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In Utero Arsenic Exposure Is Associated With Impaired Thymic Function in Newborns Possibly Via Oxidative Stress and Apoptosis

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Prenatal arsenic exposure is associated with increased infant morbidity and reduced thymus size, indicating arsenic-related developmental immunotoxicity. We aimed to evaluate effects of prenatal arsenic exposure on thymic function at birth and related mechanisms of action. In a Bangladeshi cohort, arsenic was measured in urine (U-As, gestational week (GW) 8 and 30) and blood (B-As, GW14) in 130 women. Child thymic index was measured by sonography at birth and thymic function by signal-joint T-cell receptor-rearrangement excision circles (sjTRECs) in cord blood mononuclear cells (CBMC). In a subsample (n = 44), sjTRECs content in isolated CD4$^+$ and CD8$^+$ T cells, expression of oxidative-stress defense and apoptosis-related genes in CBMC, arsenic concentrations (urine, placenta, and cord blood), and oxidative stress markers in placenta and cord blood were measured. In multivariable-adjusted regression, ln U-As (GW8) was inversely associated with ln sjTRECs in CBMC (B = −0.25; 95% confidence interval [CI] −0.48 to −0.01). Using multivariable-adjusted spline regression, ln U-As (GW30) and ln B-As (GW14) were inversely associated with ln sjTRECs in CBMC (B = −0.53; 95% CI −0.93 to −0.13 and B = −1.27; 95% CI −1.89 to −0.66, respectively) below spline knots at U-As 150 µg/l and B-As 6 µg/kg. Similar inverse associations were observed in separated CD4$^+$ and CD8$^+$ T cells. Arsenic was positively associated with 8-hydroxy-2′-deoxyguanosine in cord blood (B = 0.097; 95% CI 0.05 to 0.13), which was inversely associated with sjTRECs in CD4$^+$ and CD8$^+$ T cells. In conclusion, prenatal arsenic exposure was associated with reduced thymic function, possibly via induction of oxidative stress and apoptosis, suggesting subsequent immunosuppression in childhood.

Key Words: **sjTREC; thymus; oxidative stress; apoptosis; arsenic; cord blood.**

Disclaimer: The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

The thymus is the primary site of T-cell lymphopoiesis during fetal life and early childhood. The fetal thymus starts to produce T cells prior to midgestation and this function is almost fully developed at birth (Moore *et al.*, 2006). Therefore, the prenatal period is likely to constitute a critical window for toxic insult on the immune system (Dietert and Zelikoff, 2008). Human data, however, are scarce. We recently reported that maternal exposure to arsenic through drinking water during pregnancy is associated with reduced thymic size in infancy (Moore *et al.*, 2009; Raqib *et al.*, 2009). This may have serious consequences as a small thymic size at birth has been associated with increased risk of infection and mortality in infancy (Aaby *et al.*, 2002). Indeed, we found an arsenic-related increase in infant morbidity, especially in lower respiratory tract infection and diarrhea (Rahman *et al.*, 2010; Raqib *et al.*, 2009), and in infant mortality (Rahman *et al.*, 2007). The few previous epidemiological studies indicating adverse effects of arsenic exposure on the immune system, in particular immunosuppression, involved adults and children 6–10 years of age (Hernandez-Castro *et al.*, 2009; Selgrade, 2007; Smith *et al.*, 2011; Soto-Pena *et al.*, 2006).

Elevated concentrations of inorganic arsenic, a potent toxicant and carcinogen (Hough *et al.*, 2010; Straif *et al.*, 2009), in drinking water are common worldwide. Very little is known about the mechanisms behind the immunosuppressive effects of arsenic, particularly early in life. One possible scenario is that arsenic induces apoptosis of thymocytes and/or the produced T cells via oxidative stress. Arsenic is a known pro-oxidant (Flora, 2011), and we previously found that arsenic exposure through drinking water is associated with increased oxidative stress markers in the placenta and urine of pregnant Bangladeshi women (Ahmed *et al.*, 2010; Engstrom *et al.*, 2010). Moreover,

prenatal arsenic exposure has been associated with increased expression of genes involved in inflammation, stress response, and apoptosis in the cord blood (Fry *et al.*, 2007).

We hypothesize that arsenic exposure leads to infant immunosuppression through decreased thymic output, and the aim of this study was to elucidate the effects of prenatal arsenic exposure on infant thymic function, measured as signal-joint T-cell receptor-rearrangement excision circles (sjTRECs), and related mechanisms of action.

MATERIALS AND METHODS

Study area. The study was carried out in Matlab, a rural area, 53 km southeast of Dhaka, the capital of Bangladesh. In this area, the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) operates a health and demographic surveillance system and a central hospital with four connected health care facilities that provide health care to the residents of the area. The area is one of the most affected by arsenic in drinking water from tube wells in Bangladesh. About 70% of the tube wells exceed the WHO guideline value for arsenic in drinking water of 10 µg/l, and 50% the national standard of 50 µg/l (Rahman *et al.*, 2006).

Study design and subjects. The study was nested into a randomized population-based food and micronutrient supplementation trial (MINIMat trial, ISRCTN 16581394) that was carried out to study nutritional impact on fetal and child health and development (Persson *et al*., 2012). Pregnancy was identified by a urine test among the women who reported missing menstrual period when the community health research workers performed their monthly routine home visit. Women identified as pregnant at 6–13 weeks of gestation between November 2001 and October 2003 were invited to the closest health care facility for confirmation of pregnancy by ultrasound and to participate in the trial (N = 4436) (Rahman *et al.*, 2009). Recruited women were randomly assigned to two food groups, each of which was randomly subdivided into three micronutrient groups, receiving one of three different combinations of micronutrient supplements (1) 30 mg iron and 400 µg folic acid, (2) 60 mg iron and 400 µg folic acid, or (3) the UNICEF preparation of 15 different micronutrients including 30 mg iron and 400 µg folic acid, starting in gestational week (GW) 14 (Persson *et al*., 2012). For this study, we selected all women in the MINIMat trial who gave singleton birth in the Matlab hospital or any of the four connected health care facilities in early day time (5:00 A.M.–2:30 P.M.) from July 2002 to September 2003 (n = 300). Out of those women, we managed to collect cord blood from 286 women, but enough lymphocytes for measurements of thymic function were only available from 130 samples. Based on the preliminary results from these 130 women, additional samples were collected from 44 women who gave birth from February 2006 to February 2007 and participated in a follow-up study to the MINIMat trial assessing nutritional status in subsequent pregnancies. The main reasons for the low number of samples in both studies were the high frequency of home deliveries and delivery outside of our coverage time, which was limited due to logistic difficulties of processing and transferring samples to the laboratory. The study was approved by the Research Review Committee and Ethical Review Committee at ICBBR,B and the Regional Ethics Committee at Karolinska Institutet, Sweden. All participating women gave oral and written informed consent.

Gestational age at birth, birth anthropometry, maternal anthropometry, and morbidity data were available as a part of the MINIMat trial. In short, birth weight was measured within 72 h of delivery, using electronic scales (SECA pediatric scales, Hamburg, Germany) with a precision of 10 g. The infant's recumbent length was measured using a regularly validated locally manufactured wooden length board, precision 0.1 cm (Rahman *et al.*, 2009). Mothers were asked about their smoking (cigarettes and/or bidi) and tobacco chewing habits. The "chewing tobacco" usually consists of dried tobacco leaves locally known as "zarda" (sweetened tobacco leaves). Zarda is often taken in a mixture of sliced areca nut, lime, and a leaf of the piper betel plant (Lindberg *et al.*, 2010). The women's socioeconomic status (SES) was estimated from household assets, including the type of houses in which they reside (Saha *et al.*, 2008). During the scheduled monthly home visits, field research assistants collected information on morbidity during pregnancy (4-week recall), using a set of structured questionnaires. The questionnaires included four specific morbidity questions concerning diarrhea/dysentery, respiratory illness (in terms of cold, cough, or difficult breathing), and urinary tract infections (in terms of pain, burning, or difficulty during urination) with or without concomitant fever, including the duration of the morbidity symptoms (days of illness).

Specimen collection and preparation. In the initial study (n = 130) we collected cord blood at delivery. Data on arsenic in maternal urine samples at GW8 and GW30 and blood at GW14 were available from previous studies (Vahter *et al.*, 2006). The urine samples had been collected into trace element-free plastic cups, transferred into acid-washed 24-ml polyethylene bottles, and then transported to the laboratory for storage at −70°C. Blood samples had been collected at GW14 in Li-heparin tubes (SARSTEDT, Nümbrecht, Germany), and after centrifugation the isolated erythrocyte fractions were stored at −20°C. Urine and blood were later used for arsenic measurements. Cord blood (mixed venous and arterial blood) was collected at delivery in the health care clinics into Na-heparin-coated vials (Vacutainer System; Becton Dickinson, Rutherford, NJ) and then transported to the laboratory in Dhaka within 8–10 h. Cord blood mononuclear cells (CBMC) were separated by Ficoll (Pharmacia-Upjohn, Uppsala, Sweden) density gradient centrifugation and thereafter stored in liquid nitrogen awaiting DNA extraction with QIAamp DNA Mini Kit (QIAGEN, GmbH, Germany) and then analysis of sjTRECs.

In the follow-up study (n = 44), maternal urine was collected at GW14 (as described above), and placenta and cord blood at delivery. Cord blood samples were collected in Li-heparin tubes (SARSTEDT) for arsenic measurement in the erythrocyte fraction, and in Na-heparin-coated tubes (Vacutainer System; Becton Dickinson) for separation of plasma and CBMC. The plasma fraction was used for measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG). One portion of the CBMC was separated into $CD4^+$ and $CD8^+$ T cells with a CD4 and CD8 positive isolation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Thereafter, DNA for sjTRECs was extracted from the $CD4^+$ and $CD8^+$ T cells as described below. From the second portion of CBMC, we extracted RNA for the preparation of cDNA, using RT2 First Strand Kit (SABiosciences, Frederick, MD). The cDNA was later used for analysis of gene expression by PCR array (described below). The placenta was collected at delivery, drained from as much blood as possible. Thereafter, a 5 × 5-cm piece from the fetal portion of the placenta was cut out and immersed in 10% buffered formalin (pH 7.0) and kept at room temperature awaiting immunostaining. The remaining part of the placenta was placed in a polyethylene bag for storage at −20°C until further processing and arsenic measurement (Kippler *et al.*, 2010).

Arsenic measurements. Inorganic arsenic and its methylated metabolites were measured in urine (hereafter referred to as arsenic in urine), which reflects the actual exposure to inorganic arsenic from all sources, including drinking water and food, using hydride generation atomic absorption spectrophotometry (Vahter *et al.*, 2006). The limit of detection (LOD, 3 times the SD of the blank values) for this method was 1.30 ± 0.27 µg/l. Urine samples were adjusted for specific gravity, measured by a digital refractometer (RD712 Clinical Refractometer; EUROMEX, Arnhem, Holland), to compensate for variation in dilution (Nermell *et al.*, 2008).

Total arsenic in maternal erythrocytes (hereafter referred to as arsenic in blood), cord erythrocytes (hereafter referred to as cord blood arsenic), and placental tissue was measured (m/z 75) using inductively coupled mass spectrometry (Agilent 7500ce, Agilent Technologies, Tokyo, Japan) (Kippler *et al.*, 2010) after digestion of the samples, using a Milestone UltraCLAVE II microwave digestion system (EMLS, Leutkirch, Germany). Erythrocyte samples (~0.5 g) were mixed with 2 ml concentrated nitric acid (65% Suprapur, Merck, Darmstadt, Germany) and 3 ml deionized water, whereas the same amount of placental tissue was mixed with 5 ml concentrated nitric acid. The mixtures were thereafter heated at 250°C for 30 min in the UltraCLAVE. The LOD for arsenic

in blood, cord blood, and placenta was 0.05 µg/kg, 0.01 µg/kg, and 0.1 µg/kg, respectively. For quality control purposes, we analyzed two commercial reference materials (Seronorm Trace Elements Whole BloodL-1, REF 201505, LOT MR4206, SERO AS, Billingstad, Norway; CRM 185 bovine liver, Community Bureau of Reference, the Commission of the European Communities, Brussels, Belgium) as described in detail previously (Kippler *et al.*, 2010). The obtained arsenic concentrations (mean ± SD) of the reference materials were MR4206 whole blood ($n = 6$): 2.4 ± 0.2 µg/kg (reference, 1.8 ± 0.4 µg/kg) and CRM 185 bovine liver ($n = 8$): 23 ± 2 µg/kg (reference, 24 ± 3 µg/kg).

Thymic measurements. Thymic size was assessed by sonography at birth (usually within 24 h of birth) using a portable ultrasound machine (Toshiba SSA320 Justavision-200, Toshiba Medical Systems, Tokyo, Japan) together with a PVF-745V 5.0 to 7.0-MHz probe (Toshiba Medical System, United Kingdom) as described earlier (Moore *et al.*, 2009; Raqib *et al.*, 2009). The measurements were performed according to a validated method in which the transverse diameter of the thymus and the sagittal area of its largest lobe are multiplied to give a volume-related thymic index (TI) (Hasselbalch *et al.*, 1996).

sjTRECs. sjTRECs are by-products of T-cell receptor gene rearrangements, generated during lymphocyte maturation in the thymus (Douek *et al.*, 1998). SjTRECs are stable in thymocytes and mature T cells and do not replicate during mitosis. Thus, they are diluted during subsequent T-cell proliferation. Quantification of sjTRECs in human peripheral blood can be used to assess recent thymic emigrant T cells and thus to estimate thymus function or output. We measured sjTRECs in CBMC and separated $CD4^+$ and $CD8^+$ T cells by SYBR Green real-time quantitative PCR using a Bio-Rad CFX96 real-time PCR detection system (Hercules, CA) (Raqib *et al.*, 2007). We used the following primers: forward primer 5′ AAAGAGGGCAGCCCTCTCCAAGGCAAA 3′ and reverse primer 5′ AGGCTGATCTTGTCTGACATTTGCTCCG 3′. Real-time PCR was performed under the following conditions: denaturation (one cycle) at 95°C for 3 min; preamplification of 40–45 cycles at 95°C for 30 sec, 62°C for 30 sec, 72°C for 45 sec; and amplification (final extension step) cycle at 72°C for 5 min. Standard solutions were prepared by serial dilution of a known number of copies of a fragment of the sjTREC gene sequence in each PCR run to generate a standard curve. Samples were analyzed in duplicates. The number of copies of sjTRECs in the samples was determined automatically using standard curves and expressed as sjTREC content (number of copies/10^6 cells).

Oxidative stress markers. One of the main mechanisms of arsenic toxicity is oxidative stress induced by, for example, reactive oxygen species (ROS) and DNA damage. We used two sensitive biomarkers of oxidative DNA damage, 8-OHdG and 8-oxoguanine (8-oxoG), both of which have been previously reported to be associated with arsenic exposure (Ahmed *et al.*, 2010; Engstrom *et al.*, 2010). Cord blood plasma concentrations of 8-OHdG were analyzed by a competitive ELISA kit (Highly Sensitive 8-OHdG Check ELISA, Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). Before the analysis, the samples were filtered (Amicon Ultra-0.5, Millipore, Billerica, MA) by centrifugation at 14,000 rpm to remove proteins with molecular weight > 10 kDa, which could interfere with the measurements. Samples were measured in duplicates, avoiding the use of outermost wells of the microtitre plates to avoid edge effects. The assay was repeated if results of duplicate samples differed more than 10%. For staining and imaging of 8-oxoG in placenta, the formalin-treated pieces of the placenta were embedded in paraffin and then sectioned by Microtome (Leica, Germany) at 3 µm thickness. The sections were mounted on glass slides, treated with VECTABOND reagent (Vector Laboratories), dried overnight at 37°C, and then kept at room temperature until immunostaining with anti-human 8-oxoG antibody (Chemicon International, CA) as previously described (Ahmed *et al.*, 2010).

Arsenic in blood erythrocytes, cord blood erythrocyte, placental tissue or oxidative stress marker in cord blood plasma (8-OHdG) was measured in single batch. The inter-assay coefficient of variation of the thymic function marker (sjTRECs) and the oxidative stress marker in placenta was 7.5 and 6.0%, respectively.

Gene expression. Out of the 44 women in the follow-up study, we selected six mothers with the highest arsenic concentrations in cord blood (mean ± SD, 19.3 ± 11.4 µg/kg) and six mothers with the lowest arsenic concentrations in cord blood (1.1 ± 1.0 µg/kg) for comparison of gene expression in CBMC. The RT2 Profiler PCR Array System was used to evaluate the gene expression. We measured 84 predefined genes involved in oxidative stress and the antioxidant defense system, and 84 genes involved in the apoptosis pathways (SABiosciences). Real-time PCR array was carried out according to the manufacturer's instruction (SABiosciences). The experimental cocktail was prepared by adding 1350 µl master mix and 1248 µl H_2O to 102 µl diluted cDNA mixture. For real-time PCR detection, 25 µl of this mixture was added in each precoated primer or control well of a 96-well PCR array plate. A real-time PCR detection system (CFX96, Bio-Rad) was used to run the PCR array according to the following protocol: one cycle of 10 min at 95°C followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. SYBR Green fluorescence was used to detect PCR amplification from each well during the annealing step. Values were exported to a template excel file provided by SABiosciences for data analysis.

Statistical analyses. Statistical analyses were conducted using the software PASW 18.0 (SPSS Inc., Chicago) and Stata/IC, version 12.0 (StataCorp, College Station, TX). Data distribution patterns were evaluated using scatter plots, and normality and homogeneity of variances were formally checked by descriptive statistics. To quantify the change in sjTRECs with increasing arsenic exposure we used box plots representing the two phases indicated in the scatter plots. Spearman's correlation (for continuous variables) and Mann-Whitney *U*-test or ANOVA (for categorical variables) were used for an initial evaluation of the associations between exposure markers (arsenic in urine, blood, placenta, and cord blood), outcomes (sjTREC, 8-OHdG, and 8-oxoG), and covariates (maternal age, body mass index [BMI], maternal morbidity, parity, gestational age, birth weight and length, season of birth, sex of the child, SES, and tobacco chewing). Thereafter, we assessed the associations between exposure markers and outcomes with linear regression and found that the associations between exposure and outcome were influenced by season of birth, maternal fever (days of fever in pregnancy), and slightly by SES. When necessary, variables were ln (natural logarithm) transformed to meet the assumptions of parametric analyses. Spline knots were introduced if the visual examination (scatter plot) of the association showed deviation from a linear pattern. In sensitivity analyses, we additionally adjusted for maternal age, BMI, days of diarrhea, sex, birth weight, tobacco chewing, and supplementation.

The gene expression analyses were evaluated with a template (v3.3) provided by SABiosciences in which Student's *t*-test of the replicate $2^{(-\Delta Ct)}$ values for each gene was used to compare the high versus the low arsenic exposure groups for significant up- or downregulation. p values < 0.05 were considered statistically significant.

RESULTS

Demographic Data

The average age of the 130 women in the initial study was 25 years, whereas that of the 44 women studied in their subsequent pregnancy was 28 years ($p < 0.001$) (Table 1). These two groups of women did not differ significantly with regard to urinary arsenic concentrations. In both study samples the average gestational age at birth was 39 weeks, average birth weight was about 2800 g, and 16% of the infants had low birth weight (< 2500 g). All women were nonsmokers; however, 22% of the 130 women and 9% of the 44 women reported tobacco chewing during pregnancy. The studied women did not differ from all the women enrolled in the MINIMat trial from July 2002 to September 2003 with regard to the basic characteristics (Table 1).

TABLE 1
Descriptive Data of the Women and Their Infants in the Initial Trial (n = 130), Follow-up Study (n = 44), and All Women Enrolled in the MINIMat Trial From July 2002 to September 2003 (n = 1983)

Variables	Initial trial (n = 130) Mean ± SD or median (5–95th percentiles) or n (%)	Follow-up (n = 40) Mean ± SD or median (5–95th percentiles) or n (%)	All women (n = 1983) Mean ± SD or median (5–95th percentiles) or n (%)
Maternal characteristics			
Maternal age (years)	25.1 ± 5.9	28.1 ± 3.8	25.6 ± 5.9
BMI (kg/m^2) at GW8	20.3 ± 3.0	20.9 ± 3.3	20.2 ± 2.7
Gestational age (week)	39.0 ± 1.5	39.0 ± 1.7	39.3 ± 2.2
Parity	2 ± 1	3 ± 1	2 ± 1
SES			
Low	35 (27%)	16 (36%)	729 (37%)
Middle	27 (21%)	9 (21%)	397 (20%)
High	68 (52%)	19 (43%)	857 (43%)
Urinary arsenic at GW8 or 14 (µg/l)[a]	69 (19–441)	54 (12–504)	67 (17–481)[b]
Urinary arsenic at GW30 (µg/l)[a]	85 (20–508)		82 (18–607)[b]
Blood arsenic at GW14 (µg/kg)	4.7 (1.4–22.2)		
Placenta arsenic (µg/kg)		61 (17–598)	
Cord blood arsenic (µg/kg)		2.4 (0.8–23.5)	
Tobacco chewing			
Yes	6 (5%)	4 (9%)	161 (7%)
No	123 (95%)	40 (91%)	1722 (93%)
Fever in pregnancy			
No	70 (54%)		
Yes	58 (46%)		
Days of fever in pregnancy	10 ± 25		
Infant characteristics			
Birth weight (g)	2820 ± 398	2821 ± 384	2719 ± 401
Low birth weight (< 2500 g)	20 (16%)	7 (16%)	504 (25%)
Birth length, cm	48.1 ± 2.1	48.2 ± 2.2	47.7 ± 2.0
sjTREC/10^6 CBMC	8.0×10^3 (6.1×10^2–1.1×10^5)		
sjTREC/10^6 CD4$^+$ cells		2.4×10^5 (1.1×10^5–4.6×10^5)	
sjTREC/10^6 CD8$^+$ cells		2.4×10^5 (7.4×10^4–5.1×10^5)	

Note. Values are shown, mean ± SD or median (5–95th percentiles) or n (%).
[a]Adjusted to average specific gravity of 1.012 g/ml. Mother fever (total days of illness in pregnancy period).
[b]In the MINIMat trial, urinary arsenic was measured at GW8 and GW30 (n = 1122). Urinary arsenic referred to inorganic arsenic and its methylated metabolites in urine. Blood arsenic referred to arsenic in maternal erythrocytes and cord blood arsenic referred to arsenic in cord erythrocytes.

Arsenic Exposure, TI, and Cord Blood sjTRECs

In the initial study (n = 130), blood arsenic at GW14 was positively associated with urinary arsenic both at GW8 (r_s = 0.74, $p \leq 0.001$) and GW30 (r_s = 0.67, $p < 0.001$). Among the 44 women, we found a strong positive association between arsenic concentrations in placenta and cord blood (r_s = 0.98, $p < 0.001$), and both of these exposure markers were positively associated with urinary arsenic at GW14 ($r_s = 0.62, p < 0.001$ and $r_s = 0.63, p < 0.001$, respectively). We found that cord blood sjTRECs (n = 130) were significantly positively associated with infant TI at birth (r_s = 0.21, $p = 0.015$).

Examination of the scatter plots of sjTRECs in cord blood against maternal urinary arsenic and blood arsenic indicated clear nonlinear relationships (Figs. 1A and 1C). Comparison of median sjTRECs levels at urinary arsenic below and above 150 µg/l (above which there seemed to be no further decrease in sjTRECs with increasing arsenic) and blood arsenic below and above 6 µg/kg showed small differences related to arsenic exposure (Figs. 1B and 1D). Thus, the main difference was the absence of high sjTRECs levels at high arsenic exposure.

In order to evaluate the reason for the very high values of sjTRECs at low levels of arsenic exposure, we tested the associations between above and below median of sjTRECs with maternal illness. We found that in women with urinary arsenic concentrations (GW30) below 50 µg/l, but not above, high values of cord blood sjTRECs (above median) were positively associated with the number of days of maternal fever (r_s = 0.33, p = 0.09) during pregnancy. On the other hand, the number of days of maternal fever in pregnancy were positively associated with urinary arsenic at GW30 (r_s = 0.20, p = 0.01).

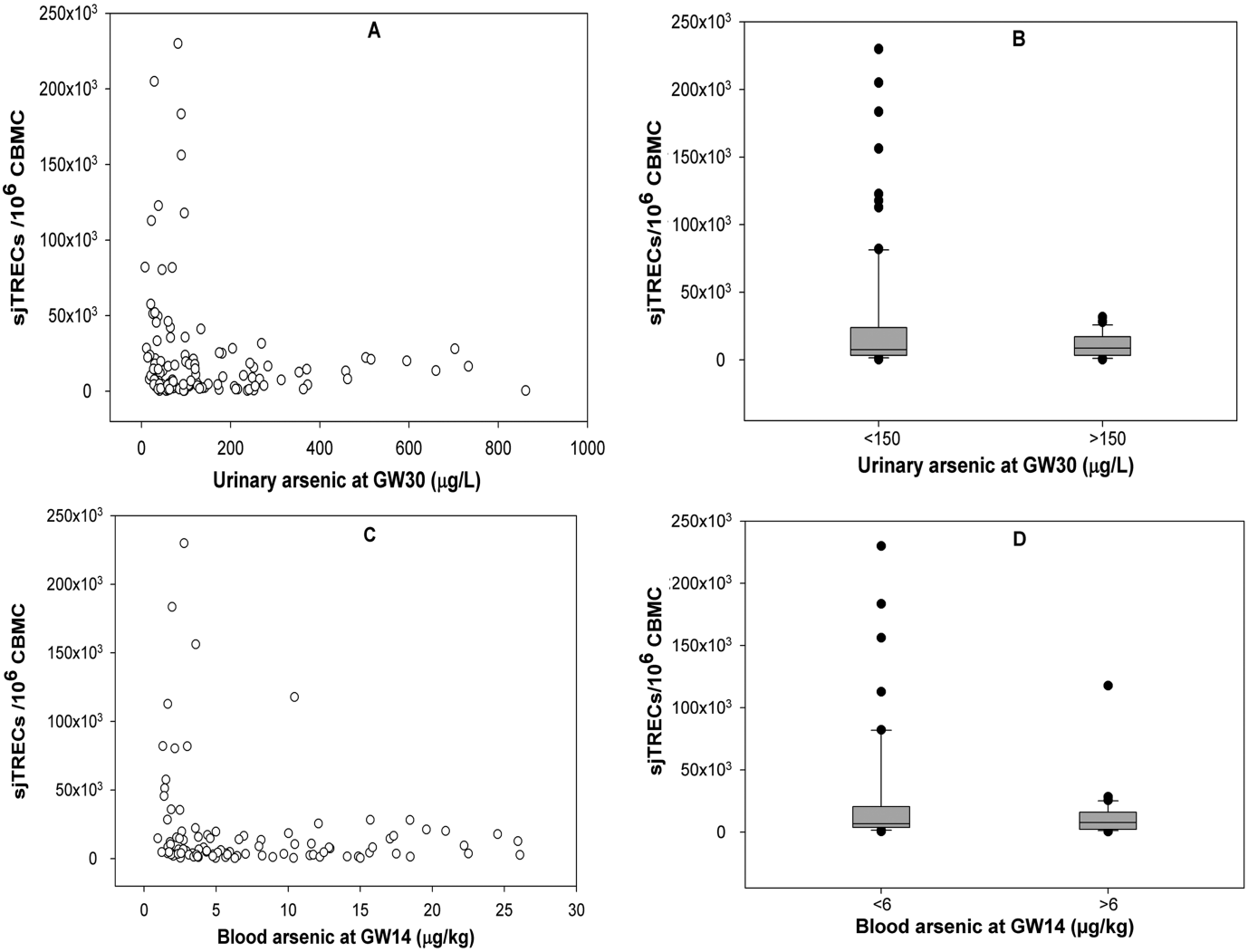

FIG. 1. (A) Association between urinary arsenic concentration at GW30 and cord blood sjTRECs. (B) Distribution of sjTRECs levels between below ($n = 92$) and above ($n = 36$) cutoff (150 µg/l) of urinary arsenic at GW30. (C) Association between blood arsenic concentration at GW14 and cord blood sjTRECs. (D) Distribution of sjTRECs levels between below ($n = 58$) and above ($n = 40$) cutoff (6 µg/kg) of blood arsenic at GW14. Groups were compared using the Mann-Whitney *U*-test.

In order to test if the observed associations between arsenic and sjTRECs could be confounded by other covariates, we used multivariable-adjusted linear regression analysis, with transformed (natural logarithm) exposure (urinary arsenic and blood arsenic) and outcome measures (sjTRECs) to fulfill the requirement of normally distributed residuals. Maternal urinary arsenic at GW8 was inversely associated with sjTRECs in cord blood (Table 2), and the estimate remained essentially unchanged after adjusting for season at birth, maternal fever, and SES. In sensitivity analyses we additionally adjusted for maternal age, BMI, days of diarrhea, sex, birth weight, tobacco chewing, and supplementation, but they did not change the estimates. Because the associations between ln urinary arsenic at GW30 or ln blood arsenic at GW14 and cord blood ln sjTRECs were nonlinear (data not shown), we used spline regression models with spline knots at ln urinary arsenic 5.0 (corresponding to 150 µg/l) and at ln blood arsenic 1.8 (corresponding to 6 µg/kg) (Table 2). We found that maternal urinary arsenic at GW30 and blood arsenic at GW14 were significantly inversely associated with cord blood sjTRECs below these cutoffs, but not above, and these associations changed very little by adjusting for season at birth, maternal fever, and SES. The slopes below and above the cutoff differed significantly (urinary arsenic in GW30, $p = 0.05$ and blood arsenic at GW14, $p = 0.04$).

We separated CBMC into $CD4^+$ and $CD8^+$ T cells in the follow-up study ($n = 44$). Examination of the scatter plots of sjTRECs in these cells in relation to arsenic exposure measures showed similar pattern as in the CBMC, i.e., absence of high sjTRECs at high exposure (data not shown). Comparing

TABLE 2
Regression Analysis of Associations of sjTREC in Cord Blood With Different Maternal Arsenic Exposure Biomarkers

	Unadjusted		Adjusted	
Predicted variables and predictors	*B* (95% CI)	*p* value	*B* (95% CI)	*p* value
ln sjTREC in CBMC (*n* = 130)				
ln urinary arsenic at GW8 (μg/l)	−0.24 (−0.47 to −0.01)	0.03	−0.25 (−0.48 to −0.01)[a]	0.03
ln urinary arsenic at GW30 (μg/l)[b]				
< 5	−0.57 (−0.96 to −0.16)	0.006	−0.53 (−0.93 to −0.13)[a]	0.009
≥ 5	0.37 (−0.32 to 1.08)	0.29	0.15 (−0.55 to 0.85)[a]	0.67
ln blood arsenic GW14 (μg/kg)[b]				
< 1.8	−1.16 (−1.78 to −0.54)	< 0.001	−1.27 (−1.89 to −0.66)[a]	< 0.001
≥ 1.8	0.59 (−0.12 to 1.31)	0.10	0.70 (−0.01 to 1.41)[a]	0.06
ln sjTRECs in CD4⁺ cells (*n* = 44)				
ln urinary arsenic at GW14 (μg/l)	−0.11 (−0.21 to −0.007)	0.03	−0.13 (−0.24 to −0.01)[c]	0.03
ln placenta arsenic (μg/kg)	−0.14 (−0.25 to −0.03)	0.01	−0.14 (−0.25 to −0.03)[c]	0.01
ln cord blood arsenic (μg/kg)	−0.14 (−0.26 to −0.02)	0.02	−0.14 (−0.26 to −0.01)[c]	0.02
ln sjTRECs in CD8⁺ cells (*n* = 44)				
ln urinary arsenic at GW14 (μg/l)	−0.14 (−0.28 to 0.001)	0.05	−0.18 (−0.33 to −0.02)[c]	0.02
ln placenta arsenic (μg/kg)	−0.19 (−0.33 to −0.04)	0.01	−0.21 (−0.36 to −0.05)[c]	0.01
ln cord blood arsenic (μg/kg)	−0.19 (−0.35 to −0.03)	0.02	−0.20 (−0.36 to −0.02)[c]	0.02

Note. B, unstandardized regression coefficients.
[a]Adjusted for season of birth, SES, and mother's fever (total days of fever in pregnancy).
[b]Spline regression model using spline knots at ln urinary arsenic 5.0 (corresponding to 150 μg/l) and at ln blood arsenic 1.8 (corresponding to 6 μg/kg).
[c]Adjusted for season of birth and SES. Urinary arsenic referred to inorganic arsenic and its methylated metabolites in urine. Blood arsenic referred to arsenic in maternal erythrocytes and cord blood arsenic referred to arsenic in cord erythrocytes.

sjTRECs levels at urinary arsenic below and above 150 μg/l (above which there seemed to be no further decrease in sjTRECs with increasing arsenic), placenta arsenic below and above 100 μg/kg, and cord blood arsenic below and above 6 μg/kg showed significant differences of sjTRECs in CD4⁺ cells with all exposure biomarkers (urine, placenta, and cord blood) and for CD8⁺ cells with urine and placenta arsenic (Fig. 2). Simple linear regression analysis showed that ln-transformed arsenic concentrations in urine, placenta, or cord blood were significantly negatively associated with ln sjTRECs in both CD4⁺ and CD8⁺ T cells (Table 2). These associations remained essentially unchanged after adjusting for season of birth and SES (we had no data on maternal morbidity in the follow-up study). With all exposure markers, the estimates for the CD8⁺ cells were 38–50% higher than the estimates of CD4⁺ cells.

Arsenic Exposure, Oxidative Stress, and Cord Blood sjTRECs

Arsenic concentrations in maternal urine, placenta, and cord blood were positively associated with cord blood 8-OHdG (*n* = 44), and the estimates increased somewhat after adjusting for season of birth and SES (Table 3). All exposure markers were also positively associated with 8-oxoG in the placenta.

Cord blood plasma 8-OHdG (*n* = 44) was negatively associated with sjTRECs levels in cord blood CD4⁺ T cells (*B* = −1.11, 95% CI = −1.85 to −0.38; *p* = 0.004) and CD8⁺ T cells (*B* = −1.65, 95% CI = −2.62 to −0.67; *p* = 0.002). The estimates remained essentially the same after adjusting for season of birth and SES. Placental 8-oxoG was not significantly associated with sjTREC in CD4⁺ (*B* = −0.12, 95% CI = −0.41 to 0.15; *p* = 0.37) or CD8⁺ T cells (*B* = −0.32, 95% CI = −0.68 to 0.04; *p* = 0.083).

Arsenic Exposure and Gene Expression in CBMC

The mothers with highest cord blood arsenic concentrations (mean ± SD, 19.3 ± 11.4 μg/kg; *n* = 6) had slightly lower SES (*p* = 0.08), compared with mothers with lowest cord blood arsenic concentrations (1.1 ± 1.0 μg/kg; *n* = 6), but there was no differences in age or BMI. The high arsenic cord blood samples had lower content of sjTRECs in both CD4⁺ (1.8×10^5 sjTRECs/10^6 cells; *p* = 0.06) and CD8⁺ (sjTRECs/10^6 cells: 1.9×10^5; *p* = 0.04) cells, compared with those with low arsenic concentrations (3.1×10^5 and 3.4×10^5 sjTRECs/10^6 cells, respectively).

We found that 18 out of 84 genes involved in oxidative stress and antioxidant defense pathways were associated with arsenic exposure (high vs. low; Fig. 3). In the cord blood samples with the highest arsenic concentrations the antioxidant-related

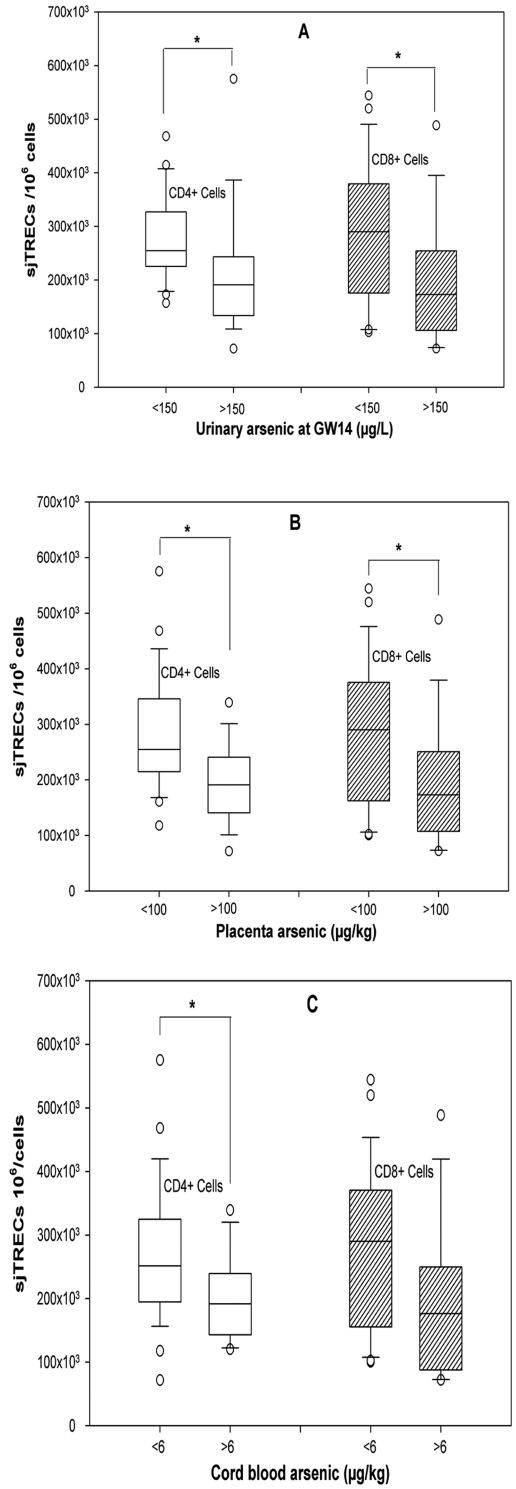

FIG. 2. In the 44 samples (A) distribution of sjTRECs levels of CD4⁺ and CD8⁺ cells between below (n = 24) and above (n = 18) cutoff (150 µg/l) of urinary arsenic at GW14. (B) Distribution of sjTRECs levels of CD4⁺ and CD8⁺ cells between below (n = 26) and above (n = 16) cutoff (100 µg/kg) of placenta arsenic. (C) Distribution of sjTRECs levels of CD4⁺ and CD8⁺ cells between below (n = 29) and above (n = 13) cutoff (6 µg/kg) of cord blood arsenic. Groups were compared using the Mann-Whitney U-test. * indicates p value < 0.05.

genes, ALB (albumin; p = 0.01), TXNDC2 (thioredoxin domain containing 2; p = 0.02), SOD3 (superoxide dismutase 3; p = 0.02), and APOE (apolipoprotein E; p = 0.007) seemed to be downregulated in CBMC compared with the lowest arsenic concentrations (Fig. 3). Similarly, the oxidative stress responsive genes ANGPTL7 (angiopoietin-like 7; p = 0.01), NME5 (nonmetastatic cells 5; p = 0.003), MBL2 (mannose-binding lectin 2; p = 0.01), and MTL5 (metallothionein-like 5; p = 0.04) seemed to be down-regulated in the high arsenic samples. We also observed that cord blood arsenic concentrations had lower expression of peroxidase-related genes in CBMC. Arsenic-related decrease in expression was found for CYGB (cytoglobin; p = 0.02), DUOX2 (dual oxidase 2; p = 0.009), EPX (eosinophil peroxidase; p = 0.005), LPO (lactoperoxidase; p = 0.003), PXDNL (peroxidasin homolog-like; p = 0.01), PXDN (peroxidasin homolog; p = 0.02), and the glutathione peroxidase gene GPX5 (glutathione peroxidase 5; p = 0.01) (Fig. 3) compared with those with low arsenic. Two other genes, NOS2A (nitric oxide synthase 2; p = 0.001), a gene involved in superoxide metabolism, and AOX1 (aldehyde oxidase 1; p = 0.003), a gene involved in ROS metabolism, seemed to be down regulated in the high arsenic cord blood samples (Fig. 3).

We also investigated associations between cord blood arsenic concentrations and expression of genes involved in apoptosis pathways in CBMC and found that 6 out of 84 genes seemed to be affected by arsenic (Fig. 4). We found that the apoptosis induction gene GADD45A (growth arrest and DNA-damage inducible alpha; p = 0.04), caspases activator gene NOD1 (nucleotide-binding oligomerization domain containing 1; p = 0.02), CASP2 (caspase 2, apoptosis-related cysteine peptidase; p = 0.04), death effector domain family gene CASP8 (caspase 8, apoptosis-related cysteine peptidase; p = 0.04), TNF ligand family gene CD70 (CD70 molecule; p = 0.04), and the death domain family gene TRADD (TNFRSF1-associated via death domain; p = 0.01) were upregulated in cord blood samples with highest compared with those with lowest arsenic concentrations (Fig. 4).

DISCUSSION

In this study, we found that maternal arsenic exposure through drinking water during pregnancy was associated with decreased sjTRECs levels in umbilical cord blood. This suggests that arsenic not only affected thymus size, as previously reported (Moore *et al.*, 2009; Raqib *et al.*, 2009) but also impaired the production of naïve T cells in the fetal thymus, particularly in response to maternal infections. This is likely to render the infants more susceptible to infections, possibly also to chronic diseases later in life (DeWitt *et al.*, 2012). Indeed, we found that arsenic exposure was associated with increased infant morbidity in the present cohort (Rahman *et al.*, 2010; Raqib *et al.*, 2009).

TABLE 3
Linear Regression Analyses of Associations of Oxidative Stress Markers in Cord Blood and in Placenta With Maternal Arsenic Exposure Biomarkers

	Unadjusted		Adjusted[a]	
Predicted variables and predictors	*B* (95% CI)	*p* value	*B* (95% CI)	*p* value
ln cord blood plasma 8-OHdG (*n* = 44)				
ln urinary arsenic at GW14 (µg/l)	0.076 (0.04 to 0.11)	< 0.001	0.094 (0.05 to 0.12)	< 0.001
ln placental arsenic (µg/kg)	0.089 (0.05 to 0.12)	< 0.001	0.096 (0.06 to 0.13)	< 0.001
ln cord blood arsenic (µg/kg)	0.094 (0.05 to 0.13)	< 0.001	0.097 (0.05 to 0.13)	< 0.001
ln placental 8-oxoG (*n* = 44)				
ln urinary arsenic at GW14 (µg/l)	0.14 (−0.01 to 0.30)	0.073	0.17 (0.007 to 0.34)	0.04
ln placental arsenic (µg/kg)	0.24 (0.07 to 0.39)	0.005	0.24 (0.08 to 0.40)	0.005
ln cord blood arsenic (µg/kg)	0.26 (0.08 to 0.44)	0.005	0.27 (0.09 to 0.43)	0.004

Note. B, unstandardized regression coefficients; 8-OHdG, 8-hydroxy-2′-deoxyguanosine (ng/ml); 8-oxoG; 8-oxoguanine (ACIA score).
[a]Adjusted for season of birth and SES. Urinary arsenic referred to inorganic arsenic and its methylated metabolites in urine. Blood arsenic referred to arsenic in maternal erythrocytes and cord blood arsenic referred to arsenic in cord erythrocytes.

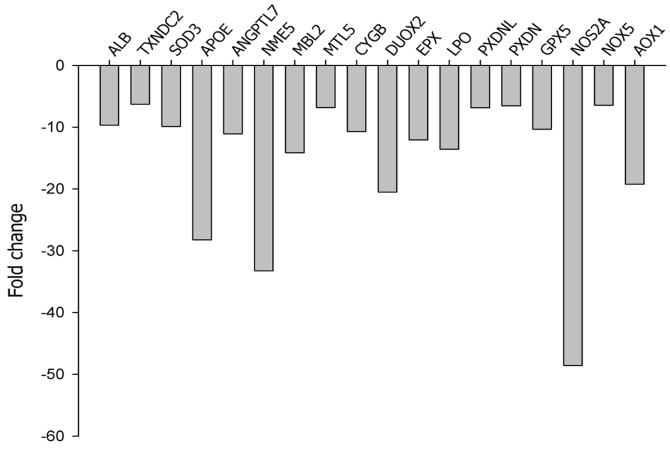

FIG. 3. Expression of genes involve in oxidative stress and antioxidant defense pathways in CBMC of six mothers (of the 44 in the follow-up) with the highest cord blood arsenic concentrations (mean ± SD, 19.3 ± 11.4 µg/kg) and in six mothers with the lowest cord blood arsenic concentrations (1.1 ± 1.0 µg/kg).

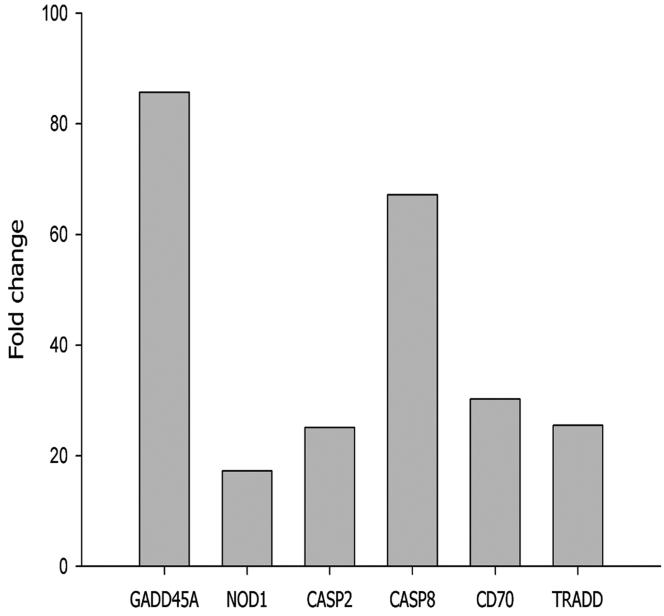

FIG. 4. Expression of genes involve in apoptosis pathways in CBMC of six mothers (of the 44 in the follow-up) with the highest cord blood arsenic concentrations (mean ± SD, 19.3 ± 11.4 µg/kg) and in six mothers with the lowest cord blood arsenic concentrations (1.1 ± 1.0 µg/kg).

The thymus is fundamental in the development of the immune system as it plays a vital role in the maturation of the T cells by providing the thymic microenvironment in which bone marrow derived progenitor cells undergo proliferation, T-cell receptor rearrangement, and thymocyte differentiation into mature T cells (Moore *et al.*, 2009; Prentice, 1999). In this study the median sjTRECs levels, a measure of thymic function, in neonatal cord blood were approximately 50% of those found in healthy U.S. children (Halnon *et al.*, 2005). Infants undergoing thymectomy, e.g., in connection with heart surgery, have approximately 10% of the TRECs levels in healthy infants, which may cause long term changes in the cellular immune system (Halnon *et al.*, 2005). Primary immunodeficiency, especially severe combined immunodeficiency disorder, is associated with extremely low TRECs, which can be fatal if not diagnosed very early in life (Puck, 2011).

To our knowledge there is no data regarding the association of arsenic exposure and thymic function. We have previously observed a nonlinear association between prenatal arsenic exposure and thymic size in infancy (Moore *et al.*, 2009; Raqib *et al.*, 2009), but no threshold was observed. Instead, the association was very similar to those in this study, with the main

decrease at low or moderate arsenic exposure and then at higher exposure levels the association was leveled out (no further decrease). In the current study, a major effect of arsenic seemed to be a block of the formation of high TRECs values, which were observed only at low arsenic concentrations, whereas the median TRECs numbers were fairly similar across arsenic concentrations. We found that the high values of sjTRECs (above median) at the lowest arsenic concentrations (below 50 µg/l) were associated with maternal fever during pregnancy. At high arsenic exposure levels, high sjTRECs values were not found; in spite of the apparent arsenic-associated higher maternal morbidity. This suggests a state of anergy of thymocytes in the high arsenic group, where the immune system appears to be unable to mount a normal immune response against infections. The reason may be an inability to produce required T cells, or excessive deletion of produced thymic T cells due to increased apoptosis. Indeed, we found that the proapoptosis genes GADD45A, NOD1, CASP2, CASP8, and TRADD were upregulated in cord blood samples with high arsenic exposure. Also, a higher apoptosis rate in peripheral blood mononuclear cells was found to be associated with arsenic exposure through drinking water in young Mexican children (Rocha-Amador *et al.*, 2011).

Decreased thymic output is usually the major contributor of lower levels of sjTRECs in $CD4^+$ T cells, low levels in $CD8^+$ T cells can be due to both decreased thymic output and increased peripheral T cell division (Ye and Kirschner, 2002). Our finding of decreased sjTRECs levels in cord blood $CD4^+$ T cells supports that prenatal arsenic exposure exerts a direct effect on the thymic microenvironment. However, the slightly stronger associations of arsenic exposure with lower TRECs levels in $CD8^+$ T cells may indicate that arsenic also increased peripheral T cell proliferation, as previously found *in vitro* (Ferrario *et al.*, 2009).

Arsenic is known to induce oxidative stress by generation of ROS and DNA damage (Jomova *et al.*, 2011) and we found increased levels of oxidative stress markers in cord blood plasma with increasing maternal arsenic exposure, which may affect the production of naïve T cells. We additionally found that many of the oxidative stress responsive genes, such as ANGPTL7, NME5, and MBL2 were reduced several folds in relation to elevated arsenic concentrations, whereas genes related to scavengers of ROS and antioxidants were many folds lower. This suggests an imbalance between the production and removal of ROS.

The strengths of our study include the prospective design and the use of several arsenic exposure biomarkers, measured at different time points during pregnancy and at delivery. The main limitation of the study is the small sample sizes.

In conclusion, fetal thymic function in rural Bangladesh seemed to be adversely affected by *in utero* arsenic exposure, probably via induction of oxidative stress, and increased apoptosis of fetal thymocytes. These events could lead to anergy of the immune system with impaired response to infections. Our study supports the concept that the prenatal period is a critical window. These children are currently being followed to assess health and development with a focus on the immune function in the children, most of whom continue to be exposed to arsenic via drinking water (Gardner *et al.*, 2011).

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