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Association of chemerin gene promoter methylation in maternal blood and breast milk during gestational diabetes

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Original Article

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Abstract

The intrauterine environment and early-life nutrition are regulated by maternal biomarkers in the blood and breast milk. We aimed to explore epigenetic modifications that may contribute to differential chemerin expression in maternal plasma, colostrum, and breast milk and find its association with fetal cord blood and infant weight at 6 weeks postpartum. Thirty-three gestational diabetes mellitus (GDM) mothers and 33 normoglycemic mothers (NGT) were recruited. Two maternal blood samples (28th week of gestation and 6 weeks postpartum), cord blood, colostrum, and mature milk were collected. Methylation-specific polymerase chain reaction and enzyme-linked immunosorbent assay were conducted. The weight of the babies was measured at birth and 6 weeks postpartum. Serum chemerin levels at the 28th gestational week and 6 weeks postpartum were significantly lower for the NGT group as compared to the GDM group; ($P < 0.05$). Higher colostrum chemerin concentrations were observed in the GDM group and remained elevated in mature milk as compared to NGT ($P < 0.05$). Colostrum and breast milk chemerin levels showed an independent association with infant weight at 6 weeks postpartum ($r = 0.270$; $P = 0.034$) ($r = 0.464$; $P < 0.001$). Forty percent GDM mothers expressed unmethylated chemerin reflecting increased chemerin concentration in the maternal blood. This pattern was also observed in newborn cord blood where 52% of samples showed unmethylated chemerin in contrast to none in babies born to normoglycemic mothers. The results of this study highlight the critical importance of altered chemerin regulation in gestational diabetic mothers and its effect during early life period and suggest a possible role in contributing to childhood obesity.

Introduction

The first 1000 days of life are very critical in the pathophysiological development of future disease states in an individual, including metabolic syndrome (MetS), diabetes mellitus (DM), and cardiovascular diseases, and therefore warrant adequate attention.[1] Placenta has been a subject of many studies as it gives the information regarding infant intrauterine experiences and conditions in situ. Large placental weight has been observed to correlate with increased blood pressure in both childhood[2] and adulthood,[3] while smaller placental weight has been linked to increased prevalence of DM.[4] Hormones and biomolecules that exist in maternal plasma may reach the child via the placenta or breast milk and thus could potentially regulate gene expression leading to childhood obesity. Higher maternal blood glucose as seen in gestational diabetes mellitus (GDM) is transported via placenta to the fetal circulation. The glucose either gets utilized as energy source or is up taken by fetal tissues for storage. Furthermore, excess glucose availability triggers fetal hyper-insulinaemia. Insulin's dual role as a growth factor and anabolic hormone in turns triggers the excess adipose tissue deposition in fetus. This whole sequence is known as "exaggerated fetal glucose steal phenomenon."[5,6] The severity of this cannot be undermined, as obesity can have harmful effects on a child's physical health, social, and emotional well-being, and self-esteem. Damage to physical health by obesity can manifest as metabolic, cardiovascular, orthopedic, neurological, hepatic, pulmonary, or renal disorders,[7] specifically MetS and DM.[8] In order to control this epidemic, its vital to understand genetic and environmental influences.[9] This study focuses on the effects of maternal gestational diabetes on intrauterine programming of fetal genes, nutrition provided by the placenta, and later, the breast milk.

One possible biomarker that could play a role in the development of childhood obesity is a 14-kDa protein called chemerin. Interestingly, chemerin is produced and secreted by the placenta,[10] as well as by other body tissues. Chemerin was initially known for its chemoattractant

properties. Later, more functions were identified, highlighting chemerin's adipokine activity and its contributions to obesity along with glucose and lipid metabolism.[11] To date, several studies have proposed a link between high chemerin levels and disease states such as MetS, DM, GDM, and increased body fat content.[12–15] The fact that high chemerin levels and childhood obesity are similarly associated with MetS invites novel exploration into chemerin's spectrum during pregnancy and lactation. During pregnancy, chemerin mediates the blood glucose level and regulates lipid metabolism.[16,17] Studies by Li et al. and Fatima et.al showed that chemerin was found to be significantly higher in GDM mothers when compared to control group.[18,19] On the other hand, Pfau et al. reported that chemerin levels were similar in both GDM and normoglycemic (NGT) women yet were exclusively related to insulin resistance.[20] Human milk is not only a source of nutrition but a consolidation of biological factors which reflect short- and long-term advantages on fetal/infant viability and health.[21] Literature suggest that diabetes can alter the composition of breast milk, and highest amounts of chemerin have been documented in colostrum and lowest in mature milk.[22] Any imbalance in the levels of this adipokine, maternal plasma, or breast milk during the critical developmental period of a child's life may predispose to childhood obesity and MetS (Fig. 1).

The involvement of epigenetic modifications, such as DNA methylation, in the development of obesity is becoming more and more evident. The influence of early nutrition starting from the embryonic–fetal period until the perinatal period of development plays a key role in the epigenetic programming of all human organs and tissues.[23] For any gene, DNA methylation occurs on position 5 of the pyrimidine ring of cytosine bases that are followed by guanine bases that are clustered together as CpG islands. Methylation of these CpG islands in the promoter region represses gene transcription and is an element of gene expression control.[24] Though an animal model suggested that chemerin DNA methylation was negatively correlated with chemerin mRNA concentration in adipose tissue[25] thus modulating the circulatory levels, the effect in humans is still unknown. For the first time, in this study, we aimed to examine any epigenetic modifications that may contribute to differential chemerin expression in maternal plasma, colostrum, and breast milk and its association with fetal cord blood and infant weight at 6 weeks postpartum.

Methodology

Recruitment

In this case control study, 167 pregnant women were recruited in the second trimester of pregnancy and followed up to 6 weeks postpartum at the Aga Khan University Hospital, Karachi. Out of these 66 pregnant women [$n = 33$ with GDM and $n = 33$ with NGT] completed the full duration of study (Fig. 2). Subjects with preexisting diabetes, hypertension, endocrinological disorders, with twin pregnancies or assisted conception, used bottle feed [$n = 22$] or who were lost to follow-up [$n = 79$] were excluded from this study. The study was approved by the institutional ethical review board of Aga Khan University (approval numbers 4211-BBS-ERC-16 and 4951-BBS-ERC-17). All subjects gave written informed consent. All experiments were performed following relevant guidelines and regulations according to the principles expressed in the Declaration of Helsinki. Participants included had the age range of 18–40 years. GDM group was identified using the International Association of the Diabetes and Pregnancy Study (IADPSG) guidelines. After 75 grams of glucose load, a 1-h oral glucose tolerance test, that is, a fasting glucose ≥92 mg/dL (5.1 mmol/L) and/or 1 h: ≥180 mg/dL (10 mmol/L) and/or 2 h: ≥153 mg/dL (8.5 mmol/L) (when any of the above plasma glucose values are exceeded, the subject was classified as having GDM) was performed between 24 and 28 weeks of gestation. All included subjects delivered at term (elective cesarean sections) and exclusively breastfed their babies until 6 weeks postpartum. The GDM women were either put on dietary modifications ($n = 22$) or were given insulin ($n = 4$) or metformin ($n = 7$). Dietary modifications were advised by the registered dietitian/nutritionist based on individual assessments and requirements.

Biophysical assessment

Serial weight assessment of the mothers was recorded at the time of the first visit (12–15 weeks of gestation), at the time of the glucose tolerance test (28 weeks of gestation), and 6 weeks postpartum. The South Asian reference range for body mass index (BMI) was used to categorize obesity status.[26] The gestational age was assessed by ultrasonography (24–28 weeks of gestation). Clinical data regarding maternal age, height, and a family history of diabetes and weight of the newborn were obtained from the patient's medical record cards.

Blood sample collection

At 28th week of gestation, 5-ml maternal venous blood was drawn. A second set of blood sample was collected at 6th week postpartum. In addition, 5 ml of arterial cord blood sample was collected at the time of delivery before separation of placenta. The blood samples were centrifuged, and plasma and buffy coat were extracted and saved at −80°C.

Breast milk collection

Within 72 h of delivery, 7 ml of breast milk sample namely colostrum was collected from mothers in sterilized tubes with a manual breast pump after an overnight fast. The second set of mature milk sample (15 ml) was collected at 6 weeks postpartum. Each of the samples was collected during a single milking procedure in the morning at 9 am, by emptying a single breast. The specimens were immediately stored at −20°C until analysis. Milk samples were processed according to a validated protocol described previously in literature.[27] Briefly, the breast milk fatty layer and cellular elements were removed by two centrifugations, at 680 g for 10 min at 4°C, after which the supernatants were removed, and then at 10,000 g for 30 min at 4°C. The resulting translucent whey was used for analysis. All assays were carried out within 2 months of storage.

Biochemical and molecular biology assays

Blood glucose and inulin levels chemerin levels were analyzed by commercially available ELISA kit (Kit Cat number 11406, Glory Science Co Ltd, USA). A spike-and-recovery experiment was designed to assess the difference in assay response according to a set published protocol and a recovery rate between 93% and 98%.[28] Arterial cord blood and maternal samples were used for DNA extraction on peripheral blood mononuclear cells using DNeasy Blood & Tissue Kit (Qiagen Cat No./ID: 69504) by following the manufacturer's guidelines. DNA concentrations were measured and 1 μg of DNA was used for bisulfite modification using EpiTect Fast Bisulfite Conversion Kit (Qiagen Cat No./ID: 59826) according to the manufacturer's instructions. Bisulfite-treated

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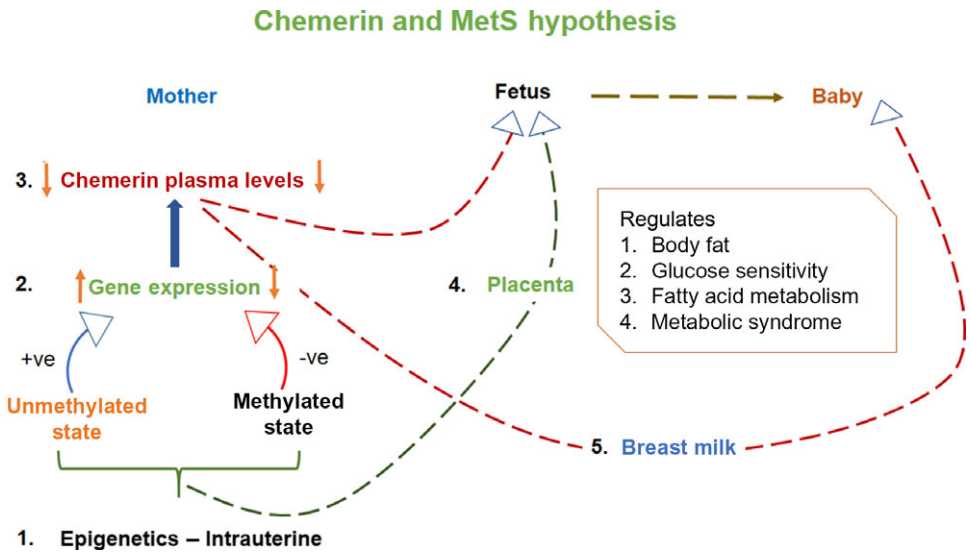

Fig. 1. The hypothesis of the mechanism of action of intrauterine programming on fetal growth, health, and disease predisposition; metabolic syndrome (MetS).

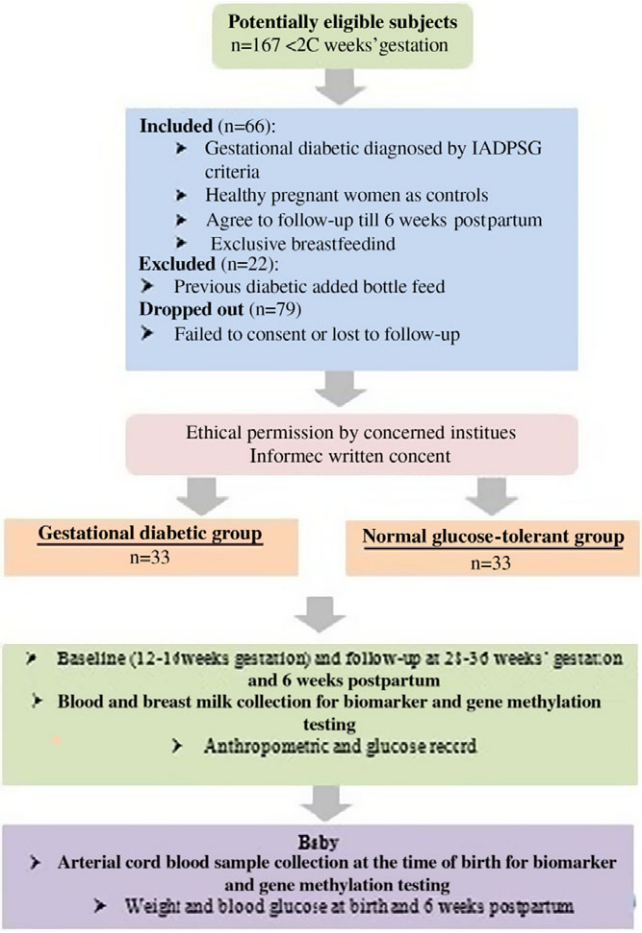

Fig. 2. Methods flow diagram.

DNA was immediately stored at −20°C. The qualitative methylation status of the chemerin gene was analyzed by methylation-specific polymerase chain reaction (MS-PCR) using specifically designed primers for MS-PCR according to a set protocol as described previously (Immunotargets Ther. 2019; 8: 29–41), and EpiTect MSP Master Mix (Qiagen Cat No./ID: 59305) was used for the reactions. The amplified PCR products were electrophoresed on 2% agarose gels and evaluated under ultraviolet light (Supplementary Figure S1). Twenty percent of the samples were randomly selected for repeat PCR experiments to validate the results by an independent researcher.

Statistical analysis

Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS version 21; IBM Corp Inc., Armonk, NY). Descriptive analysis of continuous variables was expressed as mean ± standard deviation, except for chemerin levels which were expressed as mean ± standard error of the mean. Paired *t*-test was used for comparing variables. GraphPad Prism software was used to generate scientific graphs. Correlations between chemerin level and infant weight were explored by Pearson's correlation coefficients and adjusted for maternal weight and age. In all calculations, a *P*-value of <0.05 was considered significant.

Results

The detailed results are shown in Tables 1–3, and Fig. 3 shows the methylation status of the study subjects. Higher rates of unmethylated chemerin gene were observed in gestational diabetic mothers and the cord blood of their newborns ($n = 12$ and $n = 18$) in comparison to NGT mothers and their babies ($n = 7$ and $n = 0$, respectively) (Table 2). This translated to less serum chemerin at the 28th week for the NGT group as compared to the GDM group (11.17 ± 0.48 versus 86.42 ± 7.43 ng/L, respectively). The cord blood chemerin levels were also higher in GMD babies (11.06 ± 2.86 ng/L) versus NGT babies (50.73 ± 11.67 ng/L). A similar trend was observed at 6 weeks postpartum in the NGT group when compared to that of GDM mothers (14.79 ± 1.01 versus 64.60 ± 6.17 ng/L, respectively). When we compared the chemerin levels in colostrum and breast milk, a higher concentration of chemerin was observed in GDM cases (125.34 ± 15.88 ng/L) as compared to NGT mothers (24.97 ± 2.58 ng/L). An interesting finding was that chemerin levels dropped in the breast milk samples at 6 weeks postpartum in NGT mothers (20.71 ± 2.36 ng/L) but remained elevated in GDM mothers (177.40 ± 22.49 ng/L) (Fig. 3). The weight of the babies, both at delivery and the

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Table 1. Biophysical and biochemical data of study subjects

Variables	NGT $n = 33$ Mean ± SD	GDM $n = 33$ Mean ± SD	*P*-value
Maternal data			
Age (year)	24.10 ± 1.77	24.23 ± 1.14	0.412
BMI (kg/m2)	20.55 ± 3.76	25.22 ± 4.24*	<0.001
RBG – 6 weeks postpartum (mg/dl)	107.04 ± 19.45	144.00 ± 3.66*	<0.001
HbA1c – 6 weeks postpartum (mg/dl)	5.5 ± 0.35	6.61 ± 0.92*	<0.05
Mature milk glucose (mg/dl)	24.53 ± 6.44	33.33 ± 2.34*	<0.05
Newborn data			
Fetal weight by scan (kg) at 28 weeks of gestation	1.98 ± 0.54	2.28 ± 0.59*	0.008
Baby gender	Male: 19	Male: 16	0.547
	Female: 14	Female: 17	
Baby weight at birth (kg)	3.53 ± 0.51	4.41 ± 0.97*	<0.001
Baby weight at 6 weeks (kg)	4.35 ± 0.40	5.53 ± 0.15*	<0.001
RBG – POCT (mg/dl)	91.14 ± 7.33	98.55 ± 5.77	NS
Arterial cord blood chemerin (ng/L)	11.06 ± 2.86	50.73 ± 11.67*	0.004

BMI, body mass index; RBG, random blood glucose; POCT, point-of-care testing. Values expressed as mean and standard deviation.
*significant at the 0.05 level.

Table 2. Methylation status of chemerin promoter region in our study subjects

	Methylated	Unmethylated	Both
NGT control mother	24 (72.27%)	7 (21.21%)	2 (6.06%)
NGT control child	27 (81.18%)	0	6 (18.18%)
GDM mother	2 (5.3%)	12 (36.8%)	19 (57.9%)
GDM child	10 (31.6%)	18 (52.6%)	5 (15.8%)

GDM, gestational diabetes mellitus; NGT, normoglycemic.
Data presented as absolute numbers and percentages in parenthesis.

follow-up visit, was higher for the GDM group ($P < 0.01$) (Table 1). Chemerin levels showed significant moderate positive association with maternal weight ($r = 0.574$, $P < 0.001$) and blood glucose (0.541, $P =$ <0.001). Furthermore, chemerin levels in both the colostrum and breast milk showed an independent positive association with infant weight at 6 weeks postpartum ($r = 0.270$, $P = 0.034$ and $r = 0.464$, $P < 0.001$, respectively) when adjusted for maternal BMI (Table 3).

Discussion

Literature supports the imprint of parental nutrition and diet on metabolic phenotypes in the offspring.[29] There is, however, much to be learned about the role of the intrauterine environment and early-life nutrition in the development of childhood obesity. Genomic imprinting determines the expression of alleles. Failure of proper imprinting might result in obesity by altering the expression of growth and cellular differentiation factors, causing a developmental programming of obesity.[30] This could arise due to numerous epigenetic events such as DNA hyper-/hypo-methylation

The Developmental Origins of Health and Disease (DOHaD) hypothesis narrates that adverse exposures at critical points of development may affect function as well as structure of an organ system in adulthood.[30] The review summarized changes in DNA methylation and microRNAs identified in blood cells and different tissues in obese human and rodent models. It includes information on epigenetic alterations which occur in response to fat-enriched diets.[31] Many studies focused on the hypothesis that early-life environmental factors, including placental nutrition and breast milk, induce epigenetic variation, thereby permanently affecting the metabolism and influencing chronic disease risk. Specifically, for obesity, it has been observed that obese mothers tend to have obese children.[32] Further, it has been shown that clinical intervention to cause maternal weight loss can have a positive effect on reducing the risk of obesity in the offspring.[33] The mechanisms by which nutritional challenges affect the risk of disease in later life are poorly understood. In this study, we showed that chemerin was unmethylated in the GDM group, suggesting its gain of function, which ultimately affected the babies born to those mothers. These potential interactions between the environment and epigenetic mechanisms mediating the expression of the chemerin gene might be associated with increased adiposity.

Adipose tissue is considered an endocrine organ, with the production and release of pro-inflammatory markers under the influence of adipokines. Genetic and environmental factors can impact both the number and volume of adipocytes throughout the multiple phases of growth.[34] The first year of life constitutes one of these phases,[35] where adipokines can determine lifetime risk of obesity and its associated complications including MetS and diabetes. Chemerin has been associated with both obesity and MetS in children, and weight-loss interventions have shown to decrease chemerin levels in small studies.[36] Our study supports this knowledge and shows that the expression of chemerin in the placenta and breast milk has a role to play in determining early adiposity during infancy.

We reported that not only do chemerin levels vary in maternal plasma during pregnancy, but chemerin also gets secreted into colostrum and breast milk and is associated with increased infant

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Table 3. Correlation of chemerin with maternal and infant body weight

	Unadjusted *r*					Adjusted *r*(maternal age and weight)	
	Maternal			Baby			
	Pre-pregnancy weight	Postpartum weight	Postpartum RBG	Weight at birth	Weight at 6 weeks	Weight at birth	Weight at 6 weeks
Serum chemerin at 28 gestational week(ng/L)	0.574**	0.467**	0.541**	0.473**	—	0.369**	—
Serum chemerin at 6 weeks postpartum (ng/L)	0.562**	0.442**	0.508**	0.522**	—	0.433**	—
Colostrum chemerin (ng/L)	—	—	—	—	0.347**	—	0.270*
Breast milk chemerin (ng/L)	—	—	—	—	0.555**	—	0.464**

RBG, random blood glucose level.
**Correlation is significant at the 0.01 level (two-tailed).
*Correlation is significant at the 0.05 level (two-tailed).

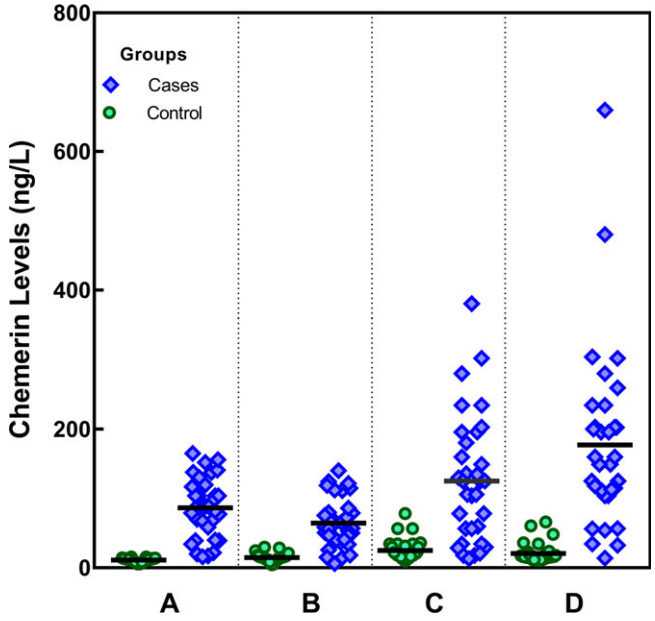

Fig. 3. (A–D) Comparison of maternal serum chemerin levels at 28th week of gestation (A), 6 weeks postpartum (B), colostrum (C), and breast milk (D). A remarkable rising trend is observed in chemerin levels in all categories for GDM women. Black bar shows the mean values.

weight at 6 weeks postpartum. A higher concentration of chemerin in colostrum of GDM cases without any drop in breast milk at 6 weeks postpartum in comparison to NGT cases and a positive association with infant weight supports the expression of chemerin in breast milk and its role in determining early adiposity during infancy. Previously, higher levels of chemerin levels were found in the milk and blood samples of gestational diabetic mothers which is likely due to an increase in inflammation as a compensatory mechanism to protect both the mother and the offspring.[22,37] It could be possible that the most likely function of chemerin in human milk is to strengthen the innate immune system in addition to regulate adipogenesis for the newborn.[38] Therefore, the newborn might be protected from infections and is prepared for adverse environmental conditions upon birth. Interestingly, a study by Mazaki-Tovi et al. also reported the presence of chemerin in cord blood samples and its association with infant birth weight.[39] On the other hand, Ustebay et.al showed no difference in breast milk levels of chemerin in a Turkish population.[22] Chemerin levels in plasma and breast milk, measured at all points in time in this study were raised in GDM mothers and were positively related to maternal BMI and blood glucose level. This difference could be due to the ethnic variations and dietary preferences in our population. In normal glucose-tolerant females, chemerin levels increased only slightly during the gestational period and decreased postpartum. Our findings can be linked to other pregnancy studies conducted on human and animal models, which reported a rise in chemerin levels in the later stages of gestation compared to early gestation periods and nonpregnant states.[10,13,40] Furthermore, previous studies showed that despite a decrement in chemerin levels postpartum, they do not revert to the original pre-pregnancy value,[41] a pattern which was also observed in our study. It is interesting to note that GDM was also related to increased baby weight at 6 weeks postpartum: this finding also agrees with the results of previous studies.[42,43]

A possible mechanistic explanation for chemerin's link with higher baby weight is its role in stimulating food intake and decreasing energy expenditure in the hypothalamus.[44] A recent study showed that chemerin infusion in animals prompts a rise in food consumption. This effect may be a complex indirect association with hypothalamic mechanisms and neuroendocrine pathways. A more tempting possibility is that chemerin exerts its effects through cellular remodeling. Furthermore, studies show that *RARRES2* (the gene encoding chemerin) has a role in the regulation of food intake and body weight in seasonal animals.[45]

A limitation of our study is the lack of reports of maternal diet, a relatively small sample size and not being able to do a full adipokine spectrum on the milk and cord blood samples. Furthermore, we were unable to record or keep track of baby weight-for-length z-score. Yet, this study gives a slight insight that early-life nutrition environment may have a lifelong effect on gene expression DNA methylation, expression of miRNAs, and epigenetic changes and may thus cause susceptibility to complex diseases in adulthood.[29] The intrauterine nutrient supply may alter DNA methylation patterns and, consequently, gene expression and organism development. This is one of the mechanisms explaining the fetal programming phenomenon. Our findings support the role of breast milk chemerin in fetal growth and metabolic reprogramming. Yet, further studies are underway to provide more evidence for a direct causal relationship between intrauterine environment and role of high levels of breast milk chemerin in newborn weight gain and childhood obesity.

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Conclusion

High levels of chemerin in serum and colostrum have been reported in GDM women which positively related with weight gain of babies. A rising trend in plasma chemerin, cord blood, colostrum, and breast milk in GDM women is reported, which further suggests that higher adipocytokines may control the mechanisms that influence infant weight gain, food intake, and modulation from the time of conception till the breastfeeding period. In addition to that, epigenetic changes reflected by the methylation status of chemerin promoter region during the intrauterine period caused weight gain in these females which may predispose them to metabolic syndrome. For the first time, our study has shown a critical relevance of methylation and expression of chemerin on early days of human development and suggests its contribution to childhood obesity.

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Conflict of interest. The authors declare no conflict of interest.

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