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Predictive epigenetic biomarkers of successful weight-loss intervention in pre-pubertal children with obesity

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Abstract

Childhood obesity represents a major public health challenge, increasing long-term risks such as type 2 diabetes and cardiovascular disease. Lifestyle interventions in prepubertal children are often more effective, but individual responses can vary, highlighting the need for predictive biomarkers to personalize treatment. This study investigated DNA methylation profiles in prepubertal children living with obesity to identify possible epigenetic markers that could predict the success of weight loss interventions. We analyzed a cohort of 26 children who underwent a six-month lifestyle intervention focused on dietary modifications. From 214 differentially methylated CpG sites between high and low responders, we narrowed these to eight key markers. These markers were used to develop a predictive model with a precision 84% in classifying children based on their BMI normalized to changes in the z-score of the age. CpGs were found in genes such as *GSDMD*, *GFRA1* and *NRP2*, all linked to pathways involved in inflammation, metabolic regulation, and lipid metabolism, which are crucial in the development of obesity. The results suggest that DNA methylation signatures can predict weight loss responses in prepubertal children, offering a novel approach to early-stage obesity intervention. These findings hold promise for the development of personalized treatment strategies in pediatric obesity. However, larger and more diverse cohort studies are needed to confirm the generalizability of these markers and refine the predictive model for broader clinical use.

Keywords Childhood obesity, Prediction model, DNA methylation, LASSO, Lifestyle intervention

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Introduction

Despite the vast efforts devoted to treatment and prevention, the worldwide prevalence of obesity has increased steadily over the past few decades [1]. Obesity in children is of particular concern, as excess weight gained during childhood can be tracked into later life. Specifically, approximately 80% of children with overweight or obesity will remain obese as adults, increasing the risk of additional comorbidities, including type 2 diabetes, cardiovascular disease, or some types of cancer [2–6], which collectively reduce life expectancy [7, 8]. In contrast, improving the status of obesity during childhood and adolescence reduces the risk of obesity and its co-morbidities later in life [9, 10]. These observations reinforce the awareness that weight loss interventions are more effective in magnitude and duration when implemented during early life (i.e., childhood) than during adulthood. However, weight loss interventions in the paediatric population are often relatively ineffective, as they are based predominantly on moderate lifestyle actions, including diet and exercise [11, 12]. This leads to excessive medical costs and increased family frustration due to clinical failure. Hence, a long-standing interest in the field is the development of novel markers and/or models aimed at predicting whether a child with overweight/obesity will positively respond to a given weight loss intervention.

Obesity arises as the complex interplay between genetic and lifestyle factors [13]. Geneticists have identified a few hundred genetic variants associated with obesity and/or increased body mass index [14]. However, they all explain less than 8% of the variation in body weight [15]. Hence, it is widely acknowledged that environmental factors, including excessive calorie intake and poor physical activity, are the major drivers of the worldwide obesity epidemic. Epigenetic mechanisms have emerged as a key element that links environmental signals with genetic activity and therefore is a solid candidate to explain the rapid increase in obesity rates [16]. Among epigenetic mechanisms, DNA methylation has been widely studied, and several laboratories have now linked genome-wide patterns of DNA methylation with childhood/adult obesity and obesity-related comorbidities [16–18]. DNA methylation in blood could be a potential good predictive marker because (i) it can be measured reliably, fast, and at low cost; (ii) it is relatively stable over time and multiple environmental conditions [19, 20]; (iii) it integrates factors of life, including nutrition interventions, with the genome [21, 22]; and (iv) it is largely established during early development, including childhood [23]. Several studies have reported that the epigenetic profile could help explain individual differences in weight loss after a dietary/lifestyle intervention in adolescents [24] and adults [25–28]. However, this type of study has not previously been carried out in prepubertal children with obesity.

The objective of this work was to explore baseline differences in DNA methylation that could be associated with an effective weight loss response after a lifestyle intervention program in Spanish prepubertal children with obesity [11]. We had previously performed a moderate lifestyle intervention based on diet and exercise in a group of prepubertal children with obesity (average age 8 years) [11, 12]. The participants showed a wide range of responses to the intervention. We therefore ranked them according to their weight-loss response, classifying individuals as Low Responders (LR) or High Responders (HR). We identified 214 CpG sites that were differentially methylated between LR and HR. Importantly, these findings allow discrimination between children with obesity who are clinically indistinguishable, identifying those who will respond adequately or poorly to the intervention based on their baseline epigenetic profile, at least in a Spanish cohort. Finally, we reduced the 214 markers to eight CpG sites that recapitulate this effect and could serve as a novel personalised predictive tool for weight-loss interventions in prepubertal children.

Materials and methods

Study population

The children included in this study were selected from a previously reported cohort recruited between January 2013 and December 2014 at the Obesity Unit at Barcelona Children's Hospital Sant Joan de Déu (Barcelona, Spain) [11, 12]. The study was approved by the hospital's ethics committee (identification code PIC-21-12), and signed informed consent was obtained from all parents. Inclusion criteria were prepubertal children (7 to 10 years old) with obesity, defined as BMI-SDS>2 SD for a given age and sex, using the reference from the World Health Organization (WHO). The prepubertal stage was defined as Tanner stage I breast development for girls and testicular volume less than 4 ml in boys. Moreover, in this study, we only included children who remained in the prepubertal stage (Tanner= 0) at the end of the intervention. Exclusion criteria included major congenital or chronic diseases, drug-induced obesity, use of drugs for weight loss, participation in another weight loss program. The study initially included 53 children with obesity. At the end of the 6-month intervention, we only had samples from 39 children due to voluntary withdrawal from the program, pubertal status at the 6-month period, and lack of blood samples at the post-intervention visit. Thus, we analyzed paired samples (baseline and 6 months) from 39 exclusively prepubertal subjects with obesity. We distributed their weight-loss responses into tertiles. The first tertiles (T1) and the third (T3) corresponded to the groups we identified as poor or low responders (LR=

T1) and high responders (HR= T3), respectively. These are the subset of samples that we have used for the epigenomic study.

Lifestyle intervention program and biological samples

The lifestyle intervention program was the routine protocol for hospitalized children with obesity and has been described in detail elsewhere [11, 12]. Briefly, we used motivational interviewing with an emphasis on promoting behavioral modifications aimed at improving lifestyle habits in both the child and their family members. To ensure long-term effectiveness, dietary counseling was tailored by nutrition professionals according to the specific needs of each patient and household. The guidance was aligned with the recommendations of the Department of Health of the autonomous government of Catalonia (Spain) and consistent with the WHO guidelines, which advocate for a Mediterranean dietary pattern. This nutritional model prescribes an energy distribution of approximately 55% from carbohydrates (with sugars contributing less than 10%), 15% from proteins and 30% from fats (with saturated fat less than 10%). Educational sessions incorporated laminated visual aids, such as food replicas and plate models, to facilitate understanding of appropriate portion sizes. Families were supported in menu planning, highlighting the importance of diversity, food quality, and cooking techniques. Furthermore, participants were advised to adopt healthier dietary choices and to participate in at least 30 min of daily physical activity. Families were free to choose the type of activity that is most compatible with their preferences and daily routines.

From each child, paired urine, blood samples and physiological data (e.g. body weight, height) were collected before (T0) and after (T6) the lifestyle intervention as fully described elsewhere [11, 12]. Blood and urine samples were taken in the morning after 8 to 10 h of fasting overnight. Blood samples were aliquoted and stored at -80°C until further analysis. The BMI z score (zBMI) was calculated using the software 'Anthro Plus' (version 1.0.4; WHO) [29]. Here, we selected the WHO BMI zscore over the Spanish reference for a number of reasons: Firstly, the most widely used Spanish reference cohort dates back to the 1980s and no longer reflects the current paediatric population of the Barcelona Metropolitan Area. Secondly, a major goal of our study was to generalize our findings. Using WHO standards facilitates comparisons with other cohorts and makes it easier to extrapolate our findings. This ensures that the study's conclusions can be interpreted more broadly and have wider clinical implications. Next, the difference in the zBMI between baseline and the end of the lifestyle treatment was calculated ($\Delta zBMI = zBMI_{T6} - zBMI_{T0}$). We selected these metrics because they are widely used in paediatric growth and intervention studies, and absolute differences provide clinically significant information that can be compared between cohorts. In contrast, other approaches, such as the percentage change relative to baseline zBMI, can introduce instability, particularly when baseline values are low, and lack standardized interpretation in paediatric settings. The response to the weight loss intervention was heterogeneous between all individuals. Thus, the initial population was stratified into tertiles according to their ΔzBMI at T6 as proposed in a similar study conducted by Salas-Pérez et al. [28]. A total of 13 high responder children (HR), that is, ΔzBMI < -0.3, and 13 low responder children (LR), that is, ΔzBMI > -0.3, were identified and analyzed.

A sensitivity power analysis was conducted to evaluate the detectable effect size given the study sample size (n = 13 per group). Under a two-sided $\alpha = 0.05$, the study had 80% power to detect a large effect between-groups (Cohen's $d > 1.15$). Consequently, the study design is primarily sensitive to larger differences between High Responders (HR) and Low Responders (LR) and is best suited for exploratory analyzes aimed at identifying epigenetic and metabolic features associated with differential clinical response.

Methylation array and pre-processing

Whole blood DNA methylation profiles were obtained using the Infinium Methylation EPIC Kit 850K [30]. We analyzed around 850,000 probes per subject and statistics of the β value and the M value were used to measure the level of methylation [31, 32]. The ChAMP R / Bioconductor package was used to process the array [33–35]. Probes with the following conditions were filtered from the analysis: a detection *P*-values above 0.01 in one or more samples, probes with a beadcount < 3 in at least 5% of samples, probes with single nucleotide polymorphism as identified in Nordlund et al. [36], probes that align to multiple locations as described elsewhere [36] and probes on the X or Y chromosome. Beta-MIxture Quantile Normalization (BMIQ) was performed to reduce the enrichment bias of both type 2 and type 1 of the probe [37]. The batch effects on the microarrays were then adjusted using the *ComBat* method [38]. To account for potential confounding due to blood cell heterogeneity, DNA methylation data were further corrected for estimated leukocyte composition. We applied the reference-based EWAS method which infers the proportions of the major white blood cell subtypes from the methylation signatures and adjusts for them in the analysis, using reference methylomes from purified leukocyte populations [39]. A total of 788373 CpGs were available for statistics after the pre-processing.

Identification of CpG sites for the prediction model

To investigate differences in DNA methylation between HR and LR children and to prioritize CpGs with predictive capacity, we began with the 788,373 CpG sites retained after quality control and preprocessing (Sect. 2.3). These were analyzed using the limma R package applied to the M-values of all 26 samples. The input consisted of a CpG-by-sample matrix of M-values, the corresponding β-values, and a phenodata table containing group labels (HR/LR and ΔzBMI) and sex. Thus, we developed a cross-validation algorithm to calculate as many sex-adjusted regression models [40] as samples were analyzed (n = 26).

Linear models were fitted to each CpGs and differential methylation was tested using limma's empirical Bayes moderation, which stabilizes variance estimates in CpGs and provides more robust test statistics in small cohorts. The CpGs were initially filtered according to statistical significance (FDR < 0.05). As a second biological filter, the effect size was calculated as the absolute difference in the mean β-values between HR and LR, and only CpGs with $|\Delta\beta| \geq 10\%$ were retained. To improve robustness and reduce overfitting, we implemented a leave-one-out (LOO) strategy. Specifically, 26 regression models were constructed, each excluding one sample at a time. In each model, differential methylation analysis was performed using ΔzBMI as the predictor (with HR/LR status considered as an alternative label). This approach rendered 26 independent lists of significant CpGs. We then prioritized only CpGs common to all lists, ensuring that the final selection represented robust and reproducible signals. This consensus process resulted in a set of CpGs (Supplementary Table S1) that consistently distinguished HR from LR children. Finally, the gene pathways associated with these CpGs were explored using the missMethyl R package [41]. Gene set tests were performed in Gene Ontology (GO) categories and KEGG pathways [42] and annotations with FDR < 0.05 were considered significantly enriched.

It is noteworthy that in this study we did not address the relationship between the intervention and adiposity (measured as waist-abdominal circumference) with DNA methylation modeling. Correlating these "crude" proxies of adiposity with high-resolution DNA methylation data (850K array) would likely introduce significant noise and lead to biologically misleading or underpowered results. Therefore, we prioritized age-and-sex-standardized zBMI as our primary outcome for predictive modeling to ensure the highest possible scientific rigor.

Classification according to ΔzBMI and diagnostic thresholding

Using the consensus set of the CpGs identified in Sect. 2.4, we next aim to derive a minimal subset of predictors suitable for classification. To this end, we implemented a partial least squares regression (PLS) framework in R (plsR package [43]), incorporating jackknife resampling to assess model stability and avoid overfitting given the small sample size. This model has previously been applied in binary classification using CpGs [44, 45]. Note that for the consensus predictive model we favored the utilization of partial least squares (PLS) regression. Although PLS is particularly appropriate for high-dimensional methylation data and provided interpretable loadings that facilitated biomarker selection, alternative algorithms such as Random Forest or support vector machines may yield complementary insights. Future studies in larger cohorts should compare different classifiers to assess the robustness and transferability of the predictive signature. A schematic of the pipeline illustrating the flow from the EPIC array to preprocessing, feature selection, and final model derivation is shown in Fig. 1.

Integration of epigenetic predictors with metabolic profiles

Following the identification of the 214 weight-loss-associated CpGs through LOO regression, we sought to evaluate the functional relevance of these epigenetic markers by exploring their baseline correlation with the corresponding metabolic signatures. For this purpose, we integrate metabolite data previously characterized in this same cohort by Leal-Witt et al. [12] This integrative approach was designed to contextualize our epigenetic findings within the broader metabolic landscape, identifying potential molecular links between long-term epigenetic regulation and immediate functional metabolic states that define the responder phenotype.

Results

Prepubertal children with obesity exhibited response variability to a lifestyle intervention

We had previously recruited a clinical cohort of prepubertal children with obesity, BMI-SDS > 2 (Fig. 2a) [11, 12]. The children were enrolled in a 6-month moderate lifestyle intervention consisting of dietary and exercise recommendations (see Sect. 2.2 for more details). The lifestyle intervention significantly decreased BMI-SDS by 0.45 units in the median (Fig. 2b). Importantly, the weight-loss intervention did not impair growth, as evaluated by height (Fig. 2c). The response to the weight loss intervention was heterogeneous among all individuals. Therefore, we stratified our population in tertiles according to their responsiveness to the intervention, and focused on low-responders (LR; ΔzBMI > −0.3), versus high-responders (HR; ΔzBMI < −0.3) (Fig. 2d, Table 1).

Interestingly, at the beginning of the intervention (T0), LR and HR children did not exhibit significant differences

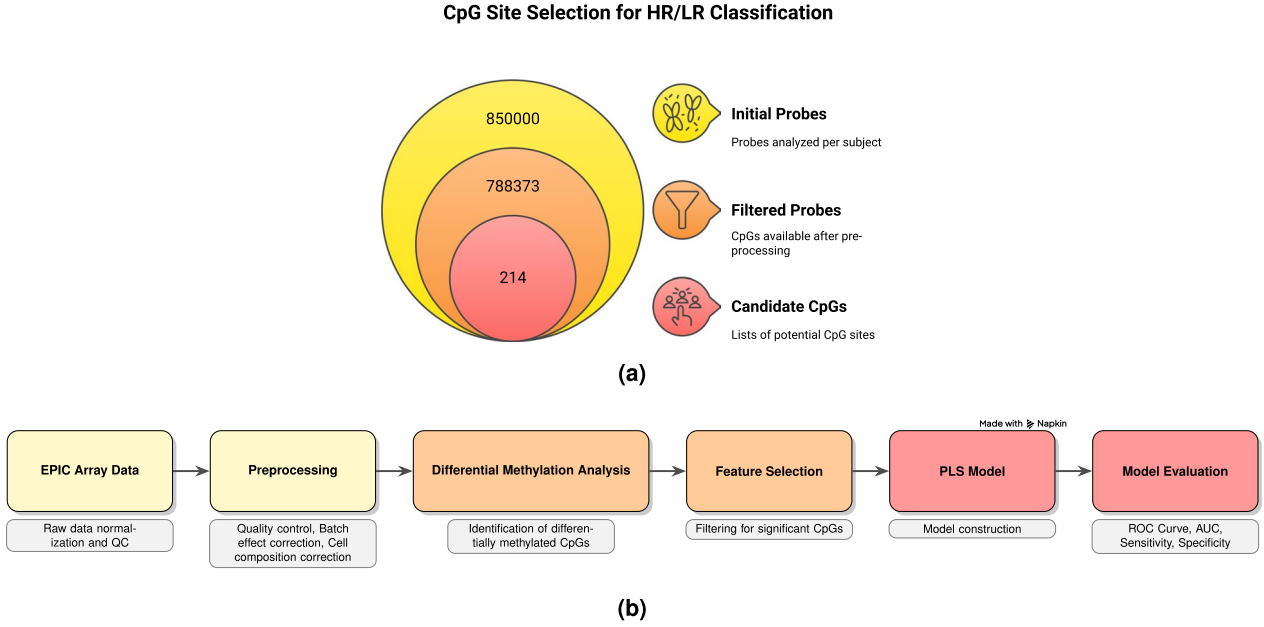

Fig. 1 Schematic overview of the predictive modeling pipeline. Panel **a** shows the number of CpGs site at each steps for HR/LR classification, while in panel **b** is a schematic overview of the predictive modeling pipeline. Here, DNA methylation was profiled using the EPIC 850K array (850,000 CpGs). After quality control and preprocessing, 788,373 CpGs were retained. Leave-one-out (LOO) regression models identified 214 CpGs consistently associated with weight-loss response (ΔzBMI)

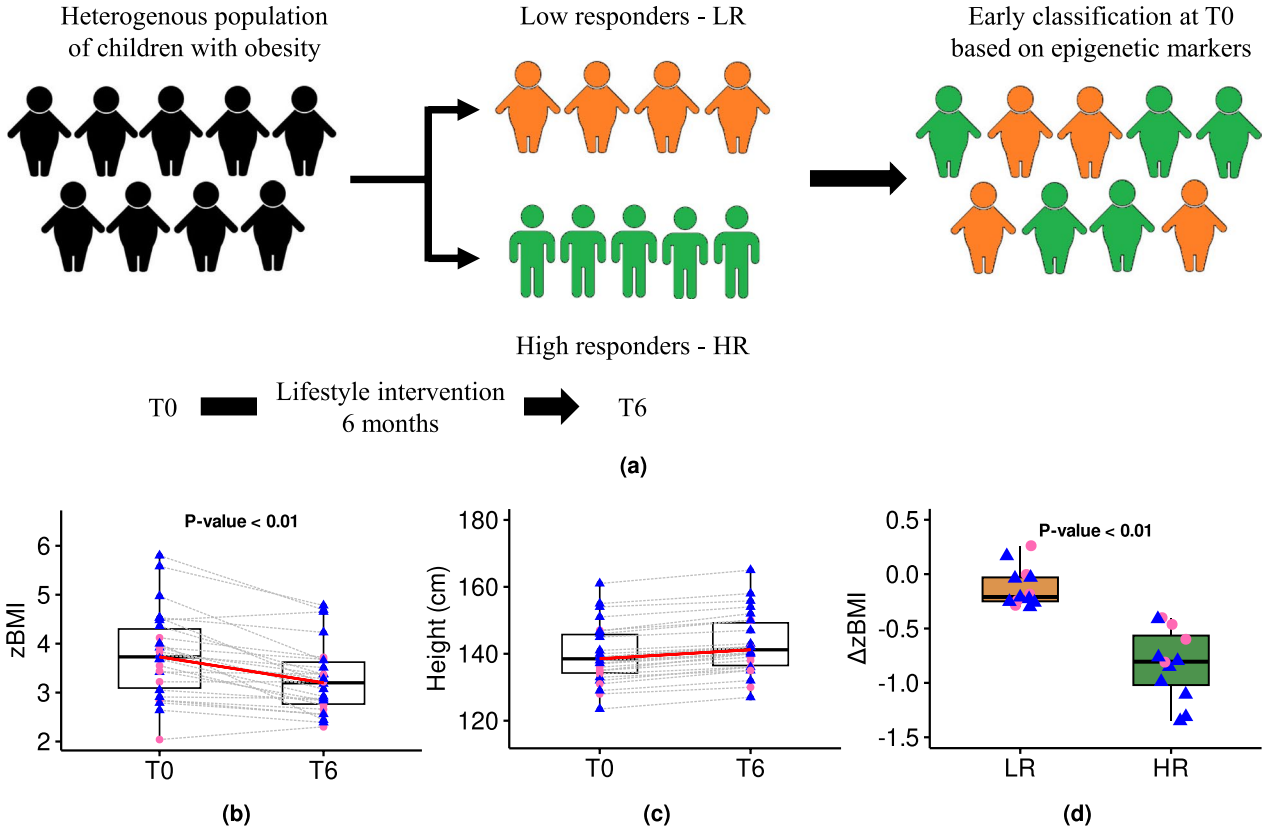

Fig. 2 Clinical outcomes and anthropometric changes following lifestyle intervention. Panel **a** summarizes the study protocol timeline. Panels **b** and **c** display individual changes in zBMI and height, respectively, with lines connecting pre- (T0) and post-intervention (T6) values for each subject. Wilcoxon signed-rank tests indicate significant changes ($p < 0.01$) across the cohort. Panel **d** illustrates the distribution of ΔzBMI for High Responders (HR) and Low Responders (LR), with group differences assessed via the Wilcoxon rank-sum test. Data points are color-coded by sex (males: blue; females: pink)

Table 1 Physiological characteristics of the 13 HR and 13 LR patients, before (T0) and after (T6) the lifestyle intervention

Variables	Unit	HR (n=13)				LR (n=13)				HR vs LR T0	
		T0	T6	*P*-val	*P*-adj	T0	T6	*P*-val	*P*-adj	*P*-val	*P*-adj
Sex	M/F	9/4		–	–	9/4		–	–	–	–
Age	Years	8.63 [8.46 9.58]	9.09 [8.95 9.56]	<**0.01**	**0.02**	9.01 [8.60 9.46]	9.56 [9.07 10.14]	<**0.001**	**0.01**	0.47	0.72
Weight	Kg	56.10 [52.20 59.20]	53.40 [49.00 57.40]	0.07	0.18	48.10 [42.70 62.40]	49.30 [44.70 65.50]	<**0.001**	<**0.01**	0.21	0.66
Height	cm	139 [135 146]	142 [140 150]	<**0.01**	**0.02**	137 [134 145]	140 [136 147]	**0.001**	**0.01**	0.77	0.88
zBMI	–	4.00 [3.69 4.53]	3.28 [2.92 3.66]	<**0.001**	<**0.01**	3.05 [2.84 3.77]	2.88 [2.56 3.51]	**0.04**	0.17	<**0.01**	0.10
Waist	cm	84 [80 90]	81 [78 86]	<**0.01**	**0.02**	84 [78 88]	80 [78 89]	0.92	1.00	0.40	0.66
Abdominal	cm	90 [89 99]	90 [82 93]	**0.02**	0.07	87 [83 92]	85 [83 96]	0.57	0.76	0.31	0.66
Hb	g/dL	13.10 [12.70 13.70]	13.30 [13.20 14.00]	**0.04**	0.15	12.90 [12.70 13.50]	13.60 [13.20 13.80]	**0.01**	0.08	0.88	0.91
HCT	%	39.40 [38.60 41.00]	39.80 [38.00 41.70]	0.31	0.52	39.40 [37.80 40.10]	40.50 [39.20 41.60]	0.06	0.20	0.61	0.81
PLT	–	287 [249 354]	269 [239 332]	0.34	0.54	305 [264 394]	303 [271 336]	0.48	0.68	0.38	0.66
WBC	–	7.10 [6.50 7.90]	7.80 [6.80 10.00]	0.66	0.89	6.60 [5.90 7.80]	7.60 [6.20 8.80]	0.31	0.53	0.37	0.65
TC	mg/dL	156 [146 164]	164 [147 168]	0.45	0.64	178 [160 199]	190.00 [178 202]	0.09	0.24	0.06	0.45
LDL-C	mg/dL	96 [85 98]	99 [92 108]	0.20	0.39	119 [103 140]	120 [103 123]	0.92	1.00	**0.03**	0.33
HDL-C	mg/dL	42 [40 47]	48 [38 53]	0.75	0.90	39 [32 48]	41 [36 43]	0.48	0.68	0.22	0.66
TG	mg/dL	75 [64 103]	70 [65 84]	0.24	0.45	73 [67 114]	89.00 [75 178]	0.13	0.29	0.41	0.66
TSH	μU/ml	2.90 [2.02 3.68]	2.98 [1.92 3.92]	1.00	1.00	2.42 [1.69 3.54]	2.78 [1.88 3.31]	0.68	0.87	0.68	0.82
AST	UI/l	21 [18 24]	23 [19 24]	0.84	0.96	22 [19 24]	23 [18 26]	0.97	1.00	0.91	1.00
ALT	UI/l	18 [17 21]	18 [15 21]	0.37	0.55	17 [16 22]	20 [19 26]	0.10	0.24	0.68	0.82
Creatinine	mg/dL	0.53 [0.49 0.57]	0.59 [0.53 0.60]	**0.02**	0.07	0.56 [0.52 0.60]	0.57 [0.51 0.60]	1.00	1.00	0.15	0.66
Urea	mg/dL	5.00 [4.50 5.60]	5.00 [4.30 5.70]	0.72	0.90	5.50 [5.00 5.85]	5.10 [4.93 5.50]	0.26	0.48	0.17	0.66
BG	mg/dL	83 [81 92]	85 [83 90]	0.94	0.98	88 [85 92]	94 [86 95]	0.05	0.17	0.57	0.81
BI	mU/ml	14.90 [12.10 17.40]	11.60 [8.30 19.00]	0.05	0.16	11.20 [7.30 17.20]	13.80 [6.70 16.60]	0.40	0.64	0.26	0.66
HbA1c	%	5.30 [5.20 5.40]	5.20 [5.10 5.40]	0.15	0.33	5.30 [5.10 5.40]	5.20 [5.10 5.30]	0.05	0.17	0.83	0.91
HOMA-IR	–	3.32 [2.47 4.21]	2.32 [1.81 3.94]	0.09	0.21	2.44 [1.42 3.59]	2.83 [1.42 3.75]	0.26	0.48	0.31	0.66
usCRP	mg/dL	3.80 [1.40 6.60]	3.30 [1.80 4.70]	0.90	0.98	1.90 [1.40 4.40]	2.30 [1.10 5.50]	0.73	0.88	0.32	0.66

Values were given as median [1*st* 3*rd*] quartiles. The Wilcoxon signed rank test was computed for each HR and LR group to compare variables at T0 and T6, while the Wilcoxon Mann Whitney's test assessed the statistical difference at T0 between HR and LR. *P*-values were adjusted by Benjamini and Hochberg (a.k.a. FDR) correction (*P*-adj). Bold font indicates *P*-adj < 0.05. Waist = Waist circumference; Abdominal = Abdominal circumference; Hb = Haemoglobin; HCT = Hematocrit; PLT = Platelets; WBC = Leukocytes; TC = Total cholesterol; LDL-C = Low-density lipoprotein cholesterol; HDL-C = High-density lipoprotein cholesterol; TG = Triglycerides; TSH = Thyroid stimulating hormone; AST = Aspartate transaminase; ALT = Alanine transaminase; BG = Glycemia; BI = Insulin; HbA1c = Glycosylated hemoglobin; HOMA-IR = Homeostatic model assessment of insulin resistance; usCRP = Ultrasensitive reactive C protein

in their biochemical or clinical variables (Wilcoxon Mann–Whitney test) (Table 1, last two columns). These findings suggest that routine physiological and biochemical parameters have limited utility in predicting an individual child's response to lifestyle intervention.

Yet, we found that baseline zBMI showed a trend toward difference between LR and HR groups and might appear to represent a potential stratification tool. However, its applicability is limited for two main reasons. First, after adjustment for multiple testing, the difference in baseline zBMI between groups did not remain statistically significant. Second, and more importantly, the classification of LR and HR was based on ΔzBMI. Since zBMI at T0 is included in the calculation of ΔzBMI, there is inherent mathematical dependence. Therefore, baseline zBMI cannot serve as a fully independent predictor, as it shares information with the outcome by construction.

In light of these limitations, we investigated whether baseline epigenetic biomarkers–identified independently of zBMI–could predict response to the lifestyle intervention. This approach allows assessment of predictors that are conceptually and statistically distinct from the outcome variable (Fig. 2a).

214 CpG sites in peripheral blood predicted the responsiveness to a weight-loss intervention

The intervention did not modify global DNA methylation, regardless of whether we analyzed the entire clinical group (Fig. 3a), or LR and HR separately (Fig. 3b). These data suggest that the methylation marks remain fairly stable in response to a relatively short and moderate lifestyle intervention.

A total of 214 CpGs appeared differentially methylated between LR and HR in the basal state (T0) before the lifestyle intervention (Figs. 4a). Most of the differentially methylated CpGs were located within gene bodies and were not associated with islands (Figs. 4d). The hierarchical clustering of the 13 HR and 13 LR subjects based on the 214 prioritized CpG allowed the two groups to be properly discriminated, with only one LR misclassified (Fig. 4f).

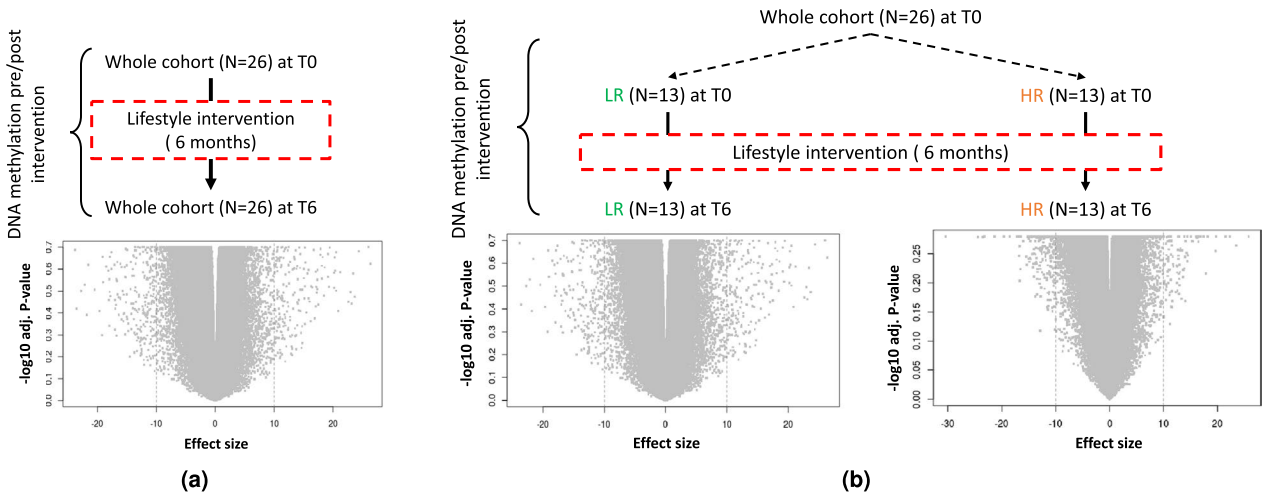

Fig. 3 Longitudinal DNA methylation changes between baseline (T0) and post-intervention (T6). Panel **a** shows analysis of differential methylation across all subjects (N = 26), while in panel **b** is the stratified analysis showing methylation changes in low responders (LR) and high responders (HR) separately

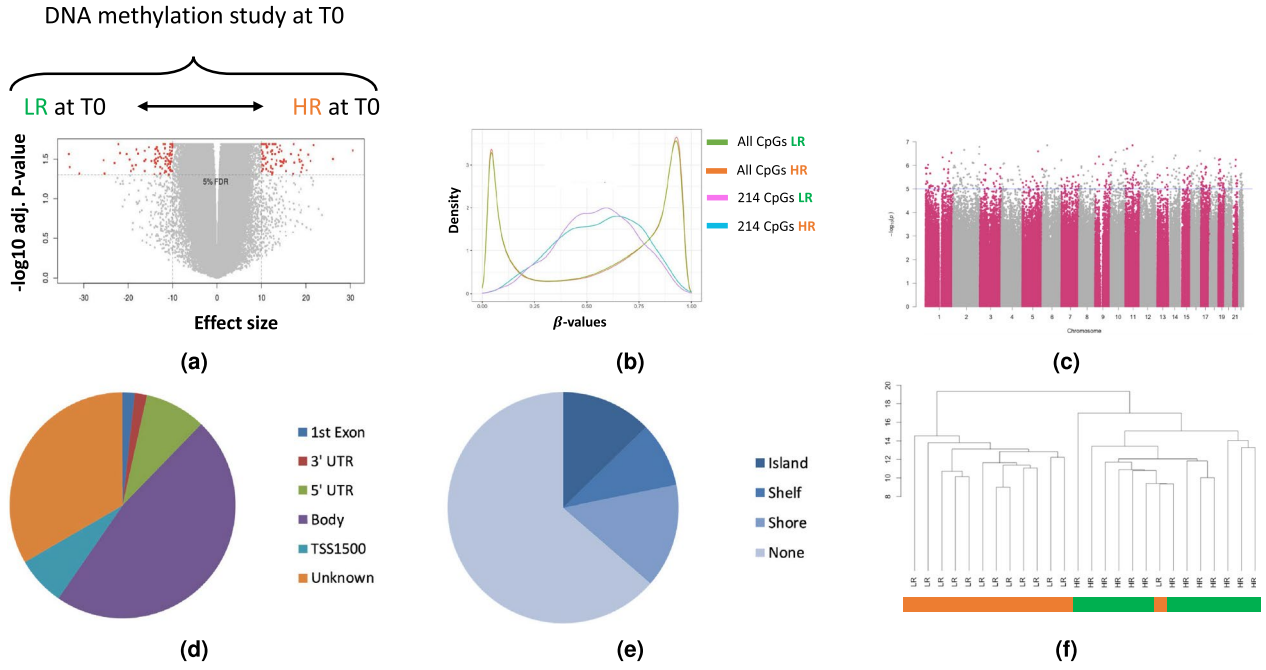

Fig. 4 DNA methylation results at T0. In panel **a** is the volcano plot shows the differential CpGs between LR and HR at T0. The negative logarithm of the association (FDR-adjusted P-value) for each CpG site is displayed on the Y-axis. CpG sites below the horizontal grey line do not reach statistical significance after false discovery rate (FDR) correction at 5%. The red dots represent the prioritised 214 CpGs. Panel **b** depicts the density distribution of DNA methylation β-values for HR and LR. The distributions are shown for all CpG sites and the 214 prioritized CpGs identified as discriminatory markers. The plot illustrates the shift in methylation profiles between HR and LR groups and the enhanced separation observed in the subset of 214 CpGs. Panel **c** shows the Manhattan plot of the 788373 CpG sites. In panel **d** is the genomic annotation of the 214 prioritized CpG sites based on UCSC RefGene regions. Panel **e** shows the distribution of the 214 prioritized CpG sites with respect to CpG island features. Here, CpGs are categorized based on their location in CpG islands, shores, shelves, or open sea regions, providing insight into their epigenomic context and potential regulatory relevance. Panel **f** depicts the dendrogram of hierarchical clustering of HR and LR using the 214 prioritised CpGs

Development of a predictive model

The previous data supports that the 214 prioritized CpGs might be clinically relevant in distinguishing between low and high responders to a lifestyle intervention. However, their direct applicability to clinical practice is curtailed by the fact that potential assays that might include so many markers would be expensive and difficult to analyze in a routine clinical setting. Therefore, here we evaluated whether a smaller subset of CpGs, selected from the initial panel, could retain a similar discriminatory power. To this aim, we systematically tested different subsets of CpGs, beginning with the full panel of 214 and iteratively

reducing the number of predictors (see Sect. 2.5). This optimization process allowed us to reduce the optimal number of final predictors of ΔzBMI from 214 to 8 CpGs (Table 2).

The selected 8 CpGs were then used to fit the PLS model, resulting in a ROC curve (Fig. 5) with an AUC of 84% and an optimal cutoff ΔzBMI of −0.426 with a sensitivity of 77% and a specificity of 85%. This means that the classification model, based on only eight CpGs, has a good overall ability to distinguish between classes, as it can accurately identify both HR and LR, reducing both false negatives and false positives. Finally, the confusion matrix for the classification according to the optimal threshold confirmed that it correctly distinguishes all children in the LR group, while only two HRs were misclassified (Fig. 5b).

Finally, we confirmed that these markers are robust and reliable as they did not change in response to the intervention, as confirmed by their M values at T0 (Fig. 5c) and T6 (Fig. 5d). In other words, the 8 CpGs were differentially methylated between the groups in the basal state (T0) and remained differentially methylated until the end of the intervention (T6). Importantly, the magnitude and direction of the changes also remained unchanged.

Enrichment analysis and biological significance

Of the 214 candidates, 149 CpG sites were found to be associated with 112 different genes (Supplementary Table S1). Thus, a third of the predictive markers were not directly related to annotated genes. These data support the notion that predictive biomarkers do not necessarily carry biological or clinical information [46, 47]. However, to provide a biological validation of this study, an enrichment analysis was performed. Neither GO (Supplementary Table S2) nor KEGG (Supplementary Table S3) pathways were statistically significant in our data set (FDR < 0.05). Yet, the sphingolipid pathway/metabolism was commonly deregulated in both databases: KEGG pathways, hsa00600, hsa0407 (Fig. 6a) and Gene Ontologies, GO:0006686 (Fig. 6b). These are a class of lipids that play important roles in the structure, signaling, or regulation of cell membrane processes, which can affect the development of obesity and metabolic syndrome [12, 48]. Three key genes appeared on these lists: the *Serine Palmitoyltransferase Long Chain Base Subunit 2* (*SPTLC2*), the *Sphingomyelin Synthase* (*SGMS1*), and the *Alpha 12 G Protein Subunit* (*GNA12*). *SPTLC2* and *SGMS1* are key limiting enzymes of the *de novo* pathway for the synthesis of ceramides and sphingolipids (Fig. 6c). Furthermore, *SPTLC2* was one of the eight markers included in the prediction model (Table 2).

Changes in these sphingolipid metabolites were associated with improvements in body weight, adiposity, and HbA1c levels. Next, we performed an integrative multi-omics approach: epigenomics/metabolomics. Specifically, we explored the correlation between the 214 CpGs and their corresponding metabolic signatures. We identified a total of 15 sphingolipid metabolites related to cg18872420 included in the classification model (Fig. 6dTogether, these data suggest that the methylation of the two ceramide markers predicts the response to the plasma variation of ceramide in response to a lifestyle intervention.

Discussion

The prevalence of childhood overweight and obesity is increasing worldwide, largely driven by unhealthy lifestyles, and represents a major modifiable risk factor for adverse long-term health outcomes [49, 50]. Although lifestyle interventions remain the main strategy to improve health in affected children, their variable effectiveness underscores the urgent need for biomarkers that can predict individual responsiveness [9, 51].

In this context, epigenetic biomarkers offer new opportunities not only for the prevention and treatment of obesity but also for the prediction of future outcomes [51–53]. In particular, DNA methylation has emerged as a promising predictive marker because (i) it can be quantitatively measured at low cost, quickly and with relative ease; (ii) it is highly stable in different environments and over time [19, 20]; and (iii) integrates lifestyle factors, including nutritional interventions, with

Table 2 Selected CpGs for classification according to ΔzBMI

ID	Chr	MAPINFO	Gene	Region	Model coefficients	Variance	*P*-value
cg00036352	8	144636448	GSDMD	5′UTR	−0.0771572	0.018	< 0.001
cg04057818	2	67487963	LOC102800447	Body	0.0817991	0.016	< 0.001
cg05387464	2	9956256	–	–	−0.0487775	0.006	< 0.001
cg08240913	10	117969024	GFRA1	Body	−0.0495441	0.009	< 0.001
cg14157435	2	206628692	NRP2	Body	−0.0798113	0.008	< 0.001
cg16007266	16	57060314	NLRC5	TSS1500	−0.0841298	0.015	< 0.001
cg18872420	14	78023429	SPTLC2	Body	−0.0676075	0.019	< 0.001
cg23743554	11	65321226	LTBP3	Body	−0.0583814	0.011	< 0.001

In the first column are the CpGs ID, followed by the indication of the chromosome, the position coordinate, gene name and the region, and the second to the fifth column, respectively. For each CpGs the corresponding PLS model coefficient, the square root of the *jackknife* test variance and the corresponding *P*-value are also provided in the sixth to the eighth column, respectively

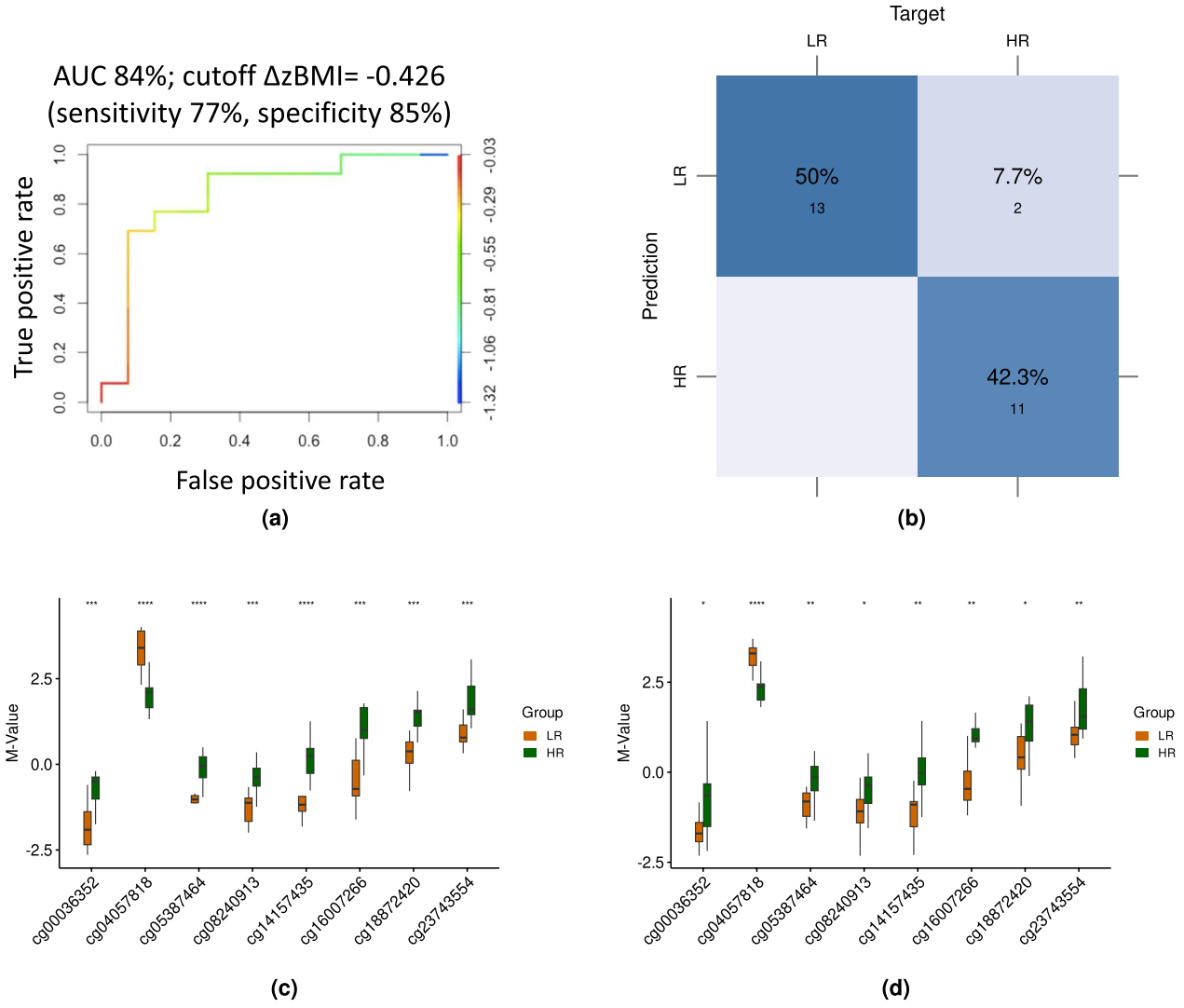

Fig. 5 Classification Model Performance and Methylation Profiles. Panel **a** shows a colored ROC curve, which illustrates the PLS classification model's performance across various threshold settings. The colour gradient indicates sensitivity (true positive rate) versus 1-specificity (false positive rate) being in green the area for optimal threshold selection. Panel **b** shows the confusion matrix illustrating the performance of the proposed model in classifying instances between HR and LR, based on the optimal threshold from ROC analysis. The diagonal elements represent the accurately classified instances, while the off-diagonal elements indicate misclassifications. Distribution of M-values of the 8 CpGs selected for classification before and after the lifestyle intervention are depicted in panel **c** and **d**, respectively. For each CpG, Wilcoxon's test significance between HR and LR groups is also provided

the genome [21–23]. In this study, we report for the first time a molecular signature comprising 214 CpG sites that can effectively predict whether prepubertal children with obesity will respond successfully to a weight loss intervention based on moderate exercise and dietary changes. More importantly, we further developed a consensus prediction model that reduced the number of predictive CpG sites from 214 to only 8. This refined model demonstrated strong classification performance, accurately distinguishing, in our study cohort, between low-responders (LR) and high-responders (HR) at the start of the lifestyle intervention. However, some caution is warranted, as broader clinical application of this predictive model requires additional validation in other representative paediatric cohorts.

Consistently, previous studies conducted in adults [25–28] and adolescents [24] have also identified baseline DNA methylation biomarkers associated with weight-loss responses. Yet, the overlap of common markers among these studies was minimal or even non-existent. For example, in adults, only four CpG sites (*CD44, ITPR1, MTSS1*, and *FBXW5*) were reported in some, but not all, of the studies. Furthermore, no overlap was observed when comparing DNA methylation biomarkers identified in adults with those found in adolescents [24]. In line with this observation, the CpG sites identified in our cohort did not overlap with those reported in previous studies.

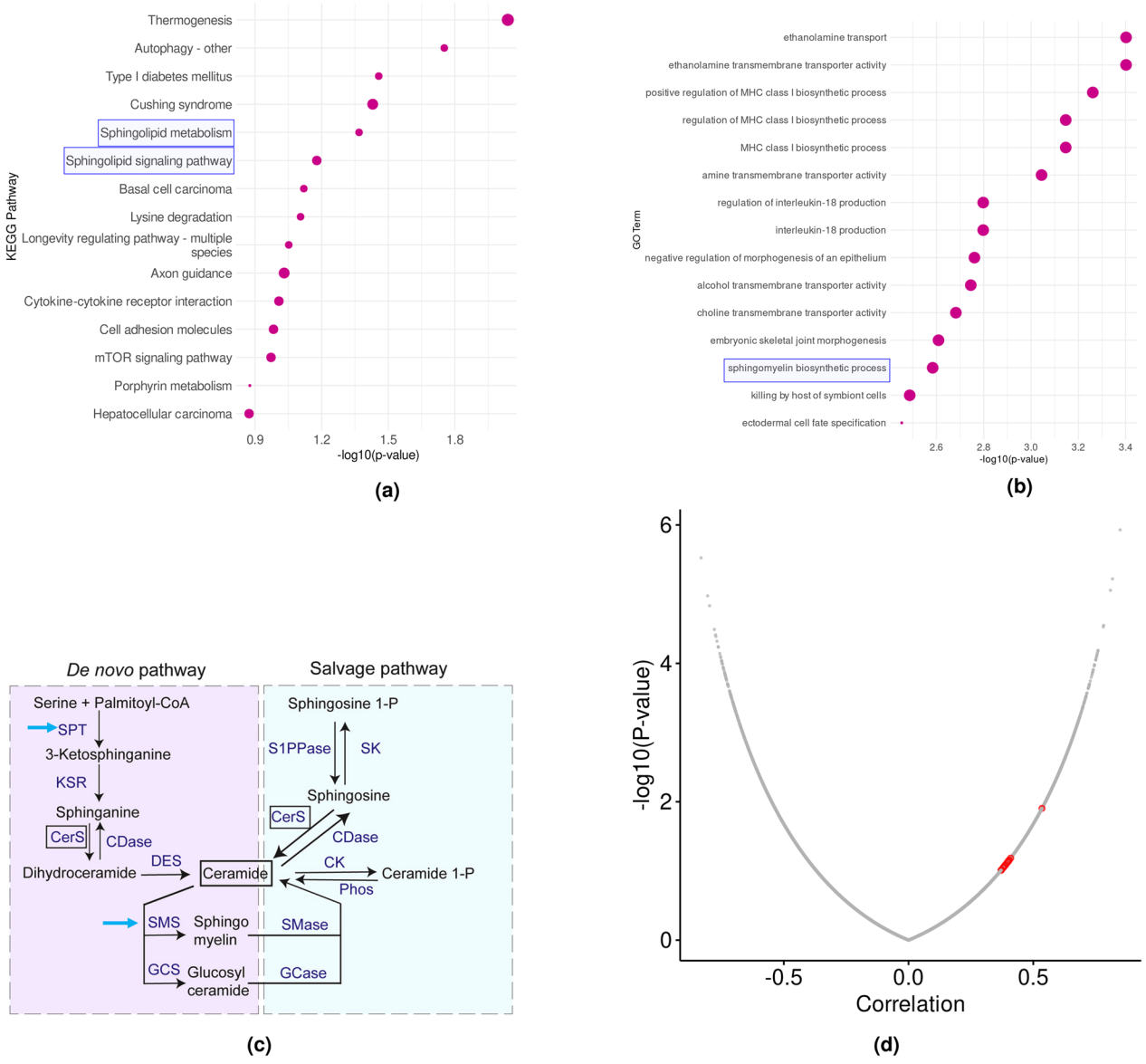

Fig. 6 Integration of epigenetic and metabolomic data highlights sphingolipid pathways. Panels **a** and **b** show the KEGG pathway enrichment analysis and Gene Ontology (GO) term enrichment analysis, respectively, for the 214 prioritized CpG sites. Terms related to sphingolipid metabolism are highlighted with blue rectangles. In panel **c** is the diagram showing the involvement of SPTLC2 and SGMS1 in the de novo and salvage pathways for ceramide and sphingolipid biosynthesis, indicating their role as limiting enzymes. Panel **d** is a volcano-plot-like graph showing the distribution of correlations between the 214 CpG sites and plasma metabolites. The sphingolipids associated with cg18872420 are highlighted in red

The lack of common biomarkers between studies can be attributed to the fact that DNA methylation is dynamic and responds to a complex interplay of factors, including genetic background [54], previous environmental exposures [55], and perhaps most significantly, the age of the participants. Regarding age, it is important to note that studies involving adults included participants ranging from 15 to over 60 years of age (reviewed in [27]). Similarly, in studies focused on adolescents, the average age of the participants was approximately 13 years [24]. In contrast, our study exclusively involved prepubertal children, with a mean age of 8 years. We argue that age might be a key variable that influences the baseline methylation profile. This condition precludes direct comparison between our data and those collected in older populations. We would like to emphasize that prepubertal children, compared to adults, constitute a particularly informative population, as obesity-associated complications have not yet manifested. Consequently, the identification of early epigenetic biomarkers in this group is more likely to reflect a predisposition to, or a causal contribution in, the development of obesity. In contrast, in adults, epigenetic alterations may arise secondarily to obesity-related comorbidities. Indeed, several studies have shown that,

in adults with obesity, most epigenetic marks appear to be a consequence of disease development rather than a primary causal factor [56, 57]. Therefore, the identification of epigenetic marks in paediatric populations may help to delineate causal pathways underlying obesity risk.

It is intriguing to note that we did not observe any changes in DNA methylation as a result of the lifestyle intervention. That is, DNA methylation profiles remained unchanged from the beginning (T0) to the end (T6) of the intervention, regardless of whether a child responded effectively to the program or not. In contrast, previous studies have reported changes in DNA methylation in response to lifestyle or weight-loss interventions [58–62]. This apparent discrepancy can be attributed to several factors. First, as previously mentioned, all prior studies were conducted in adults. Second, the nature of the interventions differed significantly. Adult studies involved a variety of protocols that lasted from 2–3 months to up to 2 years and included various strategies such as low-calorie diets, Mediterranean diets, exercise, or combinations thereof (reviewed in [22]). In contrast, our study implemented a 6-month intervention that involved moderate changes in diet and exercise, carefully designed to promote effective weight loss while maintaining normal growth trajectories (i.e., linear growth-height). Together, differences in the age, duration, and intensity of interventions of the participants could explain inconsistent findings between studies, especially when comparing adults and the paediatric cohort. Indeed, it should be noted that our intervention induced substantial effects on the BMI z-score, while changes in blood parameters were minimal (Table 1). This could be likely due to the fact that our study population consisted of young, prepubertal children. Despite their high degree of adiposity, all participants were metabolically healthy, with cardio-metabolic parameters within the normal range [63]. Therefore, it is reasonable to expect that interventions carried out during the prepubertal stage may not produce marked improvements in cardio-metabolic parameters, while such effects could become more evident after the onset of puberty.

One of the key objectives of biomedical research is the translation of discovery into clinically applicable tools. However, implementing a panel of 214 predictive CpGs sites in routine clinical practice presents several challenges: profiling such a large number of CpGs is costly, technically demanding, and yields data that are difficult to interpret outside of specialized genomic and epigenomic settings. To overcome these limitations, we developed a predictive model based on a reduced subset of CpGs that retained the discriminatory power of the full dataset. We identified eight CpG sites capable of effectively distinguishing responders from non-responders among children with obesity. To the best of our knowledge, this represents the first DNA methylation–based predictive model developed specifically for a paediatric population with obesity. Therefore, this simplified biomarker panel has strong potential for clinical translation, enabling early stratification of paediatric patients and supporting personalized intervention strategies. Nevertheless, the applicability of this panel is currently limited to the Spanish population studied and validation in independent external cohorts will be required prior to clinical implementation.

Similar types of epigenetic biomarkers have been proposed in other pathologies, particularly in cancer, where models have incorporated both DNA methylation and microRNAs (miRNAs) [64, 65]. In line with this, several studies have also suggested that miRNAs could serve as predictive biomarkers for the effectiveness of weight-loss interventions [66, 67]. Although miRNAs offer advantages such as being easy, fast, and inexpensive to analyze, they also have significant limitations. In particular, miRNAs are much more sensitive than DNA methylation to environmental and physiological perturbations, including infections, nutritional fluctuations, and other stressors, and their abundance can even be affected by sample handling and processing [68]. Given these limitations, future activities will combine multiple omics approaches to develop more robust models for predicting responses to lifestyle interventions. The concordance between methylation profiles and metabolomic signatures provides mechanistic support for the model and highlights sphingolipid metabolism as a potential modulator of treatment responsiveness.

By definition, predictive biomarkers do not necessarily have a direct biological or mechanistic role in the determination of phenotypic outcomes [51]. However, in our study, a subset of differentially methylated CpG sites was associated with genes involved in the ceramide–sphingolipid pathway. Sphingolipids, including sphingomyelins and ceramides, are fundamental components of the plasma membrane [69], and participate in various biological processes such as apoptosis, proliferation, inflammation, autophagy, and cellular differentiation. Importantly, alterations in sphingolipid metabolism have been consistently associated with obesity and insulin resistance in both humans [70–72] and rodent models [73, 74]. This pathway is particularly relevant for our study because we previously performed untargeted plasma and urine metabolomics in the same cohort [11, 12], where reductions in circulating sphingolipid-related metabolites were associated with a favourable response to lifestyle intervention [12]. To further explore this link, we performed correlation analyses between three CpG sites associated with ceramide-related genes and circulating metabolites, identifying significant associations with 15 ceramide-derived compounds. Although correlation does

not imply causation, these findings suggest that the basal DNA methylation profile may not only predict responder status but also reflect underlying biological processes–specifically involving sphingolipid metabolism–that contribute to the effectiveness of lifestyle interventions. In turn, the variation in the circulating ceramides may help explain the metabolic improvements observed in children who responded adequately to the intervention. Taken together, the integration of DNA methylation and metabolomics data supports the identification of multi-omic predictive biomarkers that could be used to robustly assess the feasibility and expected effectiveness of weight-loss interventions.

Limitations of the study

We recognize that our study has several limitations. First, the N value was relatively low (sample size= 26). However, although our study included only 26 participants, we adopted several strategies to enhance statistical robustness. Stringent FDR and effect size thresholds were applied, and a leave-one-out cross-validation framework was implemented, requiring consensus across 26 models. Jackknife resampling was also used to confirm the stability of the model. These steps reduce the likelihood of false positives and provide robustness to our study. However, larger studies will be required to further validate the predictive capacity of the identified CpGs and ensure their translational applicability.

Secondly, here, we lack an independent validation cohort. We acknowledge that replication in independent samples will be essential to confirm the generalizability of our findings. Despite an active search, we were unable to identify a comparable weight loss intervention cohort that matches the specific characteristics of this study. Nonetheless, despite this limitation, our findings provide extremely useful insights and lay the groundwork for future studies aimed at evaluating epigenetic markers as predictive tools.

Finally, physical activity was not objectively or subjectively monitored (e.g. questionnaires, pedometers, or accelerometers) during the intervention, which would have provided valuable information on the response to the intervention and the compliance of the participants. Consequently, we were unable to assess the adherence to this component of the lifestyle intervention or evaluate its independent contribution to the observed results.

Conclusions

Different whole-blood DNA methylation profiles were associated with differential responses to lifestyle intervention in prepubertal children with overweight or obesity. Notably, a small subset of eight CpG sites demonstrated strong predictive potential, underscoring the role of epigenetic variation in modulating individual responses to intervention early in life. These findings support the feasibility of using blood-based DNA methylation markers for early risk stratification and the development of personalized preventive strategies in paediatric obesity, while highlighting the need for validation in independent cohorts before clinical implementation.

Supplementary Information

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Additional file 1.

Additional file 2.

Additional file 3.

Acknowledgements

In light of the significant findings presented in this study, it is important to note that the novel methodologies and results outlined herein have been subjected to the patenting process. The patent number is EP18382873 (30/11/2018) and is owned by Sant Joan de Déu - Barcelona Children's Hospital.

Author contributions

Each author contributed substantially to the design of the study, the interpretation of the data, and the discussion. P. C.-E., F. P. and A. P. developed the algorithms and analyzed the data; C. L. M. and J. C. J. C. conceived the experiment; M. P. V. and M. R. K. conducted the experiments. All authors reviewed the manuscript.

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Data availability

The microrray methylatiion data is fully available upon request.

Declarations

Ethics approval and consent to participate

The study was approved by the Hospital Sant Joan de Déu (Children's Hospital Barcelona) Ethics Committee. Identification code PIC-21-12.

Consent for publication

Not applicable.

Competing interest

The authors declare no conflict of interest.

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