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Maternal Chrono-Nutrition and Placental DNA Methylation: The BiSC Study

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ABSTRACT

The impact of diet during pregnancy on birth outcomes and child health is well established, and epigenetic changes may be one mechanism underlying such associations, but the role of meal timing (chrono-nutrition) is unclear. We conducted an epigenome-wide association study (EWAS) of maternal meal timing and placental DNAm (plaDNAm). Data came from 389 pregnant women in the Barcelona Life Study Cohort (BiSC). Chrono-nutrition and dietary data were collected at 20 weeks of pregnancy, and plaDNAm at delivery was characterized using the Illumina EPIC array. Linear robust regression models tested associations between five chrono-nutritional behaviors (time of first and last meal, nighttime fasting duration, number of eating occasions, and eating jetlag) and plaDNAm. We identified 7 CpGs significantly associated with time of last meal (Bonferroni $p < 1E\text{-}08$) and 63 suggestive CpGs ($p < 1E\text{-}05$). Hits included cg13147785 (*E2F8*), linked to placental cell cycle regulation, cg17665505 (*DAP*) and cg18303215 (*ABCG5*), associated with smoking and lung diseases in adults. To conclude, maternal chrono-nutrition was associated with some CpGs in the placenta, particularly time of last meal. Further studies are needed to clarify how meal timing may influence fetal development and long-term health through epigenetic mechanisms.

1 | Introduction

Circadian rhythms refer to the biological processes that exhibit a 24-h oscillation, directing physiological and behavioral functions of the body [1]. These rhythms are driven by internal molecular clock mechanisms, with the superchiasmatic nucleus of the hypothalamus serving as the master pacemaker [2]. The main synchronizer of the superchiasmatic nucleus is the natural light-dark cycle, however, clocks in other organs such as liver or muscle (peripheral clocks) can respond to other environmental and behavioral time-givers, including timing of food intake. "Chrono-nutrition" has emerged as a field of research that studies the relationship between food intake, the circadian system and health [3, 4]. Studies have shown that the timing of food intake is

an important dimension to consider beyond the quantity and quality of the food consumed [5]. As diet is one of the factors synchronizing our circadian rhythm, irregular meal timings can disrupt the alignment of environmental oscillators with the central pacemaker, leading to adverse health outcomes [6].

Pregnancy is a crucial period for both health of the mother and fetal development [7]. The placenta is a vital organ for nutrient transport and hormone production, and acts as an immunologic barrier [8]. From early- to mid-pregnancy is when the placental development is mostly active [9]. Disruption in maternal physiology during this critical period of gestation influences the placental structure and function, altering nutrient and oxygen supply, hormone secretion, and the release of signaling molecules (e.g., cytokines, growth factors) into fetal circulation [10, 11].

Chrono-nutrition behaviors during pregnancy, such as breakfast skipping, short nighttime fasting, and night eating have been associated with several maternal and birth outcomes [12, 13]. Specifically, meal timing and frequency during pregnancy have been associated to gestational weigh gain [14, 15], gestational diabetes [16, 17], gestational age at birth [16], fetal growth [18, 19], and birth weight [13, 20]. The underlying biological mechanisms through which meal timing during pregnancy may affect maternal–fetal health remain poorly understood.

Epigenetic modifications, including DNA methylation (DNAm), are reported to act as potential mediators of environmental influences such as air pollution [21], maternal physical activity [22] and maternal stress [23], on fetal development. Alterations in the maternal diet during pregnancy can have lasting consequences on offspring health, as shown in historical examples such as the Dutch famine [24, 25]. DNAm, one of the most studied epigenetic marks, involves the addition of a methyl group to cytosine at CpG sites, potentially influencing gene expression. While most research has focused on the role of malnutrition and diet quality on DNAm [26, 27] very little is known about how chrono-nutrition behaviors may affect placental epigenetic programming and, by extension, fetal development.

To fill this gap in knowledge, we aimed to uncover epigenetic associations between meal timing during pregnancy and placental DNAm at delivery by conducting an Epigenome-Wide Association Study (EWAS).

2 | Methods

2.1 | Study Cohort

This study was conducted in the context of the Barcelona Life Study Cohort (BiSC), a population-based longitudinal birth cohort. The details about the BiSC recruitment process, follow-ups, and data collection procedures have been published elsewhere [28]. Briefly, it recruited 1080 pregnant women during their first routine hospital visit (weeks 11–14 of gestation), in three tertiary university hospitals (i.e., Hospital Sant Joan de Déu, Hospital de la Santa Creu i Sant Pau, and Hospital Clínic de Barcelona) in Barcelona (Spain) between October 2018 and April 2021 [28]. BiSC included pregnant women between 18 years to 45 years old with singleton pregnancy from the general population who were living in the catchment area of the recruiting hospitals and were able to communicate in Spanish/Catalan. Of the 1080 recruited pregnant women at baseline, 1032 remained in the cohort until the time of the delivery with 389 providing placenta samples for DNAm profiling as well as information on their dietary intake and meal timing at 20 weeks of gestation, who were included in this study (Figure S1). Ethics approvals were obtained from the corresponding authorities in all the participating institutions and hospitals. Clinical Research Ethics Committee of the Parc de Salut Mar (2018/8050/I), Medical Research Committee of the Fundació de Gestió Sanitària del Hospital de la Santa Creu i Sant Pau de Barcelona (EC/18/206/5272) and Ethics Committee of the Fundació Sant Joan de Déu (PIC-27–18).

2.2 | Meal Timing Assessment

A semi-quantitative 114-item food frequency questionnaire (FFQ) was completed at 20 weeks of gestation (second trimester) to assess dietary intake since the beginning of pregnancy [29]. The FFQ included a section on meal time habits, where participants reported the usual time (in 24-h format) for up to seven possible eating occasions on both weekdays and weekends, if consumed: breakfast, mid-morning snack, lunch, afternoon snack, dinner, night snack, and other (see Supporting Information). All meal time variables were calculated separately for weekdays and weekends and weighted averages were computed [30]. The time of the first and last meals was defined as the earliest and latest reported eating occasions, respectively. If breakfast was not consumed, the next earliest eating occasion was used to define the first meal of the day in a day.

Eating window (A) was defined as the number of hours between the first and the last meal of the day. Conversely, nighttime fasting (B) was the number of hours not eating, from last meal to first meal the next day [31]. Eating midpoint (C) was the time of the day at which half of the eating window is achieved. Eating jetlag (D) was defined as the difference between the eating midpoint between weekend and weekday, with a positive eating jetlag meaning a later eating midpoint at the weekend compared to weekday [32] (Figure 1).

(A) Eating window (h) = Time of last meal—time of first meal
(B) Nighttime fasting (h) = 24 – eating window
(C) Eating midpoint (h) = time of first meal + (eating window/2)
(D) Eating Jet Lag (h) = Eating midpoint on weekends – eating midpoint on weekdays.

2.3 | Covariables Assessment

The following covariates were collected at the baseline visit (11–14 weeks of gestation): maternal age (continuous, in years); body mass index (BMI), calculated as weight (kg) divided by height squared (m^2), with weight and height measured during the visit (continuous, in kg/m^2); education level, categorized as university or non-university; and physical activity, assessed as a continuous variable (MET minutes/day), based on the sum of

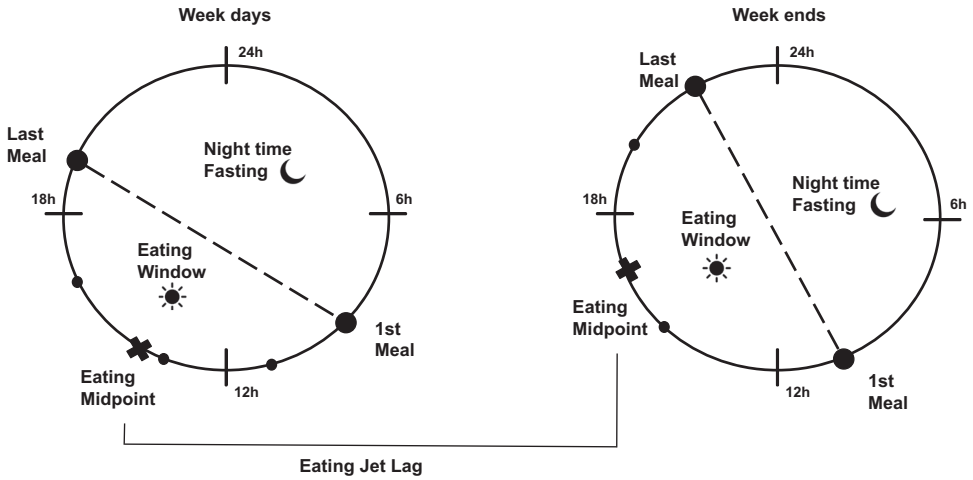

FIGURE 1 | Schematic representation of chrono-nutritional variables. The figure illustrates the different eating exposures to assess meal timing. The dots represent meal time, when the first and last one serving as cut offs for determine the eating and the fasting period.

light, moderate, and vigorous activities reported in the Pregnancy Physical Activity Questionnaire 2 (PPAQ, Supporting Information S2) [20].

Sleep duration (in hours) was calculated as the difference between self-reported bedtime and wake-up time. Data on sleep duration and quality were missing for 117 out of 389 participants. Sleep quality was assessed using the Pittsburgh Sleep Quality Index (PSQI) [33], a validated tool that evaluates multiple components of sleep, including duration, latency, and disturbances. The total PSQI score ranges from 0 to 21, with scores greater than 5 indicating poor sleep quality [34].

Mothers were considered smokers during pregnancy if they reported any smoking at either 12 or 32 weeks of gestation.

From the FFQ completed at 20 weeks of gestation, daily intake of food groups, energy, and macro- and micronutrients was calculated [35]. Energy intake was treated as a continuous variable (kcal/day). As an indicator of overall diet quality, we calculated the relative Mediterranean pregnancy diet score (rMEDp) [36], which includes eight components: cereals, vegetables, fruit, legumes, fish, meat, dairy, and olive oil (see Table S1). For each food group, tertiles of intake, expressed as a function of energy density (g/1000 kcal/day), were calculated. A score of 0, 1, or 2 was assigned to the first, second, or third tertile based on the 389 participants respectively for fruit, vegetables, legumes, cereals and fish; for meat and dairy, the scoring was inverse. For olive oil, the following scoring system was used: 0 for non-consumers, 1 for those below the median intake (among olive oil consumers), and 2 for those above the median intake [36]. The total score is the sum of all the components and ranges from 0 (low adherence) to 16 (high adherence).

Pregnancy-related variables included parity (categorized as nulliparous or multiparous), gestational age at birth (continuous, in days), gestational diabetes (yes/no), and gestational weight gain (in kilograms). Gestational age at birth was calculated based on the date of the last menstrual period and refined using estimates from the first ultrasound examination, typically performed around the 12th week of gestation.

Gestational weight gain was defined as the absolute difference in maternal weight between the first and third trimester visits, conducted approximately at 12 and 32 weeks of gestation. Although the exact timing of these visits varied slightly between participants, the calculation did not account for this variation and was based solely on the recorded weights at the two time points.

At birth, child sex was recorded as a categorical variable. Ethnicity was categorized into three groups—European, mixed European–Latin American, and other—based on self-reported maternal and paternal ethnic backgrounds.

Given that three hospitals participated in the study, hospital of recruitment was included in the main models as potential confounder. In addition, since the confinement period imposed by the COVID-19 pandemic had documented impacts on participants' daily lives and on sample collection procedures in the hospitals, whether pregnancy overlapped with the confinement was also included in the models.

2.4 | Placental DNAm

Placental tissue was collected at delivery from consenting participants. Biopsies were taken from two opposite quadrants, approximately 3–4 cm from the umbilical cord insertion site, following a standardized protocol across hospitals. Methylation profiling was performed using the Illumina Infinium MethylationEPIC BeadChip array [37].

Pre-processing of DNAm data was conducted using the PACE-Analysis R package (v0.1.9) (https://www.epicenteredresearch.com/). The pipeline included probe- and sample-level quality control, normalization, batch correction, winsorization of extreme values, and estimation of placental cell type proportions. Extreme methylation values were winsorized to the first and 99th percentiles (0.5% on each tail), with percentiles estimated from the empirical beta distribution. DNAm values are reported as beta values, ranging from 0 (completely unmethylated) to 1 (fully methylated). In addition, unreliable probes were removed

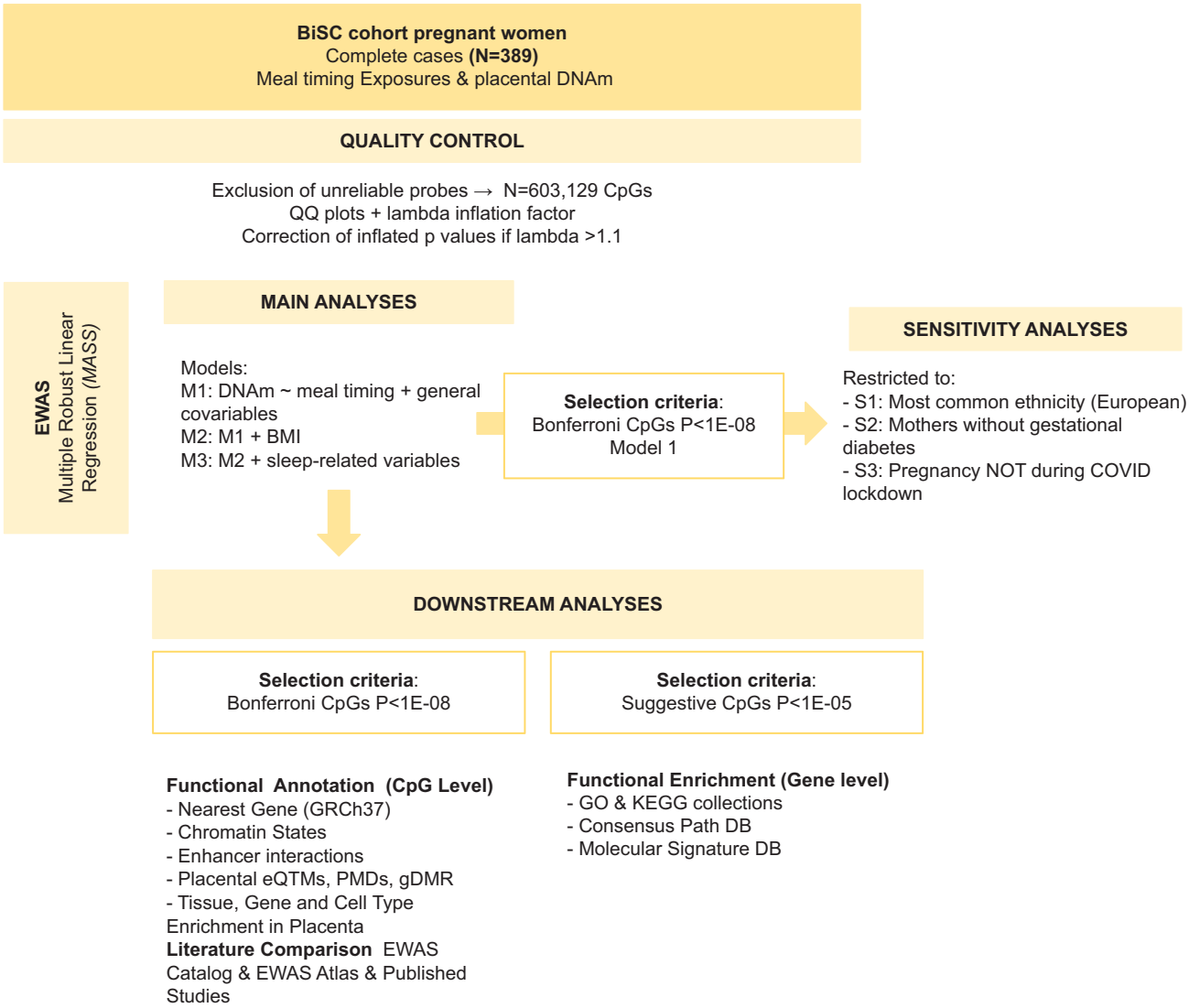

FIGURE 2 | Analysis flow diagram. Abbreviations: BMI: body mass index; EWAS: Epigenome-wide association study; eQTLs: expression quantitative trait loci; PMD: partially methylated regions; gDMR: germline differentially methylated regions; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DB: database.

prior to downstream analyses. We excluded 25% of probes from the initial array due to unreliability, reducing the total from 807 201 to 603 129 CpG sites. Further details on sample collection and DNAm processing are provided in Supporting Information S3.

DNA contamination was estimated using the *ewastools* R package [38] and defined as the mean $\log_2$ odds of contamination from external sources (e.g., maternal DNA or DNA from other participants). Proportions of six placental cell types—trophoblasts, syncytiotrophoblasts, nucleated red blood cells, Hofbauer cells, endothelial cells, and stromal cells—were estimated using the third-trimester placenta reference panel implemented in the *planet* R package [39]. Both DNA contamination and cell type proportions (with syncytiotrophoblasts being the most abundant; see Figure S1) were included as technical covariates in all statistical models.

2.5 | Statistical Analysis: Models Setup and Definitions

Workflow of all steps is shown in Figure 2. We investigated the association between five distinct chrono-nutritional variables and placental DNAm: time of first meal (h), time of last meal (h), nighttime fasting duration (h), number of eating occasions, and eating jetlag (h), all treated as continuous variables.

To examine these exposures, we adopted two complementary modeling strategies. In the first strategy, each meal time behavior was analyzed individually, without adjustment for the other behaviors. In the second strategy, selected meal time behaviors were included together in mutually adjusted models, following recommendations from previous studies [30, 40]. This approach allowed us to account for potential interdependencies among

behaviors. For example, nighttime fasting is determined by both the first and last meal of the day, while the number of eating occasions can have a direct influence on the eating window duration. Specifically, time of first meal, last meal, and number of eating occasions were mutually adjusted, and nighttime fasting was also adjusted for time of first meal and number of eating occasions.

In total, we defined seven exposure models: five individuals (Strategy 1) and two mutually adjusted (Strategy 2). For each exposure definition, we fitted three nested linear regression models (a directed acyclic graph (DAG) is shown in Figure S3). The models were defined as follows:

- Model 1: Adjusted for maternal age, parity, education, smoking during pregnancy, physical activity, energy intake, adherence to the Mediterranean diet (rMEDp), gestational age at birth, child ethnicity, delivery hospital, and placental cell type proportions.
- Model 2: Model 1 + maternal BMI in early pregnancy (12 weeks).
- Model 3: Model 2 + maternal sleep duration and sleep quality.

Due to the large number of covariates, we use Model 1 for interpretation, sensitivity analyses, and downstream functional annotation.

For sleep duration, missing values were imputed using multiple imputation by chained equations with predictive mean matching and five iterations, using the MICE package in R [41]. We additionally examined the correlation between meal timing variables and gestational weight gain using Pearson's correlation coefficients among participants with available data (Table S2). In the absence of clear associations, and to avoid overadjustment, weight gain was not included as a covariate in the final models.

This resulted in a total of 21 models (Figure S4).

Statistical analyses were carried out using R version 4.3. All models were estimated using robust linear regression models *rlm()* from the *MASS* package [42]. Effect estimates were expressed as the change in DNAm beta values per 1-h increase in timing exposure (or per one additional eating occasion, for that variable). For each EWAS model, we examined the genomic inflation factor (λ) to assess potential inflation of the test statistics and used 95% confidence intervals to evaluate the precision of effect estimates. In cases of inflation ($\lambda > 1.1$), we applied the *bacon* package, which uses a Bayesian framework to adjust for bias and inflation by modeling the expected distribution of test statistics [43]. This approach can reduce inflation while preserving true signal. To account for multiple hypothesis testing, we used a Bonferroni correction for 603 129 tests, retaining only CpGs with a Bonferroni-corrected $p < 0.05$. We also considered a suggestive significance threshold of $p <$ 1E-05 for exploratory findings.

We conducted a series of sensitivity analyses by restricting the sample to specific subgroups: S1) participants from the largest ethnic group (European origin, $N = 293$), S2) mothers without gestational diabetes ($N = 370$), S3) pregnancies that did not occur during the heavy lockdown period in Spain ($N = 297$). These sensitivity analyses were performed for model 1. To quantify the stability of effect estimates across models, we calculated the absolute difference in methylation (in percentage points) for each Bonferroni-significant CpG.

2.6 | Functional Annotation Analysis

2.6.1 | Mapping CpG-Genes

To support biological interpretation of the CpG sites associated with those chrono-nutrition indicators with significant results, we consulted several publicly available repositories to extract relevant biological information for each CpG. Annotations were based on the GRCh37/hg19 human genome assembly, which provided genomic coordinates, proximity to transcription start sites (within 1500 bp), and CpG island context.

We incorporated chromatin state data from the 15-state model of the Roadmap Epigenomics Project [44], which classifies genomic regions into functional categories (e.g., active promoters, enhancers, repressive states). These states were grouped into two broader categories for interpretation: active (i.e., promoter, enhancer, and transcribed) and inactive (i.e., bivalent, repressed, and quiescent).

2.6.2 | Genetic Regulation

To assess potential genetic regulation of methylation, we examined whether the CpGs had fetal cis-methylation quantitative trait loci (mQTLs) [37] ($\pm$1 Mb) using data from the BiSC cohort ($n = 408$). Linear regression models were applied to each CpG–single nucleotide polymorphism (SNP) pair, adjusting for sex, gestational age, ancestry (five principal components (PCs)), and estimated placental cell-type composition. Fetal genotyping was performed using the Global Screening Array v3.0 with HRC-based imputation. Genome-wide significance was defined as $p <$ 5E-08, and analysis was performed using TensorQTL [45]. In addition, we annotated overlap with known germline differentially methylated regions [46] and the placental partially methylated domains [47].

We also examined long-range regulatory interactions using EpiRaction [48], a placenta-specific enhancer–gene interaction database, to identify potential gene targets regulated by the CpGs. We assessed the relation with gene expression by inspecting previously reported expression quantitative trait methylation (eQTMs) in placenta [49]. We assessed transcriptional regulation using the Placenta Transcriptional Regulatory Network (TRN) [50], which predicts gene–TF interactions relevant to placental biology.

2.6.3 | Tissue and Cell Enrichment

Tissue and cell-type expression patterns of genes linked to CpGs were examined using the Human Protein Atlas [50], including both bulk tissue and single-cell datasets. We also assessed

placental gene enrichment using the placentome resource [51].

2.6.4 | EWAS Databases

Finally, we consulted the EWAS Catalog [52] and EWAS atlas [53] data to check whether the identified CpGs had been previously associated with specific exposures or outcomes in other EWASs. EWAS Catalog gathers reported CpGs with a nominal p-value < 1E-05 and the EWAS atlas < 1E-04.

2.7 | Functional Enrichment Analysis

We performed two functional enrichment analyses to explore the biological relevance of the findings. First, we used the *missMethyl* R package [54], which performs overrepresentation analysis (ORA) using gene sets from Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), and the Molecular Signatures Database (MSigDB), while accounting for the variable number of CpG sites per gene on the array to reduce probe-related bias. Second, we used ConsensusPathDB [55], which integrates pathway data from multiple databases (e.g., Wikipathways, Reactome, PharmGKB, BioCarta, HumanCyc, NetPath, among others). Although ConsensusPathDB does not correct for probe distribution bias, it provides access to a broader set of pathway annotations. Both analyses focused on genes annotated to suggestive meal timing-related CpGs, defined as those with p-nominal <1E-05. In all cases, we report pathway terms that met a false discovery rate (FDR) threshold of <0.05.

3 | Results

3.1 | Participants' Characteristics

Of the 1032 mother–child pairs followed until birth; placental tissue samples were collected for 611 participants. Of these, 22 samples were excluded due to poor DNA quality, resulting in 589 samples available for DNAm analysis. From these, 389 mothers had available data for meal timing and diet quality. The characteristics of the 389 mothers included in the present analysis are summarized in Table 1. On average, participants were 34.5 years old (SD = 4.42) and had a first-trimester BMI of 24.0 kg/m^2 (SD = 3.98), with a gestational weight gain of 8.7 kg (SD = 3.49). In this sample, 5% of mothers reported smoking during pregnancy, 58% were nulliparous, and 87% did not experience any pregnancy complications. Compared to the broader BiSC cohort, mothers included in this study were more likely to have higher educational attainment, engage in physical activity, and were less likely to smoke (Table S3).

Regarding diet, the mean daily energy intake was 1952 kcal (SD = 549.78). Mothers reported an average of 4.6 eating occasions per day. The median time of the first meal was 08:38, the last meal at 21:08, and the median nighttime fasting duration was 11 h and 34 min. The median eating jetlag was 45 min, indicating a tendency for later eating schedules on weekends. The full distribution of each meal timing variables on weekdays and weekends is provided in Table S4.

3.2 | Epigenome-Wide Association Studies

We identified seven CpG sites significantly associated with timing of the last meal after Bonferroni correction (p < 1E-08), shown in Table 2 and Figure 3. The genomic inflation factor (λ) for last meal timing was approximately 0.77 in the individual model and 0.90 in the mutually adjusted model (Table S5). Of the seven Bonferroni-significant CpGs, four remained significant in the mutually adjusted model, which accounted for other meal timing behaviors. Effect estimates for these CpGs were consistent in both direction and magnitude across all models, from minimally to fully adjusted. Suggestive associations (p < 1E-05) were found for the different chrono-nutritional exposures: 6 with nighttime fasting, 1 with time of first meal, 21 with number of eating occasions, 30 with time of last meal, and 5 with eating jetlag (Tables S7–S9).

Genomic inflation factors (λ) for each exposure are presented in Table S5 and Figures S5–S9. We observed inflation (λ > 1.1) for the EWAS analyses of time of first meal and number of eating occasions. Sensitivity analyses showed effects were much weaker in all subgroups (Figure 4).

3.3 | Functional Annotation Analyses

Functional annotation, shown in Table 3, revealed that five of the seven CpGs associated with time of last meal were located at annotated genes, either within the gene body (e.g., *E2F8, DAP, ART5, ABCG5*) or near transcription start sites (*NRN1*). Two CpGs (cg13147785 and cg17665505) were associated with known mQTLs, suggesting that their methylation is partially regulated by genetic variants. One CpG (cg09476611) was linked to *ETFA* and *ISL2* through enhancer–promoter interactions, suggesting a potential role in distal gene regulation. Another (cg17740417, annotated to NRN1) overlapped with a partially methylated domain in placental tissue (chr6:6003664–6491368). No CpGs overlapped with germline differentially methylated regions.

Annotated genes showed varied tissue expression: for example, *NRN1* is enriched in brain, *ART5* in ovary, skeletal muscle, and testis, and *ABCG5* in liver and intestine, while *DAP* exhibited low tissue specificity. None of the genes demonstrated strong enrichment in the placenta at the tissue level. However, single-cell expression data showed that *NRN1* is expressed in extravillous trophoblasts, a key placental cell type. Finally, using the Placental Transcriptional Regulatory Network (TRN), we found that *NRN1* is predicted to be regulated by several transcription factors implicated in placental biology, including *MEOX2*, *FOXF1*, and *HOXA13* (R^2 = 0.677), further supporting its potential functional role in placental development.

3.4 | Functional Enrichment Analyses

Functional enrichment analyses were conducted using CpGs suggestively associated with time of last meal, resulting in a total of 26 CpGs were included in this analysis. No enriched pathways were identified using Gene Ontology (GO), KEGG, or the Molecular Signatures Database (MSigDB) via the missMethyl package. However, using ConsensusPathDB, we identified six enriched

TABLE 1 | Descriptive characteristics of the sample included in the EWAS analysis, Barcelona Life Study Cohort (BiSC), $N = 389$.

	Variable name	Mean (SD)/*N* (%)
Meal timing-related behaviors	Nighttime fasting (h)	11:34 [10:42–12:17]
	Number of daily eating occasions	4.62 (0.74)
	Time of first meal (h)	8:38 [8:08–9:25]
	Time of last meal (h)	21:08 [20:38–21:38]
	Eating jet lag (h)	0:45 [0:30–1:00]
Sleep-related behaviors	Sleep duration (h)[a]	8:38 [8:06–9:17]
	Sleep midpoint (h)[a]	4:19 [4:03–4:38]
	Fasting before sleep (h)[a]	1:25 [00:21–1:57]
	Fasting after sleep (h)[a]	0:38 [00:10–1:17]
	Pittsburgh Sleep Quality Index (PSQI)[a]	5.35 (2.84)
Mother-related	Age (years)	34.45 (4.42)
	BMI (kg/m^2)	23.99 (3.98)
	Weight gain (kg)[a]	8.66 (3.49)
	Educational level: university	290 (74.6%)
	Physical activity (METs. min/day)	125.67 (84.65)
	Smoking during pregnancy	20 (5.14%)
	Energy intake (kcal/day)	1952.25 (549.78)
	rMEDp	7.50 (2.57)
	Nulliparous before this pregnancy	225 (57.84%)
	Gestational diabetes	19 (4.88%)
	Pregnancy overlaps with COVID confinement	92 (23.7%)
Child-related	Child sex: female	189 (48.59%)
	Gestational age at birth (weeks)	39.78 (1.41)
	Ethnicity	
	European	293 (75.32%)
	Mixed European and Latin-American	82 (21.08%)
	Other	14 (3.6%)
Cohort-specific	Study center	
	Hospital Sant Joan de Deu	185 (47.56%)
	Hospital Sant Pau	196 (50.39%)
	Other	8 (2.06%)

Note: Hour-type variables are presented in 24-h format (HH:MM) as medians with 25th and 75th percentiles; continuous variables are shown as medians with standard deviations (SD); categorical variables are reported as counts and percentages (*n* [%]). *N* sleep duration and midpoint = 340, *N* fasting before and after sleep = 341; *N* = weight gain 366, *N* PSQI = 272.

[a]Non-imputed mean.

pathways, all related to transcriptional regulation (Table 4). Table 4 details the total number of genes annotated to each pathway, the specific count of candidate genes from the input list that overlap with that pathway, and the statistical significance of this enrichment (p- and q-values).

Additionally, we performed an exploratory analysis to assess enrichment among all CpGs suggestively associated with any chrono-nutritional exposure ($p < 1E\text{-}05$). Using ConsensusPathDB, this broader set of gene–CpG pairs (Tables S7—S10) revealed 276 enriched pathways (Table S12 and Figure S10).

4 | Discussion

This is the first study to examine the association between chrono-nutrition behaviors during pregnancy and placental DNAm. We identified seven CpGs with differential placental DNAm associated with the time of last meal, five of them annotated to a gene. Additionally, overall meal timing was suggestively associated with methylation at 63 CpG sites at a significance threshold of 1E-05. Sensitivity analyses in restricted subsets showed a great attenuation of the observed associations, limiting the robustness of our findings.

TABLE 2 | Association between time of last meal and placental DNA methylation (Bonferroni significant CpGs) in the BiSC Cohort ($N = 389$).

		Model 1				Model 2				Model 3			
Exposure variable	**CpG**	**β**	**SE**	***p*-Value**	**BF *p*-value**	**β**	**SE**	***p*-Value**	**BF *p*-value**	**β**	**SE**	***p*-Value**	**BF *p*-value**
Time of last meal													
	cg09467508 (−)	−0.005	0.0008	1.22E-08	0.01	−0.005	0.0008	2.84E-08	0.02	−0.005	0.0008	2.35E-08	0.01
	cg09476611 (−)	−0.004	0.0004	2.12E-09	<0.01	−0.003	0.0004	1.49E-08	0.01	−0.003	0.0004	9.23E-08	0.06
	cg13147785 (+)	0.007	0.0009	1.35E-12	<0.01	0.008	0.0009	3.29E-13	0.00	0.007	0.0010	1.52E-11	0.00
	cg17233491 (−)	−0.002	0.0002	1.05E-08	0.01	−0.002	0.0002	7.14E-09	0.00	−0.002	0.0002	2.73E-09	0.03
	cg17665505 (+)	0.007	0.0011	4.90E-08	0.03	0.007	0.0011	2.52E-08	0.02	0.007	0.0011	1.16E-08	0.01
	cg17740417 (−)	−0.001	0.0001	1.95E-08	0.01	−0.001	0.0001	9.71E-09	0.01	−0.001	0.0001	2.54E-08	0.02
	cg18303215 (−)	−0.003	0.0003	2.18E-09	<0.01	−0.003	0.0003	2.79E-09	0.00	−0.003	0.0004	5.23E-08	0.03
Time of last meal adjusted for time of first meal and number of eating occasions													
	cg09467508 (−)	−0.005	0.0008	1.50E-08	0.01	−0.005	0.0008	8.06E-08	0.05	−0.005	0.0009	3.92E-08	0.02
	cg09476611 (−)	−0.004	0.0004	2.14E-09	0.00	−0.004	0.0004	8.29E-09	0.01	−0.003	0.0005	4.88E-08	0.03
	cg13147785 (+)	0.007	0.0010	2.96E-10	0.00	0.007	0.0010	1.08E-10	0.00	0.007	0.0010	1.07E-09	0.00
	cg17740417 (−)	−0.001	0.0001	4.92E-08	0.03	−0.001	0.0001	2.23E-08	0.01	−0.001	0.0001	5.74E-08	0.03

Note: CpG IDs are listed in ascending order, with direction of effect noted as (+) for hypermethylation and (−) for hypomethylation. Effect sizes reflect the change in DNA methylation (beta values) per 1-h increase in time of last meal. For each model, we report the effect estimate (β), standard error (SE), nominal p-value, and Bonferroni-adjusted p-value (BF). Model 1 adjusted for maternal age, parity, education, smoking during pregnancy, physical activity, energy intake, adherence to the Mediterranean diet (rMEDp), gestational age at birth, child ethnicity, delivery hospital, and placental cell type proportions; Model 2 further includes for maternal BMI; Model 3 further includes maternal sleep duration and quality.

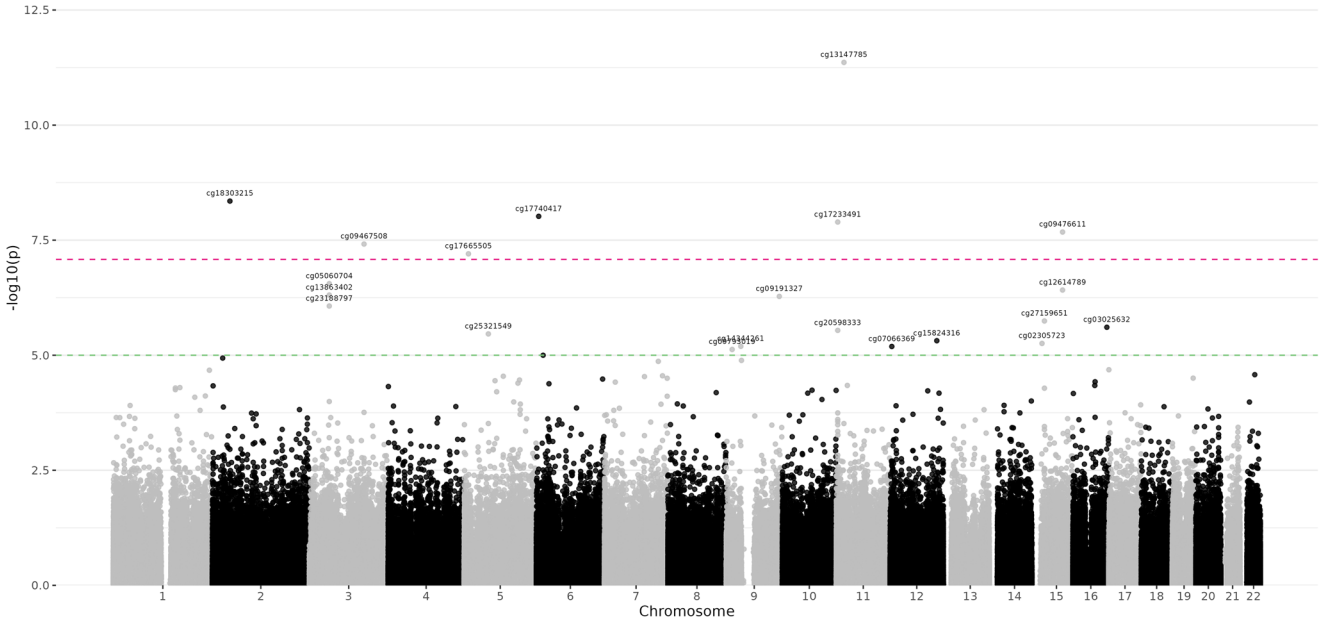

FIGURE 3 | Manhattan plot showing epigenome-wide associations between a later time of last meal and DNA methylation. Manhattan plot showing the association between time of last meal during pregnancy and placental DNA methylation. The *x*-axis represents genomic position by chromosome, and the *y*-axis shows the $-\log_{10}$ (*p*-value) for each CpG site. CpGs with $p < 1E\text{-}05$ are labelled. The green line marks the suggestive significance threshold ($p < 1E\text{-}05$) and the red line indicates the Bonferroni-corrected threshold ($p < 1E\text{-}08$).

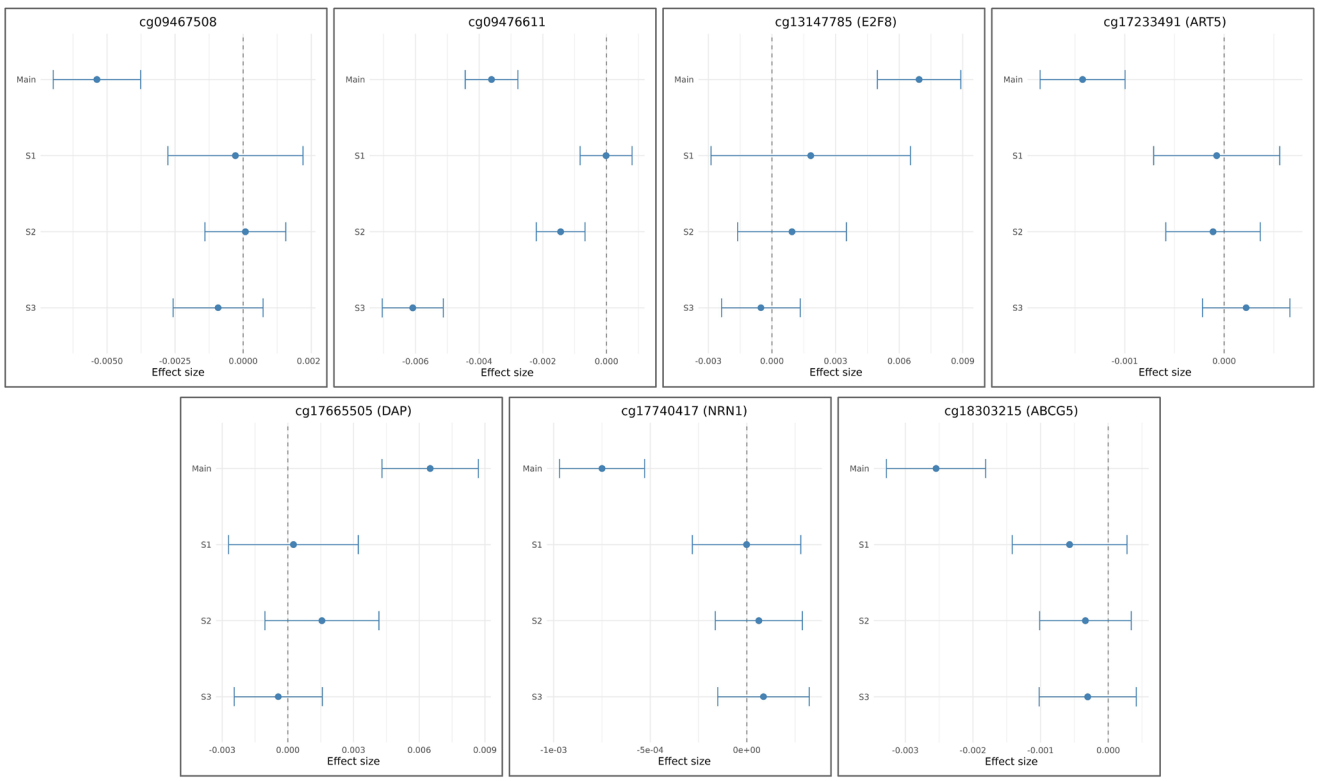

FIGURE 4 | Change of coefficients between time of last meal and DNAm in different sensitivity analyses. Effect estimates (and 95% confidence intervals) are shown for the main model and sample ($n = 389$) and a series of subgroup-specific analyses. Subgroups include participants of European origin only (S1, $N = 293$), mothers without gestational diabetes (S2, $N = 370$), and mothers whose pregnancy did not overlap with the COVID-19 lockdown period in Spain (S3, $N = 297$).

TABLE 3 | Functional annotation of CpGs associated with time of last meal in the BiSC cohort ($N = 389$).

CpG[a]	Chr: pos	Relation to island	Nearest gene	Nearest gene group	Chromatin states	Tissue specificity	Single cell type specificity	EWAS catalog (tissue [b]: exposure/outcome) associations	EWAS atlas (Biological traits) associations
cg09467508 (−)	chr3: 137486265	N Shore			Bivalent enhancer (*inactive*)			Buccal cells and peripheral blood mononuclear cells (−) [63] Age(+)(+)(+) [67]	B acute lymphoblastic leukemia (+) [64], birth weight (+) [65], gastric cardiac intraepithelial neoplasia (+) [66]
cg09476611 (−)	chr15: 76639212	Island			Flanking bivalent TSS/Enh (*inactive*)			Age (+)(+) [67] Rheumatoid arthritis (+) [69] C-reactive protein (+)(+) [70]	Breast cancer (−) [68]
cg13147785 (+)	chr11: 19247791	Open sea	*E2F8*	Body	Weak transcription (*active*)	Bone marrow, lymphoid tissue, skeletal muscle	Erythroid cells, skeletal myocytes, undifferentiated cells, plasma, squamous epithelial, gastric mucus-secreting cells	NA	NA
cg17233491 (−)	chr11: 3663266	Island	*ART5*	Body; 1st Exon; 5'UTR	Bivalent enhancer (*inactive*)	Ovary, skeletal muscle, testis	Spermatocytes, early spermatids, late spermatids, skeletal myocytes, oocytes, ovarian stromal cells	NA	NA
cg17665505 (+)	chr5: 10685203	Open sea	*DAP*	Body	Weak transcription (*active*)	Low tissue specificity	Low cell type specificity	Age (+)(−)(+) [67] Schizophrenia (+)(+) [73] Incident COPD (−) [74] C-reactive protein (+)(+) [70] GZMA protein (−) [75] LY9 protein (−) [75] Lung cancer— (+) [72]	Smoking (−) [71], lung cancer risk (+) [72]

(Continues)

TABLE 3 | (Continued)

CpG[a]	Chr: pos	Relation to island	Nearest gene	Nearest gene group	Chromatin states	Tissue specificity	Single cell type specificity	EWAS catalog (tissue [b]: exposure/outcome) associations	EWAS atlas (Biological traits) associations
cg17740417 (–)	chr6: 6007964	S Shore	*NRN1*	TSS1500; TSS200	Bivalent enhancer (*inactive*)	Brain	Adipocytes, Schwann cells, endothelial cells, lymphatic endothelial cells, extravillous trophoblasts, excitatory neurons	NA	Gastric cardiac intraepithelial neoplasia (+) [66]
cg18303215 (–)	chr2: 44059003	Island	*ABCG5*	Body	Bivalent enhancer (inactive)	Intestine, liver	Hepatocytes, proximal enterocytes, Paneth cells	Age (+)(+)(+) [67] Buccal cells and peripheral blood mononuclear cells (+) [63] Liver: fetal vs. adult liver (–) [79] Clear cell renal carcinoma tumor cells (NA) [80] Incident COPD (+) [74] Age (+) [81] C-reactive protein (+) [70] HIV infection (+) [82] Incident Lung cancer (+) [72]	Aging (+) [76] B acute lymphoblastic leukemia (+) [64], maternal smoking (-) [33], myocardial infarction (+) [77], oral squamous cell carcinoma (+) [78], smoking (+) [71]

Note: Chromosome (chr) and genomic position (pos) refer to the GRCh37/hg19 reference genome. The "Relation to island" column classifies the CpG's location relative to CpG islands (Island, Shore, or Open sea). "Nearest gene" indicates the proximal gene to which a CpG is annotated based on the GRCh37/hg19 reference genome, the full names corresponding to the gene symbols reported in the table are: Transcription Factor 8 (E2F8), ADP-ribosyltransferase 5 (ART5), Death-Associated Protein (DAP), Neuritin (NRN1), and ATP-Binding Cassette Subfamily G Member 5 (ABCG5). "Nearest gene group" indicate the closest annotated gene and its genomic context (e.g., gene body, transcription start site (TSS1500 or TSS200), first exon (1st Exon), or 5′UTR (5′ Untranslated Region)). "Chromatin states" are derived from the Roadmap Epigenomics Mapping Consortium and describe the chromatin environment in placental tissue (e.g., active transcription, bivalent enhancer). "Tissue specificity" and "Single-cell type specificity" were derived from methylation enrichment analyses across tissues and cell types, respectively. The EWAS Catalog columns shows the tissue in which the association was examined, followed by the reported exposure and outcome All studies were conducted in adults except one comparing fetal and adult liver methylation. The direction of effect is shown in parentheses: (+) or (−). If the same association appears in multiple studies, each occurrence is indicated by an additional set of parentheses (e.g., (+), (+), (−)). The next column presents exposures associated with the same CpGs as reported in EWAS Atlas studies.

[a]CpG sites are listed with their methylation direction in relation with time of last meal: (+) indicates hypermethylation and (−) indicates hypomethylation. b All tissues reported in EWAS catalog are whole blood unless specified. The direction of association of DNAm with the exposure/outcome is specified in parentheses.

TABLE 4 | Enriched pathways associated with suggestive differentially methylated CpGs related to time of last meal.

Pathway name	Set size	*N*	*p*-Value	*q*-Value
Generic transcription pathway	1178	4 (0.3%)	0.003	0.013
RNA polymerase II transcription	1307	4 (0.3%)	0.005	0.013
Transcriptional regulation by RUNX1	196	2 (1.0%)	0.006	0.013
Gene expression (transcription)	1439	4 (0.3%)	0.007	0.013
Signaling by nuclear receptors	295	2 (0.7%)	0.013	0.019
Transcriptional regulation by tumor suppressor gene encoding the p53 protein (TP53)	361	2 (0.6%)	0.019	0.022

Note: Set size indicates the number of genes annotated to each pathway; *N* is the number of gene–CpG pairs mapped to that pathway. *p*-Value represents the nominal significance of enrichment of gene–CpG pairs in a given pathway before multiple-testing correction, and *q*-value denotes the FDR-corrected *p*-value. All pathways were obtained from the Reactome database.

4.1 | Possible Biological Implications for Last-Meal Bonferroni Significant CpGs

Most EWASs reported in the EWAS Catalog and EWAS Atlas were conducted in adult populations using whole blood, limiting direct comparability with our study in placenta, given that DNA methylation is tissue specific.

Among the CpGs associated with last meal, only one (cg18303215 annotated to *ABCG5*) has been previously reported in a study in fetal tissue, whereas the other reported associations were all conducted in children, adolescents, or adults. Specifically, cg18303215 was hypomethylated in fetal liver compared to adult liver. Some of the other CpGs we identified may relate to placental function. For example, cg13147785, annotated to *E2F8*, encodes a transcription factor involved in cell cycle regulation and is expressed in key placental cell types (e.g., trophoblasts, endothelial cells). *E2F8* has been implicated in biological processes such as angiogenesis, hematopoiesis, and DNA damage response [57], which are essential for placental development.

The methylation levels at most of the CpGs we identified have been reported to change significantly from childhood to late adolescence (67): cg09467508, cg09476611, cg18303215 (*ABCG5*), and cg17665505 (*DAP*). *DAP* encodes a protein involved in programmed cell death and immune signaling and has also been linked to lung disease (COPD and lung cancer), alongside cg18303215 (*ABCG5*). *ABCG5* encodes a sterol transporter with high expression in liver and intestine and plays a role in cholesterol homeostasis [58]. Methylation changes at this CpG have also been associated with myocardial infarction, maternal smoking, and cancer-related outcomes. Additionally, cg17740417 (NRN1) has been linked to neuritin, a neurotrophic factor involved in neuronal development and synaptic plasticity [59].

4.2 | Placental Meal Timing-CpGs and Circadian Gene Clocks

Although no previous studies have directly examined the relationship between chrono-nutrition and placental DNAm, some research has linked DNAm patterns of circadian genes, primarily in blood or other tissues, with behavioral or physiological rhythms in adolescents and adults (Table S13). It was proposed that maternal circadian rhythms entrain rhythms in the fetus through factors such as glucocorticoids, dopamine, and melatonin [56]. Notably, the placenta itself expresses key components of these regulatory pathways, including glucocorticoid metabolism (e.g., 11β-HSD), melatonin synthesis and dopamine signaling [60–62]. However, in our study, we did not identify any differentially methylated CpG sites annotated either to core circadian clock genes in the placenta or at genes directly related to these hormonal pathways in association with maternal chrono-nutrition behavior.

4.3 | Strengths and Limitations

This is the first study to explore the association between meal timing behaviors and placental DNAm. One of the key strengths of our study is the well-defined nature of different chrono-nutrition domains. We had detailed information on meal consumption and timing variables, separately for weekdays and weekends. Additionally, the wealth of available data in BiSC allowed us to performed a wide range of sensitivity analyses to assess the consistency and robustness of our results. This pioneering study in its field is laying the groundwork for future research on the implications and importance of maternal meal timing and other circadian behavior exposure on placental DNAm.

However, there are also some limitations. The genomic inflation factors (λ) for the last-meal-timing EWAS were below the expected value of 1 ($\lambda = 0.77$ in the individual model and $\lambda = 0.90$ after mutual adjustment). This likely suggests a need for a bigger sample size, an overadjustment model or an over-correction for technical factors. Although with a low lambda, there is a lower risk for false positives, it can also lower statistical power and may have masked weaker methylation signals related to meal timing. Similar sub-unitary λ values have been reported in EWASs with limited sample size or heavy adjustment [56]. Given that the effect sizes for such associations (in EWASs in general) tend to be small, and that the adjustment variables are numerous, a greater sample size would have been needed. The number of covariates was likely too high for the sensitivity analyses in restricted subsets, in which the observed associations did not hold. Second, the generalizability of our findings may be limited by the characteristics of the study population. In addition, the majority of mothers in the cohort are of European ancestry, and thus the results may not be generalizable to individuals from other ethnic backgrounds. Third, although the meal timing data were carefully cleaned, they were self-reported, introducing the possibility of misclassification and measurement error. Dietary habits in the first two trimesters of pregnancy might be modified for a series of reason, including pregnancy-related symptoms or complications, change in appetite or dietary restrictions. The FFQ was administered at 20 weeks of pregnancy and asks about average meal timing since the beginning of the pregnancy. If the

meal timing during the first trimester was very different (due to nausea for example) to that of the second trimester, reporting an average at 20 weeks of pregnancy is challenging and likely carries measurement error. This might have biased the results. However, we conducted sensitivity analyses accounting for selected pregnancy complications potentially related to dietary intake and DNAm, namely gestational diabetes. We considered two additional exposures: fasting time between waking up and first meal, and fasting time between last meal and bedtime. However, due to missingness in the sleeping time values, the sample size was of $n = 341$ and we did not pursue with these exposures.

Future studies with more detailed information on meal timing at different key stages of pregnancy, using for example repeated 24 h dietary recalls and sleep diaries across the pregnancy would be ideal to accurately characterize chrono-nutritional and circadian patterns during pregnancy and their health consequences.

Fourth, we did not explore CpG sites involved in specific cell–cell interactions or regulatory networks, which may play a role in meal timing-related epigenetic regulation. Finally, the array platform used captures less than 5% of the methylome, limiting coverage, although the EPIC array remains among the most comprehensive methods currently available.

5 | Conclusions

This is the first study to explore the relationship between maternal chrono-nutrition and placental DNA methylation, identifying the timing of the last meal as the most consistently associated behavior. These results, though limited, provide a first step toward understanding how maternal meal timing may influence placental function and fetal development through epigenetic pathways. Future research should aim to replicate these findings in larger, more diverse populations, explore additional chrono-nutrition dimensions (e.g., eating windows, regularity, or chronotype alignment), and incorporate other omics layers to capture a more comprehensive view of how eating patterns during pregnancy might shape placental function and long-term offspring health.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Code for the data processing and statistical analysis is accessible at the GitHub repository: https://github.com/joanall/CHRONO_plaEWAS. Data available upon request on the BiSC website (https://projectebisc.org/en/bisc-project/publish-with-us/).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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