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Longitudinal DNA methylation profiles in saliva of offspring from mothers with gestational diabetes: associations with early childhood growth patterns

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Abstract

Background The prevalence of obesity and type 2 diabetes mellitus (T2DM) is rising globally, particularly among children exposed to adverse intrauterine environments, such as those associated with gestational diabetes mellitus (GDM). Epigenetic modifications, specifically DNA methylation, have emerged as mechanisms by which early environmental exposures can predispose offspring to metabolic diseases. This study aimed to investigate DNA methylation differences in children born to mothers with GDM compared to non-GDM mothers, using saliva samples, and to assess the association of these epigenetic patterns with early growth measurements.

Methods This study analyzed saliva DNA methylation patterns in 30 children (15 born to GDM mothers and 15 to non-GDM mothers) from the EPIDG cohort. Samples were collected at two time points: 8–10 weeks postpartum and at one year of age. Epigenome-wide analysis of over 850,000 CpG sites was conducted using the Illumina Methylation EPIC Bead Chip. Differential methylation positions (DMPs) were identified with the limma package, using a significance threshold of $p < 0.01$ and delta $\beta \geq 5\%$. Correlation analysis examined associations between methylation and growth variables (weight, height, BMI and annual growth) using Spearman tests.

Results We identified 6,968 DMPs at the postpartum stage and 5,132 after one year, with 50 sites remaining differentially methylated over time, 16 of which maintained consistent methylation directionality. Functional analysis linked several of these DMPs to genes involved in inflammation and metabolic processes, including CYTH3 and FARP2, both implicated in growth and metabolic pathways. Significant correlations were found between specific CpG sites and growth-related variables such as weight, head circumference, height, and BMI.

Conclusions This study's longitudinal design reveals stable DNA methylation patterns in saliva samples that differentiate GDM-exposed children from controls across the first year of life, highlighting the feasibility of saliva as a minimally invasive biomarker source. The persistence of these epigenetic signatures underscores their potential as early indicators of metabolic risk, offering valuable insights into the long-term impact of maternal GDM on child

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health. Although the use of saliva offers a practical and non-invasive tool for pediatric epigenetic research, further studies are necessary to validate these findings in larger populations.

Keywords Gestational diabetes, Offspring, Epigenetic, Dna methylation, Saliva, Child growth

Introduction

Obesity and diabetes are increasing globally, becoming significant public health concerns, particularly due to the rising prevalence among children. It is estimated that 29% of boys and 26% of girls are overweight or obese [1]. This means that these children are more likely to develop diabetes and other metabolic diseases later in life. Moreover, the offspring of mothers with obesity and/or diabetes are known to have an elevated risk of developing diabetes in the future [2]. This is closely linked to the Developmental Origins of Disease (DOHAD) hypothesis and the theory of the first thousand days which suggest that exposure to an adverse environment during the periconceptional and/or intrauterine period increases the lifelong risk of metabolic and related diseases in offspring. During the first 1000 days of life, the period from conception to the first two years of a person's life, there is an adaptive capacity to different stimuli and social environments that can be determinant for the individual's future [3, 4]. This timeframe represents a phase of heightened sensitivity, wherein nutritional, metabolic, and hormonal exposures can significantly influence developmental processes and future disease risk.

In recent years, significant research has focused on how gestational diabetes mellitus (GDM) influences the development of metabolic conditions like type 2 diabetes (T2DM) and obesity, both in mothers and their children. GDM is known to be associated with a range of adverse outcomes, including a higher likelihood of childhood obesity, and metabolic disorders in offspring [5]. Among the mechanisms by which prenatal environmental factors can exert long-term influences on gene expression and health outcomes, epigenetics, particularly DNA methylation, has gained substantial attention. GDM can alter the intrauterine environment, affecting fetal growth and metabolic programming, potentially predisposing the offspring to chronic conditions like obesity and T2DM [6].

Most existing studies on the epigenetic effect of GDM exposure have focused on samples from cord blood and placenta, showing that children born to mothers with GDM exhibit distinct epigenetic modifications compared to those born to non-GDM mothers [7, 8]. These epigenetic changes affect the regulation of genes involved in metabolic pathways, immune responses, and growth processes. For instance, altered DNA methylation patterns have been observed in genes related to insulin signalling, adipogenesis, and inflammation, which may contribute to the increased risk of metabolic disorders and obesity observed in these children. Furthermore, these epigenetic differences may serve as potential biomarkers helping identify children at risk for future metabolic diseases. While the potential for epigenetic markers to serve as biomarkers of future metabolic disease has been proposed, limited research has explored this in the context of accessible and non-invasive samples like saliva [9, 10]. Some studies have linked DNA methylation markers in saliva to birth weight [11]. Saliva represents a promising alternative to cord blood and placenta and offers a valuable, accessible tool for studying epigenetic biomarkers in the infant population offering promising applications for longitudinal follow-up studies. Few studies have conducted longitudinal assessments of DNA methylation in offspring, which are essential for understanding the persistence and dynamics of epigenetic modifications.

Furthermore, epigenetic modifications are not static and can be influenced by postnatal environmental factors such as diet, physical activity, and exposure to toxins. This dynamic nature of the epigenome suggests that early interventions could potentially mitigate the adverse effects of prenatal exposures.

This study aims to explore the epigenetic differences between children born to mothers with GDM and those born to non-GDM mothers, focusing on DNA methylation patterns in saliva samples. By leveraging a longitudinal design, we aim to assess the stability of these epigenetic marks over time and investigate their potential associations with childhood anthropometric measurements, thus contributing to the identifying of non-invasive epigenetic biomarkers for assessing the risk of metabolic diseases in children.

Methods

Subjects

Samples were drawn from the EPIDG cohort, comprising women with and without GDM. Details of this cohort have been published previously [12].This analysis focused on a subset of children born to mothers in this cohort. Briefly, pregnant women, with a positive O'Sullivan test, attending the Diabetes and Pregnancy unit at Hospital Universitario Virgen de la Victoria were recruited for the study. GDM was diagnosed according to National Diabetes Data Group (NDDG) criteria. The threshold values for the 100 g oral glucose tolerance test (OGTT) were 105 mg/dL for fasting glucose and 190 mg/dL, 165 mg/dL, and 145 mg/dL at 60, 120, and 180 min, respectively. A GDM diagnosis was confirmed when glucose levels met or exceeded these values at 2 or more time points

[13]. Women with a negative 100 gr OGTT were classified as controls.

Our analysis included 15 children from mothers with GDM and 15 from non-GDM mothers (control group or non-GDM). Subjects were selected based on their order of recruitment and whether they had attended both the postpartum visit (8–10 weeks after birth) and the one-year follow-up. Saliva samples were collected from the children during both visits. Anthropometric data including weight, height, BMI, head circumference and corresponding percentiles and Z-scores, were obtained from the records obtained during well-childcare visits at one month, four months and one year of age at their primary care centers. These percentiles and Z-score were calculated based on the WHO growth standards and they are specific for sex and age group [14]. Annual growth was calculated by the difference between the SD of weight at one year and at one month of age [15].

All mothers gave their consent to participate in the study. The EPIDG study was approved by the Institutional review board at the Hospital Universitario Virgen de la Victoria, Spain.

DNA extraction, and bisulfite conversion

Saliva samples were collected using swaps sample collection and stored at room temperature; DNA was extracted within 7 days of its collection (following the manufacturing protocol). Swap saliva DNA was isolated using Qiamp DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions for DNA in swaps. Quality and concentration of DNA was measured using Qubit 3.0 Fluorometer with Qubit dsDNA HS Assay Kit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). All the DNA extracted was bisulfited treated with EZ DNA Methylation kit (Zymo Research) for posterior DNA methylation analysis. After quality control, methylation analysis proceeded with 29 samples: 14 from children in the GDM group and 15 from the control group.

Epigenome wide DNA methylation analysis

The Epigenome- wide DNA methylation analysis (EWAS) was performed in a total of 29 children at both the postpartum (T1) and one year follow up (T2 visits). More than 850.000 CpGs sites were interrogated with the infinitum Methylation EPIC Bead Chip Kit (Illumina, San Diego, CA, USA) following the Infinium HD Assay Methylation protocol, and raw data (idat files) were obtained from iS (Illumina) software.

Raw data were processed using R software (version 4.0.0) and Bioconductor packages like minfi [16, 17]. Probes with a p-value greater than 0.01 in one or more samples were excluded. Probes with a beadcount of less than 3 in at least 5% of the samples were removed. We excluded non-CpG probes, probes with single nucleotide polymorphisms (SNPs) and probes aligning to multiple genomic locations [18]. **(Additional file 1).** Normalization was done using beta-mixture quantile Normalization (BMIQ) method [19]. To correct the differences in methylation resulting from differences in cellular heterogeneity, the Houseman correction was used [20].

Methylation data analysis

Differentially methylated positions (DMPs) were obtained using limma package [21] in R studio (4.0.4). To determine methylation levels, β-values and M-values were calculated. β-values represent the estimate of methylation level based on the ratio of the intensity of the methylation probe to the overall intensity. These values are used for representing results. On the other hand, M-values are obtained by logarithmically transforming the β-values and are used for performing the differential methylation analysis [22]. The significance threshold for determining DMPs was set a p-value less than 0.01 and delta $\beta \geq 5\%$ [17]. DMPs were obtained from both times, and the models were adjusted for infant sex, maternal treatment (diet or insulin) and maternal weight gain. Furthermore, to avoid possible false positives in identifying DMPs, array inflation was calculated using the RaMWAS package in the R study. This package provides the lambda (λ) value, with the following ranges considered: λ between 0.98 and 1.10: no inflation, λ between 1.10 and 1.19: slight inflation, λ between 1.20 and 1.40: moderate inflation, $\lambda > 1.4$: high inflation. A Venn diagram (Venny 2.1 tool) was used to identify common DMPs across time points (https://bioinfogp.cnb.csic.es/tools/venny/).

Functional analysis

To explore potential mechanism that may be altered in the offspring from mothers with GDM compared to the offspring from mother non-GDM, those DMPs that were maintained during time were used to perform a functional analysis.

A string network (https://string-db.org/) was performed with the genes of interest. Due to the lack of protein interaction, we settled a maximum number of interactors on 100, and an interaction score > 0.7. With the resulting net, Cytoscape (https://cytoscape.org/) was used to perform the clusterization of the net using MCODE app. Finally, ENRICHR [23] was used to figured out Kyoto encyclopedia of genes and genome (KEGG) pathways and the Gene ontology of each cluster.

Statistical analysis

Non-parametric tests (U-Mann Whitney) were used to compare quantitative variables between groups due to small sample size and variable distribution. Spearman correlation analysis explored associations between methylation levels and anthropometric variables. The

correlation matrix was obtained with corrplot and GGally packages in RStudio, the threshold was $P < 0.05$.

Results

Study population

The characteristics of these subjects are shown in Table 1. No significant differences were observed in the anthropometric measurements of the children born to mothers with GDM compared to those born to non-GDM mothers at any of the visits, except for BMI at one year of age, where a significant difference was found. Additionally, there were no significant differences in gender distribution between the two groups. In the GDM group, 35.7% of the children were boys, compared to 33.3% in the non-GDM group. Similarly, no significant differences were observed in delivery type: 64.3% of children in the GDM group and 60% in the control group were delivered via cesarean section.

Differentiated methylated analysis and correlation

A total of 6.968 DMPs and 5.132 DMPs were found at the postpartum and after one year of follow-up, between offspring from GDM and non-GDM, respectively. The CpGs most differentiated by chromosome in each time are represented in the Manhattan plot (Fig. 1). The inflation in both times was measured, in the case of postpartum the λ was 1 meaning no inflation, and we found a moderate inflation at one year follow-up analysis (λ = 1.36) (**Additional file 2).**

Among all the DMPs, 50 remained significantly differentiated over time. Of these, 16 CpGs maintained the same direction of DNA methylation levels across time points. Figure 2 displays the log2 fold change (log2FC), with an increase indicating hypermethylation and a decrease representing hypomethylation over time. This suggests that the difference in methylation levels between the two groups becomes more pronounced as time progresses but with the same trend.

Functional analysis

Of the 16 persistent DMPs, 8 were annotated in the Illumina EPIC database. We used these 8 proteins to make a functional analysis with String and ENRICHR. STRING was configured to give a network with a maximum number of interactors of 100. The resulting net was uploaded in cytoscape to perform a clusterization with the application MCODE. Four clusters were identified, two of which included some of our proteins of interest. The first cluster contained DEFB104A and was associated with the Staphylococcus aureus infection pathway and the NOD-like receptor signaling pathway, both of which are involved in the immune response to infection. The second cluster included FARP2 and CYTH3. This cluster was linked to several pathways, with the most notable being the phospholipase D signaling pathway and the VEGF signaling pathway, both of which are related to inflammatory processes. Additionally, although less significant, this cluster was also associated with type 2 diabetes and regulation of lipolysis in adipocytes (Fig. 3).

Correlation analysis

We conducted a correlation analysis to explore the relationship between DNA methylation marks and anthropometric variables related to childhood growth. 15 out of 16 CpG sites showed significant correlations with growth percentiles for weight, height, head circumference, BMI, and annual growth (Additional file 3). Some CpG sites were correlated with multiple variables at different time points, with notable differences between children exposed to GDM and controls.

In children of mothers with GDM, methylation levels at cg00124849 measured postpartum showed a strong positive correlation with head circumference at 1 month ($r = 0.762$, $p = 0.004$), 4 months ($r = 0.720$, $p = 0.008$), and one year ($r = 0.794$, $p = 0.006$), whereas in controls, cg00124849 was negatively correlated with annual growth (r=-0.580, $p = 0.04$) (Additional file 3). This relationship observed across multiple time points, suggests a

Table 1 Characteristics of the study subjects

	1 month			4 months			1 year		
	Non-GDM	**GDM**	**p**	**Non-GDM**	**GDM**	**p**	**Non-GDM**	**GDM**	**p**
N	15	14		15	14		15	14	
Weight (Kg)	4.02 ± 0.6	3.97 ± 0.7	0.979	6.87 ± 1.1	6.6 ± 0.8	0.436	9.90 ± 1.4	9.77 ± 1.7	0.683
Weight percentile	39	41	0.999	50	46	0.720	65	55	0.574
Height (cm)	52.6 ± 1.9	52.6 ± 2.5	0.776	63.0 ± 2.9	62.4 ± 2.9	0.519	74.5 ± 2.5	76.7 ± 4.3	0.384
Height percentile	36	35	0.691	44	46	0.999	49	58	0.385
Head circumference (cm)	36.2	36.6	0.207	41.4 ± 1.5	40.9 ± 1.3	0.336	45.8 ± 1.05	46.04 ± 2.1	0.392
Head circumference percentile	42	57	0.252	56	49	0.448	65	59	0.688
BMI	14.2 ± 1.6	14.2 ± 1.7	0.531	17.2 ± 2.5	16.9 ± 1.5	0.943	17.9 ± 1.7	16.5 ± 1.5	**0.033**
BMI percentile	41	45	0.865	49	51	0.999	74	51	**0.095**
Annual growth							1.007 ± 1.3	0.818 ± 1.3	0.821

Annual growth was calculated by the difference between the SD of weight at one year and at one month of age.

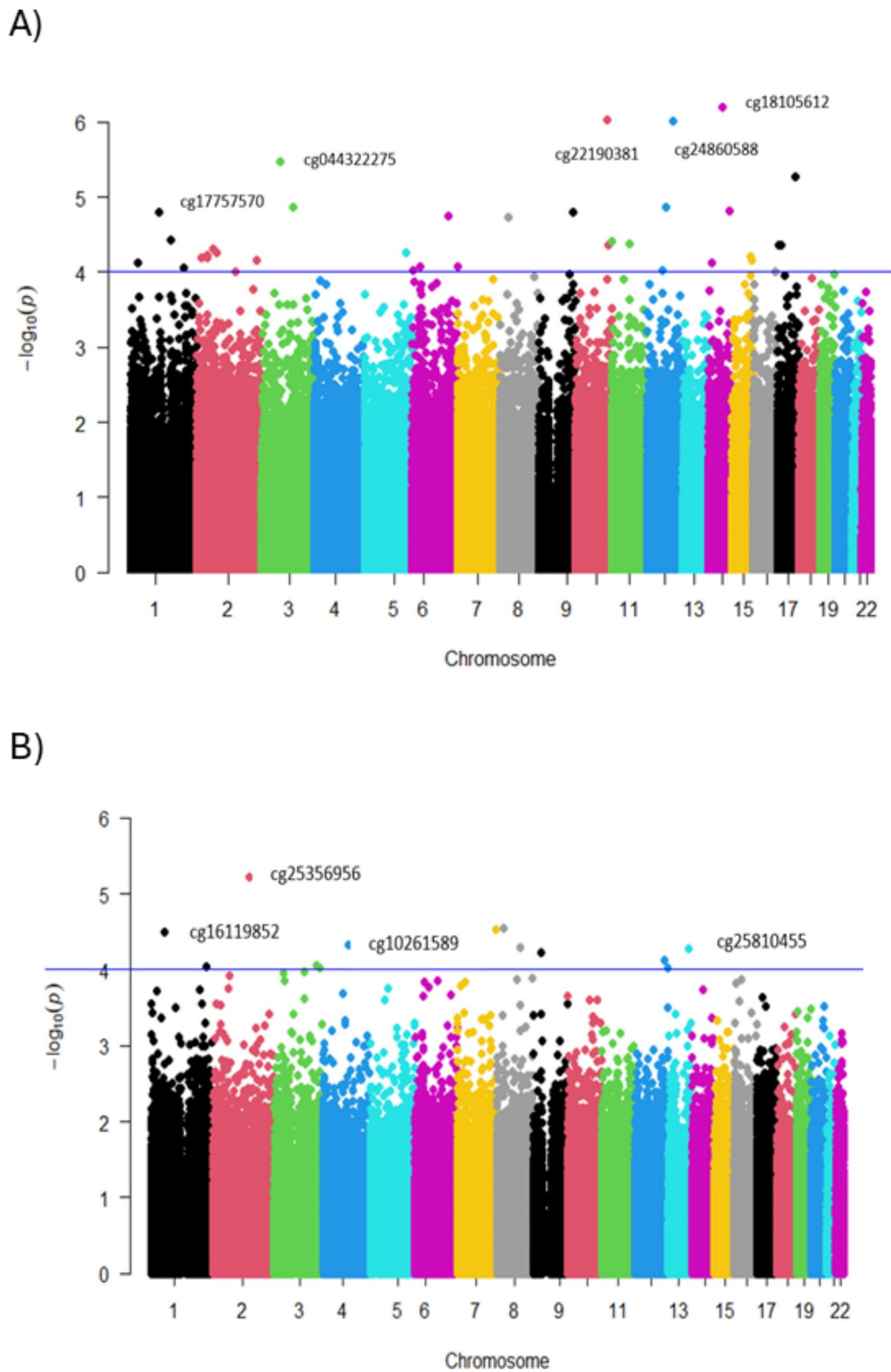

Fig. 1 **A** Manhattan plot representing the most differentiated CpGs per chromosome at the postpartum visit. **B** Manhattan plot representing the most differentiated CpGs per chromosome at the one year of follow-up visit

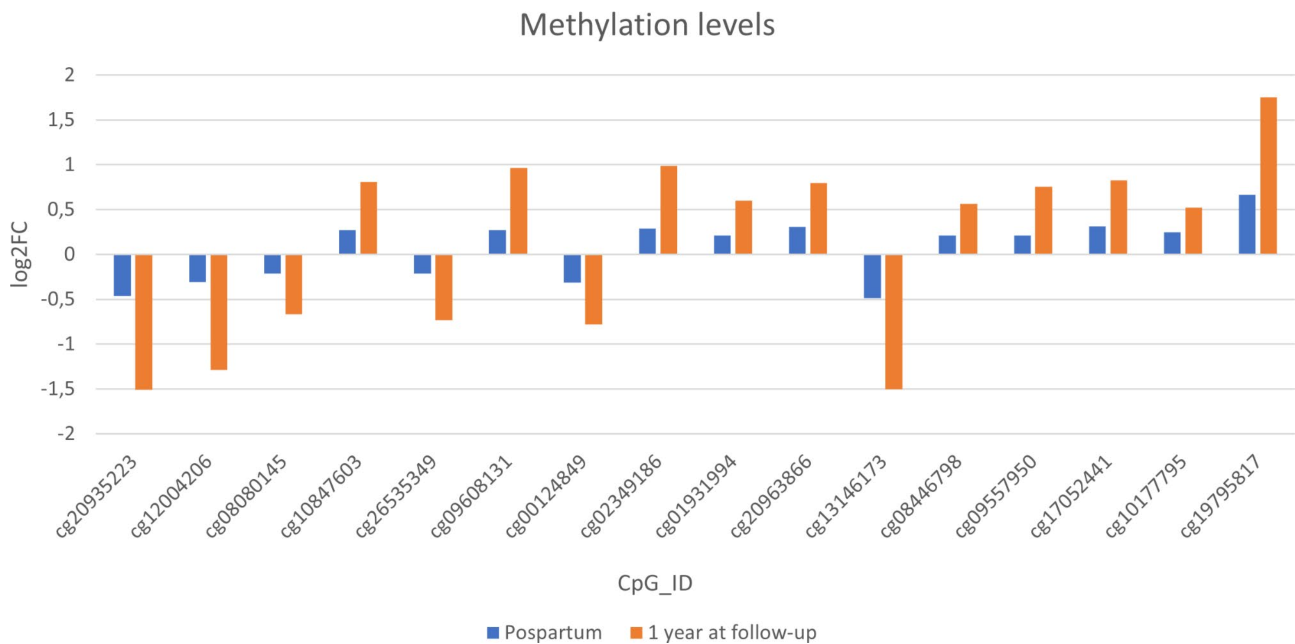

Fig. 2 CpGs that maintained the same direction of DNA methylation levels throughout the year. Postpartum: postpartum visit (8–10 weeks), Year: 1 year after birth

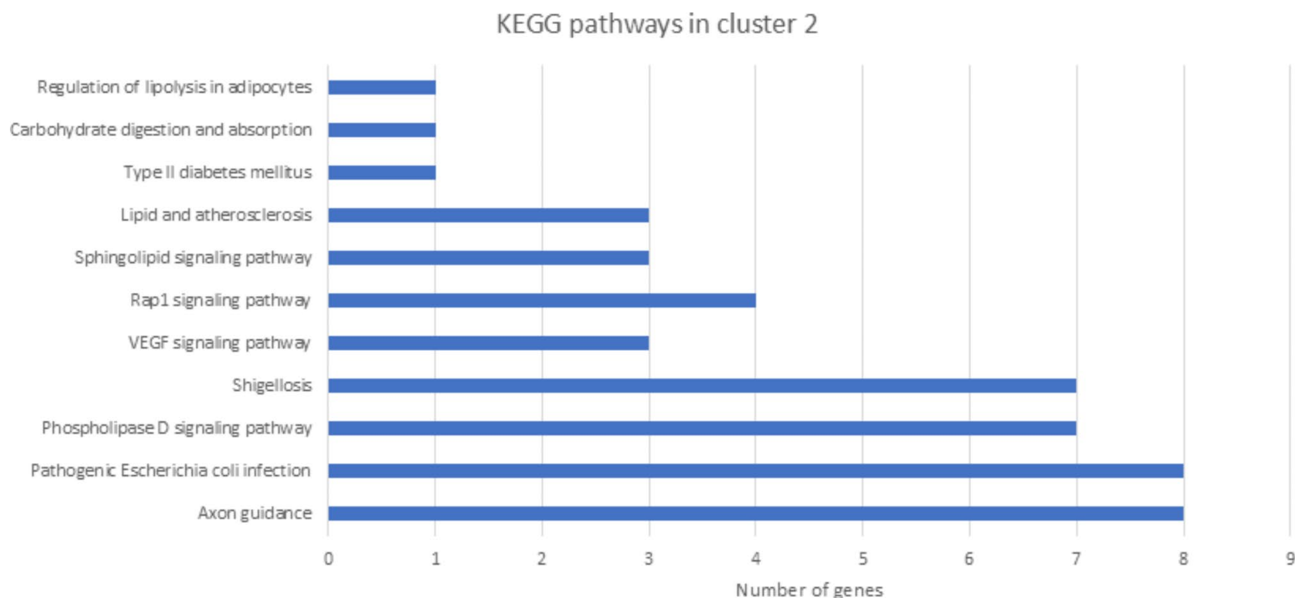

Fig. 3 Enriched signalling pathways according to KEGG

potential long-term epigenetic influence on growth patterns in children exposed to maternal GDM.

Other sites, such as cg09608131, cg20963866, and cg19795817, were significantly associated with weight, head circumference, and annual growth. cg20935223 showed a positive correlation with BMI at one year across all subjects ($r=0.455$, $p=0.025$), while cg02349186 showed a negative correlation (r=-0.510, $p=0.011$). The cg08080145 methylation levels assessed at the postpartum visit were positively associated with height percentile in offspring of mothers with GDM at 4 months (Additional file 3).

Discussion

In this study, we observed distinct epigenetic marks, specifically DNA methylation patterns, in saliva samples from children born to mothers with gestational diabetes mellitus (GDM) compared to those from non-GDM mothers. Furthermore, several of these epigenetic differences persisted over the first year of life and were

associated with anthropometric variables linked to childhood growth. Additionally, some of these DNA methylation sites are annotated in genes involved in pathways related to inflammation and type 2 diabetes, highlighting their potential role in early metabolic programming.

Previous studies have documented DNA methylation changes in cord blood and placenta of offspring born to GDM mothers, suggesting that these epigenetic modifications may reflect developmental programming of disease mechanisms and their potential role as biomarkers risk for metabolic diseases [2, 8, 24, 25].

While some research has associated DNA methylation profiles in children's saliva with birth weight, prenatal maternal stress, and potential childhood obesity predictors, studies specifically targeting the offspring of GDM mothers are limited, with most investigations performed on cord blood samples [11, 26–28]. For instance, Franzago et al. evaluated DNA methylation levels in MC4R and LPL genes, in children born to mothers with obesity and GDM in different tissues, including placenta, maternal blood, and buccal swab samples. However, their analysis did not yield significant findings in saliva [29]. A recent review carried out by Saucedo et al. [30] examined the role of DNA methylation as a potential biomarker for monitoring fetal growth during pregnancy in women with GDM [30]. They found discrepant results in more than 15 studies that evaluated the role of DNA methylation in relation to birth weight. Most of these studies were conducted using cord blood, placental tissue, and maternal blood samples, with none in buccal (oral) samples. These discrepancies are explained by multiple factors, including sample size, the techniques and approaches used, the specific regions studied, and the inclusion of potential confounding variables.

Additionally, there is a scarcity of research examining the variability of these epigenetic marks over time and their association with children's growth. The only study to date on this topic focused on GDM, conducted by Emper's group, found differential DNA methylation profiles in children born to mothers with obesity or GDM compared to controls, with differences maintained over the first year of life in whole blood samples, showing epigenetic signatures enriched in metabolic pathways [31]. Our findings align with these results, revealing that 16 DMPs, in saliva, remained differentially methylated over the first year of life, in the same direction. The observed increase in methylation levels over time likely reflects the dynamic nature of the epigenome during early development, a critical window for epigenetic remodeling. During this period, developmental and environmental factors interact to establish stable epigenetic marks, which may explain the increasing methylation observed at the 1-year follow-up. These changes could be associated with biological processes such as tissue-specific maturation, immune system development, or metabolic programming. The consistency in the direction of methylation across time points may reflect stable epigenetic programming influenced by prenatal exposures, such as gestational diabetes mellitus (GDM).

Of the 16 CpG sites identified in our study, none have previously been associated with GDM in studies conducted on cord blood or placental samples. However, through the EWAS catalogue [32], we observed that several of these CpG sites have been identified in other contexts. Specifically, two CpG sites have been linked to cancer (cg08080145, cg19795817), one has been associated with obesity (cg20935223), and the cg10847603 and cg08446798 have been related to early exposure to environmental factors, such as maternal smoking and socioeconomic status [32].

Some of these 16 CpGs were annotated in genes, such as FARP2, CYTH3, RAB37, and NACC2, which are related to pathways involved in inflammatory processes, insulin signalling, and metabolic regulation, including T2DM. Specifically, *CYTH3* has roles in Golgi apparatus function and ADP-ribosylation factor regulation, with genome-wide association studies (GWAS) suggesting its association with BMI and height [33, 34]. A recent study carried out in mice showed for first time that CYTH3 is required for full insulin signalling in mammals and might constitute a novel therapeutic target for weight reduction [35]. Previous research has identified associations between RAB37 gene and HbA1c levels, a key biomarker of long-term glucose control [36]. This suggests potential involvement in glycemic regulation, which could influence diabetes risk in the future. FARP2 enables guanylyl-nucleotide exchange factor activity, and it is involved in Rac protein signal transduction and neuronal remodelling. Polymorphisms of this gene are related to HDL concentrations and height [37, 38]. Recently, a preprint study has identified new genes associated with obesity, including FARP2 and NACC2, that showed strong associations with BMI and related phenotypes such as waist-hip ratio adjusted BMI or fasting insulin-adjusted BMI [39]. In our study, the cg10177795 annotated to FARP2 gene, and the cg12004206 annotated to NACC2 were correlated with head circumference and height percentiles, supporting findings from previous studies and their relevance for metabolic outcomes, and potential biomarkers of adiposity risk in the future.

Regarding BMI, we observed a higher BMI at one year of follow-up in the group of children born to mothers without GDM, contrary to what has been commonly described in the literature [40]. This finding could be explained by greater dietary control among women with GDM in our sample [12], the small sample size, or the shorter follow-up period. Indeed, several studies have found no significant differences in BMI between GDM

and non-GDM offspring in the first 4–5 years of life, suggesting that the potential impact of maternal GDM exposure may not manifest until later in childhood [41, 42]. Epidemiological studies have demonstrated higher rates of T2DM in offspring exposed to maternal GDM, with the HAPO follow-up study showing increased adiposity and future risk of glucose metabolism disorders, likely as a consequence of adverse intrauterine programming [43]. In our study, we identified three CpG sites (cg20963866, cg02349186 and cg17052441) that were correlated with BMI at 1 year of age. This finding underscores the potential of these CpG sites as non-invasive biomarkers for early metabolic risk. Identifying such biomarkers is particularly important, as BMI is not only a predictor of future obesity and adiposity in children but also a well-established indicator of increased risk for type 2 diabetes.

To date, most studies have focused on identifying potential epigenetic markers in newborns through cord blood samples. However, the use of saliva samples offers several advantages, including the ability to conduct longitudinal follow-ups and improved applicability in clinical practice due to their non-invasive collection method and ethical suitability for paediatric research. Increasing evidence supports the use of saliva-based methylation studies, with established links between DNA methylation patterns and maternal BMI, gestational glucose levels, and various metabolic parameters [44–46]. The correlations observed in our study between CpG sites and child anthropometric measures underscore the potential of saliva-derived epigenetic markers as accessible indicators of growth patterns, supporting their utility in non-invasive metabolic health monitoring.

Our study presents several strengths that contribute to its significance and potential clinical applicability. Firstly, the use of saliva samples provides a minimally invasive method for analyzing DNA methylation patterns, which is particularly advantageous for longitudinal studies in pediatric populations. This approach also enhances ethical considerations and facilitates the collection of samples over time, allowing for the monitoring of stable epigenetic markers. Secondly, our longitudinal design enables the assessment of DNA methylation changes across critical early life stages, revealing several persistent epigenetic differences between children of mothers with and without GDM, thus highlighting potential biomarkers of early metabolic risk. Importantly, the association of these methylation sites with anthropometric measures, such as weight, height, and BMI, underscores the potential of these markers to serve as indicators of growth patterns. Additionally, by focusing on an underexplored sample type—saliva—our study fills a gap in the literature, as most prior research has been limited to cord blood, placental, or maternal blood samples.

This study also presents some limitations. Due to the exploratory nature of our study and its small sample size, we used uncorrected p-values in our analysis. As a result, we acknowledge that the findings should be interpreted with caution. These results require replication in larger and independent cohorts to confirm their robustness and applicability. We encourage other researchers to further explore the associations identified in our study.

In conclusion, our study identifies a different DNA methylation pattern between children born to mothers with GDM and non-GDM across time, in saliva samples. Some of these epigenetic marks.

are associated with key anthropometric measurements, such as weight, height, head circumference, annual growth and BMI, indicating potential use as biomarkers risk of childhood obesity in the future. Notably, this is one of the few studies to analyze DNA methylation over time using saliva samples, which offer an accessible, non-invasive method for longitudinal studies.

The findings contribute to a growing body of evidence supporting the utility of saliva samples in epigenetic research and underscore their potential as non-invasive biomarkers for monitoring the developmental impact of maternal GDM and other prenatal exposures.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

TML-P: methodology, analysis, and wrote original draft; A-LJ: collected anthropometric variables and reviewed the results and final version of the manuscript; A-PL: contributed to the manuscript; M-P: collected sample from EPIDG cohort; M-SA: involved in data collection; MM-V: EPIDG funding acquisition, acquired data from the patients and collected samples; MJP: EPIDG funding acquisition and acquired data from the patients and collected samples; SM: EPIDG funding acquisition, designed the study, interpretation of the data and has substantively revised the work and she is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests
The authors declare no competing interests.

Institutional Review Board Statement
The study was approved by the Institutional Review Board at the Hospital Universitario Virgen de la Victoria de Málaga, Spain, in accordance with the Declaration of Helsinki. The legal guardians are Dr. Sonsoles Morcillo and Dr. María José Picón.

Informed consent
Informed consent was obtained from all subjects.

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