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Prenatal exposure to metals modified DNA methylation and the expression of antioxidant- and DNA defense-related genes in newborns in an urban area

N. Montes-Castro[a], I. Alvarado-Cruz[a,1], L. Torres-Sánchez[b], I. García-Aguiar[c], A. Barrera-Hernández[a], C. Escamilla-Núñez[b], L.M. Del Razo[a], B. Quintanilla-Vega[a,*]

[a] Department of Toxicology, Cinvestav, Ave. IPN 2508, Zacatenco, Mexico City, 07360, Mexico
[b] National Institute of Public Health-INSP, Ave. Universidad 655, Santa María Ahuacatitlán, Cuernavaca, Morelos, 62100, Mexico
[c] Department of Molecular Biomedicine, Cinvestav, Ave. IPN 2508, Zacatenco, Mexico City, 07360, Mexico

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ABSTRACT

The developmental period in utero is a critical window for environmental exposure. Epigenetic fetal programming via DNA methylation is a pathway through which metal exposure influences the risk of developing diseases later in life. Genetic damage repair can be modified by alterations in DNA methylation, which, in turn, may modulate gene expression due to metal exposure. We investigated the impact of prenatal metal exposure on global and gene-specific DNA methylation and mRNA expression in 181 umbilical cord blood samples from newborns in Mexico City. Global (LINE1) and promoter methylation of DNA-repair (*OGG1* and *PARP1)* and antioxidant (*Nrf2*) genes was evaluated by pyrosequencing. Prenatal metal exposure (As, Cu, Hg, Mn, Mo, Pb, Se, and Zn) was determined by ICP-MS analysis of maternal urine samples. Multiple regression analyses revealed that DNA methylation of LINE1, *Nrf2*, *OGG1*, and *PARP1* was associated with potentially toxic (As, Hg, Mn, Mo, and Pb) and essential (Cu, Se, and Zn) elements, and with their interactions. We also evaluated the association between gene expression (mRNA levels quantified by p-PCR) and DNA methylation. An increase in *OGG1* methylation at all sites and at CpG2, CpG3, and CpG4 sites was associated with reduced mRNA levels; likewise, methylation at the CpG5, CpG8, and CpG11 sites of *PARP1* was associated with reduced mRNA expression. In contrast, methylation at the *PARP1* CpG7 site was positively associated with its mRNA levels. No associations between *Nrf2* expression and CpG site methylation were observed. Our data suggest that DNA methylation can be influenced by prenatal metal exposure, which may contribute to alterations in the expression of repair genes, and therefore, result in a lower capacity for DNA damage repair in newborns.

1. Introduction

According to the WHO, air pollution causes approximately 600,000 deaths per year in children under five years of age [1]. Environmental pollutants, such as metals, are considered a major public health problem because they are known risk factors for several chronic diseases, including cardiovascular, neurological, and immune system disorders, as well as cancer. Likewise, *in utero* metal exposure has been associated with genetic damage and low birth weight in newborns [2,3].

In vitro studies have shown that metal exposure can contribute to genetic damage through the induction of double-strand breaks (DSBs) or by affecting critical proteins of various DNA-repair pathways [4].The mechanisms underlying the damage from metal exposure can be linked to oxidative stress and altered gene expression in key cellular pathways related to DNA-repair and the regulation of oxidative stress [5]. Base excision repair (BER) is responsible for removing oxidized DNA bases, which can be recognized and removed by DNA glycosylases. The major form of oxidized purine, 8-oxoG, is repaired by the 8-oxoguanine DNA glycosylase (OGG1), leaving an abasic site that is further processed by short- or long-patch repair, depending on various factors, such as the specificity and availability of the initiating glycosylase [6]. Another essential protein involved in the initial stages of the BER pathway is

Abbreviations: DSBs, double-strand breaks; BER, base excision repair; OGG1, 8-oxoguanine DNA glycosylase; PARP1, poly (ADP-ribose) polymerase 1; Nrf2, nuclear factor erythroid 2-related factor 2; LINE1, long interspersed repetitive elements; 5-mC, 5-methylcytosine; DNMTs, DNA methyltransferases; MCMA, Mexico City Metropolitan Area

* Corresponding author.
E-mail address: mquintan@cinvestav.mx (B. Quintanilla-Vega).
[1] Current affiliation: Therapeutic Radiology Department, School of Medicine, Yale University, 15 York Street, Hunter Building 308, New Haven, 06520, CT, USA.

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poly (ADP-ribose) polymerase 1 (PARP1), which recognizes DNA damage and contributes to the recruitment of other enzymes. DNA damage caused by metal exposure is primarily due to the generation of reactive oxygen species (ROS), with the nuclear factor erythroid 2-related factor 2 (Nrf2) being the major protein responsible for regulating cellular redox balance and antioxidant protection [7].

Epigenetic modifications in the promoter regions of genes and in long interspersed repetitive elements (LINE1) have been associated with alterations in gene expression and genomic instability [8]. The most studied epigenetic mechanism is DNA methylation, which involves the addition of a methyl group to carbon 5 in the cytosine pyrimidine ring (5-methylcytosine, 5-mC) via DNA methyltransferases (DNMTs) [9]. DNA hypermethylation has been associated with repressed gene expression; however, it is a complex process that has also been related to transcriptional activation [10]. Recent reports in Mexican newborns and school children revealed modifications in the methylation of LINE1 and DNA-repair genes in response to toxic metal exposure [11,12]. Furthermore, DNA methylation associated with toxic metal exposure can be altered according to the concentrations of essential elements [13]. Interestingly, both an antagonistic and synergistic relationship between arsenic (As) and selenium (Se) have been reported [14,15]. However, a few studies have evaluated changes in global and gene-specific methylation in newborns exposed to metals and there are no reports describing the impact of the concentration of potentially toxic and essential elements.

The Mexico City Metropolitan Area (MCMA) is one of the most densely populated cities, with more than 21 million inhabitants. The assessment of metals in the MCMA air has demonstrated the presence of copper (Cu), manganese (Mn), lead (Pb), zinc (Zn), and vanadium (V), among others, as well as As [12]. Additionally, in Mexico City, low birth weight and chromosomal abnormalities are the primary causes of infant death [16]. Therefore, we aimed to evaluate alterations in the methylation of DNA-repair and antioxidant genes caused by metal exposure and the association between such methylation and gene expression in newborns of the MCMA.

2. Materials and methods

2.1. Study population and sample collection

This study was performed in the Gyneco-Pediatrics 3A Hospital from the Mexican Social Security Institute (IMSS) in Mexico City in 181 women who were neither diabetic nor hypertensive, who were aged 18 to 35 years, and who delivered a live newborn (≥ 37 gestational weeks) without the diagnosis of intrauterine growth restriction (IUGR) or malformations. The study participants had been residents of the MCMA for at least 1 year (including the duration of the pregnancy), with no history of antiepileptic prescription or anemia during the third trimester of pregnancy (hemoglobin concentration > 11.5 g/dL) and without complications during pregnancy. Through direct interviews, we obtained information on sociodemographic characteristics, maternal age, place of residence, pregnancy progress, and smoking status during pregnancy (active or second-hand). Because all women answered not be active smokers during pregnancy, those women who reported have been exposed to second-hand smoking during their pregnancy, either at home or at work were classified as passive smokers.

At delivery, trained nurses collected fasting maternal blood and umbilical cord blood samples. Information on the conditions of the newborns at birth was collected from the medical histories. This study was approved by the Social Security Mexican Institute (IMSS; Ref. 90-B5-61-2800/201300/2056) as part of an initial project where our study was nested and by the Bioethics Committee for the Study in Human Beings of Cinvestav (COBISH, Folio 015/2014). Prior to their enrollment in the study, all participants provided written informed consent.

2.2. Analysis of DNA methylation by pyrosequencing

Umbilical cord blood was collected in EDTA-coated vacutainer tubes at the time of birth. DNA was isolated using phenol/chloroform, and 1 μg of DNA was bisulfite-treated using the EZ-96 DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's recommendations. The DNA was then PCR amplified, and pyrosequencing was performed using the PyroMark® Q24 pyrosequencing system according to the manufacturer's instructions (Qiagen, Hilden, Mettmann, Germany). We evaluated the repetitive element LINE1 as a marker of global methylation [17] and the gene-specific methylation in *OGG1* (4 CpG sites) and *PARP1* (12 CpG sites) using primers previously described by Alvarado-Cruz et al. [12]. We used the UCSC Genome Browser and MethPrimer tool to identify transcriptionally important CpG sites within the promoter region of *Nrf2* located in the CpG island closest to the transcription start site, with transcriptional regulatory marker binding regions, and acetylation marks (i.e., H3K27). Polymorphisms and repetitive sequences were avoided. The following PCR and sequencing primers were used for *Nrf2* (3 CpG sites): biotin-GGGTTAAAGATTTGGATTTAGAT (forward primer) and AAATAAACAATACTAACCACTCTCC (reverse primer). The DNA methylation status of each CpG was quantified using the PyroMark® Software version 2.0.7 (Qiagen, Hilden, Mettmann, Germany). As quality control, the software incorporates internal controls to assess the efficiency of the bisulfite conversion (at least 3 points in each plate) and adequate signal over background noise. We used the PyroMark Control Oligo as part of the quality control (0 and 50% methylation); additionally, as internal control, samples with known low methylation (0–5%) levels were used, which were previously obtained from a Mexican children population [12]. The samples were run in duplicate and reads were averaged, with a low coefficient of variation (< 5%). Methylation was expressed as the percentage of 5mC (%5mC) of each CpG site. Fig. 1 shows the location of the evaluated gene promoters, amplified regions, and CpG sites.

2.3. RNA extraction and real time-PCR

Mononuclear cells were isolated from cord blood samples using Histopaque®-1077 separation medium (Sigma Aldrich, St. Louis, MO, USA). Total RNA was extracted from mononuclear cells using TRIzol® (Sigma Aldrich, St. Louis, MO, USA); after the lysis of the cells, chloroform (Sigma Aldrich, St. Louis, MO, USA) was added, RNA was collected from the aqueous phase, and then precipitated with isopropanol. RNA was quantified, and the purity and integrity were assessed on a 1% agarose gel. cDNA synthesis was performed in a 25-μL reverse transcription reaction including 3 μg of RNA. TaqMan® PCR assays for *Nrf2* (Hs00975961_g1), *PARP1* (Hs00242302_m1), and *OGG1* (Hs01114116_g1) were performed in triplicate on cDNA samples in 96-well optical plates using a StepOne Plus™ system (ThermoFisher Scientific, Waltham, MA, USA). For each TaqMan reaction, 1 μL of cDNA was mixed with 6.25 μL of 2× TaqMan Universal Master Mix, 0.625 μL of TaqMan probe (20X), and 0.625 μL of the HPRT housekeeping gene for normalization of the gene expression levels (Applied Biosystems, Foster City, CA, USA). The reaction was completed with 12 μL of PCR-grade water. The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The mRNA expression levels relative to those of the housekeeping gene were determined using the $2^{-\Delta Ct}$ method [18].

2.4. Metal determination in maternal urine by ICP-MS

Maternal urine samples were collected at the time of birth and stored at −70 °C until analysis. Urine samples were thawed and aliquots of 200 μL were directly diluted 1:10 with nitric acid 65% (Merck, Darmstadt, Hesse, Germany) and ultrapure water to obtain a final concentration of 0.16%, and then the samples were analyzed with an

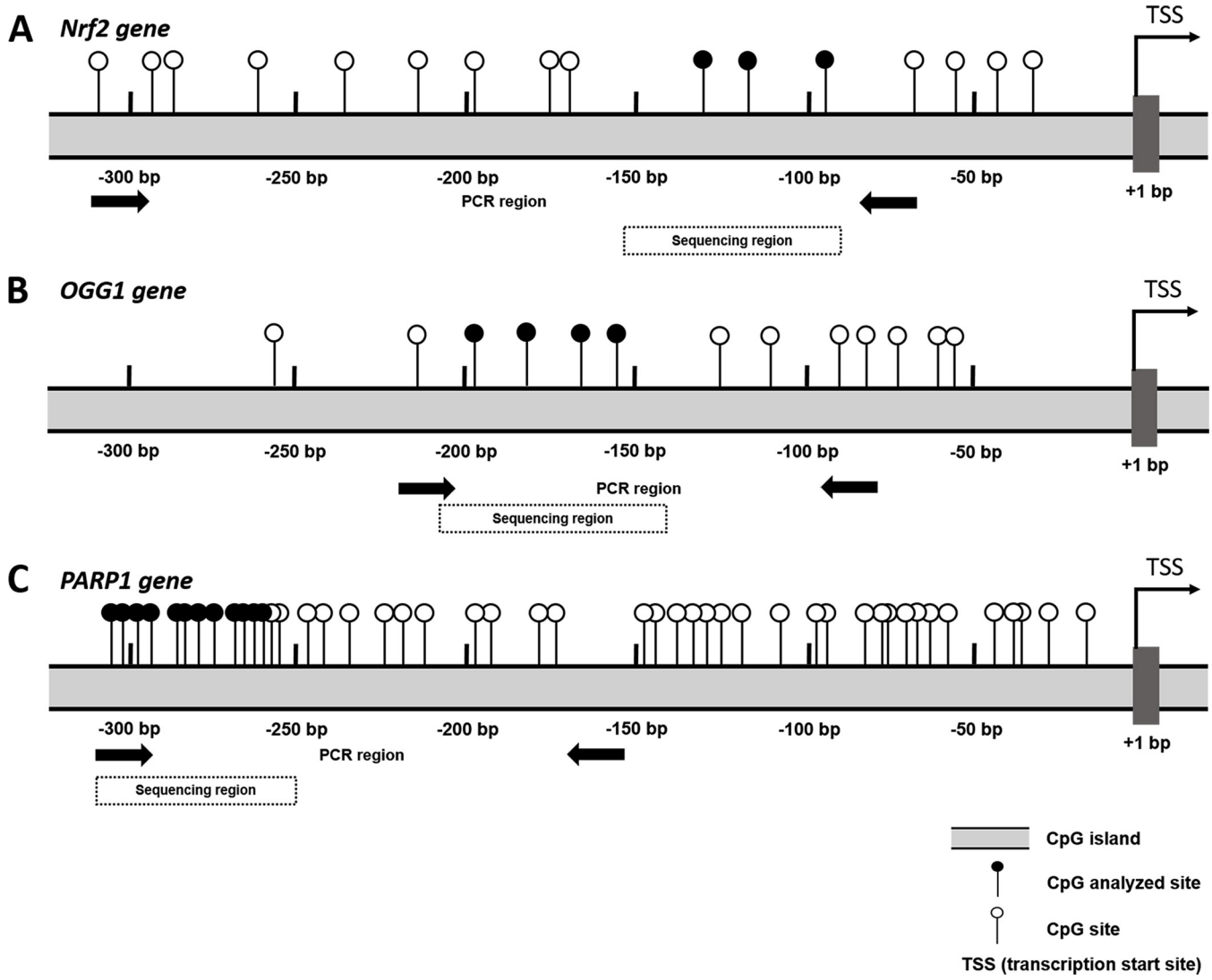

Fig. 1. Cytosine-guanine dinucleotide (CpG) sites on the promoter region of the analyzed genes. A) *Nrf2*, B) *OGG1*, and C) *PARP1*.

ICP-MS NexION 300D (Perkin Elmer, Waltham, MA, USA). The equipment was optimized for each run according to the manufacturer's instructions. Urine-certified reference material (QM-U-Q1706) of the National Public Health Institute of Quebec was used as a quality control and the percentage of recovery and relative standard deviation were calculated. Any undetected samples were assigned the value of the detection limit according to the Guidance for Data Quality Assessment [19]. This method (LISTO-MET-PRO-TEC-016) has been validated by the Metal Laboratory of the Laboratory of Research and Service in Toxicology of Cinvestav. The calibration graphs were generated at 0.5, 1, 5, 10, 25, 50, and 100 ppb from the multi-element calibration standards™ 2, 3, 4, and 5 (Perkin Elmer, Waltham, MA, USA). Twenty-one elements were quantified in the maternal urine samples; however, 7 elements exhibited values below the detection limit in more than 15% of the samples [19]. Creatinine was measured in the maternal urine using a commercial kit based on the Jaffé reaction (CR510, Randox Laboratories, San Diego, CA, USA) for the final calculations. All analyses, including those of the samples, standards, and reference materials, were carried out in duplicate using Syngistix™ software (Perkin Elmer, Waltham, MA, USA).

2.5. Statistical analyses

Descriptive statistics, including the arithmetic mean, standard deviation, frequency and percentage, were used to describe the study population based on sociodemographic, clinical, and genetic characteristics. Out of the fourteen elements with detectable values in more than 15% of the samples, only eight were associated with DNA methylation (As, Hg, Mn, Mo, Pb, Cu, Se, and Zn). The metal concentrations in the maternal urine were expressed as the geometric mean and an interquartile range of 25–75, and later, these variables were subsequently transformed to natural logarithms. The relationship between metal exposure and cord blood DNA methylation (%5mC) of LINE1, *Nrf2*, *OGG1,* and *PARP1* at each CpG site was estimated using independent linear regression models. Those variables associated with changes in global or specific DNA methylation, such as newborn sex [20,21], maternal age [22], second-hand smoking during pregnancy [23], maternal serum folic acid and vitamin B12 levels [24–26], and hemoglobin levels at delivery [24,27] were selected as potential confounders. The maternal concentrations of essential elements (Cu, Se, and Zn) were also considered in each final model. Those variables that changed the coefficient in more than 10% remained in the final models. We explored the possible modification effect of the selected potentially toxic metals (As, Hg, Mn, Mo, and Pb) on DNA methylation caused by the concentrations of Cu, Se, and Zn, incorporating the corresponding interaction terms into the regression model. Molybdenum and Mn are essential elements and cofactors of certain enzymes [28,29]; however, considering that these elements can easily reach toxic concentrations

Table 1
Sociodemographic, clinical and genetic characteristics of the study population.

Characteristic	n	Percentage or arithmetic mean ± SD
Maternal		
Maternal age (years)	181	26 ± 4
Hemoglobin (g/dL)	181	13.15 ± 0.91
Second-hand smoking[a]		
No	139	77
Yes	42	23
Vitamin B12[b]		
≥187 pg/mL[c]	102	57
< 187 pg/mL	77	43
Folic acid (ng/mL)[b]	179	12.34 ± 2.26
Neonate		
Gestational age (weeks)	181	39 ± 1
Newborn weight (kg)	181	3.2 ± 0.37
Newborn sex		
Male	92	51
Female	89	49
DNA methylation (%5mC)		
LINE1	179	72.85 ± 1.56
Nrf2	179	3.16 ± 0.52
OGG1	180	0.75 ± 0.19
PARP1	177	1.78 ± 0.37
mRNA expression ($2^{-\Delta CT}$)		
Nrf2	180	0.41 ± 0.21
OGG1	180	0.15 ± 0.17
PARP1	178	0.30 ± 0.27

[a] During pregnancy.
[b] Serum concentrations determined at the time of delivery.
[c] Reference value for vitamin B12. Some biomarkers present a different number of individuals due to missing data due to technical problems or a reduced sample size to perform all determinations.

due to environmental exposure, they were included in the group of potentially toxic metals for this study. Similarly, As is a metalloid [30], but this element was included in the potentially toxic metals group. The association between gene expression (mRNA levels) and DNA methylation (%5mC) was evaluated by multiple linear regression. We performed regression diagnostics to evaluate outliers and to reduce the possibility of type I error we performed the bootstrapping regression with 1000 repetitions; we reported only those estimators that remained significant. A p value < 0.05 was considered to be significant. STATA 12.0 software was used for all the analyses.

3. Results

3.1. General characteristics of the study population

Table 1 shows the general characteristics of the mother-newborn pairs. The mean maternal age was 26 ± 4 years, the mean maternal hemoglobin concentration was 13.15 ± 0.91 g/dL, and most mothers (n = 135, 77%) reported that they had never smoked during pregnancy. Serum folic acid and vitamin B12 concentrations were determined and 77 mothers (43%) showed levels below the reference value for vitamin B12. The average gestational age was 39 ± 1 weeks and the mean birth weight was 3.2 ± 0.37 kg. The proportion of boys and girls was similar in this sample.

3.2. DNA methylation and gene expression in newborns

Regarding DNA methylation, the average global methylation of the CpG sites analyzed in LINE1 was 72.85% and methylation in the promoter was 0.75% for *OGG1*, 1.78% for *PARP1*, and 3.16% for *Nrf2* (Table 1). The values of %5mC for each CpG site are shown in Supplementary Table A1 and the mean values of each CpG site of LINE1 and of the three genes showed significant differences among them (data not shown), suggesting a high variability. The mRNA values of the four genes are also shown in Table 1. The $2^{-\Delta CT}$ value of *Nrf2*, *OGG1*, and *PARP1* was 0.41 ± 0.21, 0.15 ± 0.17, and 0.30 ± 0.27, respectively.

3.3. Concentrations of the ionic elements in maternal urine

The average concentrations of the elements evaluated in 181 maternal urine samples are presented in Table 2. All elements exhibited wide concentration ranges. Molybdenum was the most abundant element quantified, while As was the most abundant toxic element, followed by Hg. In contrast, Pb and Mn concentrations were below the detection limit in 6.6 and 2.7% of the samples, respectively. Regarding the essential elements, the order of abundance was Zn > Se > Cu. According to the available data from human biomonitoring programs, 52% of the samples of this study had As concentrations above the established value of 15 ng/mL (95% reference value (RV_{95}); German Human Biomonitoring Commission) [31] (Supplementary Table A2), and 55% of the samples had Pb concentrations above the established value of 1.9 ng/mL (RV_{95} of the Human Biomonitoring of the Canadian Health Measures Survey) [32]. Regarding the Mo concentration, 53% of the samples had concentrations higher than the value established by the Biomonitoring Equivalent for urinary Mo (BE: 22 ng/mL) [33]. Finally, approximately 20% of the urine samples had Hg concentrations ≥ 35 μg/g creatinine, which is the value established by the Mexican Official Norm that corresponds to the biological exposure index (BEI) for occupationally exposed adults [34] (Table 2).

3.4. Effects of ionic elements on global and gene-specific DNA methylation in cord blood samples

As an initial approach, we evaluated the effect of exposure to each metal on global and gene-specific DNA methylation. A significant

Table 2
Maternal urinary metal concentrations.

Metal (μg/g of creatinine)	LOD[a] (μg/L)	n = 181		Geometric mean (IQR)[b]
		< LOD[a] n (%)	≥ LOD[a] n (%)	
Potential toxic				
Arsenic (As)	0.041	NA	180 (100)	22.94 (14.76; 36.32)
Manganese (Mn)	0.065	5 (2.76)	176 (97.24)	4.10 (3.00; 6.63)
Mercury (Hg)	0.079	NA	181 (100)	16.44 (9.54; 28.56)
Molybdenum (Mo)	0.071	NA	181 (100)	36.37 (25.63; 51.10)
Lead (Pb)	0.065	12 (6.63)	169 (93.37)	2.97 (2.04; 5.84)
Essential				
Copper (Cu)	0.112	NA	181 (100)	32.25 (21.18; 47.67)
Selenium (Se)	0.124	8 (4.42)	173 (95.58)	37.35 (33.44; 69.49)
Zinc (Zn)	0.368	NA	181 (100)	579.75 (360.43; 875.29)

[a] LOD: limit of detection.
[b] Interquartile range 25–75. NA: no values were found < LOD.

Table 3
Global and promoter DNA methylation in umbilical cord blood according to prenatal metal exposure.

Gene	Site	Metal (μg/g of creatinine)	β (95% CI)	*p* value[a]
LINE1	CpG1	Mo	−0.58 (−1.14; −0.02)	0.04[bc]
	CpG2	Mn	−0.25 (−0.50; −0.01)	0.04[b]
		Zn	0.34 (0.04; 0.63)	0.02
	CpG3	Zn	0.54 (0.16; 0.91)	0.004
Nrf2	CpG2	Cu	0.27 (0.10; 0.44)	0.002
	CpG3	Hg	0.12 (0.001; 0.24)	0.04
OGG1	CpG4	As	−0.23 (−0.43; −0.03)	0.01
		Hg	−0.32 (−0.47; −0.16)	< 0.001
PARP1	All	Pb	−0.06 (−0.12; −0.007)	0.04
	CpG2	Pb	−0.07 (−0.13; −0.01)	0.01[b]
	CpG5	Zn	−0.13 (−0.26; −0.003)	0.04
	CpG7	Hg	0.25 (0.08; 0.41)	0.003
		Mn	−0.21 (−0.39; −0.04)	0.01[b]
		Pb	−0.19 (−0.35; −0.02)	0.02[b]
	CpG9	Hg	0.12 (0.02; 0.22)	0.01[b]

Only those CpG sites or the average of all that exhibited significant changes in methylation are shown. [a]Models were adjusted for newborn sex, folic acid, vitamin B12, maternal hemoglobin, maternal age, and passive smoking status. In cases where the variation coefficient was greater than 10%, models were adjusted for essential metals ([b]Zn and [c]Cu). The regression coefficient or β value and the 95% confidence interval in parenthesis were obtained using multiple regression models.

negative relationship between LINE1 CpG1 methylation and Mo concentration ($p = 0.04$) was observed, as well as between methylation at the CpG2 site and Mn concentration ($p = 0.04$) (Table 3). In contrast, there was a positive relationship between Zn concentration and methylation at the CpG2 ($p = 0.02$) and CpG3 ($p = 0.004$) sites of LINE1. The analyses of individual *Nrf2* CpG sites revealed a positive relationship between DNA methylation and Cu ($p = 0.002$) and Hg ($p = 0.04$) concentrations. A decrease in the methylation of certain CpG sites of *PARP1* was associated with exposure to Pb, Mn, and Zn. Furthermore, a negative association was observed between the DNA methylation at all CpG sites in *PARP1* with exposure to Pb ($p = 0.04$). In contrast, Hg was positively associated with methylation at the CpG7 ($p = 0.003$) and CpG9 ($p = 0.01$) sites of *PARP1*. Finally, a significant reduction in *OGG1* methylation associated with As ($p = 0.01$) and Hg ($p < 0.001$) exposure was observed only at the CpG4 site. The complete analyses are shown in Supplementary Tables A3–A6.

3.5. Modifications in DNA methylation by potential toxic and essential elements

DNA methylation is affected by a wide variety of chemicals. Although very little is known about the mechanisms through which contaminants alter DNA methylation, it is known that certain metals increase DNA methylation while others have the opposite effect [11,35]. We evaluated changes in DNA methylation due to the exposure to individual metals, as well as the effect of the interaction between potentially toxic metals and essential elements on DNA methylation, which has not been previously reported. Changes in DNA methylation at various CpG sites due to potentially toxic and essential elements are shown in Fig. 2–4. It was observed that As ($p = 0.01$) and Hg ($p = 0.002$) significantly increased the methylation of LINE1 at low Zn concentrations; however, when Zn concentrations increased, LINE1 methylation was reduced (Fig. 2a–b). In addition, changes in LINE1 methylation at the CpG2 and CpG3 sites due to Mn and Mo were modified according to the concentration of Cu (Fig. 2c–f). DNA methylation at all CpG sites of LINE1 was modified due to the association between As or Hg with Zn concentrations (Supplementary Table A7).

Regarding the *Nrf2* gene, certain potentially toxic metals, such as As, Pb, and Hg, increased methylation at the CpG2 site, which was reduced according to the Se concentration (Fig. 3a–c). Interestingly, a significant alteration in methylation at the CpG2 site resulting from the association between Hg and Zn concentrations ($p = 0.04$) (Fig. 3d) was observed in our study. The interaction between Hg and Zn has not been previously demonstrated. In addition, *Nrf2* methylation at all CpG sites due to Hg exposure was modified according to Zn concentrations (Supplementary Table A7).

Of the twelve CpG sites evaluated in *PARP1*, four CpG sites were significantly modified by the potentially toxic and essential elements (Fig. 4). The CpG5, CpG10, and CpG11 sites were hypermethylated by maternal As exposure; however, a significant decrease in methylation at the CpG5 ($p = 0.01$) (Fig. 4a) and CpG11 ($p = 0.02$) (Fig. 4b) sites was observed as the concentration of Se increased, and methylation at the CpG10 site decreased as the Zn concentration increased ($p = 0.009$) (Fig. 4h). Furthermore, increased methylation at the CpG11 site of *PARP1* ($p = 0.01$) was associated with exposure to Hg, which was attenuated at high Se concentrations (Fig. 4c). Our results revealed a significant association between the concentrations of Mo and Cu and alterations in DNA methylation at the CpG9 ($p = 0.009$), CpG10 ($p = 0.006$), and CpG11 ($p = 0.01$) sites in the *PARP1* promoter (Fig. 4d–f). In addition, our data revealed that Mn exposure increased methylation at the CpG10 site of *PARP1*; however, hypomethylation was observed in the presence of Cu ($p = 0.01$) (Fig. 4g) and methylation at all CpG sites of *PARP1* due to As exposure was modified by Cu concentrations (Supplementary Table A8). Finally, only Mn exposure was associated with detectable changes in the DNA methylation pattern at the CpG4 site of *OGG1*, and these changes were modified according to the Cu concentration ($p = 0.04$) (Fig. 4i). The complete analysis of each CpG site of all genes is shown in Supplementary Tables A9-A12.

3.6. Relationship between mRNA expression of analyzed genes and DNA methylation

To investigate whether the observed increases in DNA methylation caused by metal exposure could reduce gene expression, we evaluated the relationship between the mRNA levels of *Nrf2*, *OGG1,* and *PARP1* and the methylation of the CpG sites of these genes (Table 4). Although changes in methylation at the CpG4 site due to metal exposure were observed for *OGG1* only, a significant reduction in *OGG1* expression was associated with methylation at all CpG sites ($p = 0.007$) and at the CpG2 ($p = 0.03$), CpG3 ($p = 0.02$), and CpG4 ($p < 0.001$) sites. Furthermore, an increase in DNA methylation at the CpG5, CpG8, and CpG11 sites was associated with a reduction in *PARP1* expression of 9, 7, and 13%, respectively. In contrast, we observed that an increase in *PARP1* expression was associated with methylation at the CpG7 site ($p = 0.04$). No associations were observed between CpG methylation patterns and *Nrf2* expression (Table 4).

4. Discussion

Epigenetic modifications due to environmental pollutants, such as metals, play an important role in health problems. Some experimental and epidemiological studies have reported that metal exposure alters DNA methylation; however, to our knowledge, no studies exist regarding the alterations in DNA methylation caused by the presence of potentially toxic and essential elements. Particularly, the most novel findings from our study are the interaction between potentially toxic metals and essential elements and the global (LINE1) and gene-specific methylation of DNA-repair (*OGG1* and *PARP1*) and antioxidant (*Nrf2*) genes in newborns prenatally exposed to environmental metals. It is worth noting that newborns may be exposed to toxic metals, such as As, Hg, and Pb at concentrations higher than those reported by certain human biomonitoring programs or those established by national regulations.

Recent reports have demonstrated associations between metal exposure and global DNA methylation in newborns; however, the results are inconsistent. Pilsner et al. [11] performed a study in Mexican

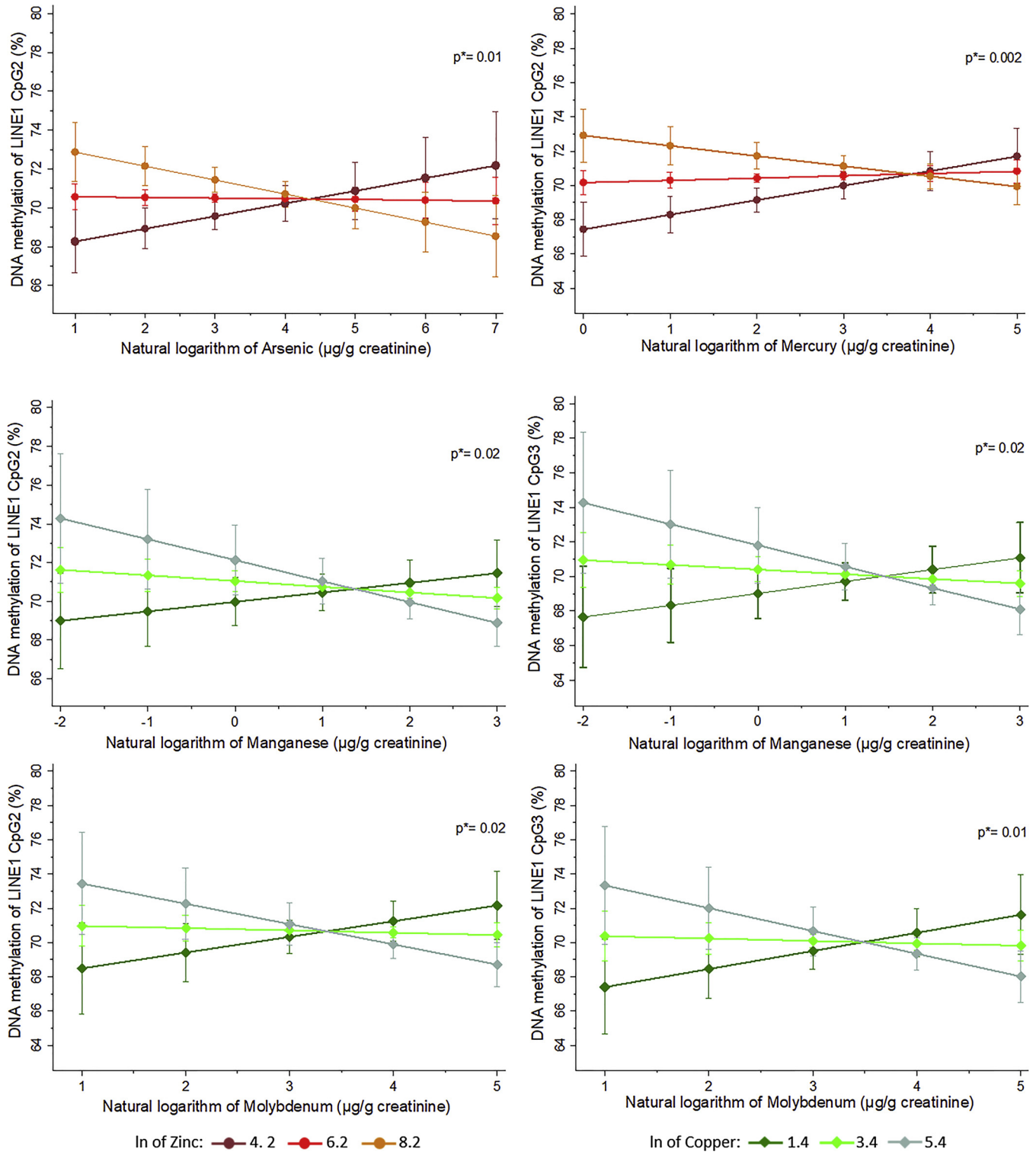

Fig. 2. Effects of potentially toxic and essential element interactions on DNA methylation of LINE1 CpG sites. Interaction between (a) As and Zn, (b) Hg and Zn, (c, d), Mn and Cu, and (e, f) Mo and Cu. ln: Natural logarithm. *p value of the interaction between metals using multiple regression models.

newborns wherein LINE1 methylation in cord blood was inversely correlated with maternal patella lead concentrations. Additionally, Kile et al. [35] demonstrated an increase in LINE1 methylation in newborns whose mothers had the highest urine levels of As. In a genome-wide methylation analysis of placental DNA, Maccani et al. [36] identified 5 CpG loci of crucial importance in the differentiation and development of the central nervous system that were differentially methylated and associated with the levels of Mn present in the toenails of infants. On the other hand, adults exposed to As exhibited an increase in global DNA methylation, which decreased at the highest As concentrations [37]. Regarding *in vitro* and *in vivo* studies, deficiencies in essential metals, such as Zn, significantly decreased DNA methylation in rat liver [38] and toxic metals, such as Cd, initially induced DNA hypomethylation, although prolonged Cd exposure resulted in DNA hypermethylation and increased DNMT activity [39]. The mechanisms by which metals alter DNA methylation are unknown; however, the results

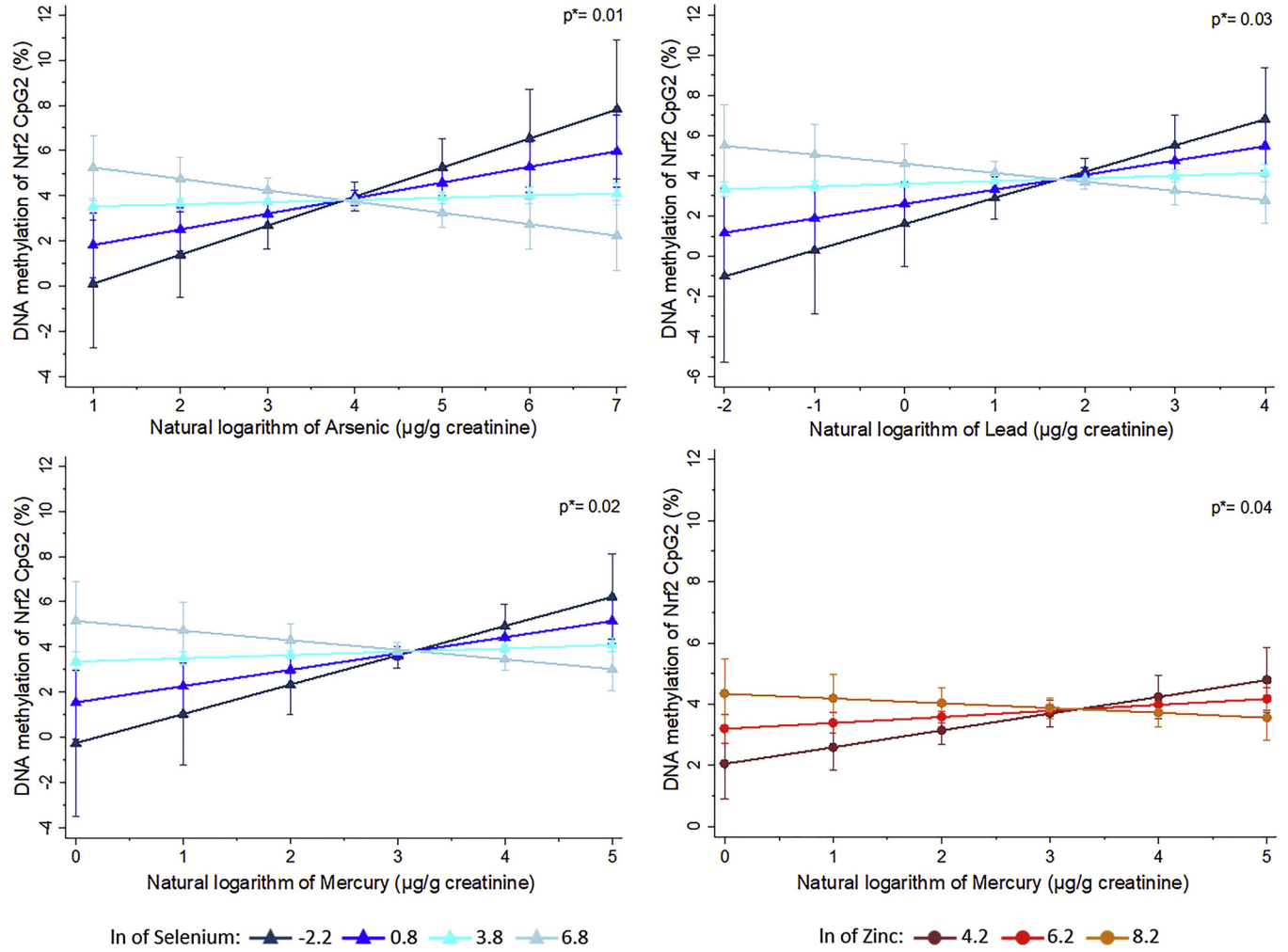

Fig. 3. Effects of potentially toxic and essential element interactions on DNA methylation of *Nrf2* CpG sites. Interaction between (a) As and Se, (b) Pb and Se, (c) Hg and Se, and (d) Hg and Zn. ln: Natural logarithm. *p value of the interaction between metals using multiple regression models.

suggest a nonlinear exposure-response relationship.

Regarding gene-specific methylation, information about changes in DNA methylation of evaluated genes by prenatal exposure to metals is null, however, in As-exposed newborns, Intarasunanont et al. [40] reported a slight increase in the methylation in the *p53* promoter. The aberrant promoter methylation of tumor suppressor genes has been related to cancer [41]. In addition, a recent study reported that nine CpG sites on chromosome 7 in the paraoxonase 1 (*PON1*) gene were hypomethylated from 1 to 3.8% due to prenatal Hg exposure [42]. PON1 is considered an antioxidant enzyme that can mitigate the pro-oxidant effects produced by metals [43]; therefore, changes in the DNA methylation of this gene can inhibit its protective effect. Pyrosequencing is a very sensitive and validated tool in a variety of epidemiological studies. Baccarelli et al. [44] reported changes below 3% in LINE1 methylation and in some specific genes including DNA-repair genes, which agrees with Hou et al. [45], who showed a discrete rise in the DNA methylation levels (1.8 to 6%) of DNA-repair genes associated to in utero exposure to particulate matter (PM). Similarly, Goodrich et al. [46] observed a positive association between Pb exposure and increments of 0.2 to 0.6% in gene-specific methylation.

Interestingly, the modifications in DNA methylation associated with exposure to potentially toxic metals can be attenuated by essential elements; however, this interaction has been scarcely investigated. We evaluated the effect of possible interactions between potentially toxic metals (As, Hg, Mn, Mo, and Pb) and essential elements (Cu, Se, and Zn) on DNA methylation. Our results showed that changes in LINE1 methylation caused by Mo and Mn were modified by Cu, while As and Hg caused an increase in LINE1 methylation, which, in turn, was decreased at high Zn concentrations. Zinc has diverse biological functions, primarily acting as a catalytic metal ion in enzymes, such as methionine synthase, or as a structural metal ion of certain transcription factors (i.e., Sp1), and as a Zn/ATP cofactor of certain kinases that epigenetically modify DNA and regulate gene expression [47].

In this study, we also investigated the effect of potentially toxic and essential elements on gene-specific methylation. Arsenic, Hg, and Pb increased methylation at the CpG2 site of *Nrf2* at low Se concentrations. The behavior of Se in biological systems is influenced by many metals, and Se can interact with toxic metals, modifying its effect [48]. Walton et al. [49] demonstrated that selenite inhibits arsenite methylation via a direct interaction with arsenite methyltransferase (AS3MT). Likewise, Yang et al. [50] identified the *TLR2* (toll-like receptor 2) and *ICAM1* (intercellular adhesion molecule 1) genes as targets of Se-dependent epigenetic regulation; the authors suggest that Se may alter the expression of DNMT1, leading to the epigenetic silencing of *TLR2* and *ICAM1*. In addition, a study in rodents reported that long-term Se consumption affects the exon-specific methylation of *p53* in a dose-dependent manner in rat liver and colon mucosa [51]; moreover, the toxic effects of Se depend on its chemical form and concentration [52]. This finding may indicate that Se participates in the regulation of various genes. Although the mechanism by which potentially toxic and essential elements alter *Nrf2* methylation in newborns is unknown, it may be related to the oxidative stress generated by metal exposure.

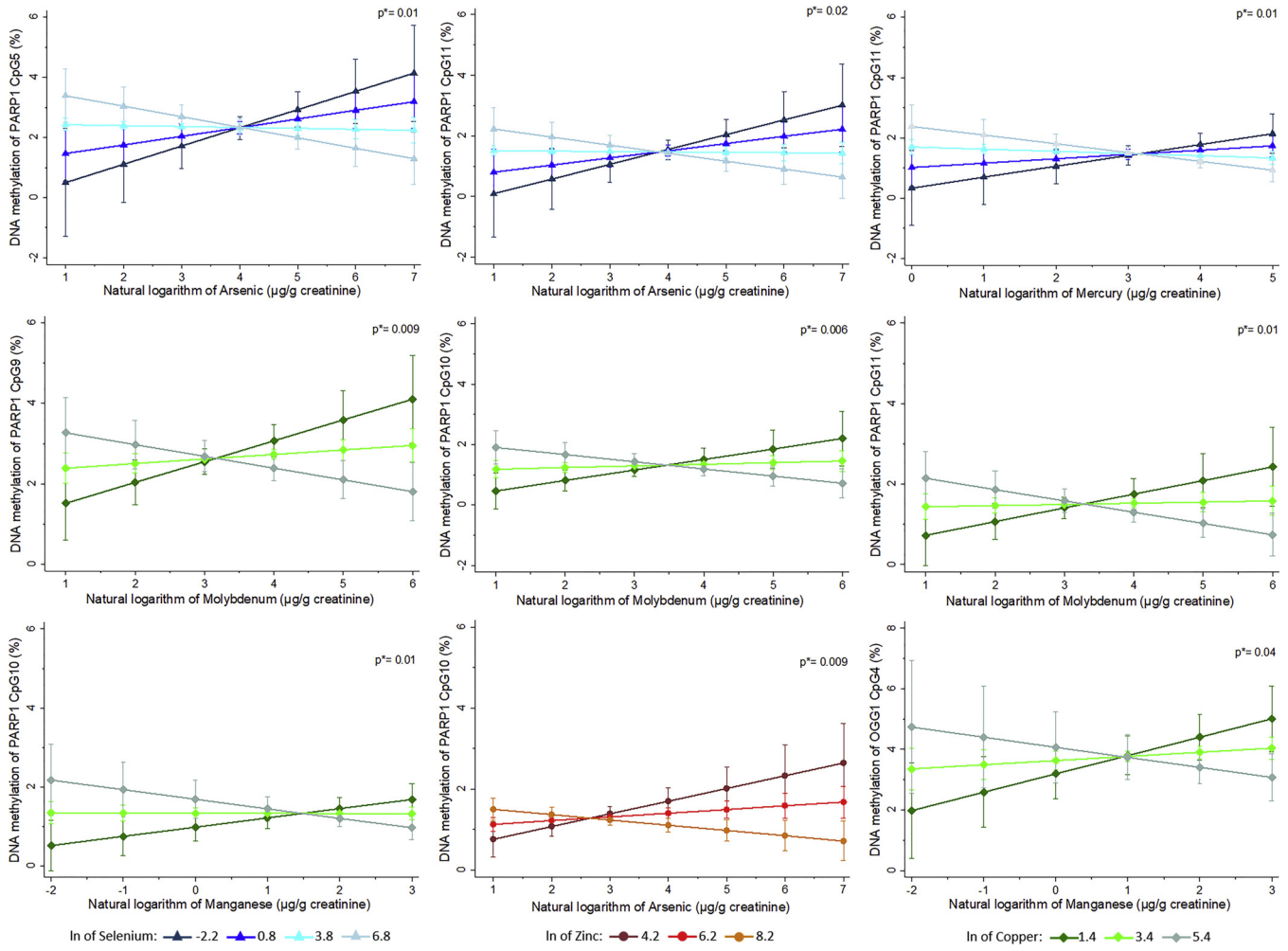

Fig. 4. Effects of potentially toxic and essential element interactions on DNA methylation of *PARP1* and *OGG1* CpG sites. Subsections (a) to (h) show the modifications in the methylation of *PARP1* CpG sites and subsection (i) shows the modifications in the methylation of *OGG1* CpG4 site. Interaction between **(a, b)** As and Se, **(c)** Hg and Se, **(d, f)** Mo and Cu, **(g)** Mn and Cu, **(h)** As and Zn, and **(i)** Mn and Cu. ln: Natural logarithm. **p* value of the interaction between metals using multiple regression models.

Arsenic, Hg, and Pb are potent neurotoxic elements due to their induction of oxidative stress and these elements have been shown to modify the activity of Nrf2, a key factor in the antioxidant response [30,53,54]. Reactive oxygen species may induce site-specific hypermethylation via either the upregulation of DNMT expression or the increased formation of DNMT complexes [55]. Janasik et al. [56] reported that occupational exposure to As induces *Nrf2* hypermethylation, which may be related to the carcinogenicity of As.

The *PARP1* and *OGG1* genes are essential in the BER pathway of DNA repair. Information regarding the effects of methylation changes in these genes caused by the exposure to metals in newborns is very limited. Neven et al. [57] found that exposure to $PM_{2.5}$ induced placental *OGG1* methylation, whereas *PARP1* methylation was not associated with $PM_{2.5}$ exposure. Additionally, our group recently reported that V adsorbed in $PM_{2.5}$ was correlated with an increase in the methylation of certain *PARP1* CpG sites in the blood cells of Mexican children [12]. In this study, the hypermethylation of *PARP1* at various CpG sites due to Mo was affected by the Cu concentration. Although Mo is a relatively nontoxic element, it has been shown that the interaction of Mo with Cu leads to its accumulation in rat liver and kidney tissues [58]; however, no explanation exists as to how Mo interacts with Cu to affect DNA methylation. In addition, we found that the Zn concentration affected the hypermethylation at the CpG10 site of *PARP1* caused by As. Vidal et al. [13] showed that the association between maternal Cd concentration and CpG site methylation in newborn imprinted genes (*PLAGL1* and *PEG3*) varies according to the Zn concentration. Furthermore, certain CpG sites within the *PARP1* promoter were shown to be hypermethylated in newborns according to the maternal urine concentration of As; however, hypomethylation was observed when the concentration of Se was increased.

The results of experimental and epidemiological studies that evaluated the influence of Se on DNA methylation have been inconsistent [59,60]. Several metals, such as As, Cu, Pb, Mn, and Mo, have been shown to interact with Se [48], although their interaction with As has been more thoroughly studied. Arsenic and Se can be methylated, and this methylation depends on the availability of methyl groups donated by S-adenosylmethionine (SAM) [61]. These methylated metabolites may inhibit or stimulate the activity of DNMT by binding to their active sites [62].

Modifications in the methylation of promoter regions of genes are generally related to changes in gene expression. We observed alterations in *OGG1* expression that were associated with methylation at the CpG2, CpG3, and CpG4 sites. There is no information available about changes in *OGG1* methylation in response to metal exposure; however, in a recent study conducted by our group on mice exposed to the pesticide methyl parathion, hypermethylation was observed at two CpG sites within the *OGG1* promoter in sperm cells with a concomitant lack of mRNA expression of this gene, suggesting a lack of repair after the

Table 4
Association between gene expression in umbilical cord blood and gene-specific DNA methylation.

Gene	Site	β (95% CI)	*p* value[a]
Nrf2	All	−0.007 (−0.07; 0.05)	0.81
	CpG1	0.01(−0.03; 0.07)	0.51
	CpG2	−0.02 (−0.06; 0.01)	0.24
	CpG3	0.006 (−0.03; 0.04)	0.77
OGG1	All	−0.17 (−0.29; −0.04)	0.007
	CpG1	−0.06 (−0.19; 0.06)	0.33
	CpG2	−0.17 (−0.33; −0.01)	0.03
	CpG3	−0.07 (−0.13; −0.01)	0.02
	CpG4	−0.06 (−0.08; −0.03)	< 0.001
PARP1	All	−0.04 (−0.15;0.06)	0.42
	CpG1	−0.03 (−0.13; 0.07)	0.52
	CpG2	−0.07 (−0.15; 0.01)	0.12
	CpG3	−0.03 (−0.12; 0.05)	0.42
	CpG4	−0.05 (−0.15; 0.04)	0.29
	CpG5	−0.09 (−0.14; −0.03)	0.001
	CpG6	−0.003 (−0.04; 0.03)	0.85
	CpG7	0.04 (0.001; 0.07)	0.04
	CpG8	−0.07 (−0.13; −0.01)	0.01
	CpG9	0.01 (−0.05; 0.09)	0.60
	CpG10	−0.06 (−0.15; 0.03)	0.20
	CpG11	−0.13 (−0.20; −0.07)	< 0.001
	CpG12	0.02 (−0.03; 0.08)	0.42

[a] Models were adjusted for newborn sex, folic acid, vitamin B12, maternal hemoglobin, maternal age and passive smoking status. The regression coefficient or β value and the 95% confidence interval in parenthesis were obtained using multiple regression models.

exposure to the pesticide [63]. This finding suggests that *OGG1* expression is regulated by the methylation of these CpG sites.

Regarding *PARP1* expression, upregulated expression was associated with hypermethylation at the CpG7 site in the promoter. In contrast, metal exposure did not alter the expression of *Nrf2*, despite the changes in DNA methylation observed in the *Nrf2* promoter. Likewise, gene-specific methylation and its relationship with gene expression have also been described in newborn mice. Xie et al. [64] revealed a loss of DNA methylation in GC-rich regions following the *in utero* exposure to As, along with the enhanced expression of genes encoding for glutathione production and a decrease in the expression of genes related to methyl metabolism, such as thioether *S*-methyltransferase (TEMT) and betaine-homocysteine methyltransferase (BHMT). Information on changes in DNA methylation of repair genes in response to metal exposure and the subsequent alterations in the expression of such genes is limited. However, in F32 lymphoblastic cells exposed to benzene, *PARP1* hypermethylation was related to low mRNA expression levels [65]. In addition, reduced *OGG1* expression was correlated with hypermethylation of this gene in age-related cataract patients [66].

It is important to note that DNA hypermethylation has been generally associated with the repression of gene expression; however, DNA methylation is a complex process that has also been related to transcriptional activation. Yu et al. [10] reported the dichotomous roles of DNA methylation in CpG islands during development. Likewise, CpG methylation within a gene body has been both positively and negatively associated with gene expression [67]. Our results suggest that hypomethylation and hypermethylation events in the evaluated genes are different and are caused by independent mechanisms.

It is also important to highlight that changes observed in this work are discrete and can be improved by applying more novel sequencing techniques that allow a deeper analysis (for example, new generation sequencing), which would probably allow to observe greater changes in methylation. In addition, short regions of the promoters were selected in this study; therefore, it will be necessary to evaluate longer sequences, which include the 3′ and 5′ UTR terminal ends to better explore the role of methylation changes on gene transcription. Our findings extend the knowledge about the effect of DNA methylation on gene regulation; however, the underlying mechanisms still require clarification.

For the interpretation of our results, the following should be taken into account. We reported the alterations on DNA methylation in whole blood cells by prenatal exposure to metals, individually and/or their interactions; however, we are aware that DNA methylation in leukocytes should be a weighted measure of the modifications [68] observed in this study. We were not able to consider the leukocyte populations since many of the cord blood samples were insufficient to do this determination. It is important to note that mothers with pregnancy complications that could contribute to variations in the white blood cell types were not included. In addition, we cannot rule out the possibility that metals may induce small changes in the white blood cell subpopulations, which may have influenced our findings. Because humans are constantly exposed to a mixture of toxic metals that can interact with each other and give composite effects, we do not rule out the possibility that the effects observed in DNA methylation may be different because of the interactions between metals. The evaluation of the effects of metal mixtures represents a challenge and the small sample size of our study did not allow to evaluate their toxicity on DNA methylation. In addition, the present study was focused on evaluating the impact of essential elements on the effects of potentially toxic elements. Finally, the urine samples were taken at delivery for metal determination and we assumed that metal concentrations were representative of the total pregnancy, since women lived in the same place during their whole pregnancy and were exposed to similar (constant) environmental exposures, causing changes in DNA methylation in the newborns. Unfortunately, we did not collect the information about the residence of participants in MAMC before pregnancy to estimate the influence of previous exposures.

In conclusion, we observed that exposure to potentially toxic metals during pregnancy modifies the methylation of DNA-repair and antioxidant genes in newborns and interestingly, their interaction with essential metals affected these modifications. Our findings suggest that hypermethylation in response to metal exposure can activate or repress gene expression. It is known that metals can cross the placental barrier and significantly affect the fetus; therefore, more studies are warranted to understand the influence of potentially toxic and essential elements on DNA methylation and the regulation of genes related to cellular defense as risk factors for developing diseases later in life.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.06.014.

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