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DNA methylation in cord blood partially mediates the effects of prepregnancy BMI on early childhood offspring BMI

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Abstract

Objective: We investigated whether prepregnancy BMI (prePregBMI) in women with obesity was associated with differential DNA methylation (DNAm) in cord blood (CB) and whether DNAm may mediate the association of prePregBMI and early childhood BMI *z* score (BMIz).

Methods: From the Treatment of Obese Pregnant Women (TOP) study, 232 mother–child pairs were included. We conducted an epigenome-wide association study on prePregBMI and CB DNAm (450k array), followed by causal mediation analyses to test whether DNAm may mediate effects of prePregBMI on BMIz at age 36 months (BMIz36).

Results: DNAm at 5345 CpG sites annotated to 2842 genes, which were overrepresented in biological processes linked to carbohydrate metabolism and plasma lipoprotein particle clearance, was associated with prePregBMI (false discovery rate < 10%). Causal mediation analyses of 168 methylation sites associated with BMIz36 ($p < 0.05$) and overlapping with the 5345 prePregBMI-associated sites identified two sites on *SYT7* and *DEAF1*, partially mediating the effect of prePregBMI on BMIz36

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($p \leq 0.01$). After cross-validation, a methylation risk score including these two sites could predict the highest quartile of BMIz36 and fat mass (in grams) with area under the curve = 0.72 (95% CI: 0.58–0.85) and area under the curve = 0.71 (95% CI: 0.58–0.85), respectively.

Conclusions: CB DNAm at birth may partially mediate effects of prePregBMI on early childhood BMIz36, supporting its plausible role in influencing individual future obesity risk.

INTRODUCTION

Childhood obesity is one of the most serious public health challenges, with the prevalence of overweight and obesity now reaching ~30% in European countries [1]. The intrauterine environment plays a key role in offspring metabolic programming, as well as the risk of developing obesity and related metabolic diseases later in life [2, 3]. Higher prepregnancy BMI (prePregBMI) has been associated with obesity and obesity-related disorders in offspring [4–6]. A putative causal factor linking these associations is epigenetics, and, so far, DNA methylation (DNAm) is the most extensively studied and accessible epigenetic modification [2, 7, 8].

Notably, previous epigenome-wide association studies (EWAS) have reported an association between prePregBMI and DNAm in cord blood (CB) [9–14]. However, insights remain limited, and evidence from clinical studies has been controversial [15]. Also, differences in maternal obesity levels, ethnicity, and methodological approaches have made replication challenging [13]. To the best of our knowledge, only one EWAS investigation has examined longitudinal follow-up and the impact of prePregBMI on the risk of obesity in childhood in prospective cohorts using causal mediation analysis, but this was done in a cohort including urban, low-income ethnic minority groups (i.e., Black and Hispanic individuals) [14].

CB is safer and easier to obtain than other intrauterine tissues, making it an excellent tissue for the potential detection of early biomarkers predicting future development of obesity, with important implications for primary prevention. Additionally, it has been shown that DNAm levels in leucocytes are quite stable and that associations between prePregBMI and global DNAm in children at age 36 months still exist [16].

The aims of our study were as follows: 1) to investigate whether prePregBMI in women with obesity was associated with DNAm in CB; and 2) to test whether methylation at some of the associated sites mediates the association of prePregBMI and BMI *z* score at age 36 months (BMIz36) using causal mediation analyses (Figure 1A).

Study Importance

What is already known?

- Maternal obesity before and during pregnancy has been associated with offspring obesity and with DNA methylation (DNAm) in cord blood (CB).
- The intrauterine environment appears to play a key role in offspring metabolic programming through epigenetic mechanisms.

What does this study add?

- CB DNAm at birth partially mediates the effects of prepregnancy BMI on BMI *z* score (BMIz) at age 36 months.
- A methylation risk score can predict the highest quartile of BMIz and fat mass at 36 months after cross-validation.

How might these results change the direction of research or the focus of clinical practice?

- Our study contributes to understanding the biological pathways leading from prepregnancy BMI to offspring obesity through epigenetic programming, stressing the importance of periconceptional factors.
- Our study provides new insights supporting the promising development of early CB epigenetic markers of obesity for risk stratification of individuals at birth from a precision medicine perspective.

METHODS

Research cohort

From the Treatment of Obese Pregnant Women (TOP) study, 232 mother–child pairs with available DNAm in CB were included. The TOP study is a randomized controlled trial of 425 pregnant women with obesity (BMI > 30 kg/m^2), the details of which have been described elsewhere [5, 17], that has been approved by the Ethics Committee for the Capital Region of Denmark (in January 2009, H-D-2008–119; Hillerød, Denmark) and registered at ClinicalTrials.gov (NCT01345149).

Detailed information on enrollment, conduct of the trial, clinical measurements, and data collection has been previously reported [5, 8, 17]. Maternal age, educational level, prePregBMI (kilograms per meters squared), gestational weight gain (GWG; kilograms), and smoking status during pregnancy and offspring sex, gestational age (weeks), and birth weight (grams) measurements were considered for

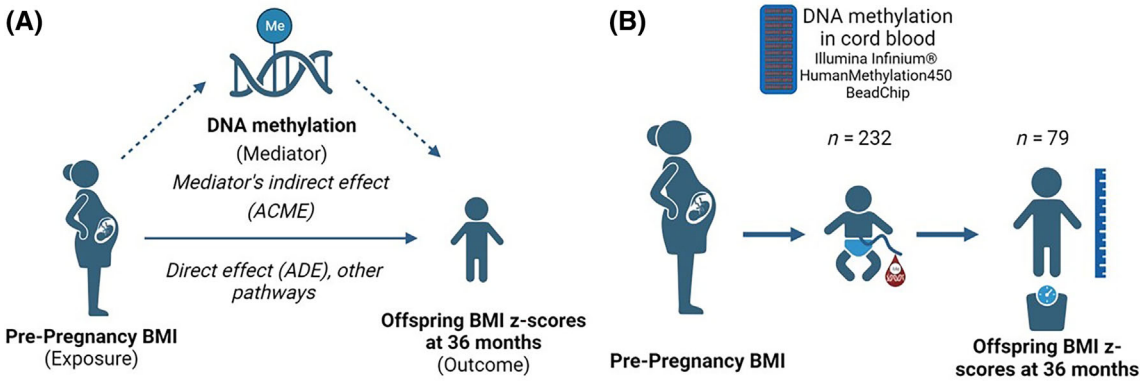

FIGURE 1 Aims and methods of the study. (A) The aims of the study were as follows: 1) to test whether prepregnancy BMI (prePregBMI) was associated with DNA methylation (DNAm) in cord blood (CB); and 2) to use a causal mediation analysis to test whether DNAm at any of the discovered sites could partially explain the effect that prePregBMI exerts on BMI *z* score at age 36 months (BMIz36). The solid blue arrow represents the effect of prePregBMI on children's anthropometric measurements that operate directly (average direct effect [ADE]) or through a pathway different from the mediator analyzed in the current study (DNAm in CB). The dotted blue arrows represent a suggested alternative pathway, in which an indirect effect (average causal mediator effect [ACME]) of prePregBMI on children's anthropometric measurements is mediated by CB DNAm at each respective CpG site chosen as the mediator. Created with BioRender.com. (B) The cohort and data collection are illustrated. Created with BioRender.com.

this study. A total of 79 of the 232 offspring were followed until age 3 years, and anthropometric data, including measures of fat mass (FM) by bioelectrical impedance analysis, were collected (Figure 1B). Details are available in online Supporting Information Methods.

DNAm analysis

DNA was extracted from CB collected from the umbilical vein of the clamped umbilical cord at birth and was bisulfite-converted, as further described in online Supporting Information Methods.

Genome-wide DNAm analysis was performed using Illumina Infinium HumanMethylation450 BeadChips (Illumina Inc., San Diego, California), covering 485,577 sites, as previously described [8]. The bioinformatic pipeline is described in online Supporting Information Methods.

Methylation data were acquired from 460,729 probes. Because CB contains multiple cells, a reference-based method was employed to correct for any potential effects of cellular heterogeneity [18]. This deconvolution technique allows for estimation of relative proportions of CB cell types using CB-derived signatures of CD8T, CD4T, natural killer cells, B cells, monocytes, and neutrophils DNAm.

The statistical analyses were performed on 213 out of 232 participants because 19 samples were removed for failed entry quality control, bisulfite conversion, sex mismatch, or missing data regarding prePregBMI or other covariates used in the regression models (Figure 2).

For genome-wide bioinformatic and statistical analyses, β values were converted into M values to eliminate heteroscedasticity using the following formula: $M = \log2(\beta/[1-\beta])$.

Statistical analysis

Statistical analyses were performed using R Project for Statistical Computing Software version 4.2.1 (2022-06-23, Vienna, Austria) and SPSS Statistics version 29 (IBM Corp., Armonk, New York). Data are presented as mean (SD) unless stated otherwise.

The BMIz36 was calculated according to the World Health Organization using the *anthro* package (R Project for Statistical Computing).

Associations of BMIz36 with maternal and offspring clinical factors were assessed using linear regression models.

To test whether CB DNAm was associated with prePregBMI, linear regression models adjusted for maternal age, educational level, assignment to TOP intervention, GWG, and smoking status during pregnancy and offspring gestational age, sex, and cell-type composition were run. We then performed a linear regression model adjusting for the same covariates, except smoking status, to test whether DNAm was associated with BMIz36 (Figure 2). Associations between prePregBMI and CB DNAm were corrected for multiple testing using false discovery rate (FDR) analysis (Benjamini-Hochberg), and associations with $q < 0.1$ are presented.

When comparing our results with other studies, enrichment calculations were performed using the two-sided binomial test from the *MutationalPatterns* package (R Project for Statistical Computing).

Gene ontology (GO) analysis was performed to analyze possible biological functions of differentially methylated CB methylation sites associated with prePregBMI ($q < 0.1$), as described in online Supporting Information Methods.

Causal mediation analysis

To investigate whether the identified prePregBMI-associated CB methylation sites may mediate the association between prePregBMI and offspring BMIz36 (Figure 1A), we performed

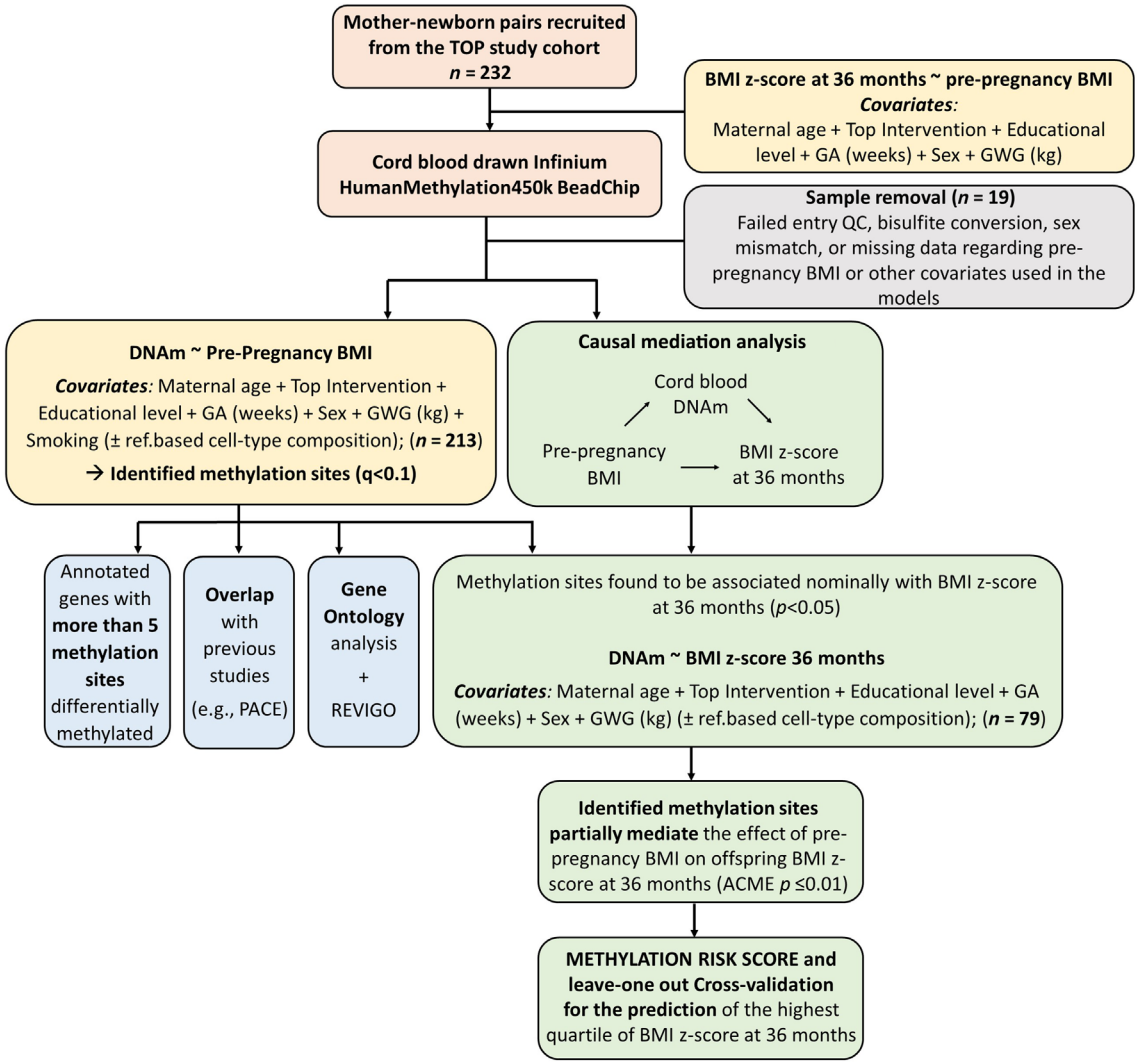

FIGURE 2 Study design and analytic workflow. All the analyses considering BMI *z* score at age 36 months were not adjusted for smoking because only two mothers smoked during pregnancy in the subset of 79 children with anthropometric follow-up at 36 months. ACME, average causal mediator effect; DNAm, DNA methylation; GA, gestational age; GWG, gestational weight gain; TOP, Treatment of Obese Pregnant Women; PACE, Pregnancy and Childhood Epigenetics Consortium; QC, quality control.

nonparametric causal mediation analyses using the *mediation* package (R Project for Statistical Computing) [19]. The bootstrap (sims = 1000) was used for inferences. We analyzed the identified prePregBMI-associated methylation sites ($q < 0.1$) that overlapped with methylation sites associated with BMIz36 based on $p < 0.05$. The effect was estimated for each association between prePregBMI and BMIz36. DNAm of each respective site was designated as the mediator and BMIz36 as the outcome. We adjusted the model for maternal age, educational level, TOP intervention assignment, and GWG and offspring gestational age and sex. We estimated the extent to which the association between prePregBMI and BMIz36 (average total effect) was attributable to the mediating effect of the methylation level at a specific locus (average causal mediation effect [ACME]).

Prediction of the risk of obesity using methylation risk score and cross-validation

The methylation risk score (MRS) was calculated as the sum of the methylation value for each methylation site found to partially mediate the association between prePregBMI and BMIz36, weighted by multiplying the effect size of the association with BMIz36 (estimate), as follows: ([BMIz36_Estimate1 × cpg1_M value] + [BMIz36_Estimate2 × cpg2_M value] + [BMIz36_Estimate(*n*)] × [cpg(*n*)_M value]) [20].

We used logistic regression and leave-one-out cross-validation to estimate the predicted probability of being in the highest quartile of BMIz36 using the MRS. The binary outcome in our model was the highest quartile of BMIz36 versus the lower three quartiles (reference group), and the MRS was the main predictor.

TABLE 1 Clinical characteristics of the mothers and offspring at birth and at age 36 months and associations between clinical factors and BMIz36.

	Offspring at birth ($n = 213$)	Offspring at 36 mo ($n = 79$)	β[a]	*p* value[a]
Maternal age (y)	31.0 (4.5)	31.9 (4.4)	0.041	0.720
Maternal educational level 1/2/3[b]	23/88/105	6/28/45	0.029	0.797
Prepregnancy BMI (kg/m^2)	34.2 (3.9)	33.8 (3.6)	0.235	**0.036**
Gestational weight gain (kg)	9.6 (6.3)	9.8 (6.4)	0.187	0.096
TOP intervention (yes)	143 (65.3%)	52 (66%)	0.142	0.208
Smoking status during pregnancy (yes)	16 (7.3%)	2 (2.5%)	0.067	0.553
Sex (M)	104 (47.5%)	30 (37.5%)	0.009	0.934
Gestational age (wk)	40.0 (1.5)	40.3 (1.3)	−0.089	0.435
Birth weight (g)	3688.8 (516.5)	3736.7 (523.1)	0.086	0.447
BMIz at birth	−0.05 (1.01)	−0.03 (1.05)	0.222	**0.049**
BMIz36		0.54 (0.9)		
Fat mass (g) at 36 mo		2810.9 (889.9)	0.831	**<0.001**
Fat mass (%) at 36 mo		18.1 (4.3)	0.778	**<0.001**

Note: Data given as mean (SD) or number (percentage) for continuous and categorical variables, respectively. Body composition (i.e., fat mass in grams and percentage) has been measured by BIA at 36 months ($n = 72$).
Abbreviations: BIA, bioelectrical impedance analysis; BMIz, BMI *z* score; BMIz36, BMI *z* score at 36 months; M, male; TOP, Treatment of Obese Pregnant Women.
[a]Standardized β and *p* values of the associations of clinical factors, as predictors, with BMIz36, as outcome, using linear regression models. Bold values indicate significant *p* values.
[b]Educational level was categorized into the following three categories: 1) grammar school for 10 years; 2) secondary school for 12 years, vocational training school, and further education for 1 to 2 years; and 3) tertiary education for 3 to 4 years (bachelor level) and advanced education (postgraduate).

We adopted quartiles of BMIz36 rather than focusing on incident obesity based on the low prevalence of overweight or obesity at 36 months (~8%, defined as BMIz ≥2 SD) within our cohort. We also tested the predicted probability of being in the highest quartile of BMIz36 using the clinical factors associated with BMIz36 alone and in combination with the MRS and the predicted probability of being in the highest quartile of FM (grams) at 36 months using the MRS. Iterating through each of the *n* observations ($n = 79$), we fit the model to *n*-1 observations and obtained a prediction for the observation that was left out at that iteration, giving a predicted risk for each individual. The prediction was evaluated using the receiver operating characteristics (ROC) curve and the area under the ROC (AUC) using the *pROC* package (R Project for Statistical Computing).

RESULTS

Baseline characteristics of pregnant women with obesity included from the TOP study and their children at birth and age 36 months are reported in Table 1.

BMIz36 was associated with prePregBMI and with BMIz at birth (Table 1). The association with prePregBMI was also significant independent of maternal age, educational level, TOP intervention assignment, and GWG and offspring gestational age and sex ($\beta = 0.252$; $p = 0.033$).

DNAm associated with prePregBMI

DNAm at 5345 sites, annotated to 2842 unique genes, in CB at birth was associated with prePregBMI independent of the same plausible covariates mentioned earlier and smoking status during pregnancy ($q < 0.1$; Table S2). The Manhattan plot indicated that these 5345 methylation sites were distributed across the whole genome (Figure 3A). In most of these associated sites, an inverse correlation between prePregBMI and DNAm was detected: as prePregBMI increased, methylation levels decreased (Table S2).

GO and REViGO (http://revigo.irb.hr/) analysis of the 5345 methylation sites highlighted 30 enriched biological processes ($p < 0.01$; Figure 3B; Table S3), including carbohydrate metabolism, plasma lipoprotein particle clearance, cytoskeleton organization, nitric oxide-mediated signal transduction, and extracellular matrix organization. All of these pathways are potentially involved in metabolic programming of obesity risk and metabolic health.

Among the 5345 methylation sites, 48 annotated protein-coding genes had at least five methylation sites associated with prePregBMI ($q < 0.1$; Table S4). Several of those were related to metabolic pathways, such as phosphatidylinositol signaling (e.g., *INPP5A*, *PTPRN2*, *ZFYVE28*) and insulin signaling (e.g., *INS-IGF2*, *RPTOR*, *BAIAP2*); to cytoskeleton structure and function (e.g., *AGAP1*, *PLEC1*); to exocytosis or membrane trafficking pathways (e.g., *AGAP3*, *ATP11A*, *JPH3*, *TAP2*, *TSNARE1*); and to the extracellular matrix (e.g., *COL11A2*, *TNXB*). Four genes had more than 10 hypomethylated sites associated with prePregBMI (i.e., *ADARB2*,

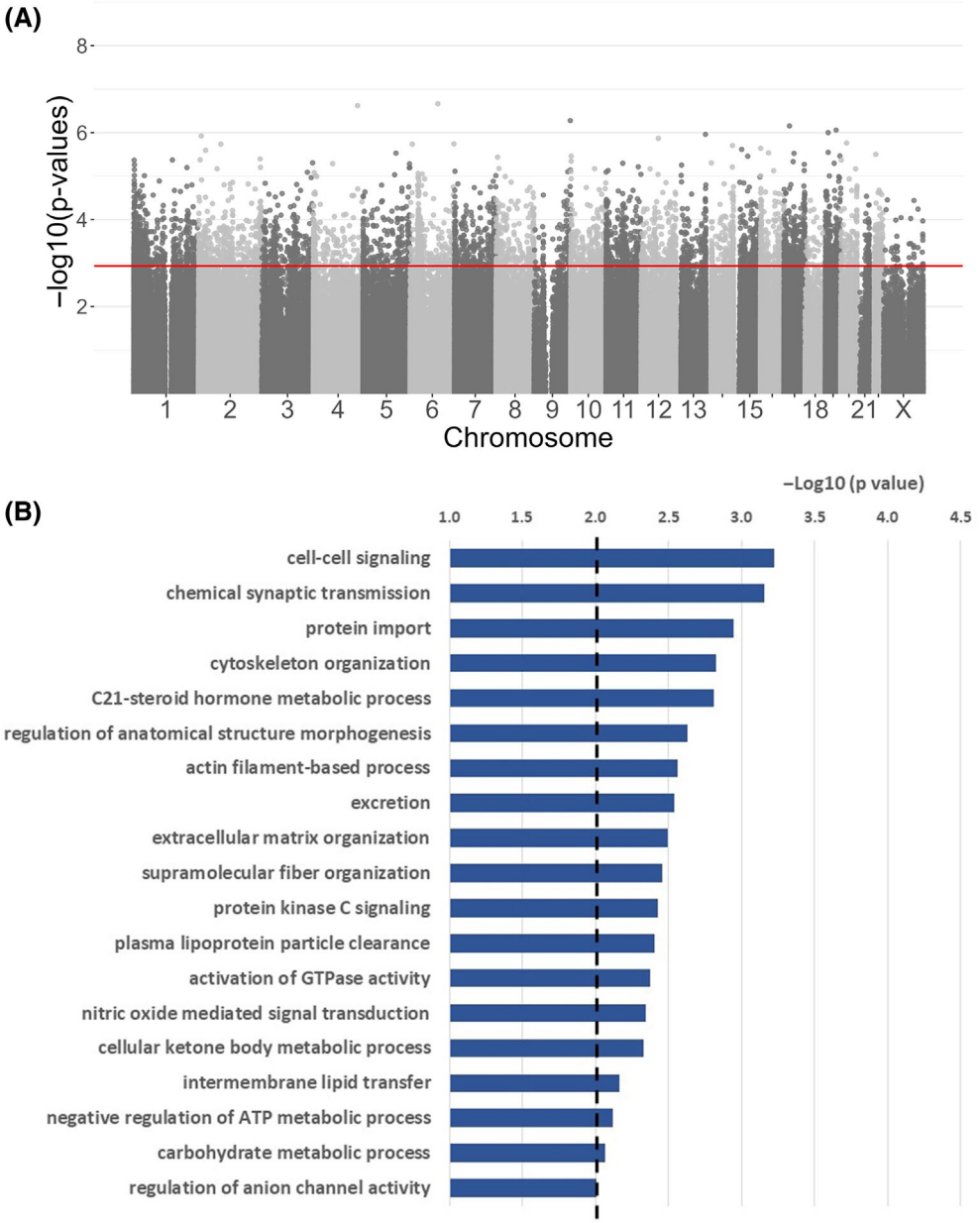

FIGURE 3 Manhattan plot and Gene Ontology (GO) analysis. (A) The Manhattan plot represents the distribution of methylation sites across the genome for the association between prepregnancy BMI and DNA methylation in cord blood, after adjustment for covariates. The red line shows $q < 0.1$. (B) GO analysis of the 5345 ($q < 0.1$) methylation sites after the selection of biological processes (BP) with $p < 0.01$ and the removal of the redundant terms using REViGO. Some of the most relevant nonredundant enrichment items in the BP retained in REViGO analysis with the corresponding p value of these items. The dotted line represents $p = 0.01$. All the 30 nonredundant enrichment items in the BP retained in the REViGO analysis are shown in Table S3.

DIP2C, *LMF1*, and *MCF2L*), and four genes had more than 20 significant hypomethylated sites (i.e., *COL11A2*, *PRDM16*, *PTPRN2*, and *TNXB*).

Overlap based on two previously published studies

We then compared our prePregBMI-associated methylation sites with the results of two previously published studies [10, 14]. First, we investigated overlap with the results from the Pregnancy and Childhood Epigenetics (PACE) Consortium, which comprises several birth cohorts of mostly European ancestry with a total of 7523 mother–child pairs who were meta-analyzed and in whom CB DNAm with the 450k array was studied [10]. Notably, we found a significant enrichment ($p < 0.001$) among our 5345 methylation sites associated with prePregBMI ($q < 0.1$) in mothers with obesity and sites identified by Sharp et al. in mothers with normal weight [10]:

705 sites overlapped out of the 9044 methylation sites associated with maternal BMI in an unadjusted model for estimated cell counts [10]. All were consistent in the direction of the effect (Table S5), with most of them showing an inverse relationship between prePregBMI and the DNAm at identified sites. However, no significant overlap was found between our results and the recent study by Si et al. [14], who analyzed DNAm using the Infinium MethylationEPIC BeadChip array in children born to predominantly Black mothers living in urban, disinvested communities in Boston, Massachusetts, representing a low-income minority group with the highest rates of childhood obesity in the United States [14]. We attributed the lack of overlap to vastly different levels of obesity, differences in the ethnic composition, and low probe reliability as reported by Olstad et al. in analyzing concordance between 450k and EPIC arrays in CB [21].

Causal mediation analysis and prediction of the risk of obesity using MRS

We tested whether CB DNAm may mediate the association between prePregBMI and BMIz36. Among the 5345 methylation sites associated with prePregBMI in CB, 168 sites (annotated to 119 protein-coding genes) were also nominally associated with BMIz36 ($p < 0.05$) after adjustment for the same putative confounding variables mentioned earlier and were selected for the causal mediation analysis (Table S6).

We found that DNAm at two of these 168 shared methylation sites partially mediates (proportion mediated by CpG = 38%–68%) the effect of prePregBMI on BMIz36 (ACME $p < 0.01$; proportion-mediated $p < 0.05$), i.e., the cg03608093 on *DEAF1* and the cg13395854 on *SYT7* (Table 2), which are both in the gene body,

TABLE 2 Causal mediation analysis of the significant associations between prePregBMI and BMIz36-related methylation sites as mediators and BMIz36 as outcome (ACME $p < 0.01$ and proportion-mediated $p < 0.05$)

Target ID	Gene	ACME estimate of mediator CpG (95% CI)	ACME *p* value	ADE estimate (95% CI)	Total effect (95% CI)	Proportion mediated by CpG (95% CI)	Proportion-mediated *p* value
cg03608093	*DEAF1*	0.02 (0.01; 0.05)	0.004	0.04 (−0.02; 0.08)	0.06 (0.002; 0.11)	0.38 (0.02; 2.17)	0.044
cg13395854	*SYT7*	0.04 (0.01; 0.08)	0.006	0.02 (−0.04; 0.07)	0.06 (0.002; 0.11)	0.68 (0.11; 2.97)	0.044

Note: Models adjusted for maternal age, TOP intervention assignment, education level, and gestational weight gain and offspring gestational age and sex.
Abbreviations: ACME, average causal mediator effect; ADE, average direct effect; BMIz36, BMI *z* score at 36 months; prePregBMI, prepregnancy BMI.

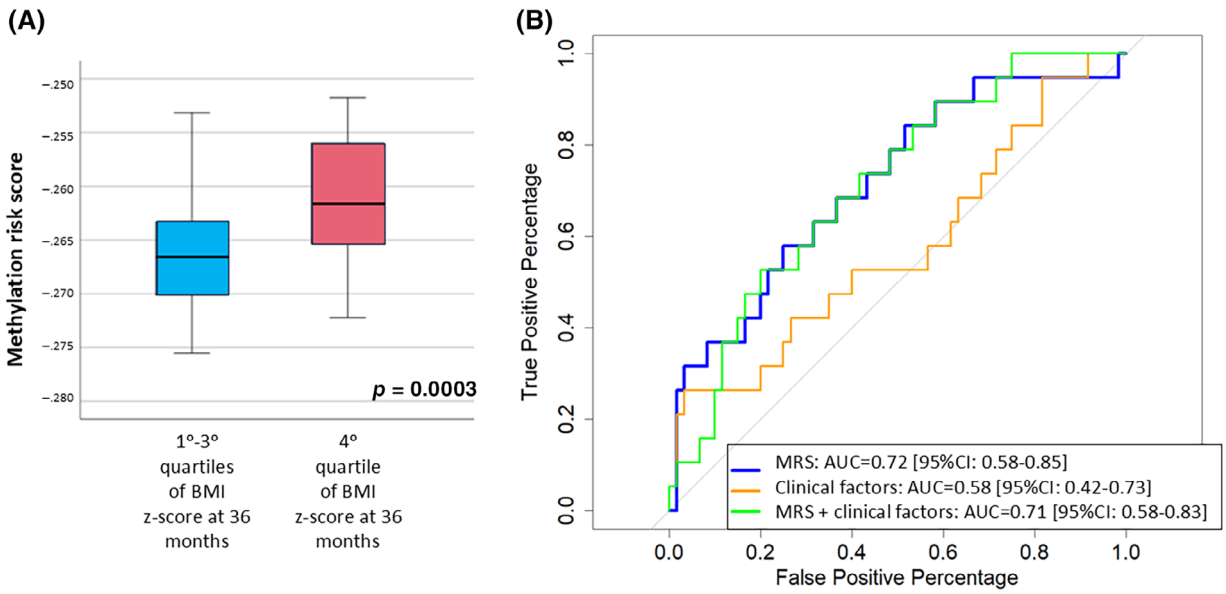

FIGURE 4 Box plot of methylation risk score (MRS) and receiver operating characteristic (ROC) curves. (A) Box plot of MRS generated by DNA methylation levels of cg13395854 (*SYT7*) and cg03608093 (*DEAF1*) in cord blood of 79 mother–child pairs. Comparison between the highest quartile (4°) of BMI *z* score at 36 months (BMIz36) and the other quartiles considered together (1°–3°) (reference group). An independent *t* test was used for the analysis. (B) ROC curves show the predictive ability of MRS to predict the highest quartile of BMIz36 after leave-one-out cross-validation (area under the ROC [AUC] of 0.72). The clinical factors alone (i.e., prepregnancy BMI and BMIz at birth) and in combination with MRS could predict the highest quartile of BMIz36 with an AUC of 0.60 (95% CI: 0.47–0.74) and 0.73 (95% CI: 0.60–0.85), respectively, after leave-one-out cross-validation.

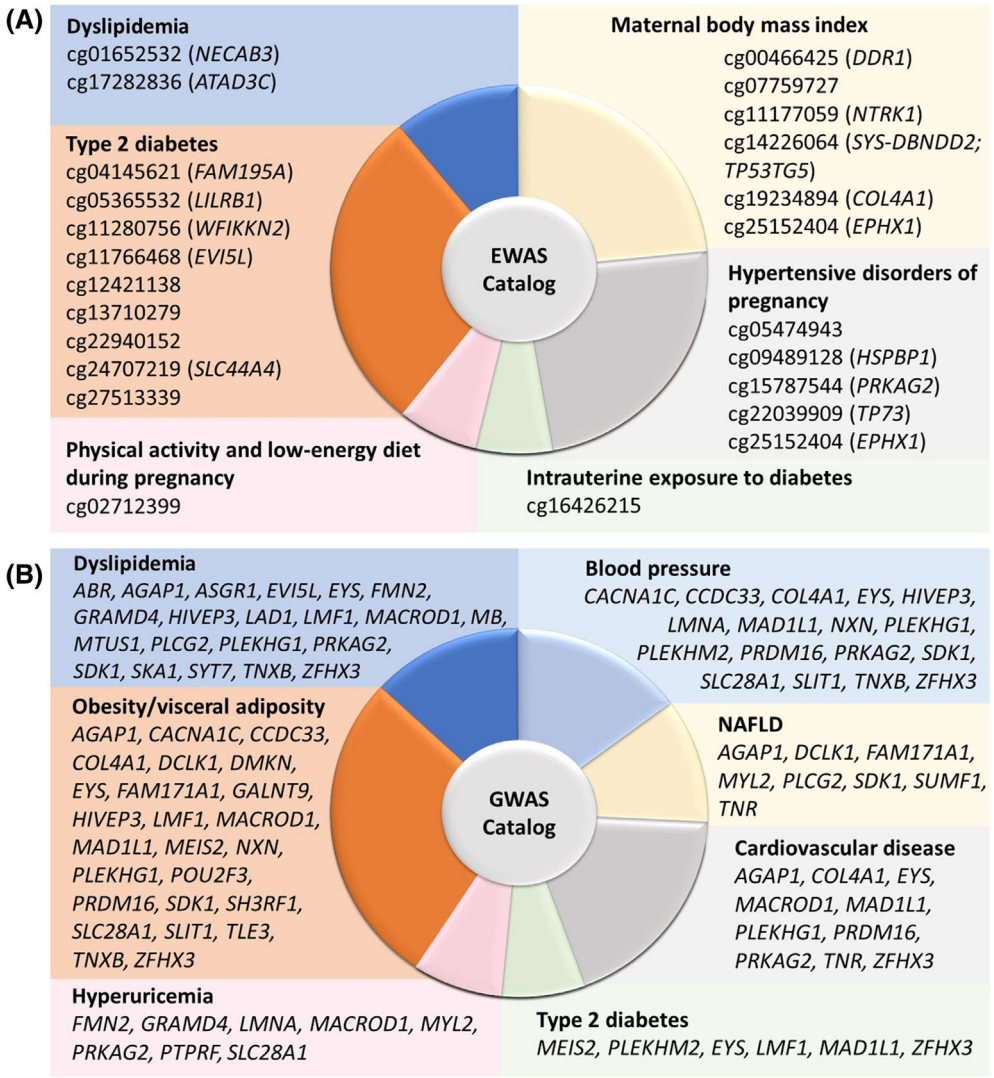

FIGURE 5 Epigenome-wide association studies (EWAS) and genome-wide association studies (GWAS) catalog search. (A) EWAS catalog traits wheel of the 168 methylation sites associated with prepregnancy BMI (prePregBMI; $q < 0.1$) and with BMI *z* score at 36 months (BMIz36; $p < 0.05$; covariate-adjusted models). The figure shows the methylation sites and annotated genes, where available, that have been previously associated with traits related to metabolic health (i.e., maternal BMI, hypertensive disorders of pregnancy, physical activity and low-energy diet during pregnancy, cholesterol esters to total lipids ratio in chylomicrons and very low-density lipoprotein, ratio of omega 3 fatty acids to total fatty acids, and incident or prevalent type 2 diabetes [T2D]) (accessed December 2023). (B) GWAS catalog traits wheel of the 119 protein-coding genes annotated to the 168 methylation sites associated with prePregBMI ($q < 0.1$) and with BMIz36 ($p < 0.05$; covariate-adjusted models). The figure shows the genes annotated to the 168 methylation sites and to the single-nucleotide polymorphisms that have previously been associated with obesity or obesity-related traits (i.e., obesity or visceral adiposity, dyslipidemia, blood pressure, T2D, nonalcoholic fatty liver disease, cardiovascular disease, and hyperuricemia) (accessed December 2023).

suggesting that a significant proportion of the total effect is mediated.

Next, using these two methylation sites, we calculated an MRS that was significantly associated with BMIz36 ($\beta = 0.461$; $p < 0.001$) and significantly higher in the group of participants in the highest quartile of BMIz36 compared with the participants in the lower three quartiles (reference group; $p = 0.0003$; Figure 4A). The MRS was also associated with FM (grams) at age 36 months ($\beta = 0.284$; $p = 0.016$), but not with FM percentage ($\beta = 0.211$; $p = 0.076$).

We then examined whether the MRS could predict the highest quartile of BMIz36 compared with the reference group. We ran a leave-one-out cross-validation and performed a logistic model with the MRS as the main predictor and the highest quartile as the binary outcome. We also performed logistic models with the associated clinical factors alone (i.e., prePregBMI and BMIz at birth) and in combination with the MRS. The ROC curve analysis showed that the MRS was good at predicting the highest quartile of BMIz36 with an AUC of 0.72 (95% confidence interval [CI]: 0.58–0.85; Figure 4B), whereas the clinical factors alone and in combination with the MRS could

predict it with an AUC of 0.60 (95% CI: 0.47–0.74) and 0.73 (95% CI: 0.60–0.85), respectively, after cross-validation. The addition of the clinical factors did not significantly improve the prediction of the highest quartile of BMIz36 by the MRS ($p = 0.812$; Figure 4B), and the 95% CI of the clinical factors indicated considerable uncertainty about this model discriminatory power. We also examined whether the MRS could predict the highest quartile of FM (grams) compared with the reference group. The MRS could predict FM (grams) with an AUC = 0.71 (95% CI: 0.58–0.85).

Biological relevance assessment

To further explore the biological relevance of the 168 methylation sites, we performed an EWAS catalog search (accessed December 2023). We found that 23 sites of the 168 (annotated to 16 genes) have been previously associated with related metabolic traits such as maternal BMI (e.g., *DDR1*, *NTRK1*, *COL4A1*, *EPHX1*), intrauterine exposure to diabetes (cg16426215), hypertensive disorders of pregnancy (e.g., *HSPBP1*, *PRKAG2*, *TP73*, *EPHX1*), dyslipidemia (e.g., *ATAD3C*, *NECAB3*), and incident types 2 diabetes (T2D; e.g., *FAM195A*, *LILRB1*, *WFIKKN2*, *EVI5L*, *SLC44A4*; Table S7; Figure 5A). We also used the genome-wide association studies (GWAS) catalog (accessed December 2023) to determine whether the 119 protein-coding genes annotated to the 168 methylation sites have any single-nucleotide polymorphisms (SNPs) that have been previously associated with obesity or related metabolic traits in GWAS. Here, we found that SNPs annotated to 43 of 119 genes have already been associated with metabolic traits related to obesity (e.g., *AGAP1*, *CACNAC1*, *COL41A*, *GALNT9*, *SLIT1*, *TNXB*) and/or obesity-related diseases such as T2D (e.g., *MEIS2*, *EYS*, *MAD1L1*), dyslipidemia (e.g., *HIVEP3*, *LMF1*, *PLCG2*), blood pressure (e.g., *PLEKHM2*, *PRDM16*, *ZFHX3*), nonalcoholic fatty liver disease (e.g., *DCKL1*, *SUMF1*, *TNR*), hyperuricemia (e.g., *FMN2*, *LMNA*, *PTPRF*), and cardiovascular disease (e.g., *MACROD1*, *PLEKHG1*, *PRKAG2*; Table S8; Figure 5B).

Additionally, using the methylation quantitative trait loci (mQTL) database (https://mqtldb.org, accessed December 2023) [22], we found that 45 CB methylation sites out of 168 have been associated with SNPs, so-called mQTLs, at birth (Table S9). Among these 45 mQTLs, the following six SNPs have been associated with disease traits related to obesity (i.e., visceral adiposity or BMI) in the GWAS catalog: rs10910018, rs12562437, and rs9662633 annotated to *TP73*; rs2013105 annotated to *HIVEP3*; rs7956193 annotated to the pseudogene *IMMP1LP2*; and rs76209954 annotated to *LINC00971* (a long intergenic non-protein-coding RNA gene; Table S10). All populations involved in the reported GWAS were of European ancestry.

DISCUSSION

Our study highlights that prePregBMI is associated with childhood BMIz36 and that CB DNAm at individual sites at birth is associated with prePregBMI. Notably, CB DNAm of *SYT7* and *DEAF1* partially mediated the effect of prePregBMI on BMIz36. Moreover, combined into an MRS, these two methylation sites could discriminate the highest quartile of BMIz36 and FM (grams) with an AUC of 0.72 and 0.71, respectively, after cross-validation, sustaining a potential role of these new and early epigenetic risk factors of childhood obesity.

To the best of our knowledge, this is the first prospective study to link prePregBMI-associated CB DNAm with BMIz36 from mothers with obesity and European ancestry. BMI at age 3 years is a known predictor of rebound adiposity, which is a risk factor for childhood obesity and the persistence of obesity into adulthood [23]. A recent study supported the crucial role of BMIz36 in predicting the risk of developing obesity later in life. A single BMI measure is more practical and provides more information on subsequent BMI levels than rebound age calculated through repeated measurements of BMIz during early age [23]. Indeed, in our study, BMIz36 was highly correlated with bioelectrical impedance analysis FM measurements.

Additionally, our causal mediation analyses revealed that two prePregBMI-associated methylation sites partially mediated the effect of prePregBMI on childhood BMIz36. This contributes to understanding the biological pathways implicated in this association. Moreover, it provides new possible early markers of obesity for detecting individuals who are at risk already at birth and allows for further advances in preventive and early therapeutic strategies from a precision medicine point of view.

Several of the methylation sites found to be associated with prePregBMI in our cohort were annotated to genes overrepresented in biological processes potentially involved in the development of obesity and metabolic derangements (e.g., carbohydrate metabolic process, plasma lipoprotein particle clearance, cytoskeleton organization, nitric oxide-mediated signal transduction, and extracellular matrix organization).

Additionally, we found several hypomethylated sites within known genes of the leptin signaling pathway (i.e., *CCND*, *H19*, *INS*, *IRS*, *SRC*, and *STAT3*). Several studies have consistently reported increased leptin levels in newborn CB from women with obesity, which is a known adipokine that may play an integrative role in metabolic and immune processes, supporting the relationships among maternal obesity, inflammation, and CB methylation of these pathway genes [24].

Although the number of probes for different genes on Illumina arrays can vary and is influenced by different factors, we found 23 sites all hypomethylated in the *PTPRN2* gene associated with prePregBMI. The CB methylation levels of *PTPRN2* have already been associated with maternal BMI [14]. This gene, which encodes a protein acting as phosphatidylinositol phosphatase and regulating insulin secretion, has been associated with energy intake and the pathophysiology of childhood obesity [25]. Additionally, we recently showed that DNAm of *PTPRN2* is associated with circulating triglyceride levels [26]. Furthermore, among the list of 48 protein-coding genes with ≥five methylation sites associated with prePregBMI, we interestingly found two genes, i.e., *PRKCZ* and *PRKAR1B*, exhibiting a blood DNAm profile that has previously been linked to prePregBMI and gestational diabetes during childhood [11]. The *PRKCZ* gene encodes a serine–

threonine kinase participating in proliferation, differentiation, and secretion, and *PRKAR1B* encodes the regulatory subunit of cyclic AMP-dependent protein kinase A that is involved in many cellular signaling pathways, including transport of ions, metabolic functions, and transcription [11]. Both of these genes have also been reported to be differentially methylated in CB [27] and linked to the subsequent risk of metabolic disease. *PRKCZ* has also been shown to be differentially methylated in leucocytes [28], adipose tissue [29], and pancreatic islets [30] from adults with T2D. Moreover, we found five methylation sites associated with prePregBMI on the *INS-IGF2* gene, which has been reported as extremely important for placenta nutrient transfer and, thereby, fetal growth [31]. Twelve methylation sites were hypomethylated on the *LMF1* gene body, encoding lipase maturation factor 1, which is involved in the folding and expression of lipoprotein lipase and has been frequently reported to cause or predispose to hypertriglyceridemia [32]. Twenty-one methylation sites were hypomethylated on the *PRDM16* gene, a key transcriptional regulator that drives brown and beige adipogenesis and UCP-1 expression [33]. Twenty and twenty-six sites were significantly hypomethylated on the *COL11A2* and *TNXB* genes, respectively, both of which encode extracellular matrix proteins. Recently, the role of the extracellular matrix in obesity has been identified, playing a crucial role in the expansion of healthy adipose tissue with important metabolic implications for obesity and related complications [34].

Finally, among the genes annotated to the two methylation sites partially mediating the effect of prePregBMI on BMIz36, we found *SYT7*, encoding a protein that mediates calcium-dependent regulation of membrane trafficking in synaptic transmission and exocytosis. It has been involved in cholesterol transport [35] and insulin secretion in β cells and human pancreatic islets [36, 37]. Regarding the transcription factor encoded by *DEAF1*, it has recently been identified as a new interactor for glycogen synthase kinases (i.e., GSK3A and GSK3B) as well asPI3K-mTOR pathway components associated with insulin resistance and T2D [38], and it is differentially methylated in adults with T2D [29].

Collectively, these findings provide evidence that prePregBMI in mothers with obesity affects the CB epigenome of genes linked to metabolism and metabolic disease and that the methylation level of *SYT7* and *DEAF1* might plausibly be involved in the biological pathway leading from maternal obesity to offspring obesity.

The strengths of our study are the ethnic homogeneity of our cohort of European ancestry and the deep characterization of the clinical trial participants, mitigating the risk of bias. This is the first study, to our knowledge, attempting to investigate causality between prePregBMI–DNAm-associated sites and childhood obesity in European individuals. Although proving a causal intrauterine effect of maternal obesity on offspring health is complex, a causal mediation analysis approach can be helpful in inferring causality [39], especially using longitudinal cohorts. Additionally, this is the first study, to our knowledge, considering the predictive ability of a CB MRS at birth for childhood obesity risk, considering not only BMI but also measures of adiposity composition, and comparing its predictive ability with potential clinical factors. Finally, the associations found in our study between prePregBMI and DNAm are supported by the replicated findings reported by the most recent comprehensive study (i.e., the PACE Consortium meta-analysis) [10].

Some limitations also need to be acknowledged. We performed multiple testing correction based on the Benjamini-Hochberg method, which is known to be less strict than Bonferroni correction. Concurrently, Bonferroni may be too conservative for EWAS, as DNAm values in nearby probes are known to be correlated, and many array sites showed minimal variability, supporting our choice of the Benjamini-Hochberg method [40]. The lack of analysis of differential methylated regions and the application of the Illumina 450k array, which only covers ∼1.7% of CpG sites of the human genome, provides limited coverage of some genes and genomic regions. We therefore encourage more studies on this topic to use platforms with better coverage and take into consideration probe reliability to uncover consistent and replicable CB EWAS results [21]. Adjusting for cell composition is crucial in epigenetic studies, as different cell types have distinct methylation profiles, and stressors such as maternal obesity have been shown to alter offspring cell-type composition [41] and impair inflammatory response in CB monocytes [42]. Reference-based methods offer reliable and stable estimates, but their accuracy relies on the quality and completeness of the reference methylation profiles that can limit their applicability and introduce biases based on population specificity, inability to capture dynamic changes, and exclusion of novel or rare cell types. Although data-processing steps are complex and can introduce variability, standardized protocols are necessary to account for technical artifacts and batch effects, and they could mitigate these limitations, enhancing the reliability and applicability of methylation studies.

The modest size of our follow-up cohort and the absence of an independent validation cohort necessary to generalize our findings to different populations are additional limitations. Therefore, we applied cross-validation to generate the ROC curve and limit overfitting.

Moreover, we adopted quartiles of BMIz36 due to the low prevalence of overweight or obesity at 36 months in our cohort. This occurrence could be attributed in part to the effectiveness of the follow-up process and the relatively lower prevalence of childhood obesity in Denmark compared with other southern European countries [43]. Regardless of whether the mothers were allocated to lifestyle interventions, we adjusted the models for the TOP intervention assignment and GWG. The fact that we can still discover DNAm associations independent of these covariates strengthens the robustness of our results and the role of prePregBMI in offspring metabolic programming. PrePregBMI was collected from the mothers' self-reported measurements at the first pregnancy visit. However, measured BMI and self-reported BMI before and during early pregnancy are strongly correlated and reliable [44].

Although small epigenetic changes in different genes or regulatory regions collectively have cumulative effects contributing to complex phenotypes, the biological significance of small methylation changes is challenging to interpret. We recognize the need for and encourage the validation of our results in independent cohorts with a

higher prevalence of childhood obesity, larger sample sizes, and different ethnicities, as well as the need for downstream functional studies of the methylation sites identified to elucidate and verify the biological mechanisms potentially involved in mediating prePregBMI effects on offspring BMI.

CONCLUSION

This study supports that CB DNAm at birth partially mediates the effects of prePregBMI on BMIz36. These findings highlight the potential critical role of maternal obesity and the periconceptional environment in determining future metabolic health, providing suggestive evidence of epigenetic involvement in the intergenerational risk of obesity.

In addition, we found that an MRS composed of two methylation sites could discriminate the highest quartile of children's BMIz and FM (grams) at age 36 months with an AUC of 0.72 and 0.71, respectively, after cross-validation. These data demonstrate that CB-based epigenetic markers could be promising for identification and stratification at birth of children who are at higher risk of developing obesity later in life, encouraging its potential future use for precision medicine.O

AUTHOR CONTRIBUTIONS

Alice Maguolo designed and planned the current study, analyzed data, performed the statistical analyses, and drafted and revised the manuscript. Josefine Jönsson, Kristina M. Renault, Allan Vaag, Paul W. Franks, and Charlotte Ling designed and planned the current study and edited and discussed the manuscript. Kristina M. Renault, Emma Malchau Carlsen, and Kirsten Nørgaard designed and planned the TOP study and the 3-year follow-up. Kristina M. Renault and Emma Malchau Carlsen conducted the TOP study and collected data. Alexander Perfilyev performed the DNA methylation bioinformatics analyses. Marlena Maziarz performed the cross-validation analysis. Josefine Jönsson, Paul W. Franks, and Charlotte Ling supervised the analyses. All authors reviewed and provided critical comments on the manuscript and approved the final version of the manuscript. Alice Maguolo, Josefine Jönsson, Kristina M. Renault, Paul W. Franks, and Charlotte Ling are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the data's integrity and the data analysis's accuracy.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflicts of interest.

DATA AVAILABILITY STATEMENT

DNA methylation data from cord blood of the Treatment of Obese Pregnant Women (TOP) study (accession number LUDC2020.08.14) are deposited in the Lund University Diabetes Centre repository (https://www.ludc.lu.se/resources/repository) and are available to academic researchers upon request through the repository portal.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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