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Obesity and insulin resistance trigger the early onset of adolescent-like sexually dimorphic metabotypes in middle childhood

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Abstract

Background The pathophysiology of obesity and insulin resistance are differentially affected by sexual dimorphisms along lifespan, being female children often at higher metabolic risk although presenting lower obesity rates, whereas puberty induces a switch in their prevalence. However, scarce data is available in middle childhood, when crucial hormonal maturation processes begin.

Methods In this study, we recruited a cohort of prepubertal children (6–12 years, Tanner I) of both sexes, comprising subjects with obesity and insulin resistance ($N = 19$ girls, 20 boys), children with obesity without insulin resistance ($N = 10$ girls, 12 boys), and healthy controls ($N = 12$ girls, 16 boys). Plasma and erythrocyte samples were collected for exhaustive clinical biochemistry assessment (insulin/glucose metabolism, inflammatory markers, hormonal profile) and state-of-the-art metabolomics. Then, sex-stratified multivariate and univariate statistical analyses were applied to decipher obesity- and insulin resistance-driven metabolic impairments separately in boys and girls.

Results Surprisingly, we found boys to exhibit a more unfavorable metabolic profile, with exacerbated insulin resistance and raised inflammatory markers compared to girls, as expected for adolescents rather than prepubertal children. This was accompanied by a sexually dimorphic increase of steroid levels in children with obesity (i.e., androgens in boys, but also estrogens and progestogens in girls) compared to controls, as well as by male-specific alterations in metabolites associated with the pathophysiology of obesity and insulin resistance (e.g., carbohydrate, lipid, and protein metabolism, oxidative stress, phospholipid turnover).

Conclusions Altogether, we hypothesize that premature obesity-driven hormonal development may induce an adolescent-like metabotype in mid-aged children (i.e., protective estrogenic profile in girls vs. deleterious androgenic profile in boys), which could be responsible for sex differences in related metabolic complications.

Keywords Childhood obesity, Hormones, Insulin resistance, Metabolomics, Sexual dimorphism

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Background

Obesity is a chronic disease primarily associated with adipose tissue hypertrophy and hyperplasia, causing the release of pro-inflammatory cytokines, reactive oxygen species, and lipotoxic mediators, which may ultimately lead to disruptions in insulin signaling and metabolism (i.e., insulin resistance – IR) [1]. In turn, IR has been postulated as the trigger of other cardiometabolic impairments (e.g., dyslipidemia, hypertension) that altogether constitute the so-called metabolic syndrome. The efficacy of population-wide strategies for approaching obesity has often been proven to be modest because of its complex and multi-factorial pathophysiology, which put the focus on the need of precision medicine tools to address the influence of inter-individual variability [2]. Among the multiple factors that might modulate the susceptibility of developing obesity, sexual dimorphisms have received great attention because of the pivotal role of genetic and hormonal determinants in the onset of metabolic disorders along lifespan. At early ages, obesity prevalence is usually lower in girls, plausibly as a result of hormonal (e.g., higher leptin levels, leading to appetite suppression and improved energy disposal) and sociocultural (e.g., weight-related concerns) influences [3]. In contrast, it has paradoxically been reported that young girls are more insulin resistant, since several genes linked to insulin dysfunction are located on the X chromosome [4, 5]. However, sex differences in prevalence of obesity and metabolic syndrome switch during puberty due to profound hormonal and metabolic remodeling processes. Sex steroids regulate the expression of genes involved in adipose tissue development, distribution, and function, with estrogens promoting accumulation of subcutaneous adipose tissue (SAT) in women, whereas men tend to produce greater visceral adipose tissue (VAT) depots [6]. This differential fat distribution has crucial health repercussions, as SAT is better evolutionarily adapted for large and long-term storage of lipids with the ultimate aim of preserving female reproductive functions [6]. On the other hand, it has consistently been described that the secretion of pro-inflammatory cytokines is mainly associated with VAT rather than with SAT [7]. Moreover, estrogens are known to improve insulin sensitivity through the activation of receptors present in various tissues (e.g., pancreas, adipose tissue, liver, skeletal muscle) [8]. Accordingly, adolescent and adult females are generally characterized by higher total body fat contents and obesity rates, but males are more susceptible to cardiometabolic risk. Interestingly, this sexual dimorphism is blurred after menopause, thereby reinforcing the outstanding involvement of sex hormones in the pathophysiology of obesity-related complications [6].

To better understand the influence of inter-individual variability factors in modulating the susceptibility to disease development, metabolomics has proved great potential because of its capacity for providing thorough phenotypic readouts by comprehensively examining complex metabolic interactions at molecular level [9]. On the topic at hand, it has been repeatedly reported that male subjects typically display stronger associations between obesity, cardiometabolic traits, and metabolomics disturbances in adolescent [10–13] and adult [14–16] populations, but these sex differences are largely attenuated at older ages (> 50 years) [15–17]. On the contrary, obesity-related metabolic impairments have been found to be exacerbated among girls during infancy and early childhood [18–20], in line with the rationale aforementioned. However, to our knowledge, no metabolomics studies have been performed in middle childhood, when crucial hormonal maturation processes begin. In this sense, it should be noted that existing literature about sex differences on metabolic complications (e.g., IR) during childhood is rather inconsistent [21], plausibly because this is a very dynamic life period. Thus, further research considering more homogeneous populations (i.e., narrower age ranges) is crucial to get deeper insights into the age-dependent influence of sex in modulating the susceptibility of disease development along childhood. For this purpose, we recruited a sex-stratified cohort encompassing mid-aged (6–12 years) children with obesity and IR, children with obesity without IR, and healthy control children, from whom plasma and erythrocyte samples were collected. The complementarity of both biological matrices has been demonstrated in previous publications, with plasma being one of the most common biofluids for non-invasive biomedical research, while erythrocytes can serve a reliable sensor of relevant pathogenic events (e.g., energy dyshomeostasis, oxidative stress, inflammation) [22]. Then, state-of-the-art metabolomics approaches were employed to comprehensively explore the impact of sexual dimorphisms on the interplay between obesity and IR during this transition prepubertal period.

Methods

Study design

The study population consisted of children of both sexes, aged between 6 and 12 years. The prepubertal stage was first assessed by pediatricians through meticulous physical exploration according to the Tanner scale (i.e., genital developmental stage G in boys, breast developmental stage B in girls), and then confirmed on the basis of hormonal determinations (i.e., follicle-stimulating hormone < 4 mU/mL; luteinizing hormone < 0.3 mU/mL) [23]. The anthropometric characterization in terms of height, weight, body mass index (BMI), and waist circumference was performed by using a precision stadiometer, a bioimpedance body composition analyzer, and a tape

measure. Then, their corresponding Z-scores were calculated with respect to sex- and age-adjusted Spanish reference values [24]. On this basis, the participants were categorized as children with obesity (i.e., BMI Z-score above 2) or healthy control children (i.e., BMI Z-score below 1). Children with obesity underwent an oral glucose tolerance test (OGTT), which allows diagnosing IR when at least one of the following criteria is fulfilled: (i) homeostasis model assessment of insulin resistance (HOMA-IR) score above 3.5, (ii) fasting insulin levels above 15 mU/L, (iii) insulin levels above 75 mU/L at 120 min of the OGTT, (iv) insulin levels above 150 mU/L at any time point of the OGTT [25]. Complementarily, age- and sex-standardized HOMA-IR Z-scores were calculated based on reference data from the IDEFICS study [26]. Accordingly, the study groups considered herein were as follows: children with obesity and IR (ObIR+, N = 39), children with obesity without IR (ObIR-, N = 22), and healthy control children (CNT, N = 28). The recruitment was performed at "Hospital Universitario Puerta del Mar" (Cádiz, Spain) among patients attending the Pediatric Unit for routinary health assessment. Exclusion criteria were suffering from other known chronic systemic diseases or acute infectious processes. Written informed consent to participate in the study was provided by legal guardians. The "Hospital Puerta del Mar" ethical committee approved the study protocol (Ref. PI22/01899).

Sample collection and biochemical analysis

Venous blood samples were collected using BD Vacutainer EDTA tubes, in the morning after overnight fasting to minimize differences allocated to the circadian rhythm and diet. The blood tubes were centrifuged at 1500 g for 10 min at 4 °C to obtain plasma samples. Afterward, cell pellets were rinsed three times with cold saline solution (9 g/L NaCl, 4 °C) to get the erythroid fraction after centrifugation (1500 g, 5 min, 4 °C). An aliquot of fresh blood was employed for determining general biochemical parameters using an Alinity analyzer (Abbot, Madrid, Spain), including glucose, insulin, glycated hemoglobin, lipids (total cholesterol, high- and low-density lipoprotein cholesterol, triglycerides), and C-reactive protein. Hormones (follicle-stimulating hormone, luteinizing hormone, 17β-estradiol, progesterone, testosterone, androstenedione, dehydroepiandrosterone sulfate, cortisol) were measured by chemiluminescent immunoassays using the same analyzer (the analytical performance is shown in Table S1, according to manufacturer's specifications). The HOMA-IR score was calculated as: HOMA-IR = (Glc×Ins)×0.055/22.5, where Glc and Ins refer to fasting glucose (mg/dL) and insulin (μU/mL), respectively. Finally, white blood cells were counted using an automated hematology analyzer to compute the following inflammatory indices: systemic immune inflammation index (SII) = N × P/L; systemic inflammation response index (SIRI) = N × M/L; aggregate index of systemic inflammation (AISI) = N × P × M/L; platelet-to-lymphocyte ratio (PLR) = P/L; monocyte-to-lymphocyte ratio (MLR) = M/L; and neutrophil-to-lymphocyte ratio (NLR) = N/L; where, N, P, L, and M refer to neutrophil, platelet, lymphocyte, and monocyte counts, respectively. The rest of samples were stored at − 80 °C until metabolomics analysis.

Untargeted metabolomics analysis

The biological samples (plasma and erythrocytes) were subjected to protein precipitation and then analyzed by reversed-phase ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS), using the operational conditions described elsewhere (details in Supplementary Material) [27]. A quality control (QC) sample was prepared by pooling equal aliquots of each individual sample, which was analyzed at intermittent points throughout the batch to check system stability and data quality, according to the QComics guidelines [28]. For raw data preprocessing, we implemented the "notame" workflow in MS-DIAL software, comprising peak picking, peak alignment, and QC-based signal drift correction [29]. To conclude, molecular features of interest were annotated according to the Metabolomics Standards Initiative recommendations: (i) matching spectral data (i.e., accurate m/z and tandem MS/MS spectrum, maximum error: 10 ppm) against open access databases (i.e., Human Metabolome Database, METLIN); (ii) analysis of pure standards when available [30]. Complementarily, the identification of phospholipids and conjugated metabolites was performed on the basis of their distinctive MS fragmentation [31, 32].

Statistical analysis

Descriptive statistics of demographic, anthropometric, and biochemical variables were expressed as the mean ± standard deviation. Analysis of variance (ANOVA) with post hoc Fisher LSD test was employed to look for within-sex differences between obesity-stratified groups, whereas between-sex differences within each obesity-stratified group were investigated by the Student's t-test. For metabolomics data analysis, sex-stratified comparisons between study groups (i.e., ObIR + vs. ObIR- vs. CNT) were accomplished by combining multivariate and univariate tools, using a statistical pipeline well-established within the metabolomics community [33]. Preliminary data processing started with the removal of variables containing more than 20% missing values. The remaining missing data were subjected to kNN imputation, as this method has been proven to provide better performance, robustness, and an overall marginal type 1 error rate [34], especially when proportion of missing

data across samples for a molecular feature is relatively low (as in our datasets, with only 1.8% of imputable variables). Then, data was filtered based on the interquartile range to remove non-informative variables. Finally, log transformation was employed to increase the symmetry of skewed distributions, and Pareto scaling to adjust for differences in concentration scales (this method has the advantage of correcting for different dynamic ranges in metabolite concentrations without amplifying noisy variables so much [33]). Orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to explore the discriminant power of metabolomics data in a multivariate manner (i.e., Variable Importance for the Projection parameter greater than 1). Then, the significance of associations observed in multivariate models was validated by ANOVA with Fisher LSD post hoc tests and false discovery rate (FDR) correction for multiple testing. Additional linear models with covariate adjustment were computed to control for age as a confounder. All statistical analyses were conducted in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/).

Results

This study leverages a clinically well-characterized cohort of children (N = 89) in terms of demographic, anthropometric, and biochemical variables (Table 1). The participants were on average 9.2 years-old (age range: 6–12 y) and 46.1% were female. As defined by inclusion criteria, children with obesity showed greater BMI, weight, and waist circumference compared to controls, with Z-scores above 2. Noteworthy, these anthropometric measures were comparable between ObIR- and ObIR+ children, as well as between female and male subjects, which enables us to discard that differences in other variables under investigation are driven by obesity degree. A gradual increase in fasting insulin levels and HOMA-IR scores was found when comparing healthy controls, ObIR-, and ObIR+ children in sex-stratified analyses, but without affecting glycemia. Interestingly, insulin homeostasis impairments were found to be exacerbated among males (higher insulin levels and HOMA-IR scores compared to female counterparts within the ObIR- and ObIR+ groups). Children with obesity also had lower high-density lipoprotein cholesterol and higher triglyceride content, regardless of IR status or sex. Regarding inflammatory markers, elevated C-reactive protein levels and white blood cell-based indices (i.e., SII, SIRI, AISI, NLR) were observed in children with obesity of both sexes, especially among those with concomitant IR. In this respect, it should be noted that boys exhibited a sharpened inflammatory milieu. Finally, hormonal determinations evidenced that our population is in the onset of adrenarche (i.e., dehydroepiandrosterone sulfate > 40 µg/dL, as would be expected for their age range), but without reaching widely adopted pubertal thresholds (i.e., follicle-stimulating hormone < 4 mU/mL; luteinizing hormone < 0.3 mU/mL) [23]. Childhood obesity was associated with increased androgen (i.e., testosterone, androstenedione, dehydroepiandrosterone sulfate) and corticosteroid (i.e., cortisol) contents in both sexes, whereas pituitary hormone production (i.e., luteinizing hormone, follicle-stimulating hormone; this latter without reaching statistical significance) was solely up-regulated in girls. Unfortunately, estrogens (17β-estradiol) and progestogens (progesterone) were unquantifiable due to the low concentrations normally present in prepubertal children and the limited sensitivity of laboratory assays employed in the clinical practice [35].

Metabolomics also evidenced a sex-dependent interplay between obesity, IR, and steroid hormones (Fig. 1, Table S2). As stated above, children with obesity of both sexes showed higher plasma levels in a multitude of androgens, but sexually dimorphic association patterns were observed depending on the concomitance of IR and phase II conjugation of the species under consideration. Among males, obesity-driven androgen increases were comparable regardless of the presence or not of IR, while ObIR- subjects were hormonally more similar to healthy controls within the female subpopulation. Moreover, this androgen raise was slightly more pronounced in boys for sulfated species, whereas the over-production of glucuronidated steroids was predominant in girls. Estrogens and progestogens were exclusively increased in female ObIR+ children (but not in male counterparts), with control and ObIR- subjects showing similar levels. Finally, childhood obesity was also associated with higher corticosteroid contents, with these differences being more pronounced in females. The influence of insulin in this corticosteroid surplus was species-specific, since sulfated metabolites exhibited gradual changes between the study groups (ObIR + > ObIR- > CNT), whereas glucuronide derivatives were unaffected by IR concomitance (ObIR + ≈ ObIR- > CNT).

Besides this profound hormonal remodeling, sexually dimorphic differences were also detected in many other metabolites at circulating (plasma) and cellular (erythrocytes) levels after complementary multivariate (Figures S1-S2) and univariate statistical analysis, as detailed in Tables S3-S4. Overall, the total number of differential metabolites between obesity groups was fewer among females (11 plasma and 10 erythroid metabolites in females, 32 plasma and 45 erythroid metabolites in males) (Fig. 2). The statistical significance of most of these associations was maintained after adjusting for age as a confounding factor. Alterations in a number of metabolites were only significant, or at least exacerbated, in males with obesity (albeit weaker associations were observed in the same direction among girls

Table 1 Demographic, anthropometric, and biochemical characteristics of the study cohort, expressed as mean ± standard deviation. Different superscript letters within each row denote within-sex significant differences between obesity-stratified groups (i.e., ObIR + vs. ObIR- vs. CNT), according to ANOVA and subsequent post hoc Fisher LSD test. † Denotes between-sex significant differences within each obesity-stratified group, according to Student's t-test (p-values in brackets within the same cell).

	Female subjects				**Male subjects**			
	CNT	**ObIR-**	**ObIR+**	***p*-value (ANOVA)**	**CNT (*p*-value M vs. F)**	**ObIR- (*p*-value M vs. F)**	**ObIR+ (*p*-value M vs. F)**	***p*-value (ANOVA)**
Demographic and anthropometric characteristics								
N	12	10	19	-	16	12	20	-
Age (years)	9.1 ± 1.5	8.9 ± 1.4	9.4 ± 1.1	NS	9.2 ± 1.2	9.3 ± 1.7	9.4 ± 1.5	NS
Weight (kg)	27.0 ± 3.8 [a]	54.6 ± 18.8 [b]	59.0 ± 10.2 [b]	6.4×10^{-12}	27.7 ± 6.2 [a]	55.2 ± 13.3 [b]	57.8 ± 9.1 [b]	7.8×10^{-13}
Weight Z-score	−0.1 ± 0.8 [a]	4.5 ± 1.5 [b]	4.8 ± 1.7 [b]	8.5×10^{-9}	0.0 ± 0.7 [a]	4.4 ± 1.5 [b]	5.0 ± 1.6 [b]	2.2×10^{-7}
Body mass index (kg/m^2)	16.3 ± 1.4 [a]	27.5 ± 5.1 [b]	29.0 ± 3.5 [b]	1.4×10^{-13}	16.8 ± 1.6 [a]	28.1 ± 3.5 [b]	29.1 ± 4.0 [b]	3.1×10^{-16}
Body mass index Z-score	−0.4 ± 0.8 [a]	4.3 ± 1.1 [b]	4.5 ± 1.6 [b]	5.0×10^{-11}	−0.2 ± 0.7 [a]	4.4 ± 1.5 [b]	4.6 ± 1.8 [b]	8.7×10^{-12}
Waist circumference (cm)	58.1 ± 3.9 [a]	92.3 ± 9.5 [b]	91.1 ± 8.4 [b]	1.8×10^{-11}	58.2 ± 6.8 [a]	92.7 ± 13.2 [b]	96.0 ± 10.4 [b]	2.1×10^{-14}
Waist circumference Z-score	−0.2 ± 0.4 [a]	4.6 ± 0.8 [b]	4.5 ± 1.6 [b]	1.3×10^{-9}	−0.4 ± 1.0 [a]	4.9 ± 1.9 [b]	4.8 ± 1.7 [b]	1.2×10^{-9}
Biochemical characteristics								
Glucose (mg/dL)	84.3 ± 5.4	85.1 ± 5.9	84.8 ± 7.7	NS	84.0 ± 3.6	82.2 ± 6.2	88.1 ± 8.3	NS
Insulin (μU/mL)	5.1 ± 2.0 [a]	9.8 ± 3.2 [b]	18.3 ± 9.4 [c]	3.7×10^{-7}	4.4 ± 2.0 [a]	12.9 ± 2.7 [b,†] (2.7×10^{-2})	23.4 ± 9.6 [c,†] c	1.1×10^{-5}
HOMA-IR	1.1 ± 0.4 [a]	2.1 ± 0.7 [b]	4.0 ± 2.0 [c]	8.2×10^{-7}	0.9 ± 0.4 [a]	2.7 ± 0.6 [b,†] (4.9×10^{-2})	5.2 ± 2.6 [c,†] (4.5×10^{-2})	1.6×10^{-5}
HOMA-IR Z-score	0.0 ± 0.6 [a]	1.5 ± 1.2 [a, b]	3.3 ± 2.9 [b]	3.9×10^{-5}	−0.3 ± 0.5 [a]	2.0 ± 1.2 [a, b]	5.6 ± 4.3 [b,†] (4.9×10^{-2})	4.6×10^{-4}
Glycated hemoglobin (%)	5.2 ± 0.1	5.4 ± 0.1	5.3 ± 0.2	NS	5.1 ± 0.2	5.4 ± 0.3	5.4 ± 0.3	NS
Total cholesterol (mg/dL)	173.5 ± 26.9	172.4 ± 30.9	169.8 ± 38.3	NS	161.9 ± 25.0	162.5 ± 22.5	157.2 ± 21.6	NS
High-density lipoprotein cholesterol (mg/dL)	63.3 ± 14.3 [a]	48.6 ± 4.7 [b]	46.1 ± 8.2 [b]	5.7×10^{-4}	70.1 ± 19.4 [a]	50.7 ± 12.5 [b]	44.1 ± 7.2 [b]	3.0×10^{-6}
Low-density lipoprotein cholesterol (mg/dL)	103.0 ± 24.2	107.0 ± 26.6	108.6 ± 35.3	NS	87.3 ± 19.8	95.0 ± 16.2	94.8 ± 18.3	NS
Triglycerides (mg/dL)	51.5 ± 20.3 [a]	78.5 ± 36.6 [b]	82.1 ± 36.6 [b]	8.5×10^{-3}	47.1 ± 18.0 [a]	75.9 ± 37.5 [b]	79.4 ± 33.9 [b]	1.7×10^{-3}
C-Reactive protein (mg/dL)	0.5 ± 0.3 [a]	2.8 ± 2.2 [b]	3.1 ± 2.8 [b]	2.5×10^{-3}	0.5 ± 0.3 [a]	3.2 ± 0.7 [b]	3.3 ± 2.8 [b]	1.3×10^{-5}
Systemic immune inflammation index (SII)	218.3 ± 56.6 [a]	340.5 ± 161.1 [a, b]	413.8 ± 159.8 [b]	3.3×10^{-3}	318.6 ± 128.5 [a,†] (3.3×10^{-2})	373.9 ± 167.6 [a]	529.2 ± 239.2 [b]	7.0×10^{-3}
Systemic inflammation response index (SIRI)	0.4 ± 0.2 [a]	0.6 ± 0.3 [a, b]	0.8 ± 0.5 [b]	4.5×10^{-3}	0.6 ± 0.2 [a,†] (3.5×10^{-2})	0.7 ± 0.3 [a]	1.0 ± 0.4 [b,†] (4.9×10^{-2})	1.8×10^{-4}
Aggregate index of systemic inflammation (AISI)	111.9 ± 43.3 [a]	205.2 ± 118.6 [a, b]	253.3 ± 155.5 [b]	1.3×10^{-2}	181.3 ± 86.3 [a,†] (2.5×10^{-2})	236.6 ± 128.8 [a]	356.7 ± 184.4 [b,†] (2.4×10^{-2})	8.2×10^{-4}
Platelet-to-lymphocyte ratio (PLR)	97.6 ± 25.0	112.0 ± 44.7	111.8 ± 31.3	NS	119.7 ± 40.2	113.2 ± 25.0	133.6 ± 53	NS
Monocyte-to-lymphocyte ratio (MLR)	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	NS	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1 † (1.8×10^{-2})	NS
Neutrophil-to-lymphocyte ratio (NLR)	0.8 ± 0.2 [a]	1.1 ± 0.4 [b]	1.4 ± 0.5 [b]	2.8×10^{-4}	1.0 ± 0.3 [a,†] (4.9×10^{-2})	1.1 ± 0.3 [a]	1.6 ± 0.6 [b]	5.1×10^{-3}
Follicle-stimulating hormone (mU/mL)	1.5 ± 0.9	1.3 ± 0.8	2.0 ± 1.3	NS	1.1 ± 0.5	1.0 ± 0.7	1.2 ± 1.0 † (1.5×10^{-2})	NS
Luteinizing hormone (mU/mL)	0.12 ± 0.0081 [a]	0.11 ± 0.011 [a]	0.27 ± 0.029 [b]	3.1×10^{-5}	0.13 ± 0.039	0.12 ± 0.030	0.19 ± 0.14 † (8.5×10^{-4})	NS
Testosterone (ng/dL)	8.9 ± 5.3 [a]	11.4 ± 2.8 [a]	18.3 ± 5.4 [b]	1.3×10^{-6}	9.3 ± 4.6 [a]	13.2 ± 1.2 [a,†] (2.3×10^{-2})	17.3 ± 8.5 [b]	3.6×10^{-6}
Androstenedione (ng/mL)	0.3 ± 0.3 [a]	0.3 ± 0.2 [a]	1.8 ± 0.4 [b]	5.2×10^{-12}	0.6 ± 0.1 [a,†] (1.7×10^{-9})	0.7 ± 0.1 [a,†] (1.6×10^{-11})	1.7 ± 0.3 [b,†] (5.5×10^{-5})	8.3×10^{-13}

Table 1 (continued)

	Female subjects				Male subjects			
	CNT	ObIR-	ObIR+	*p*-value (ANOVA)	CNT (*p*-value M vs. F)	ObIR- (*p*-value M vs. F)	ObIR+ (*p*-value M vs. F)	*p*-value (ANOVA)
Dehydroepiandrosterone sulfate (μg/dL)	97.6 ± 46.9^{a}	103.0 ± 33.7^{a}	177.8 ± 60.5^{b}	3.7×10^{-12}	91.9 ± 30.3^{a}	$115.8 \pm 24.6^{b,\dagger}$ (1.1×10^{-2})	$223.1 \pm 71.9^{c,\dagger}$ (4.4×10^{-5})	1.2×10^{-27}
Cortisol (μg/dL)	6.6 ± 1.8^{a}	6.5 ± 4.5^{a}	10.7 ± 5.0^{b}	3.9×10^{-3}	7.0 ± 2.1^{a}	8.3 ± 4.0^{a}	11.5 ± 5.1^{b}	3.5×10^{-5}

CNT: control children; F: female; M: male; NS, non-significant; ObIR-: children with obesity without insulin resistance; ObIR+: children with obesity with insulin resistance

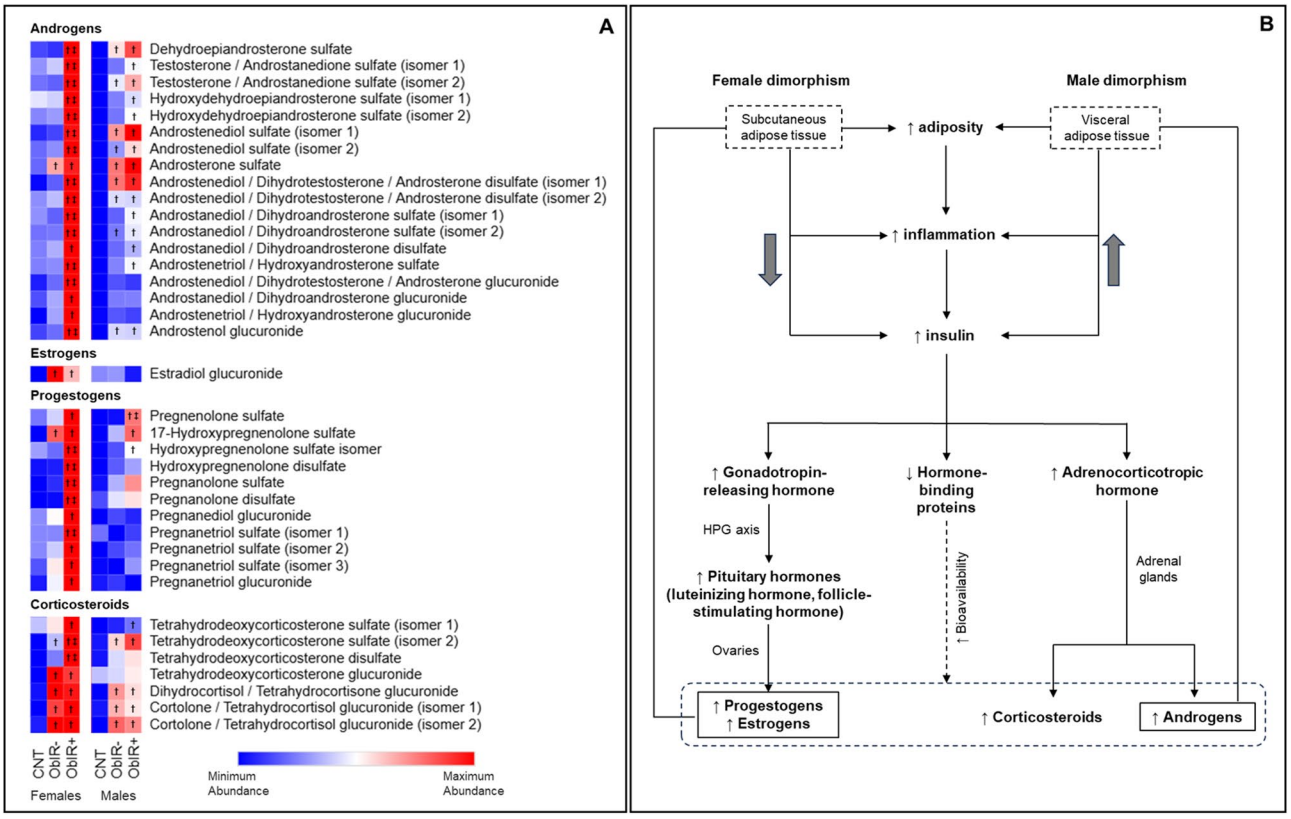

Fig. 1 **A** Heatmaps of differential steroid hormones between children with obesity and insulin resistance (ObIR+), children with obesity without insulin resistance (ObIR-), and healthy control children (CNT) in sex-stratified analyses. † and ‡ denote significant differences with respect to CNT and ObIR- groups, respectively, according to ANOVA with post-hoc Fisher LSD test. **B** Sexually dimorphic mechanisms behind obesity-driven enhanced steroidogenesis

without reaching statistical significance), including glycolytic intermediates, branched-chain and aromatic amino acid derivatives, nitrogenous compounds, and oxidative stress byproducts in both biological matrices. Regarding lipids, we found childhood obesity to be characterized by distinctive metabolic patterns in fatty acid oxidation (i.e., lower plasma levels of hydroxylated fatty acids and N-acetylglycine, higher erythroid levels of free fatty acids and carnitine precursors) and phospholipid turnover (i.e., higher saturation degree of phospholipid-containing fatty acids in both matrices, lower plasma lyso-phospholipids), differences that were again more pronounced in boys. Apart from the aforementioned male-specific changes, we observed children with obesity of both sexes to exhibit decreased xanthosine 5-triphosphate and increased hypoxanthine circulating levels, but their final oxidation to uric acid was up-regulated in girls. Children with obesity also showed sex-dependent raises in plasma bile acids, being the increase in precursors (i.e., 7α-hydroxy-cholesten-3-one, 7α-hydroxy-3-oxocholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid) solely detected among females, while primary bile acids' accumulation (i.e., cholic acid, chenodeoxycholic acid) was predominant in males. In this line, some diet-related xenobiotics were found to be associated with obesity only within females. Noteworthy, many of these

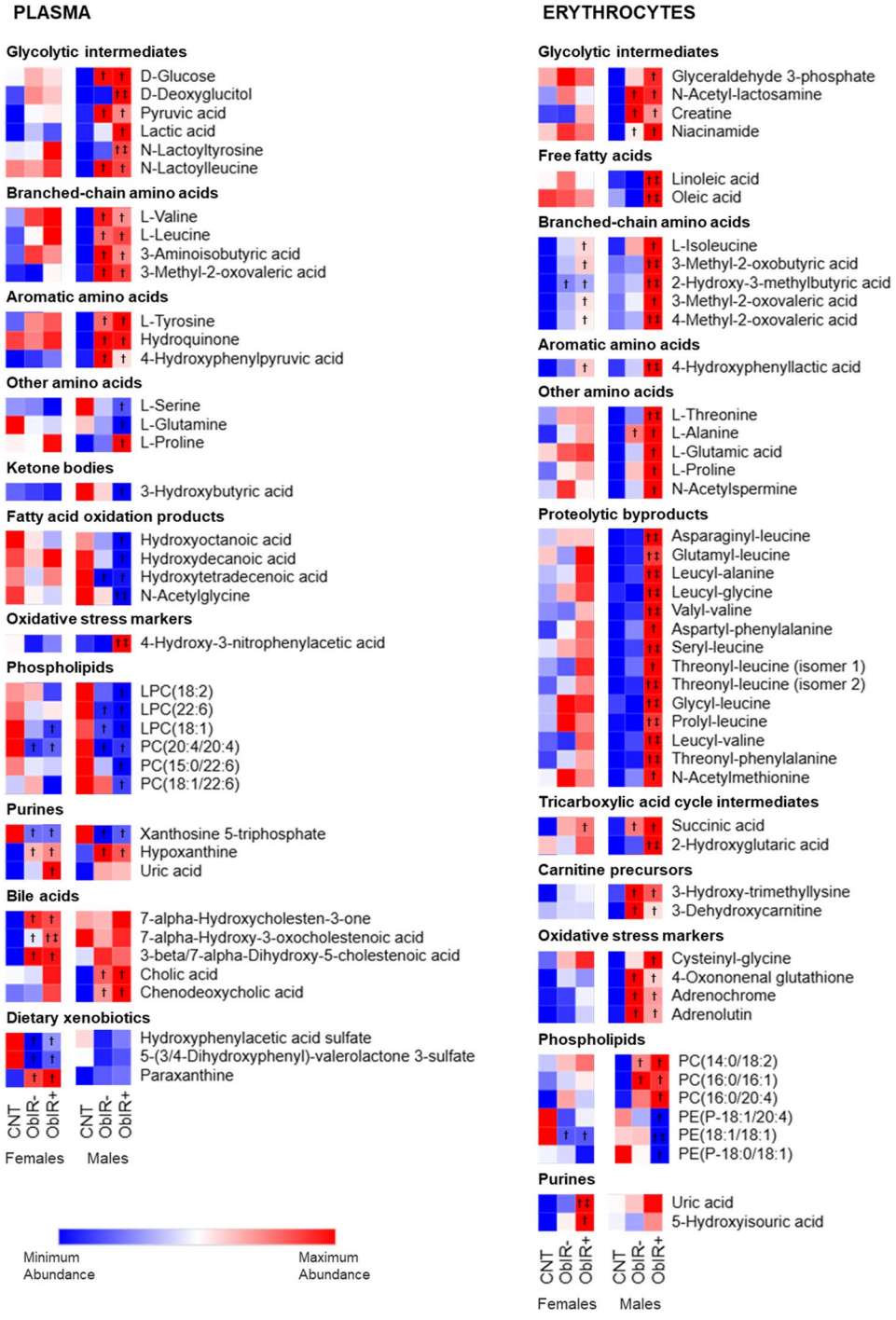

Fig. 2 Heatmaps of differential plasma and erythroid metabolites between children with obesity and insulin resistance (ObIR+), children with obesity without insulin resistance (ObIR-), and healthy control children (CNT) in sex-stratified analyses. † and ‡ denote significant differences with respect to CNT and ObIR- groups, respectively, according to ANOVA with post-hoc Fisher LSD test

obesity-metabolite associations were generally stronger among ObIR+ subjects compared to the ObIR- group. Interestingly, some metabolites enabled us to perfectly discriminate between both obesity groups but only when analyzing the erythroid fraction, whereas analog plasma metabolites showed similar levels regardless of IR status (e.g., branched-chain amino acids and catabolites).

Discussion

The pathophysiology of obesity and related metabolic complications are well-known to be tightly modulated by sexual dimorphisms in an age-dependent manner, being female subjects more susceptible to metabolic risk factors during infancy although presenting lower obesity rates, while puberty induces a shift in these sex differences [6]. However, few studies have been conducted during middle childhood, when hormonal development begins. Herein, we have demonstrated that mid-aged boys exhibit a more unfavorable metabolic profile compared to girls with similar obesity degree, as reflected in exacerbated IR (i.e., higher fasting insulin levels and HOMA-IR scores) and inflammatory status (i.e., higher C-reactive protein and white blood cell counts). These results are apparently contradictory to some studies conducted in larger cohorts (e.g., IDEFICS) [36], which report higher metabolic syndrome prevalence in girls. Nevertheless, as reviewed by Aa et al., literature about sex differences on IR during childhood is rather inconsistent [21], plausibly because this is a very dynamic life period. Accordingly, these findings could suggest that our population is metabolically more similar to adolescents than to prepubertal children (although they were categorized as Tanner I by experienced pediatricians). Supporting this hypothesis, we found children with obesity and IR to show sexually dimorphic enhanced steroidogenesis (i.e., higher levels of estrogens, progestogens, androgens, and corticosteroids in girls; higher levels of androgens and corticosteroids in boys), as summarized in Fig. 1A. This concurs with scientific evidence reporting that insulin can boost the production of steroid hormones through various pathways in females, such as secretion of the gonadotropin-releasing hormone (responsible for the synthesis of progestogens and estrogens in ovaries) and secretion of the adrenocorticotropic hormone (responsible for the synthesis of androgens and corticosteroids in adrenal glands) [37]. On the contrary, adrenal activity is the only mechanism known to be up-regulated in prepubertal boys with obesity, thus explaining why male subjects solely had higher contents of androgens and corticosteroids. In addition, hyperinsulinemia and chronic inflammation have been proven to lower hormone transport protein levels (sex hormone-binding globulin, corticosteroid-binding globulin, albumin), thereby further increasing the bioavailability of sex steroids in obesity [38, 39]. This differential raise of steroid hormones, primarily mirrored in an androgen-to-estrogen surplus in boys, may in turn bidirectionally modulate metabolic health in a sex-dependent manner, since estrogens preserve insulin function in females [8], while androgens promote the accumulation of pro-inflammatory VAT in males [7], in line with our results (Fig. 1B). In this respect, we found that most of the obesity-driven hormone increases were only significant in the ObIR+ group among females, but also in non-IR individuals when considering males. This reinforces our hypothesis that girls exhibit better metabolic flexibility during middle childhood, since concomitance of IR was a *sine qua non* condition to induce hormonal changes in them, whereas obesity itself (regardless of the presence or not of IR) was able to provoke a deleterious steroid profile in boys. Another remarkable finding in this regard was the different pattern of association between obesity, IR, and hormones depending on their conjugation, particularly for androgens (predominant production of sulfated and glucuronidated species in males and females, respectively) and corticosteroids (greater differences between ObIR + and ObIR- individuals in sulfated species). The absence of unconjugated species among these differential steroids shown in Fig. 1A could be in part due to methodological issues, since they are predominantly bound to transport proteins while in circulation because of their lipophilic nature. As sample treatment for metabolomics analysis is based on protein precipitation, these species are often lost during extraction and, thus, largely undetectable by subsequent LC-MS analysis. However, it should be noted that steroid sulfation reactions are essential for endocrine balance and metabolic control, so our observation that sulfated conjugates were the most discriminant steroid species also suggests that phase II metabolites could provide additional information about health status [40]. Altogether, we hypothesize that obesity in middle childhood induces an adolescent-like metabotype driven, at least in part, by premature hormonal development (i.e., protective estrogenic profile in girls vs. deleterious androgenic profile in boys), which may in turn be responsible for sexual dimorphisms in other metabolic complications.

As a direct reflection of this sexually dimorphic metabolic risk, we found that many of the obesity-related differential metabolites identified in this study exclusively differed within male subjects, albeit similar (but weaker) associations were observed in females (Fig. 2). Most of these metabolites are well-known to participate in pathways strictly regulated by insulin, thus suggesting that the aforementioned aggravation of IR in boys could be a major triggering factor of these sex differences. Within the male subpopulation, childhood obesity was primarily characterized by plasma and erythroid increases in a myriad of metabolites involved in carbohydrate, lipid,

and protein disposal, plausibly pointing to an excessive intake and inefficient expenditure of calories as the main etiological cause of this disorder. On the one hand, we observed a raise in circulating levels of simple sugars (i.e., glucose, deoxyglucitol), accompanied by the accumulation of various intermediates from their catabolism via glycolysis (i.e., glyceraldehyde 3-phosphate, pyruvic acid), anaerobiosis (i.e., lactic acid, N-lactoyl-amino acids), and glycosylation (i.e., N-acetyl-lactosamine), as well as essential cofactors (i.e., creatine, niacinamide). These metabolic increments have repeatedly been reported as obesity hallmarks, indicative of boosted glycolytic metabolism to counteract deficits in other energy-related pathways [22, 41]. Secondly, elevated erythroid free fatty acids could serve as direct markers of higher lipid intake, but also impaired mitochondrial β-oxidation and enhanced release from adipose tissue [42]. These lipids may in turn disrupt insulin signaling due to their lipotoxic nature, thus accounting for the higher levels detected among subjects with IR compared to ObIR- and control groups. To close the loop regarding the association between obesity and macronutrient metabolism, our metabolomics results evidenced a consistent increase in blood levels of proteolytic byproducts (i.e., peptides, N-acetylated amino acids), free amino acids, and downstream catabolites, which could be allocated to a dietary protein surplus and impaired capacity to inhibit protein turnover [43]. Among them, branched-chain amino acids and their derivatives (e.g., α-ketoacids) have received great attention because of their ability to exacerbate IR through various mechanisms (acting as insulin secretagogues, mammalian target of rapamycin complex activation) [44], while aromatic amino acids serve as precursors of uremic toxins and inflammatory mediators [45]. Altogether, as a consequence of this close interplay between obesity, IR, and energy homeostasis, we found that differences in many of above-mentioned metabolite classes were often more pronounced in the ObIR+ group, but only when analyzing the erythroid fraction, which highlights the utility of erythrocytes as sensors to investigate IR-specific disturbances [22].

This nutrient oversupply may in turn elicit additional deleterious health repercussions, since it overloads the mitochondrial oxidative capacity and, at long term, provokes oxidative stress [46]. In the present study, this was mirrored in altered levels of multiple metabolites suggesting mitochondrial dysfunction in energy-related pathways, such as the tricarboxylic acid cycle (i.e., higher erythroid succinic and 2-hydroxyglutaric acids), ketogenesis (i.e., lower plasma 3-hydroxyburtyric acid), and fatty acid β-oxidation (i.e., lower plasma hydroxylated fatty acids and N-acetylglycine, higher erythroid free fatty acids and carnitine precursors), as previously reported [22, 41]. Furthermore, children with obesity had raised contents in markers resulting from oxidative damage to proteins (i.e., 4-hydroxy-3-nitrophenylacetic acid), lipids (i.e., 4-oxononenal glutathione), and catecholamines (i.e., adrenochrome, adrenolutin). Together with these disturbances reflecting unbalanced redox milieu, we also observed deficits in glutathione metabolism, with increased glutamate-to-serine ratios (indicative of impaired biosynthesis) [43] and cysteinyl-glycine levels (a breakdown product) [47]. Besides its crucial role in glutathione synthesis, glutamic acid is also essential for controlling nitrogen homeostasis through its conversion into glutamine. In this sense, it has been described that rates of glutamine formation are reduced in obesity, leading to the activation of alternative mechanisms for assimilating excess nitrogen pools, such as synthesis of polyamines via arginine-proline metabolism [48], in line with our results. Finally, another family of metabolites that was influenced by obesity, predominantly within males, were phospholipids. Children with obesity showed higher saturated-to-polyunsaturated ratios in fatty acids contained in the structure of plasma and erythroid phospholipids, as well as reduced plasma lyso-phospholipids. This concurs with recent findings suggesting that phospholipid impairments in obesity could be allocated to altered lecithin cholesterol acyltransferase activity and cell membrane composition changes [22]. Overall, our male-specific results are in close agreement with other metabolomics investigations conducted in adolescents [10–13], which reinforces our hypothesis that obesity-driven enhanced steroidogenesis might induce profound homeostasis remodeling already during middle childhood, bringing forward the metabolic shifts that are expected to occur during puberty.

Unlike the aforementioned male-specific metabolic changes, a few other metabolites showed stronger associations with obesity within female subjects. The degradation of purines into uric acid and 5-hydroxyisouric acid was significantly more pronounced in girls with obesity, especially among ObIR+ individuals, compared to male counterparts. Although underlying causes are still unclear, but probably mediated by sex differences in xanthine oxidoreductase activity, it has consistently been reported that women with obesity have greater odds of suffering from hyperuricemia, which in turn closely relates to higher cardiovascular risk [49]. A remarkable sexual dimorphism was also observed in plasma bile acids, with increased levels of precursor species (i.e., 7α-hydroxy-cholesten-3-one, 7α-hydroxy-3-oxocholestenoic acid, 3β,7α-dihydroxy-5-cholestenoic acid) being only detected in girls with obesity, whereas the production of primary bile acids (i.e., cholic acid, chenodeoxycholic acid) was over-expressed in boys. In this respect, it is well-known that obesity stimulates cholesterol metabolism toward the generation of bile

acids with the final aim of facilitating the management of excess body fat [50]. However, estrogens may partially reverse this situation by inhibiting cytochrome P450 enzymes involved in their synthesis as well as by impairing their transport [51], which explain why females tended to accumulate biosynthetic intermediates rather than bile acid end-products. To conclude, we also found children with obesity to have lower plasma contents of metabolites resulting from polyphenol-rich food consumption (i.e., hydroxyphenylacetic acid sulfate, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone 3'-sulfate), together with higher paraxanthine (a caffeine metabolization product), but these results were only significant for females. This could be indicative of lower adherence to healthy dietary patterns among girls due to sex/gender-biased eating behaviors, but the lack of dietary assessment data in our population impedes us corroborating this hypothesis.

Although the recruitment of a clinically-well characterized cohort, with available biological samples for exhaustive clinical biochemistry assessment and cutting-edge metabolomics analysis, has enabled us to get deeper insights into the sexually dimorphic impact of obesity and IR during mid-childhood, some limitations deserve to be mentioned. The relatively modest sample size limits the generalizability of our results, so future studies in larger and independent cohorts are needed for validation purposes. Moreover, as this transition prepubertal period is highly dynamic, both at hormonal and metabolic levels, further research stratified by narrower age ranges could help to minimize inter-individual variability. In this respect, it is worth mentioning that assessing pubertal status in clinical practice may have several drawbacks, such as the difficulty of evaluating breast gland development in girls with obesity due to breast adiposity, or the lack of consensus cut-off hormonal values (in part attributable to great fluctuations of their basal levels). This could explain our observation that mid-aged children with obesity are in an "early subclinical pubertal status" (undetectable by existing clinical and biochemical tests), thus highlighting the need of more reliable and sensitive methods to diagnose puberty onset. To complement these cross-sectional findings, additional longitudinal studies will enable to explore how early metabolic impairments may predict later cardiometabolic risk. Finally, combination of classical metabolic assessment and metabolomics with other phenotypic (e.g., MRI, bone health) or lifestyle (e.g., dietary habits) information would be of great interest to better understand the interplay between obesity, IR, and sex.

Conclusions

This study demonstrates for the first time that sexual dimorphisms in mid-aged children with obesity and IR are more similar to those typically displayed by adolescents than to clinically prepubertal subjects. This could be allocated to early obesity-driven hormonal development and, consequently, the induction of an androgenic unfavorable metabolic profile in males, as reflected in exacerbated IR and chronic inflammation. As a result of this greater cardiometabolic risk, many metabolite markers known to be associated with pathophysiology of obesity and IR (e.g., energy-related intermediates in carbohydrate, lipid, and protein metabolism, oxidative stress indicators, phospholipids) showed more significant changes within the male subpopulation. These findings are of great relevance to better understand the age-dependent influence of sex in modulating the susceptibility of disease development. Further studies in larger and independent cohorts are needed to validate these results, with the final aim of providing personalized recommendations to prevent and treat obesity and related complications.

Supplementary Information

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Supplementary Material 1.

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Author contributions

Conceptualization, R.G.-D.; methodology, Á.G.-D., O.S., and R.G.-D.; formal analysis, Á.G.-D., O.S., and R.G.-D.; investigation, Á.G.-D., J.D.-R., O.S., A.L.-S., R.L., and R.G.-D.; resources, J.D.-R., R.L., A.L.-S., and R.G.-D.; data curation, R.G.-D.; writing—original draft preparation, R.G.-D.; writing—review and editing, Á.G.-D., R.L., A.L.-S., and R.G.-D.; supervision, R.G.-D.; project administration, R.G.-D.; funding acquisition, R.G.-D. All authors have read and agreed to the published version of the manuscript.

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Data availability

The datasets generated and/or analyzed during the current study are available in Zenodo repository, https://zenodo.org/records/13983276.

Declarations

Ethics approval and consent to participate
The "Hospital Puerta del Mar" ethical committee approved the study protocol (Ref. PI22/01899). Written informed consent to participate in the study was provided by all participants and/or legal guardians.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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