

# DNA methylations of MC4R and HNF4 $\alpha$ are associated with increased triglyceride levels in cord blood of preterm infants

Eun Jin Kwon, BS<sup>a,b</sup>, Hye Ah Lee, PhD<sup>c</sup>, Young-Ah You, PhD<sup>d</sup>, Hyesook Park, MD, PhD<sup>c</sup>, Su Jin Cho, MD, PhD<sup>e</sup>, Eun Hee Ha, MD, PhD<sup>b</sup>, Young Ju Kim, MD, PhD<sup>a,\*</sup>

## Abstract

The association of preterm birth with obesity and metabolic syndrome later in life is well established. Although the biological mechanism for this association is poorly understood, epigenetic alterations of metabolic-related genes in early life may have important roles in metabolic dysfunction. Thus, we investigated the associations of DNA methylations of melanocortin 4 receptor (MC4R) and hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ) with metabolic profiles in cord blood of term and preterm infants.

We measured metabolic profiles in cord blood samples of 85 term and 85 preterm infants. DNA methylation and mRNA expression levels of MC4R and HNF4 $\alpha$  in cord blood cells were quantified using pyrosequencing and real-time PCR. Triglyceride (TG) levels were grouped by percentile as low (<10th percentile), mid (11th–89th percentiles), and high (>90th percentile). A multiple linear regression model was used to assess the differential effects of DNA methylation on metabolic indices in cord blood between term and preterm infants.

The beta-coefficients for associations between TG levels and methylation statuses of MC4R-CpG3 and HNF4 $\alpha$ -CpG2 in the P1 promoter differed significantly between term and preterm infants ( $P=0.04$  and  $P=0.003$ , respectively). DNA methylation statuses of MC4R-CpG3 and HNF4 $\alpha$ -CpG2 in the P1 promoter were significantly lower in preterm infants in the high-TG group compared with those in the mid- and low-TG groups ( $P=0.01$ ). Notably, preterm infants in the high-TG group had higher TG levels in cord blood than term infants in the high-TG group (60.49 vs 54.57 mg/dL). In addition, MC4R and HNF4 $\alpha$  expression levels were higher in preterm infants than in term infants ( $P<0.05$ ).

Epigenetic alterations of the newly identified genes MC4R and HNF4 $\alpha$  in early life might contribute to metabolic profile changes, especially increased TG levels, in the cord blood of preterm infants.

**Abbreviations:** AS = Apgar score, GA = gestational age, HDL-c = high-density lipoprotein cholesterol, HNF4 $\alpha$  = hepatocyte nuclear factor 4 alpha, HOMA-IR = homeostasis model assessment of insulin resistance, LDL-c = low-density lipoprotein cholesterol, LMP = last menstrual period, MC4R = melanocortin 4 receptor, PTB = preterm birth, T2D = type 2 diabetes, TC = total cholesterol, TG = triglyceride, TSS = transcription start site.

**Keywords:** cord blood, DNA methylation, hepatocyte nuclear factor 4 alpha, melanocortin 4 receptor, metabolic indices, preterm infants, triglyceride

Editor: Bing-Fang Hwang.

**Funding/support:** This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2004833) and Ministry of Health & Welfare, Republic of Korea (H14C0306) through the Korea Health Industry Development Institute (KHIDI).

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

<sup>a</sup>Department of Obstetrics and Gynecology, <sup>b</sup>Department of Occupational and Environmental Medicine, <sup>c</sup>Department of Preventive Medicine, <sup>d</sup>Medical Research Institute, <sup>e</sup>Department of Pediatrics, School of Medicine, Ewha Womans University, Seoul, Republic of Korea.

\*Correspondence: Young Ju Kim, Department of Obstetrics and Gynecology, School of Medicine, Ewha Womans University, 1071 Anyangcheon-ro, Yangcheon-ku, Seoul 158-710, Republic of Korea (e-mail: kkyj@ewha.ac.kr).

Copyright © 2016 the Author(s). Published by Wolters Kluwer Health, Inc. All rights reserved.

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

Medicine (2016) 95:35(e4590)

Received: 17 February 2016 / Received in final form: 11 July 2016 / Accepted: 22 July 2016

<http://dx.doi.org/10.1097/MD.0000000000004590>

## 1. Introduction

Preterm birth (PTB; <37 weeks gestation) is a major cause of neonatal death and has long-term adverse effects on health.<sup>[1]</sup> About 28% of early neonatal mortality has been reported to result from PTB.<sup>[1,2]</sup> The rate of PTB increased from 2.6% in 1993 to 5.9% in 2010 in Korea,<sup>[3]</sup> and rates are reportedly increased rapidly in developing countries.<sup>[4]</sup> Children born preterm may have a higher risk of metabolic abnormalities associated with type 2 diabetes (T2D) and cardiovascular disease later in life.<sup>[5,6]</sup> Changes in lipid metabolism, such as elevated triglyceride (TG) and total cholesterol (TC) levels, in early life may lead to metabolic dysfunction and disease susceptibility.<sup>[7]</sup> Although the molecular mechanisms underlying the associations remain unknown, epigenetics has recently emerged as a key factor contributing to the development of chronic diseases.<sup>[8]</sup>

Epigenetics can affect gene activity independent of the DNA sequence and is crucial for development and cellular differentiation.<sup>[9,10]</sup> Epigenetic alterations induced by environmental stimuli may influence gene expression and may be transmitted across generations, although the mechanisms of epigenetic inheritance remain unclear.<sup>[7,11,12]</sup> Some of these epigenetic changes appear to be relatively stable and persist throughout life, but others are potentially reversible.<sup>[13]</sup> Subtle differences in

DNA methylation patterns of some genes associated with gestational age (GA) at birth (e.g., *DUOX2*, *TMEM176A*, and *CASP8*) may influence long-term health outcomes.<sup>[14,15]</sup> Moreover, exposure to prenatal famine was found to lead to altered DNA methylation of genes associated with growth and metabolism (e.g., *INSR* and *CPT1A*).<sup>[16]</sup>

Several previous studies have showed that melanocortin 4 receptor (*MC4R*), which controls the appetite, and hepatocyte nuclear factor 4 alpha (*HNF4α*), which regulates lipid homeostasis, are involved in the development of obesity and metabolic-related disorders.<sup>[17–21]</sup> *MC4R* signaling regulates insulin sensitivity and glucose utilization.<sup>[17]</sup> In addition, *MC4R* activity contributes to increased fat mass by increasing the synthesis of TG and the assembly and secretion of hepatic lipoprotein in the liver.<sup>[18]</sup> Recently, decreased methylation of *MC4R*, with corresponding increases in gene expression, was found to lead to obesity in mice fed a high-fat diet.<sup>[19]</sup> *HNF4α* affects glucose and lipid metabolism and contributes to the regulation of pivotal metabolic processes in the liver.<sup>[20]</sup> *HNF4α* alteration induces impaired insulin signaling, resulting in increased lipid profiles.<sup>[21]</sup> In animals, maternal chronic exposure to a high-fat diet induced increased *HNF4α* expression in the fetal liver and contributed to lipid accumulation.<sup>[22]</sup> In humans, differences in DNA methylation patterns of *HNF4α* in response to the intrauterine environment have been identified between twins with and without T2D.<sup>[23]</sup> Most PTB studies have analyzed DNA methylation using cell-free DNA, which can enable the discovery of biomarkers for prediction due to its accessibility and reliability.<sup>[24,25]</sup> An epigenome-wide association study identified some differentially methylated loci, including the previously known *HNF4α*, in cord blood of infants with intrauterine growth restriction.<sup>[26]</sup> On the basis of this evidence, *MC4R* and *HNF4α* are plausible biological candidate genes underlying the associations between PTB and metabolic-related disorders.

To our knowledge, no study has found a link between epigenetic alterations of metabolic-related genes and metabolic indices in cord blood. Thus, we investigated the associations between DNA methylations of *MC4R* and *HNF4α* and metabolic indices in cord blood of preterm infants, compared with term infants.

## 2. Methods

### 2.1. Study population

This case-control study was conducted from 2007 to 2010 at Ewha Womans University Hospital, Seoul, Korea. Pregnant women who received prenatal examinations between 24 and 28 weeks of gestation at the hospital were recruited. The study population included women aged 20 to 42 years, with GAs of 25 to 42 weeks and no complication during pregnancy. A total of 85 preterm infants (GA < 37 weeks) were delivered at the hospital, and 85 term infants (GA 37–42 weeks) were selected randomly from women who delivered healthy infants during the same period as preterm infants. We excluded infants from multiple births and those with major congenital abnormalities or medical problems. All subjects provided clinical data and cord blood samples. Participants gave their written informed consent prior to enrollment in the study. This study was approved by the Institutional Review Board of the Ewha Womans University Hospital.

Trained nurses collected information regarding potential confounding factors, including maternal age, height, weight at

delivery, education, mode of delivery, GA, birth weight, height, ponderal index, and Apgar score (AS) 1 and 5 minutes after birth using medical charts. GA was calculated based on the onset of the last menstrual period (LMP). Ultrasonographic estimation of the GA was used when the LMP data were unreliable or when the GA determined by LMP and true fetal size differed by >10 days. The ponderal index was calculated as weight in kilograms divided by cube of height in meters (kg/m<sup>3</sup>). Maternal education level was dichotomized as high school (<12 years) and university or higher (≥12 years).

### 2.2. Biochemical assessments

Umbilical cord blood samples were collected into Vacutainer<sup>®</sup> tubes containing EDTA (BD Biosciences, San Jose, CA) immediately after delivery. The blood samples were centrifuged at 3000 rpm for 10 minutes, and the serum was stored at –80 °C until chemical analysis. Serum glucose, TG, TC, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were measured by automatic analyzer (Model 7180; Hitachi, Tokyo, Japan). The average intraassay coefficients of variation (CVs) and sensitivity limits for the glucose, TG, TC, HDL-c, and LDL-c assays were 1.15% and 2 mg/dL, 1.85% and 9 mg/dL, 1.43% and 4 mg/dL, 2.51% and 3 mg/dL, and 2.71% and 4 mg/dL, respectively. Insulin was analyzed using an immunoradiometric assay kit (Biosource Europe, Nivelles, Belgium) according to the manufacturer's instructions, with an average intraassay CV of 2.40% and a sensitivity limit of 0.2 μU/mL. Insulin resistance levels were determined by the widely used homeostasis model assessment of insulin resistance (HOMA-IR) method, which was calculated as follows: (plasma glucose [mmol/L] × insulin [μU/mL])/22.5.

### 2.3. DNA methylation analysis by pyrosequencing

Genomic DNA samples were isolated from whole cord blood using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. The total DNA concentration and purity were assessed by spectrophotometer (ND2000; Nanodrop Technologies, Wilmington, DE).

The promoter regions of the *MC4R* and *HNF4α* genes were amplified by a primer set designed using the PSQ Assay Design software (Biotage AB, Uppsala, Sweden) (Supplemental Digital Content-Table S1, <http://links.lww.com/MD/B242>). In *MC4R*, the 3 CpG sites are located in the promoter region up to 775 to 792 bp upstream of the transcription start site (TSS). The *HNF4α* gene has 2 promoters (P1 and P2). The 4 CpG sites in the P1 promoter region are located up to 88 to 115 bp, and the 4 CpG sites in the P2 promoter region are located up to 44,903 to 44,920 bp upstream of the TSS (Supplemental Digital Content-Figure S1 and Table S3, <http://links.lww.com/MD/B242>). Of these, the CpG2 site in the P2 promoter of *HNF4α* was almost always methylated (>99%). Thus, it was excluded from this study. Methodological details of DNA methylation analysis are reported in our previous study.<sup>[27]</sup>

### 2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from blood samples of term (n=38) and preterm (n=20) infants using the Easy-BLUE<sup>™</sup> total RNA extraction kit (iNtRON Biotechnology, Sungnam, Korea) according to the manufacturer's protocol. The concentrations and purities of extracted RNA samples were quantitated by

bioanalyzer (Agilent Technologies, Santa Clara, CA). Complementary DNA (cDNA) synthesis was performed using 1 µg RNA sample in a 25-µL reaction mixture containing 1 µL of 10 pM oligonucleotide primer, 5 µL 10 × RT buffer, 5 µL 2.5 mM dNTP, 1 µL 40 U RNase inhibitor, and 1 µL 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) for 60 minutes at 42 °C and 5 minutes at 95 °C. Quantitative real-time polymerase chain reaction (PCR) was carried out in a 20-µL reaction mixture containing 2 µL cDNA, 10 µL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1 µL each 10-pM primer, and 6 µL sterile water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control. Amplification was conducted under the following conditions: denaturation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minutes on an ABI PRISM 7000 sequence detection system (Applied Biosystems). All PCR reactions were performed in duplicate, and no template controls were included in any run. We then tested primer specificity by RT-PCR, confirmed using melting curve analysis. Comparative quantitation of each target gene was performed based on the cycle threshold (CT), which was normalized against the CT of GAPDH using the  $\Delta\Delta CT$  method. Primer sets and melting temperature for quantitative real-time PCR are described in Supplemental Digital Content-Table S2, <http://links.lww.com/MD/B242>.

## 2.5. Statistical analysis

Continuous variables with normal distributions were assessed using Student *t* test or analysis of variance, followed by the Bonferroni test, and were expressed as means and standard deviations (SD). Categorical variables with nonnormal

distributions were assessed using the Wilcoxon rank sum test and expressed as numbers and percentages. To satisfy statistical assumptions for normality, TG, TC, HDL-c, LDL-c, insulin levels, and HOMA-IR data were log transformed. The results for log-transformed data are presented as back-transformed means with 95% confidence intervals. DNA methylation levels of *MC4R* and *HNF4α* genes were rescaled using the means and SD to satisfy normality. TG levels were grouped by percentile as low (<10th percentile), mid (11th–89th percentiles), and high (>90th percentile), according to a previous study.<sup>[28]</sup>

To assess the significant differential effects of DNA methylation statuses of *MC4R* and *HNF4α* at each CpG sites on metabolic indices in cord blood between term and preterm infants, a multiple linear regression model was used. The model controlled for maternal height, weight at delivery, education, mode of delivery, GA, ponderal index, and 5-minute AS. The selected confounding variables were marginally related to DNA methylation levels or metabolic indices in all univariate analyses ( $P < 0.20$ ). Statistical analyses were conducted using the SAS software (ver. 9.3; SAS Institute Inc., Cary, NC). All analyses were 2-tailed, and  $P$  values  $< 0.05$  were considered to indicate statistical significance.

## 3. Results

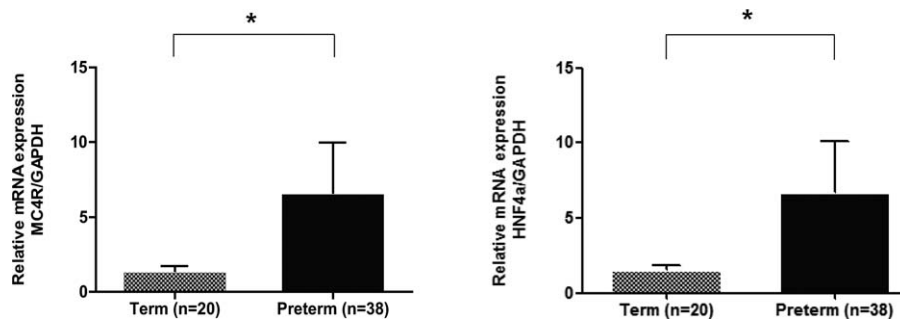
### 3.1. General characteristics of study subjects

Eighty-five term and 85 preterm infants were included in this study (Table 1). The 2 groups were similar in maternal age, gender, and parity. However, women who had preterm infants were markedly shorter ( $P = 0.04$ ) and had a higher occurrence of cesarean section ( $P < 0.001$ ) than women who had term infants.

**Table 1**  
Demographic characteristics of study subjects.

Characteristics	Term (N = 85)		Preterm (N = 85)		P
	Mean (SD)	N (%)	Mean (SD)	N (%)	
Mothers					
Age at delivery, years	31.64 (3.78)		32.09 (4.39)		0.47
Maternal height, cm	161.73 (4.62)		160.16 (5.39)		0.04
Weight at delivery, kg	67.21 (8.52)		68.58 (10.97)		0.36
Education, years					
≤12		21 (24.71)		31 (36.47)	0.10
>12		64 (75.29)		54 (63.53)	
Mode of delivery					
Vaginal		71 (83.53)		43 (50.59)	<0.001
C-section		14 (16.47)		42 (49.41)	
Infants					
Gender					
Male		51 (60.00)		53 (62.35)	0.75
Female		34 (40.00)		32 (37.65)	
Parity					
Nulliparous		46 (54.12)		44 (51.76)	0.76
parous		39 (45.88)		41 (48.24)	
Gestational age, weeks	39.30 (1.05)		33.91 (2.32)		<0.001
Birth weight, g	3249.29 (336.89)		2230.47 (606.02)		<0.001
Birth height, cm	49.86 (1.73)		44.47 (3.59)		<0.001
Ponderal index, kg/m <sup>3</sup>	26.25 (2.15)		24.91 (3.69)		<0.01
1-minute AS	9.33 (1.19)		7.75 (1.86)		<0.001
5-minute AS	9.86 (0.71)		9.09 (1.17)		<0.001

Continuous variables are expressed as mean (SD) and categorical variables are expressed as n (%).  $P$  values were calculated using Student *t* test or the Wilcoxon rank sum test. AS = Apgar score, C-section = caesarean section, SD = standard deviation.



**Figure 1.** The mRNA expression levels of the MC4R (A) and HNF4 $\alpha$  (B) genes in cord blood of preterm infants (n=20) and term infants (n=35). MC4R=melanocortin 4 receptor, HNF4 $\alpha$ =hepatocyte nuclear factor 4 alpha.

### 3.2. Quantitation of DNA methylation and mRNA expression levels in cord blood cells

We measured DNA methylation levels in cord blood cells at 3 CpG sites in the *MC4R* promoter, and at 7 CpG sites in the 2 *HNF4 $\alpha$*  promoter regions (Supplemental Digital Content-Table S3, <http://links.lww.com/MD/B242>). To compare differences in methylation patterns in cord blood cells between term and preterm infants, we analyzed the average DNA methylation levels of each CpG sites in the *MC4R* and *HNF4 $\alpha$*  genes. The median methylation status of the CpG2 and CpG3 sites located within the promoter of *MC4R* (*MC4R*-CpG2 and *MC4R*-CpG3, respectively) was significantly lower in cord blood cells of preterm infants compared with term infants ( $P < 0.01$ ). In addition, the median methylation status of *HNF4 $\alpha$*  at 4 CpG sites in the P1 promoter was significantly lower in cord blood cells of preterm infants ( $P < 0.01$ ), whereas the methylation status of the CpG sites within the P2 promoter was similar between the 2 groups (Supplemental Digital Content-Table S4, <http://links.lww.com/MD/B242>). The *MC4R* and *HNF4 $\alpha$*  promoter regions contain putative binding sites for transcription factors, including C/EBP alpha and SP1, which are involved in the regulation of lipid metabolism (Supplemental Digital Content-Figure S1, <http://links.lww.com/MD/B242>).<sup>[29,30]</sup> The expression levels of *MC4R* and *HNF4 $\alpha$*  genes were significantly increased in cord blood cells of preterm infants compared with term infants ( $P = 0.04$  and  $P = 0.048$ , respectively; Fig. 1).

### 3.3. Metabolic profiles in cord blood from term and preterm infants

Table 2 shows the average metabolic indices in cord blood of 2 groups. The mean TG level was significantly lower ( $P = 0.001$ ),

whereas the average TC, HDL-c, LDL-c levels, and HOMA-IR were significantly higher in cord blood of preterm infants compared with term infants ( $P < 0.05$ ). No significant difference in other metabolic indices, such as glucose and insulin levels, was observed between groups.

### 3.4. Associations between DNA methylation status of CpGs and metabolic indices in cord blood of term and preterm infants

To evaluate the effects of different DNA methylation patterns on metabolic indices between groups, we identified associations between methylation statuses of CpG sites in *MC4R* and *HNF4 $\alpha$*  and metabolic indices in cord blood of term and preterm infants. We selected 6 CpG sites and 5 metabolic indices that differed significantly between groups. The beta coefficients for associations between log-TG levels and methylation statuses of the *MC4R*-CpG3 and the CpG2 site located within the P1 promoter of *HNF4 $\alpha$*  (*HNF4 $\alpha$* -CpG2) in cord blood differed significantly between term and preterm infants ( $P = 0.04$  for *MC4R* and  $P = 0.003$  for *HNF4 $\alpha$* ; Fig. 2). These associations remained unchanged after adjusting for maternal height, weight at delivery, education, mode of delivery, GA, ponderal index, and 5-minute AS (Table 3).

### 3.5. DNA methylation levels of CpG sites in cord blood cells from term and preterm infants according to TG group

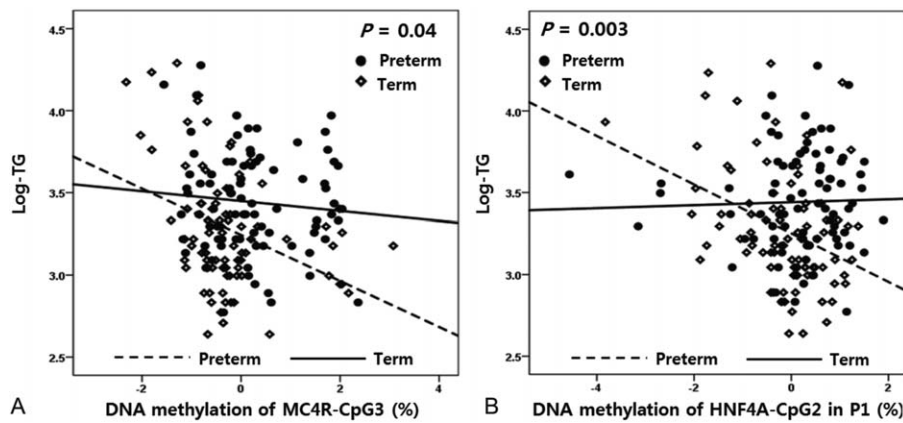
DNA methylation levels of *MC4R*-CpG3 and *HNF4 $\alpha$* -CpG2 in the P1 promoter differed significantly among preterm infants

**Table 2**  
Metabolic indices in cord blood of study subjects.

Metabolic indices	Term			Preterm			P
	Mean	SD	95% CI	Mean	SD	95% CI	
Glucose, mg/dL	67.35	26.68		68.61	23.16		0.75
TG, mg/dL*	31.25		29.19–33.45	26.55		24.50–28.78	0.001
TC, mg/dL*	61.20		58.07–64.50	75.35		70.86–80.14	<0.001
HDL-c, mg/dL*	20.70		19.10–22.43	23.17		21.38–25.12	0.04
LDL-c, mg/dL*	16.46		15.07–17.98	28.82		25.98–31.97	<0.001
Insulin, $\mu$ U/mL*	2.44		2.01–2.97	3.01		2.41–3.75	0.23
HOMA-IR*	0.36		0.29–0.46	0.50		0.38–0.65	0.05

TG, TC, HDL-c, LDL-c, insulin levels, and HOMA-IR were log-transformed. Glucose is expressed as mean  $\pm$  SD, with  $P$  value from Student  $t$  test. Log-transformed data (TG, TC, HDL-c, LDL-c, insulin, and HOMA-IR) are expressed as mean s with 95% confidence interval, with  $P$  values from the Wilcoxon rank sum test. TG=triglyceride, TC=total cholesterol, HDL-c=high density lipoprotein cholesterol, LDL-c=low density lipoprotein cholesterol, HOMA-IR=omeostasis model assessment of insulin resistance, SD=standard deviation, CI=confidence interval.

\*The results presented are back-transformed values of the means.



**Figure 2.** Association between methylation statuses of *MC4R* and *HNF4α* at CpG sites and metabolic indices in cord blood of preterm and term infants. DNA methylation of (A) *MC4R*-CpG3 and TG levels and (B) *HNF4α*-CpG2 in the P1 promoter and TG levels. Open lines (--) denote preterm infants and closed lines (—) denote term infants. *MC4R*=melanocortin 4 receptor, *HNF4α*=hepatocyte nuclear factor 4 alpha, TG=triglyceride.

**Table 3**

**Associations between methylation status of *MC4R* and *HNF4α* at CpG sites and metabolic indices in cord blood of term and preterm infants.**

DNA methylation site	Metabolic indices	Term	Preterm	P
		β (SE)*	β (SE)*	
MC4R-CpG 3	TG	-0.06 (0.03)	-0.14 (0.04)	0.048
HNF4α-CpG 2 in P1	TG	-0.03 (0.03)	-0.14 (0.05)	0.007

*MC4R*=melanocortin 4 receptor, *HNF4α*=hepatocyte nuclear factor 4 alpha, P=promoter region, TG=triglyceride, SE=standard error.

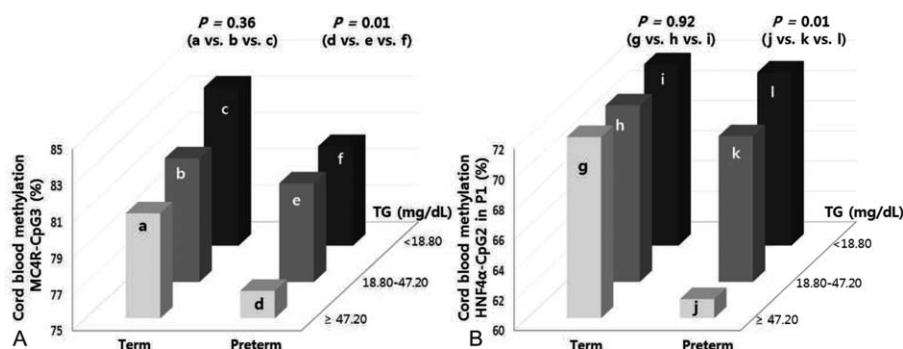
\*β (beta) and SE estimated by multiple linear regression adjusted for maternal height, weight at delivery, education, mode of delivery, gestational age, ponderal index, and Apgar score 5 minutes.

according to TG group ( $P=0.01$ ; Fig. 3). Methylation statuses of *MC4R*-CpG3 and *HNF4α*-CpG2 in the P1 promoter were significantly lower in preterm infants in the high-TG group than in those in the low-TG group ( $P=0.04$  for *MC4R* and  $P=0.01$  for *HNF4α*). In addition, methylation statuses of *MC4R*-CpG3 and *HNF4α*-CpG2 in the P1 were significantly lower in preterm infants in the high-TG group compared with the mid-TG group (both  $P=0.01$ ). Notably, preterm infants in the high-TG group were found to have higher TG levels in cord blood than term infants in the high-TG group (60.49 vs 54.57 mg/dL; Table 4). These preterm infants also had significantly lower GA, birth

weight, and height than term infants of the high-TG group ( $P < 0.001$ ). No significant difference in cord blood of term infants was observed according to TG group, except TG level.

#### 4. Discussion

This study is the 1st to investigate the associations between DNA methylation statuses of metabolic-related genes and metabolic profiles in cord blood. Our findings showed that DNA methylations of *MC4R* and *HNF4α* were associated significantly with TG level in cord blood of preterm infants compared with



**Figure 3.** DNA methylation status by TG group of *MC4R*-CpG3 (A) and *HNF4α*-CpG2 (B) in the P1 promoter in cord blood cells from term and preterm infants. Preterm infants in the high-TG group show the lowest methylation levels of *MC4R*-CpG3 and *HNF4α*-CpG2 in the P1 promoter. *P* values were calculated using the Bonferroni post-hoc test. a: High-TG group of term infants in A, b: mid-TG group of term infants in A, c: low-TG group of term infants in A, d: high-TG group of preterm infants in A, e: mid-TG group of preterm infants in A, f: low-TG group of preterm infants in A, g: high-TG group of term infants in B, h: mid-TG group of term infants in B, i: low-TG group of term infants in B, j: high-TG group of preterm infants in B, k: mid-TG group of preterm infants in B, and l: low-TG group of preterm infants in B. *MC4R*=melanocortin 4 receptor, *HNF4α*=hepatocyte nuclear factor 4 alpha, TG=triglyceride.

**Table 4****DNA methylation status of CpG sites in MC4R and HNF4 $\alpha$  in term and preterm infants by TG group.**

	Term			P	Preterm			P
	Low (n=4)	Mid (n=71)	High (n=9)		Low (n=12)	Mid (n=64)	High (n=7)	
Cord blood TG, mg/dL	16.95 [15.73–18.34]	30.13 [28.56–31.79]	54.57 <sup>*,†</sup> [48.81–61.03]	<0.001	16.52 [15.56–17.54]	26.52 [25.01–28.13]	60.49 <sup>‡,§</sup> [53.13–68.86]	<0.001
Gestational age, weeks	38.73 $\pm$ 0.88	39.27 $\pm$ 1.05	39.92 $\pm$ 0.99	0.11	34.21 $\pm$ 2.23	33.85 $\pm$ 2.40	33.44 $\pm$ 1.92 <sup>  </sup>	0.79
Birth weight, kg	3.39 $\pm$ 0.26	3.24 $\pm$ 0.34	3.24 $\pm$ 0.35	0.69	2.42 $\pm$ 0.65	2.23 $\pm$ 0.60	1.84 $\pm$ 0.42 <sup>  </sup>	0.13
Birth height, cm	50.50 $\pm$ 1.29	49.76 $\pm$ 1.68	50.30 $\pm$ 2.35	0.49	45.92 $\pm$ 3.15	44.44 $\pm$ 3.72	42.29 $\pm$ 2.63 <sup>  </sup>	0.11
Ponderal index, kg/m <sup>3</sup>	26.34 $\pm$ 1.62	26.33 $\pm$ 2.20	25.46 $\pm$ 2.16	0.53	24.82 $\pm$ 4.84	24.96 $\pm$ 3.54	24.01 $\pm$ 3.14	0.81
MC4R-CpG 3	83.70 $\pm$ 3.95	81.80 $\pm$ 3.24	80.80 $\pm$ 4.12	0.36	80.45 $\pm$ 2.81	80.41 $\pm$ 3.43	76.52 $\pm$ 2.05 <sup>‡,§</sup>	0.01
HNF4 $\alpha$ -CpG 2 in P1	72.06 $\pm$ 5.37	71.68 $\pm$ 8.84	72.93 $\pm$ 4.86	0.92	71.46 $\pm$ 3.44	69.63 $\pm$ 6.96	61.27 $\pm$ 12.32 <sup>‡,§</sup>	0.01

Data are means  $\pm$  SD. P value obtained from analysis of variance. Log-transformed TG levels are expressed as means and 95% confidence interval, with P values from the Kruskal–Wallis test. MC4R = melanocortin 4 receptor, HNF4 $\alpha$  = hepatocyte nuclear factor 4 alpha. P = promoter region, TG = triglyceride.

\* P values from Bonferroni post-hoc test, comparison between low- and high-TG groups in term infants.

† P values from Bonferroni post-hoc test, comparison between mid- and high-TG groups in term infants.

‡ P values from Bonferroni post-hoc test, comparison between low- and high-TG groups in preterm infants.

§ P values from Bonferroni post-hoc test, comparison between mid- and high-TG groups in preterm infants.

|| P values from Bonferroni post-hoc test, comparison between high-TG groups of term and preterm infants.

term infants. Interestingly, preterm infants in the high-TG group were observed to have higher TG levels, despite lower GA and worse birth outcomes, than term infants in the high-TG group. These preterm infants could have disrupted lipid metabolism in utero, and altered lipid profiles may affect metabolic processes and disease susceptibility, which could be mediated partly through epigenetic mechanisms at specific loci.

Epidemiological studies have reported a close link between PTB and the development of metabolic disorders in later life.<sup>[31–33]</sup> Approximately 35% of patients with T2D are known to have had low birth weight.<sup>[34]</sup> Impaired fetal growth caused by an adverse prenatal environment leads to metabolic adaptations in the fetus, which could influence long-term risks of metabolic diseases. For instance, a study indicated that extremely low birth weight infants was linked strongly to high TG and low HDL-c levels in 2-year-olds.<sup>[35]</sup> Young adults who were born prematurely have been observed to have higher blood pressure and LDL-c levels than those who were born at term.<sup>[36]</sup> In addition, adults who were born preterm with very low birth weight have been shown to have high insulin and glucose levels.<sup>[37]</sup>

Our findings showed that average TG levels were significantly lower in cord blood of preterm infants compared with term infants, whereas mean TC, HDL-c, and LDL-c levels were significantly higher, consistent with previous studies.<sup>[38–41]</sup> However, some of these findings are inconsistent with our results that TG, TC, and LDL-c levels in cord blood were higher in late preterm infants than in term infants.<sup>[42]</sup> Haridas and Acharya reported that preterm infants had higher TG levels than term infants, although the difference was not statistically significant.<sup>[43]</sup> Notably, the discrepancy in TG levels between the 2 groups may be due to variations in GA (near-full term vs very preterm) of the study subjects.<sup>[44]</sup> Moreover, this difference may be due to differences in consumption of nutrients, especially fat, associated with higher birth weight.<sup>[45,46]</sup> During the last trimester of pregnancy, which is accompanied by increased GA and total body fat, TG levels increase and TC levels decrease.<sup>[39,45]</sup>

Increased TG level is a well-established major risk factor for cardiovascular disease, which is the leading cause of death in many countries. Elevated lipid profiles at birth may affect the regulation of gene expression concerning growth and cell signaling pathways, and contribute to increased risk of diseases.<sup>[47]</sup> Abnormal lipid profiles in childhood could be

predictive of dyslipidemia and cardiovascular disease in adulthood.<sup>[48]</sup> In addition, altered metabolic profiles triggered by prenatal conditions may be explained partly by epigenetic mechanisms. These epigenetic alterations during prenatal development may persistently affect long-term health outcomes.<sup>[15]</sup>

Our results provided supporting evidence that higher TG levels were related significantly to decreased methylation of MC4R-CpG3 and HNF4 $\alpha$ -CpG2 in the P1 promoter in cord blood of preterm infants, compared with term infants. MC4R, which is relevant to the regulation of energy homeostasis, was found to elevate fatty acid uptake and TG synthesis in adipose tissue.<sup>[18,49]</sup> Another study implicated that activity of MC4R affects TG levels in cardiovascular patients.<sup>[50]</sup> To date, very few studies have been conducted to investigate the association between DNA methylation of MC4R and metabolic components in cord blood, although most genetic studies have shown that common variants near MC4R were associated with elevated TG levels and an increased risk of hypertriglyceridemia in obese children and adolescents.<sup>[51]</sup> In addition, genetic variants of MC4R have been found to lead to substantially higher postprandial response of TG levels in healthy subjects.<sup>[52]</sup> The active forms of MC4R decrease the activity of stearoyl-coenzyme A desaturase 1, which is involved in TG synthesis and affects LDL levels via the liver, resulting in decreased TG synthesis.<sup>[50]</sup> Consistent with our findings, the recent study by Widiker et al<sup>[19]</sup> showed hypomethylation of MC4R and increased expression levels in mice following a high-fat diet, which resulted in obesity. Rats fed a high-fat diet had 43% higher expression levels of MC4R in the paraventricular nuclei than those fed a low-fat diet, which was associated positively with fat mass.<sup>[53]</sup>

The other gene, HNF4 $\alpha$ , is known to be relevant to glucose and lipid metabolism.<sup>[54,55]</sup> In subjects with hyperlipidemia, variants of HNF4 $\alpha$  were involved in the elevation of TG and glucose levels.<sup>[56]</sup> In offspring of rats fed a high-fat diet, the expression levels of HNF4 $\alpha$  were substantially increased in the liver.<sup>[22]</sup> Many studies have shown that mutations in the P1 promoter of HNF4 $\alpha$  are related to the risk of metabolic disorders.<sup>[57,58]</sup> In a recent study, changes in DNA methylation of CpG sites, including in the known HNF4 $\alpha$ , were identified in subjects with T2D.<sup>[23]</sup> Collectively, we suggest that increased TG levels are associated closely with DNA methylation status of the MC4R and HNF4 $\alpha$  loci in cord blood cells.

Of note, our findings identified higher TG levels in high-TG preterm infants compared with term infants, whereas DNA methylation levels of *MC4R*-CpG3 and *HNF4α*-CpG2 in the P1 promoter in cord blood cells were significantly decreased in comparison with those with other TG levels. These high-TG preterm infants also had significantly lower GA and birth weight than term infants of the high-TG group. However, no significant association was found in term infants with high TG levels. Cress et al.<sup>[59]</sup> reported that TG levels >65 mg/dL in cord blood were related to fetal distress and adverse perinatal environment. On the basis of this evidence, we suggest that preterm infants with high TG levels may be at greater risk of metabolic-related disorders, although longitudinal studies are needed to clarify these findings.

In this study, we found the associations between DNA methylation of *MC4R* and *HNF4α* and metabolic components in cord blood, and differences between term and preterm infants, although in a relatively small sample. Further studies are needed to confirm the differences in methylation of metabolic-related genes as potential contributing factors for the development of metabolic-related diseases, and long-term follow-up studies are also needed.

DNA methylation patterns have tissue and cell-type specificity. However, many studies of DNA methylation have been performed on DNA extracted from whole blood, which contains a heterogeneous cell population; the use of cell-free circulating DNA could enable the discovery of accurate biomarkers due to its accessibility.<sup>[24,25,60]</sup> Recently, an altered DNA methylation status of *HNF4α* induced by the intrauterine environment was identified in cord blood from neonates with intrauterine growth restriction.<sup>[26]</sup>

Overall, this evidence indicates that epigenetic variations of *MC4R* and *HNF4α* established in early life may lead to altered metabolic profiles, such as increased TG levels, in cord blood of preterm infants. In particular, high TG levels in preterm infants may represent a greater risk of metabolic-related diseases in adulthood. Particular attention is required for sensitive subjects, such as babies born prematurely, especially those with increased TG levels, although the values are within normal ranges. Additional long-term follow-up studies are needed to gain further information regarding DNA methylation and metabolic components during each period of life.

## Acknowledgments

The authors thank Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2004833) and Ministry of Health & Welfare, Republic of Korea (HI14C0306) through the Korea Health Industry Development Institute (KHIDI) for the support.

## References

- [1] Beck S, Wojdyla D, Say L, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010;88:31–8.
- [2] Lawn JE, Wilczynska-Ketende K, Cousens SN. Estimating the causes of 4 million neonatal deaths in the year 2000. *Int J Epidemiol* 2006;35:706–18.
- [3] Lim JW. The changing trends in live birth statistics in Korea, 1970 to 2010. *Korean J Pediatr* 2011;54:429–35.
- [4] Lawn JE, Cousens SN, Darmstadt GL, et al. 1 year after The Lancet Neonatal Survival Series—was the call for action heard? *Lancet* 2006;367:1541–7.

- [5] Evensen KA, Steinshamn S, Tjonna AE, et al. Effects of preterm birth and fetal growth retardation on cardiovascular risk factors in young adulthood. *Early Hum Dev* 2009;85:239–45.
- [6] Singhal A, Fewtrell M, Cole TJ, et al. Low nutrient intake and early growth for later insulin resistance in adolescents born preterm. *Lancet* 2003;361:1089–97.
- [7] Menon R, Conneely KN, Smith AK. DNA methylation: an epigenetic risk factor in preterm birth. *Reprod Sci* 2012;19:6–13.
- [8] Zhang Y, Cerjak D, Ali O. Finding epigenetic determinants of the syndrome. *Austin J Endocrinol Diabetes* 2014;1:1029.
- [9] Paulsen M, Ferguson-Smith AC. DNA methylation in genomic imprinting, development, and disease. *J Pathol* 2001;195:97–110.
- [10] Ginder GD, Gnanapragasam MN, Mian OY. The role of the epigenetic signal, DNA methylation, in gene regulation during erythroid development. *Curr Top Dev Biol* 2008;82:85–116.
- [11] Waterland RA, Michels KB. Epigenetic epidemiology of the developmental origins hypothesis. *Annu Rev Nutr* 2007;27:363–88.
- [12] Heard E, Martienssen RA. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* 2014;157:95–109.
- [13] Bishop KS, Ferguson LR. The interaction between epigenetics, nutrition and the development of cancer. *Nutrients* 2015;7:922–47.
- [14] Schroeder JW, Conneely KN, Cubells JC, et al. Neonatal DNA methylation patterns associate with gestational age. *Epigenetics* 2011;6:1498–504.
- [15] Parets SE, Conneely KN, Kilaru V, et al. Fetal DNA methylation associates with early spontaneous preterm birth and gestational age. *PLoS One* 2013;8:e67489.
- [16] Tobi EW, Goeman JJ, Monajemi R, et al. DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nat Commun* 2014;5:5592.
- [17] Chambers JC, Elliott P, Zabaneh D, et al. Common genetic variation near *MC4R* is associated with waist circumference and insulin resistance. *Nat Genet* 2008;40:716–8.
- [18] Nogueiras R, Wiedmer P, Perez-Tilve D, et al. The central melanocortin system directly controls peripheral lipid metabolism. *J Clin Invest* 2007;117:3475–88.
- [19] Widiker S, Karst S, Wagener A, et al. High-fat diet leads to a decreased methylation of the *Mc4r* gene in the obese B6F1 and the lean B6 mouse lines. *J Appl Genet* 2010;51:193–7.
- [20] Stoffel M, Duncan SA. The maturity-onset diabetes of the young (MODY1) transcription factor HNF4α regulates expression of genes required for glucose transport and metabolism. *Proc Natl Acad Sci U S A* 1997;94:13209–14.
- [21] Miura A, Yamagata K, Kakei M, et al. Hepatocyte nuclear factor-4α is essential for glucose-stimulated insulin secretion by pancreatic beta-cells. *J Biol Chem* 2006;281:5246–57.
- [22] McCurdy CE, Bishop JM, Williams SM, et al. Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates. *J Clin Invest* 2009;119:323–35.
- [23] Ribel-Madsen R, Fraga MF, Jacobsen S, et al. Genome-wide analysis of DNA methylation differences in muscle and fat from monozygotic twins discordant for type 2 diabetes. *PLoS One* 2012;7:e51302.
- [24] Levenson VV. DNA methylation as a universal biomarker. *Expert Rev Mol Diagn* 2010;10:481–8.
- [25] Parets SE, Bedient CE, Menon R, et al. Preterm birth and its long-term effects: methylation to mechanisms. *Biology* 2014;3:498–513.
- [26] Einstein F, Thompson RF, Bhagat TD, et al. Cytosine methylation dysregulation in neonates following intrauterine growth restriction. *PLoS One* 2010;5:e8887.
- [27] Yoo JY, Lee S, Lee HA, et al. Can proopiomelanocortin methylation be used as an early predictor of metabolic syndrome? *Diabetes Care* 2014;37:734–9.
- [28] Genest JJr, McNamara JR, Ordovas JM, et al. Lipoprotein cholesterol, apolipoprotein A-I and B and lipoprotein (a) abnormalities in men with premature coronary artery disease. *J Am Coll Cardiol* 1992;19:792–802.
- [29] Desvergne B, Michalik L, Wahli W. Transcriptional regulation of metabolism. *Physiol Rev* 2006;86:465–514.
- [30] Blondet A, Gout J, Durand P, et al. Expression of the human melanocortin-4 receptor gene is controlled by several members of the Sp transcription factor family. *J Mol Endocrinol* 2005;34:317–29.
- [31] Burdge GC, Hanson MA, Slater-Jefferies JL, et al. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr* 2007;97:1036–46.
- [32] Parkinson JR, Hyde MJ, Gale C, et al. Preterm birth and the metabolic syndrome in adult life: a systematic review and meta-analysis. *Pediatrics* 2013;131:e1240–63.

- [33] Desai M, Jellyman JK, Ross MG. Epigenomics, gestational programming and risk of metabolic syndrome. *Int J Obes* 2015;39:633–41.
- [34] Boyko EJ. Proportion of type 2 diabetes cases resulting from impaired fetal growth. *Diabetes Care* 2000;23:1260–4.
- [35] de Jong M, Lafeber HN, Cranendonk A, et al. Components of the metabolic syndrome in early childhood in very-lowbirth-weight infants. *Horm Res Paediatr* 2014;81:43–9.
- [36] Sipola-Leppänen M, Väärasmäki M, Tikanmäki M, et al. Cardiometabolic risk factors in young adults who were born preterm. *Am J Epidemiol* 2015;181:861–73.
- [37] Hovi P, Andersson S, Eriksson JG, et al. Glucose regulation in young adults with very low birth weight. *N Engl J Med* 2007;356:2053–63.
- [38] Donegá S, Oba J, Maranhão RC. Concentration of serum lipids and apolipoprotein B in newborns. *Arq Bras Cardiol* 2006;86:419–24.
- [39] Bansal N, Cruickshank JK, McElduff P, et al. Cord blood lipoproteins and prenatal influences. *Curr Opin Lipidol* 2005;16:400–8.
- [40] Nagano N, Okada T, Yonezawa R, et al. Early postnatal changes of lipoprotein subclass profile in late preterm infants. *Clin Chim Acta* 2012;413:109–12.
- [41] Kelishadi R, Badiie Z, Adeli K. Cord blood lipid profile and associated factors: baseline data of a birth cohort study. *Paediatr Perinat Epidemiol* 2007;21:518–24.
- [42] Ghaemi S, Najafi R, Kelishadi R. Cord blood lipoprotein profile in term, preterm, and late preterm newborns. *J Res Med Sci* 2014;19:1038–40.
- [43] Haridas N, Acharya PT. Serum lipid status in neonates. *Indian Pediatr* 1984;21:327–34.
- [44] Bastida S, Cuesta C, Perea S, et al. Lipid and lipoprotein changes throughout the term-period in neonates from the Toledo Study. *Rev Esp Fisiol* 1996;52:23–9.
- [45] Lane DM, McConathy WJ. Factors affecting the lipid and apolipoprotein levels of cord sera. *Pediatr Res* 1983;17:83–91.
- [46] Radunovic N, Kuczynski E, Rosen T, et al. Plasma apolipoprotein A-I and B concentrations in growth-retarded fetuses: a link between low birth weight and adult atherosclerosis. *J Clin Endocrinol Metab* 2000;85:85–8.
- [47] Laker RC, Wlodek ME, Connelly JJ, et al. Epigenetic origins of metabolic disease: the impact of the maternal condition to the offspring epigenome and later health consequences. *Food Sci Hum Wellness* 2013;2:1–1.
- [48] Albisetti M, Chan AK, McCrindle BW, et al. Impaired fibrinolytic activity is present in children with dyslipidemias. *Pediatr Res* 2004;55:576–80.
- [49] Fan W, Boston BA, Kesterson RA, et al. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;385:165–8.
- [50] Brönnér G, Sattler AM, Hinney A, et al. The 103I variant of the melanocortin 4 receptor is associated with low serum triglyceride levels. *J Clin Endocrinol Metab* 2006;91:535–8.
- [51] Fernandes AE, de Melo ME, Fujiwara CT, et al. Associations between a common variant near the MC4R gene and serum triglyceride levels in an obese pediatric cohort. *Endocrine* 2015;49:653–8.
- [52] Perez-Martinez P, Garcia-Rios A, Delgado-Lista J, et al. A variant near the melanocortin-4 receptor gene regulates postprandial lipid metabolism in a healthy Caucasian population. *Br J Nutr* 2011;106:468–71.
- [53] Huang XF, Han M, South T, et al. Altered levels of POMC, AgRP and MC4R mRNA expression in the hypothalamus and other parts of the limbic system of mice prone or resistant to chronic high-energy diet-induced obesity. *Brain Res* 2003;992:9–19.
- [54] Hayhurst GP, Lee YH, Lambert G, et al. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* 2001;21:1393–403.
- [55] Yamagata K, Furuta H, Oda N, et al. Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature* 1996;384:458–60.
- [56] Weissglas-Volkov D, Huertas-Vazquez A, Suviolahti E, et al. Common hepatic nuclear factor-4alpha variants are associated with high serum lipid levels and the metabolic syndrome. *Diabetes* 2006;55:1970–7.
- [57] Marcil V, Amre D, Seidman EG, et al. Hepatocyte nuclear factor 4 alpha polymorphisms and the metabolic syndrome in French-Canadian youth. *PLoS One* 2015;10:e0117238.
- [58] Bonnycastle LL, Willer CJ, Conneely KN, et al. Common variants in maturity-onset diabetes of the young genes contribute to risk of type 2 diabetes in Finns. *Diabetes* 2006;55:2534–40.
- [59] Cress HR, Shaher RM, Laffin R, et al. Cord blood hyperlipoproteinemia and perinatal stress. *Pediatr Res* 1977;11:19–23.
- [60] Jacoby M, Gohrbandt S, Clausse V, et al. Interindividual variability and co-regulation of DNA methylation differ among blood cell populations. *Epigenetics* 2012;7:1421–34.