	31.4	<LOD	<LOD	0.10	0.67	3.11

LOD: Limit of detection

Table S4. Sexual maturation status by cohort.				
Outcomes	**Granada (age: 15-17 yrs)**		**Menorca (age: 14-15 yrs)**	
	N	**n (%)**	**N**	**n (%)**
Genital Tanner stage	139		36	
G=3		3 (2.2)		7 (19.4)
G=4		78 (56.1)		19 (52.8)
G=5		58 (41.7)		10 (27.8)
Pubic hair Tanner stage	139		42	
PH=2		1 (0.7)		2 (4.5)
PH=3		5 (3.6)		16 (36.4)
PH=4		41 (29.5)		16 (36.4)
PH=5		92 (66.2)		8 (18.2)
Overall pubertal development*	146		51	
Pre + early + mid-pubertal		94 (64.4)		41 (80.4)
Late + post-puberty (PDS≥4)		52 (35.6)		10 (19.6)
Gonadal development**	146		50	
Pre + early + mid-pubertal		66 (45.2)		32 (64)
Late + post-puberty (PDS≥4)		80 (54.8)		18 (36)
Adrenal development**	146		51	
Pre + early + mid-pubertal		71 (48.6)		34 (66.7)
Late + post-puberty (PDS≥4)		75 (51.4)		17 (33.3)
TV≥25 mL	139	65 (46.8)	0	–
*Based on PDS-Carskadon and Acebo algorithm;				
**Based on PDS-Shirtcliff et al. algorithm				

Table S5. Association between urinary pesticide metabolites and Tanner stage in boys from Granada (N=139).

Outcomes	Pesticide metabolites	OR (95% CI)
		Granada
Tanner G=5	**TCPy: ≥ *vs.* <LOD**	1.44 (0.68-3.06)
	IMPy (ref: <LOD)	
	LOD-P75	2.02 (0.82-4.96)
	>P75	1.67 (0.60-4.62)
	p-Trend	
	MDA (ref: <LOD)	
	LOD-P75	0.95 (0.32-2.85)
	>P75	0.65 (0.18-2.29)
	p-Trend	
	DETP (ref: <LOD)	
	LOD-P75	0.84 (0.34-2.07)
	>P75	0.26 (0.09-0.76)*
	p-Trend	
	DCCA (ref: <LOD)	
	LOD-P75	1.66 (0.72-3.82)
	>P75	0.82 (0.29-2.31)
	p-Trend	
	ETU (ref: <LOD)	
	LOD-P75	0.80 (0.33-1.96)
	>P75	0.90 (0.34-2.35)
	p-Trend	
	1-NPL: ≥ vs. <LOD	0.92 (0.43-1.93)
Tanner PH=5	**TCPy: ≥ *vs.* <LOD**	1.15 (0.50-2.65)
	IMPy (ref: <LOD)	
	LOD-P75	0.72 (0.28-1.85)
	>P75	0.82 (0.28-2.43)
	p-Trend	
	MDA (ref: <LOD)	
	LOD-P75	1.33 (0.42-4.23)
	>P75	0.90 (0.24-3.39)
	p-Trend	
	DETP (ref: <LOD)	
	LOD-P75	1.23 (0.47-3.26)
	>P75	0.95 (0.33-2.76)
	p-Trend	
	DCCA (ref: <LOD)	
	LOD-P75	1.47 (0.62-3.47)
	>P75	2.37 (0.74-7.57)
	p-Trend	
	ETU (ref: <LOD)	
	LOD-P75	0.90 (0.34 -2.37)
	>P75	1.01 (0.35-2.93)
	p-Trend	
	1-NPL: ≥ vs. <LOD	1.59 (0.69-3.65)

LOD: Limit of detection; P75: 75th percentile.

Models are adjusted for cohort, age, urinary creatinine, maternal education, and child BMI z-score (continuous)

*p-value<0.05

Table S6. Association between urinary concentration of pesticide metabolites and development adjusted by co-exposure.

Outcomes	Pesticide metabolites	OR (95% CI)
Tanner G=5 (N=175)	TCPy >LOD	1.49 (0.74-3.04)
	IMPy: LOD-P75	1.63 (0.72-3.67)
	IMPy >P75	1.26 (0.49-3.26)
	ETU: LOD-P75	0.82 (0.36-1.87)
	ETU >P75	0.84 (0.34-2.10)
	1-NPL >LOD	0.88 (0.42-1.85)
Tanner PH=5 (N=181)	TCPy >LOD	1.17 (0.54-2.53)
	IMPy: LOD-P75	0.56 (0.24-1.40)
	IMPy >P75	0.57 (0.21-1.59)
	ETU: LOD-P75	0.55 (0.22-1.37)
	ETU >P75	0.69 (0.25-1.93)
	1-NPL >LOD	1.53 (0.68-3.41)
Overall pubertal development PDS≥4 (N=196)	TCPy >LOD	0.61 (0.27-1.29)
	IMPy: LOD-P75	1.55 (0.67-3.59)
	IMPy >P75	0.66 (0.25-1.77)
	ETU: LOD-P75	1.74 (0.75-4.04)
	ETU >P75	1.82 (0.70-4.70)
	1-NPL >LOD	1.92 (0.92-4.00)
Adrenal development PDS ≥4 (N=196)	TCPy >LOD	0.80 (0.41-1.58)
	IMPy: LOD-P75	1.44 (0.67-3.11)
	IMPy >P75	0.94 (0.39-2.26)
	ETU: LOD-P75	1.03 (0.48-2.21)
	ETU >P75	2.01 (0.85-4.75)
	1-NPL >LOD	2.70 (1.33-5.49)*
Gonadal development PDS≥4 (N=195)	TCPy >LOD	0.50 (0.25-0.98)*
	IMPy: LOD-P75	1.51 (0.71-3.21)
	IMPy >P75	0.58 (0.78-1.86)
	ETU: LOD-P75	0.92 (0.43-1.94)
	ETU >P75	1.40 (0.60-3.24)
	1-NPL >LOD	1.09 (0.54-2.20)
TV≥25 mL (N=139)	TCPy >LOD	0.87 (0.38-1.95)
	IMPy: LOD-P75	1.04 (0.40-2.71)
	IMPy >P75	0.79 (0.27-2.31)
	ETU: LOD-P75	2.45 (0.93-6.46)
	ETU >P75	1.66 (0.59-4.75)
	1-NPL >LOD	0.36 (0.16-0.82)*

Models are adjusted for age, cohort, urinary creatinine, maternal education, BMI z-score, and urinary concentration of others pesticides metabolites.

9.7 ANEXO VII: Material suplementario del artículo: "Organophosphate pesticide exposure, hormone levels, and interaction with pon1 polymorphisms in male adolescents"

SUPPLEMENTARY MATERIAL

Table S1. General characteristics of study participants and of subjects not included in the current analysis.

Characteristic	Boys included (N=117)	Boys not included[a] (N=38)
Age (years), %		
16	78.6	7.1
17	21.4	2.9
Season of blood sampling, %		
Spring	19.7	28.6
Summer	12.8	19.5
Fall	46.2	36.1
Winter	21.4	15.8
Area of residence, %		
Urban	70.1	57.0
Sub-urban/rural	29.9	43.0
Family income (euros), %		
<25000	34.2	50.1**
25000-35000	47.0	33.1
>35000	18.8	16.8
BMI (kg/m^2), mean	23.05	24.33
Body fat mass (%), mean	13.4	18.7

[a]Subjects with data on levels of urinary OP pesticide metabolites but not on serum hormones or PON1 polymorphisms.

**p<0.05, *p<0.10 (difference between boys included and not included).

Table S2. Selected reaction monitoring (SRM) transitions, retention times, and analytical parameters of the calibration curves.

Metabolite	Q-SRM	Tr (min)	a	b	R^2	LOD (µg/L)	LOQ (µg/L)	Mean accuracy (%)	% RSD
TCPy	197.91	3.21	-0.76826	0.91352	0.997	0.039	0.130	88.29	13.03
IMPy	151.09	1.74	-0.80231	0.60619	0.994	0.117	0.391	92.77	10.99
DETP	169.01	3.14	-1.22351	0.84444	0.996	0.116	0.387	101.98	14.74
DEDTP	184.98	2.29	-1.53302	0.84401	0.996	0.142	0.474	99.46	8.58

Q-SRM: Selected reaction monitoring; Tr: Retention time; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

Table S3. Hormone assay sensitivities.

Hormones	Limit of detection	Repeatability range CV (%)	Intermediate precision range CV (%)
TT	0.087 nmol/L	1.2-4.7	2.8-8.4
E_2	18.4 pmol/L	1.9-8.5	2.5-11.9
DHEAS	0.003 µmol/L	1.7-2.8	2.4-4.7
SHBG	0.350 nmol/L	2.1-2.7	2.6-5.6
LH	0.100 mU/mL	0.8-1.8	1.9-5.2
FSH	<0.100 mU/mL	1.4-2.0	2.9-5.3
Prolactin	1.00 µU/mL	1.8-4.0	2.8-5.0
AMH	0.07 pmol/L	1.0-1.8	2.9-4.4
IGF-1	0.50 µg/L	1.9-9.8	3.8-12.2
FT4	0.5 pmol/L	1.6-5.7	3.0-10.7
TT3	0.300 nmol/L	3.5-5.3	4.1-5.4
TSH	0.005 µU/mL	1.5-8.6	1.8-8.7

CV: coefficient of variation

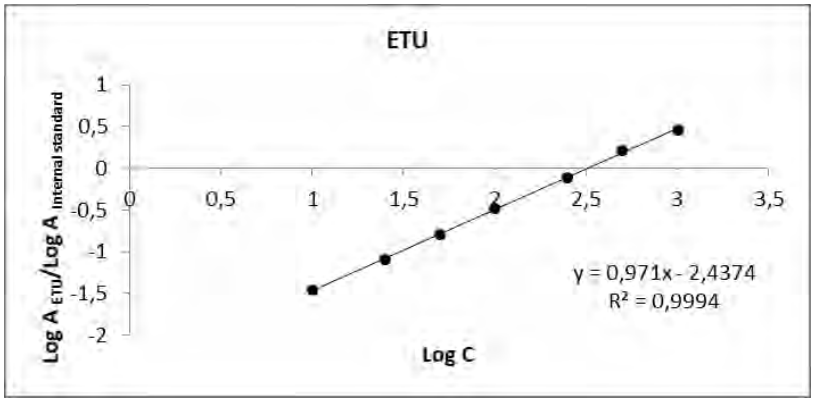
9.8 ANEXO VIII: Material suplementario del artículo: "Urinary metabolites of non-persistent pesticides and serum hormones in spanish adolescent males"

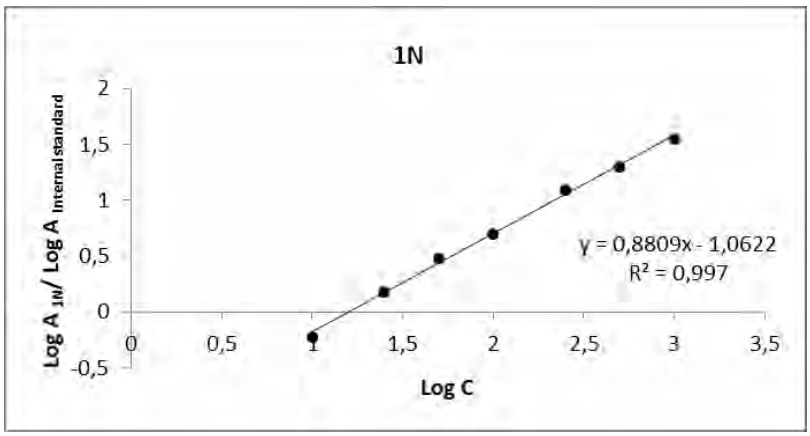
Table 1. Selected reaction monitoring (SRM) transitions, retention time, and analytical parameters of the calibration curves.

Metabolite	Q-SRM	tR (min)	a	b	R^2	LOD (µg/L)	LOQ (µg/L)	Linear range (µg/L)	Mean accuracy (%)	% RSD
ETU	103.03	1.73	-2.43736	0.97101	0.999	0.072	0.241	0.241-1000	94.64	13.28
3-PBA	213.05	3.36	-0.58052	0.83928	0.994	0.117	0.389	0.389-50	93.58	14.19
1N	143.04	1.45	-1.06224	0.88090	0.997	0.156	0.527	0.527-1000	101.68	9.11

Q-SRM: Selected reaction monitoring; tR: Retention time; LOD: Limit of detection; LOQ: Limit of quantification.

Figure 1. Calibration curves.





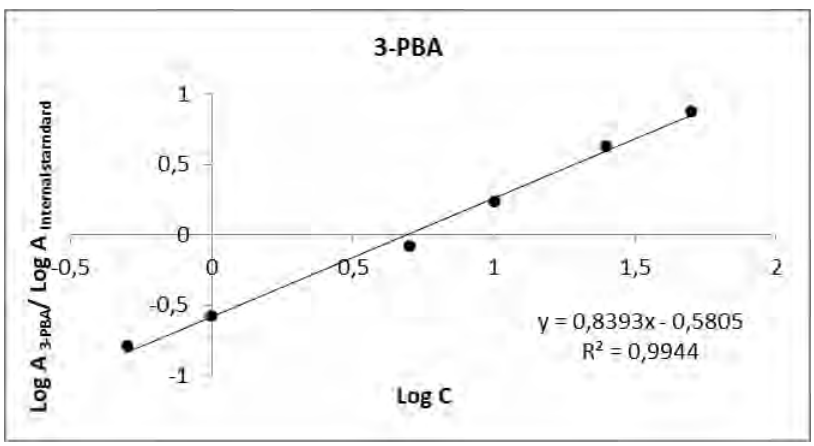


Table 2. General characteristics of study participants and of subjects not included in the current analysis.

Characteristics	Boys included (N=134)	Boys not included[a] (N=21)
Age (years), %		
16	79.9	7.3
17	20.1	2.7
Season of blood sampling, %		
Spring	21.6	29.0
Summer	11.9	20.1
Fall	44.8	36.0
Winter	21.6	14.9
Area of residence, %		
Urban	71.6	55.0
Sub-urban/rural	28.4	45.0
Family income (euros), %		
<25000	35.8	49.5**
25000-35000	44.8	36.6
>35000	19.4	13.9
BMI (kg/m^2), mean	23.09	24.30
Body fat mass (%), mean	13.4	18.5

[a]Subjects with data on levels of urinary pesticide metabolites but not on serum hormones.

**$p<0.05$ (difference between boys included and not included).

Table 3. Percentage variation in hormone concentrations associated with urinary levels of pesticide metabolites – models adjusted for the three pesticide metabolites simultaneously.

Hormones	ETU				3-PBA		1N	
	Detected *vs.* undetected		Continuous		Detected *vs.* undetected		Detected *vs.* undetected	
	%var	*95%CI*	*%var*[a]	*95%CI*	*% var*	*95%CI*	*% var*	*95%CI*
Testosterone	-6	-24; 61	0	-4; 4	12	-11; 41	-9	-26; 12
E_2	-13	-38; 21	-1	-8; 5	-9	-36; 31	-24	-45; 5*
DHEAS	-11	-25; 6	-1	-4; 2	-3	-19; 17	18	1; 41**
SHBG	2	-12; 18	0	-3; 4	8	-9; 27	0	-14; 16
LH	5	-16; 31	-1	-5; 3	0	-21; 27	12	-10; 38
FSH	-2	-37; 52	3	-5; 11	-20	-50; 30	-27	-52; 5*
AMH	-17	-37; 11	-2	-6; 4	-4	-30; 31	-17	-37; 10
Prolactin	5	-10; 22	0	-1; 3	9	-7; 28	-5	-18; 10
IGF-1	-1	-11; 10	6	-2; 3	-4	-14; 8	1	-9; 13
ACTH	7	-17; 36	1	-2; 7	8	-17; 42	0	-21; 27
Cortisol	8	-11; 32	-1	-4; 3	15	-7; 42	-1	-18; 20
FT4	-2	-8; 4	-1	-2; 1	5	-1; 12	2	-4; 8
TT3	3	-4; 9	0	-1; 1	8	2; 16**	4	-2; 10
TSH	1	-16; 22	1	-2; 5	0	-19; 23	4	-12; 25

All models are adjusted for the three pesticide metabolites simultaneously and for urinary creatinine (continuous), age (16 or 17 yrs), BMI (continuous), timing of blood/urine samples by season (spring, summer, fall, or winter), and time of day (continuous).

%var: percentage variation in hormone concentration for detected *versus* undetected pesticide metabolite;

[a]Percentage variation in hormone concentration per each doubling increment in ETU level.

**p<0.05; *p<0.10

Table 4. P-values of the interaction effects between detectable urinary pesticide metabolites and CYP polymorphisms on hormone concentrations (models adjusted for urinary creatinine, age, BMI, season, and time of day) (N=117)

Hormones	CYP2C19*2	CYP2D6*4	Hormones	CYP2C19*2	CYP2D6*4
Testosterone			**IGF-1**		
ETU*CYP	0.03	0.96	ETU*CYP	0.42	0.45
3-PBA*CYP	0.31	0.35	3-PBA*CYP	0.97	0.44
1N*CYP	0.59	0.12	1N*CYP	0.73	0.56
E_2			**Prolactin**		
ETU*CYP	0.88	0.78	ETU*CYP	0.10	0.68
3-PBA*CYP	0.54	0.14	3-PBA*CYP	0.13	0.82
1N*CYP	0.52	0.45	1N*CYP	0.29	0.36
DHEAS			**ACTH**		
ETU*CYP	0.02	0.80	ETU*CYP	0.55	0.72
3-PBA*CYP	0.78	0.06	3-PBA*CYP	0.74	0.45
1N*CYP	0.48	0.74	1N*CYP	0.30	0.02
SHBG			**Cortisol**		
ETU*CYP	0.24	0.43	ETU*CYP	0.27	0.21
3-PBA*CYP	0.71	0.44	3-PBA*CYP	0.69	0.18
1N*CYP	0.55	0.51	1N*CYP	0.42	0.41
LH			**FT4**		
ETU*CYP	0.56	0.97	ETU*CYP	0.52	0.56
3-PBA*CYP	0.42	0.48	3-PBA*CYP	0.83	0.68
1N*CYP	0.62	0.14	1N*CYP	0.63	0.66
FSH			**TT3**		
ETU*CYP	0.04	0.61	ETU*CYP	0.90	0.55
3-PBA*CYP	0.17	0.37	3-PBA*CYP	0.28	0.63
1N*CYP	0.78	0.82	1N*CYP	0.68	0.47
AMH			**TSH**		
ETU*CYP	0.11	0.32	ETU*CYP	0.02	0.51
3-PBA*CYP	0.40	0.41	3-PBA*CYP	0.78	0.32
1N*CYP	0.83	0.91	1N*CYP	0.78	0.97