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Prenatal Arsenic Exposure and Shifts in the Newborn Proteome: Interindividual Differences in Tumor Necrosis Factor (TNF)-Responsive Signaling

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Exposure to inorganic arsenic (iAs) early in life is associated with adverse health effects in infants, children, and adults, and yet the biological mechanisms that underlie these effects are understudied. The objective of this research was to examine the proteomic shifts associated with prenatal iAs exposure using cord blood samples isolated from 50 newborns from Gómez Palacio, Mexico. Levels of iAs in maternal drinking water (DW-iAs) and the sum of iAs and iAs metabolites in maternal urine (U-tAs) were determined. Cord blood samples representing varying iAs exposure levels during the prenatal period (DW-iAs ranging from <1 to 236 μg As/l) were analyzed for altered expression of proteins associated with U-tAs using a high throughput, antibody-based method. A total of 111 proteins were identified that had a significant association between protein level in newborn cord blood and maternal U-tAs. Many of these proteins are regulated by tumor necrosis factor and are enriched in functionality related to immune/inflammatory response and cellular development/proliferation. Interindividual differences in proteomic response were observed in which 30 newborns were "activators," displaying a positive relationship between protein expression and maternal U-tAs. For 20 "repressor" newborns, a negative relationship between protein expression level and maternal U-tAs was observed. The activator/repressor status was significantly associated with maternal U-tAs and head circumference in newborn males. These results may provide a critical groundwork for understanding the diverse health effects associated with prenatal arsenic exposure and highlight interindividual responses to arsenic that likely influence differential susceptibility to adverse health outcomes.

Key words: **arsenic; arsenic metabolism; *in utero*; metals; pregnancy; birth outcomes.**

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

Over 100 million people worldwide, including pregnant women and their unborn children, are exposed to levels of inorganic arsenic (iAs) in their drinking water that exceed the World Health Organization's recommended limit 10 μg As/l and are considered detrimental to human health (Uddin and Huda, 2011; WHO, 2006). Long-term exposure to iAs is associated with the development of diverse chronic health conditions in adults including cardiovascular disease, peripheral vascular disease, neurological effects, diabetes mellitus, and cancers in several organs including the lungs, liver, urinary bladder, prostate, and skin (NRC, 2001; Rahman *et al.*, 2009a). In addition to adverse health effects in adults, exposure to high levels of iAs during pregnancy is also associated with considerable risks to maternal and fetal health including increased rates of spontaneous abortion, preterm birth, stillbirth, decreased birth weight, and decreased size at birth (as reviewed in Vahter, 2009). In addition, prenatal and/or early childhood exposure to iAs is associated with delayed health effects, including increased rates of mortality in young adults from both noncancerous diseases (e.g., bronchiectasis, acute myocardial infarction) and cancers of the lung, urinary bladder, larynx, and liver (as reviewed in Farzan *et al.*, 2013). Together, these results suggest that the prenatal period represents a particularly sensitive time to the toxic effects of iAs and the latent nature of several of these outcomes suggests their impacts are likely underestimated (Vahter, 2009).

A limited number of studies have examined the effects of prenatal iAs exposure in newborn cord blood at the molecular level. Previous studies have shown that newborns exposed to iAs *in utero* have increased expression of proinflammatory genes at both the transcriptional level and protein level in umbilical cord blood (Ahmed *et al.*, 2011; Fry *et al.*, 2007). There is also substantial evidence that *in utero* iAs exposure can act as an immunosuppressant, as prenatal exposure is associated with

increased morbidity and mortality and reduced thymic index in infants (Ahmed *et al.*, 2012; Rahman *et al.*, 2010; Raqib *et al.*, 2009). Taken together, these studies indicate that iAs can act as an immunomodulatory agent *in utero*, a phenomenon which may play a role in the development of the diverse adverse health effects associated with iAs exposure.

We hypothesized that prenatal arsenic exposure is likely to perturb the expression levels of proteins involved in a variety of cellular processes in addition to immune function. In order to better understand the molecular perturbations associated with prenatal arsenic exposure, we conducted a proteomics assessment of newborn cord blood from our recently established prospective pregnancy cohort, herein termed the Biomarkers of Exposure to ARsenic (BEAR) cohort. BEAR participants consist of 200 mother-child pairs residing in Gómez Palacio, Durango State, in the Lagunera region of northern Mexico. Importantly, iAs-associated health effects such as diabetes mellitus (Del Razo *et al.*, 2011) and skin lesions (Valenzuela *et al.*, 2009) have been reported in other northern and/or central Mexican populations, and iAs concentrations in the drinking water within Region Lagunera have been detected at levels that exceed 600 μg/l (Mendez-Gomez *et al.*, 2008). In the present study, we describe the proteomic shifts in newborn cord blood associated with concentrations of maternal urinary arsenic and identify significant changes in proteins involved in inflammation and immune function, cellular development and proliferation, and proteins with known relationships with iAs-associated diseases. Tumor necrosis factor (TNF), which plays a critical role in inflammation and cellular growth/development-related signaling, was identified as an upstream regulator of many of these proteins. Interestingly, interindividual differences in the proteomic shifts were observed among the newborns and were associated with birth outcome in a sex-dependent manner.

MATERIALS AND METHODS

Study subjects and sample collection. This study was approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill (no. 10-1583) and Universidad Juárez del Estado de Durango (UJED). A total of 200 pregnant women residing in Gómez Palacio, in the State of Durango, Mexico, were recruited at the General Hospital of Gómez Palacio to participate in the BEAR prospective pregnancy cohort. Requirements for participation in the study included (1) a 1 year minimum residence in the Gómez Palacio region, (2) confirmation of a singleton, intrauterine pregnancy without pregnancy complications such as eclampsia or pre-eclamsia, and (3) good overall health status (e.g., no signs of chronic or acute disease). Each participant gave written, informed consent to participate and agreed to provide urine samples, drinking water samples, and to donate umbilical cord blood at delivery. Participants also completed detailed questionnaires that gathered information on various socioeconomic factors (e.g., age, education, occupation, smoking status, and alcohol consumption) as well as potential sources of iAs exposure (e.g., source of drinking water and volume consumed). Women could not be informed of their iAs exposure during pregnancy as arsenic levels in drinking water and urine were determined after delivery. However, they were informed of their exposure status within 3 months after the births of their children.

Within 4 weeks of newborn delivery, a representative drinking water sample was collected by the research team at the homes of each of the study participants. Maternal spot urine samples were collected at the time of delivery, immediately placed in a cryovial, and stored under liquid nitrogen. Birth outcomes were assessed by the delivering physician. Fetal cord blood was collected from newborns immediately after delivery using a BD Vacutainer tube with clot activator (Becton, Dickinson and Company, Franklin Lakes, NJ). After clot formation, each tube was centrifuged and the supernatant was collected and stored at −70°C until proteomic analyses were performed. All samples were shipped on dry ice to the United States.

Determination of arsenic concentrations in drinking water and maternal urine. The concentrations of iAs in drinking water samples (μg/l; DW-iAs) were measured at the Faculty of Medicine, UJED, Gómez Palacio, Durango, Mexico using a hydride generation-atomic absorption spectrometry (HG-AAS) system (FIAS-100) as described previously (Le and Ma, 1998). The limit of detection (LOD) for iAs in drinking water by HG-AAS was 0.456 μg/l.

All urine analyses were conducted at the Nutrition Obesity Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC. The specific gravity (SG) of each urine sample was determined using a handheld refractometer (Reichert TX 400 no. 13740000; Reichert Inc., Depew, NY). Concentrations of the sum of trivalent and pentavalent iAs (μg As/l urine; U-iAs) and the monomethylated and dimethylated metabolites of iAs, namely, the sum of trivalent and pentavalent MMAs (μg As/l urine; U-MMAs) and the sum of trivalent and pentavalent DMAs (μg As/l urine; U-DMAs), respectively, were determined by HG-AAS with cryotrapping (CT) as previously described (Hernandez-Zavala *et al.*, 2009). The LOD of urinary iAs, MMAs, and DMAs by HG-AAS-CT were 0.2, 0.1, and 0.1 μg As/l urine, respectively.

To account for differences in water composition between urine samples, U-iAs, U-MMAs, and U-DMAs were adjusted by SG using the following equation: iAs × (mean SG-1)/(individual SG-1) as previously described (Nermell *et al.*, 2008). Therefore U-iAs, U-MMAs, and U-DMAs were reported as SG-adjusted values (μg As/l urine). Total urinary arsenic (U-tAs) was defined as the sum of SG-adjusted U-iAs, U-MMAs, and U-DMAs in each urine sample (μg As/l urine).

Assessment of protein expression in cord blood. The present study for cord serum proteomic analyses examined 50 samples obtained from mother-newborn pairs selected from the larger

BEAR cohort ($n = 200$). The samples used in this study were selected to include subjects exposed to varying levels of arsenic as determined by both the sum of the levels of iAs and iAs metabolites in maternal urine and iAs levels in drinking water. The relative expression levels of 507 proteins across the 50 cord serum samples were determined using the Biotin Label-based Human Antibody Array I, L series 507 (RayBiotech, Norcross, GA). Targets of the array include proteins involved in various aspects of cellular signaling and include cytokines, chemokines, growth factors, angiogenic factors, soluble receptors, and soluble adhesion molecules. Protein labeling and hybridization were carried out according to the manufacturer's instructions using 70 μl of each cord serum sample. Briefly, this procedure involved biotinylation of the primary amines of serum proteins and hybridization of the labeled sample to a membrane array containing antibodies specific for each of the 507 protein targets. Following incubation with a horseradish peroxidase (HRP)-streptavidin conjugate, membrane-bound proteins were revealed by chemiluminescence following incubation with a detection buffer containing an appropriate HRP substrate. Two types of positive controls are present on the array. The first is a biotin-labeled protein that is independent of the sample that is spotted on each array in a series of known concentrations and therefore used to normalize signal intensities across arrays. There is also an internal positive control which is an exogenous, nonmammalian protein that is added to the serum sample prior to biotinylation. This protein serves as a control for the biotin labeling and sample incubation steps as well as serving as an additional point of reference for normalization across multiple arrays.

Statistical analyses. Statistical analyses were performed using SAS 9.3 (SAS Institute Inc., Cary, NC), Partek Genomics Suite software (version 6.6; Partek, Inc., St Louis, MO), and Spotfire software (TIBCO software, Somerville, MA). All data were analyzed for their distribution patterns and homogeneity. Maternal urine samples with concentrations of iAs, MMAs, or DMAs below the LOD and DW samples with iAs below the LOD were assigned a value equal to the LOD/$\sqrt{2}$. A regression model was used to quantify the relationship between SG-adjusted maternal U-tAs as the independent variable and the normalized, background-subtracted signal intensities of each of the 507 protein array targets as the dependent variable. Potential confounders (e.g., maternal education, maternal age, gestational age, newborn sex, and birth weight) were examined for significant association with both the independent (U-tAs) and dependent (protein expression) variables. Both Spearman's rank correlation and Pearson's correlation were used to evaluate associations between potential confounders and dependent and independent variables. Significantly correlated variables were further analyzed in multivariable adjusted regression models. The models controlled for covariates identified based on correlations ($p < 0.1$) with the exposure (U-tAs) and outcome (protein levels). Based on these criteria, both newborn sex and birth weight were included in the final regression model. The regression model was used to identify all proteins that had a statistically significant relationship between expression level and U-tAs ($p < 0.05$) where false discovery (type II error) was controlled using a q-value <0.1.

A supervised analysis was performed to identify the activator and repressor proteomic groupings. First, newborns were classified into a low exposure class (LEC) or a high exposure class (HEC) based on median maternal U-tAs (30.7 μg/l). Average protein expression levels were determined within these classes and the expression level of each protein was compared to the average protein expression in the exposure categories. Activators were classified as newborns where the majority of assayed protein demonstrated quantitatively higher expression levels relative to the average protein expression for the HEC. Repressors were classified as newborns where the majority of assayed proteins demonstrated quantitatively lower expression levels relative to the average protein expression for the HEC. The relationship between newborn activator/repressor status and maternal characteristics, arsenic exposure indicators, and birth outcomes were determined using a Wilcoxon Rank Sum test and Chi-Square tests.

Functional analyses of proteins. Fetal cord blood proteins with statistically significant associations between protein level and U-tAs were analyzed for associated biological functions, canonical pathways, predicted upstream regulatory molecules, and interacting molecular networks using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). The proteins were also analyzed for associated canonical pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg). Benjamini-Hochberg-corrected p-values were reported for all canonical pathways.

RESULTS

Characteristics of Study Population

Maternal demographic characteristics, indicators of iAs exposure, and pregnancy and birth outcomes of the mother-child pairs selected for analysis here ($n = 50$) are described in Supplementary table 1. Indicators of iAs exposure included the concentration of iAs in maternal drinking water (μg As/l; DW-iAs) and urinary concentrations of iAs and its monomethylated and dimethylated metabolites, MMAs and DMAs, respectively, in maternal urine (μg As/l; U-iAs, U-MMAs, and U-DMAs, respectively). For comparative purposes, these data are also provided for the overall BEAR cohort ($n = 200$; Supplementary table 1).

The levels of iAs in drinking water samples of the cohort ($n = 50$) ranged from below the LOD of 0.456 up to 236 μg As/l. Of these drinking water samples, 30 (60%) had DW-iAs that exceeded the WHO standard of 10 μg As/l and 25 (50%) ex-

ceeded Mexico's standard of 25 μg As/l. The mean and median DW-iAs of the cohort were 53.9 and 24.2 μg As/l, respectively.

As observed with DW-iAs, there was a range of U-iAs, U-MMAs, and U-DMAs ($n = 50$; Supplementary table 1). Most of the urine samples demonstrated detectable levels of U-iAs and U-MMAs with ranges of <LOD–23.0 μg As/l and <LOD–18.2 μg As/l, respectively. DMAs was detected in all urine samples and ranged from 5.3 to 292.5 μg As/l. Total maternal urinary arsenic (U-tAs), defined as the sum of U-iAs, U-MMAs, and U-DMAs, ranged from 6.18 to 319.74 μg As/l. The average proportions of U-tAs comprising iAs, MMAs, and DMAs were 6.5% iAs, 6.5% MMAs, and 87.0% DMAs, respectively. There was a significant positive correlation between DW-iAs and U-tAs in the cohort ($n = 50$; $r = 0.88$; $p < 0.001$).

Analysis of the cohort for various birth outcomes showed that all pregnancies resulted in a live, singleton birth of which 54% were males. More detailed birth information is found in Supplementary table 1.

Identification of Cord Blood Proteins Associated with Maternal U-tAs

Multivariable regression analyses identified 111 proteins for which there was a statistically significant association between protein abundance in newborn cord blood and maternal U-tAs (Supplementary table 2). These analyses demonstrated that although these proteins were associated with maternal U-tAs across all subjects ($p < 0.05$, $q < 0.1$), there were two distinct groups of newborns that could be identified based on differences in proteomic response. Specifically, a supervised analysis was used to compare each newborn's individual proteomic response to the average proteomic response, analyzed in the context of prenatal arsenic exposure (i.e., U-tAs). These analyses resulted in the identification of two classes of newborns, those who demonstrated general increased expression levels of the 111 proteins as maternal U-tAs increased ($n = 30$) and were subsequently termed "activators" (Fig. 1A, Supplementary table 2). Similarly, there were newborns who in general showed decreased expression of the 111 proteins as maternal U-tAs increased ($n = 20$) and were subsequently referred to as "repressors" (Fig. 1B, Supplementary table 2).

Associations Between Activator/Repressor Status with Maternal Characteristics, Arsenic Exposure, and Birth Outcomes

The activator/repressor status was analyzed for association with various maternal characteristics, indicators of iAs exposure and metabolism, and selected birth outcomes. There was no difference in maternal characteristics, namely age at delivery, education level, smoking status, or alcohol consumption between activators and repressors (Supplementary table 3).

Overall, DW-iAs was similar between the activator and repressor groups ($p = 0.35$) with activators ranging in exposure levels from <LOD (<0.456 μg As/l) up to 212.1 μg As/l and repressors ranging in exposure levels from <LOD up to 235.6 μg As/l (Supplementary table 3). When the sex of the newborns was considered separately for analyses, there was no significant relationship between DW-iAs and activator/repressor status ($p = 0.42$ for males; $p = 0.60$ for females). Likewise, U-tAs was not significantly different between the activator/repressor groups (Supplementary table 3).

When newborn males and females were analyzed separately, there was a significant difference in U-tAs between the activators and repressors in newborn males ($p = 0.02$) but not in newborn females ($p = 0.90$) (Supplementary table 3). As observed with U-tAs, there was a statistically significant difference in U-DMAs and activator/repressor status in newborn males ($p = 0.03$) but not in newborn females ($p = 0.82$). A comparison between activator males versus activator females revealed there was no significant difference in U-tAs ($p = 0.07$) or U-DMAs ($p = 0.56$) between them. There were no statistically significant differences between indicators of iAs exposure or indicators of iAs metabolism (e.g., %MMAs, DMAs/MMAs) with activator/repressor status observed across all newborns, newborn males only, or newborn females only (Supplementary table 3).

Of the measured birth outcomes, a significant difference in head circumference was observed between activator and repressor newborn males ($p = 0.01$), in which activator males had a smaller head circumference (mean = 34.5 cm; median = 35 cm; range 32–37 cm) than repressor males (mean = 36.1 cm; median 36 cm; range 35–37 cm). There was no significant difference observed between activator and repressor newborn females ($p = 0.90$). There were no other statistically significant differences between activator/repressor status and any of the other measured birth outcomes including method of delivery, sex of newborn, gestational age, newborn weight, five minute Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score, placental weight, newborn crown to heel length, and newborns classified as small for gestational age (SGA), large for gestational age (LGA), or born at low birth weight (LBW) (Supplementary table 3).

Pathway and Network Analysis Reveals Enrichment of Proteins Involved in Development, Proliferation, and Inflammation

To gain insight into the biological role of the 111 proteins, functional analyses were performed. Analysis of the most significant enriched canonical pathways revealed they could be summarized into two major functional categories, namely those involved in (1) cellular growth/proliferation and/or development and those involved in (2) immune response/inflammation. Specifically, the pathways associated with growth/proliferation and/or development included the transforming growth factor beta (TGF-β) signaling pathway ($p = 5.7 \times 10^{-13}$) and the mitogen-activated protein kinase (MAPK) signaling pathway ($p = 5.6 \times 10^{-2}$), among others (Supplementary table 4A and B). Additionally, inflammation-associated pathways were identified including the nuclear factor kappa-light-chain-enhancer

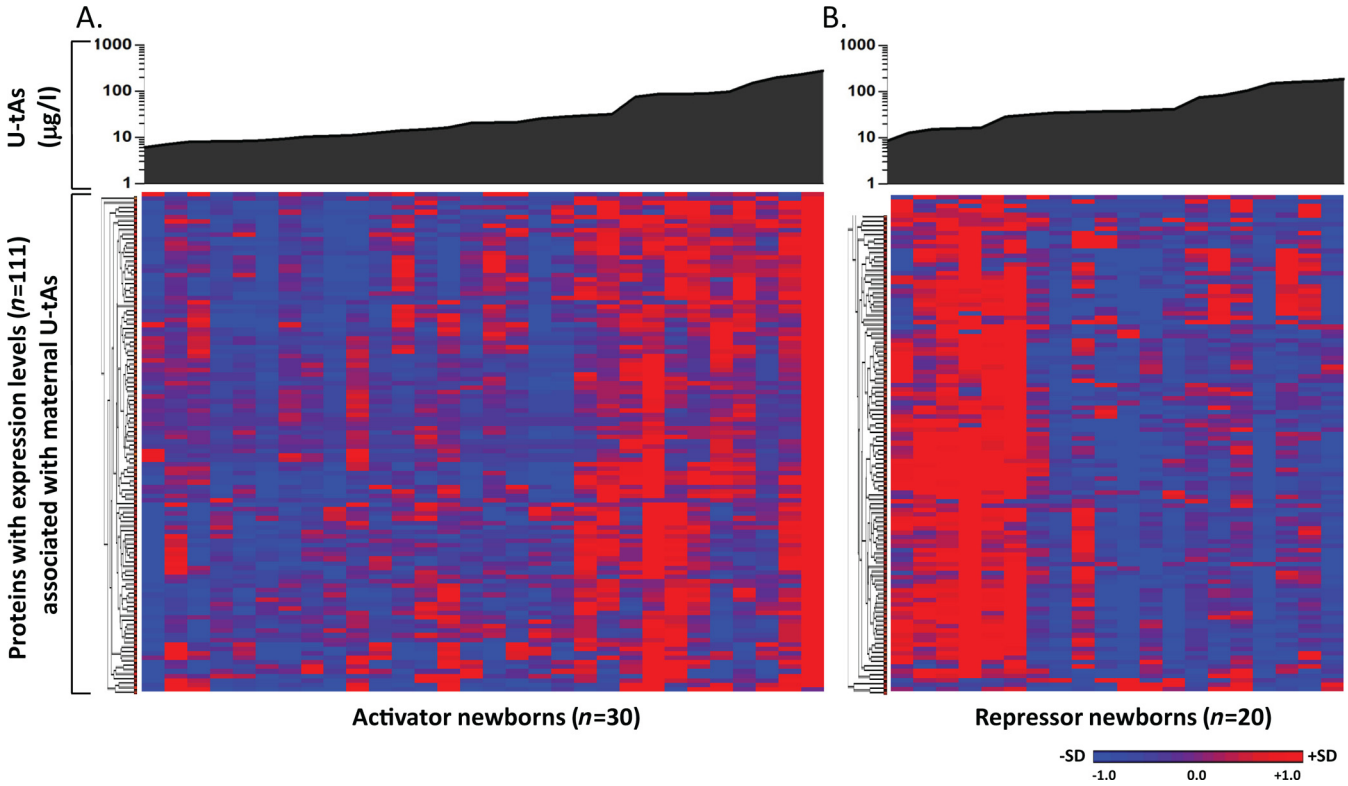

FIG. 1. Relative expression levels of U-tAs-associated cord blood proteins differ between newborns. Heat maps illustrate the relative protein expression levels of 111 U-tAs-associated cord blood proteins in activator newborns (A) and repressor newborns (B). Expression values for each protein are mean centered with high relative expression indicated in red and low relative expression indicated in blue. U-tAs: sum of inorganic arsenic (iAs) and its monomethylated and dimethylated metabolites (MMAs and DMAs, respectively) in maternal urine (µg As/l urine).

of activated B cells (NF-κB) signaling pathway ($p = 1.36 \times 10^{-5}$) (Supplementary table 4A and B).

In support of these results, network analyses revealed five significant protein clusters with functions associated with development and immune response (Supplementary table 4C). The two most significant networks had central hubs consisting of stress-responsive molecules, namely the transcription factor NF-κB and extracellular signal-regulated kinases 1/2 (ERK1/2) (Supplementary fig. 1A and B respectively; Supplementary table 4C). The most significant molecular network containing NF-κB as a central hub comprises several proteins involved in immune response (Supplementary fig. 1A).

The molecular network centered around ERK1/2 MAPK also contains several proteins involved in immune response/inflammation (Supplementary fig. 1B) as well as several proteins associated with development including bone morphogenic protein 2 (BMP2), bone morphogenic protein receptor 2 (BMPR2), neuregulins 1 and 3 (NRG1 and NRG3, respectively), and transforming growth factor beta receptor 2 (TGFBR2).

The 111 proteins were also analyzed for associated diseases and disorders, in which diseases related to the immune system and inflammation were among the most significant (Table 1). The pathway-level analyses identified several inflammation-associated diseases associated with arsenic exposure, including cancers, atherosclerosis, and liver fibrosis (Supplementary table 4A and B).

TNF Predicted to Regulate the Fetal Proteome

Analysis was performed on the 111 proteins to identify key upstream molecules including signaling molecules and/or transcription factors that are predicted to regulate their expression. This analysis uncovered a significant regulatory network ($p = 6.14 \times 10^{-25}$) predicted to mediate 62 (~56%) of the U-tAs-associated proteins. This network contains known interactions among 16 proteins or protein complexes consisting primarily of cytokines and transcription factors that play a role in inflammation/immune response, the majority of which (12; 75%) are predicted to be activated based on the expression profiles of the U-tAs proteins in the activator group (Fig. 2). This analysis highlights the potential role of TNF as a key regulator in the U-tAs-associated proteomic response in newborn cord blood and indeed of the 111 U-tAs-associated proteins, 45 (~40%) are known regulatory targets of TNF (Fig. 3). These proteins include those involved in immune response such as chemokine (C-X-C motif) ligand 16 (CXCL16), chemokine (C-X-C motif)

TABLE 1
Most Significant Diseases and Disorders of the 111 U-tAs-Associated Cord Blood Proteins

Disease/disorder	Average *p*-value	Number of proteins
Inflammatory response	1.21×10^{-05}	57
Cardiovascular disease	7.93×10^{-06}	35
Connective tissue disorders	1.93×10^{-05}	31
Immunological disease	1.50×10^{-05}	42
Inflammatory disease	1.00×10^{-05}	44

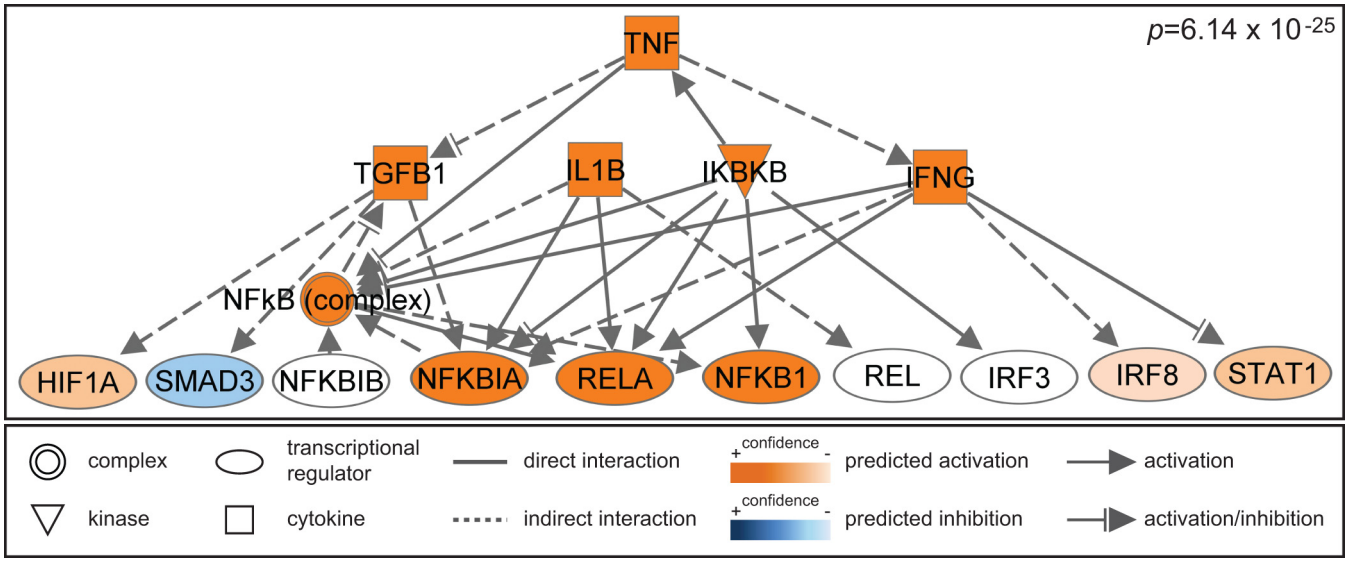

FIG. 2. Regulatory network of U-tAs-associated cord blood proteins. An upstream network predicted to regulate the expression of the majority (~56%) of the 111 U-tAs-associated cord blood proteins was identified. Proteins are displayed as predicted to be activated (orange) or inhibited (blue) with increasing maternal U-tAs, a trend representative of the activator newborn response. HIF1A: hypoxia inducible factor 1, alpha subunit; IFNG: interferon gamma; IKBKB: inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta; IL1B: interleukin 1 beta; IRF3: interferon regulatory factor 3; IRF8: interferon regulatory factor 8; NFKB complex: nuclear factor kappa light chain enhancer of activated B cells complex; NFKB1: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NFKBIA: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; NFKBIB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta; REL: *v*-rel reticuloendotheliosis viral oncogene homolog (avian); RELA: *v*-rel reticuloendotheliosis viral oncogene homolog A (avian); SMAD3: SMAD family member 3; STAT1: signal transducer and activator of transcription 1; TGFB1: transforming growth factor beta 1; TNF: tumor necrosis factor.

ligand 1 (CXCL1), and macrophage migration inhibitory factor (MIF) as well as proteins involved in development such as epiregulin (EREG) and TGFBR2.

Comparison with Reported Cord Blood Gene/Protein Changes in Other Human Cohorts

The 111 proteins identified here were compared with known prenatal arsenic-responsive genes (Fry *et al.*, 2007) and prenatal arsenic-responsive proteins (Ahmed *et al.*, 2011). Fry *et al.* (2007) identified 447 transcripts that were differentially expressed in cord blood leukocytes from newborns born to arsenic-exposed versus arsenic-unexposed mothers in Thailand (Fry *et al.*, 2007). Of these 447 transcripts, 30 represented genes in common with the 507 proteins probed on the array in the present study, and six (5%) were among the 111 U-tAs-associated proteins. The proteins encoded by these six genes were: chemokine (C-C motif) receptor 2 (CCR2), chemokine (C-C motif) receptor 3 (CCR3), CXCL1, chemokine CXCL16, CXCR4, and EREG. In the present study, each of these proteins displayed increased expression levels in activator newborns as U-tAs increased.

Ahmed *et al.* (2011) analyzed 18 cord blood proteins for associations with maternal U-tAs in Bangladeshi newborns. Of these 18 proteins, 15 were represented on the arrays in the current study, however, none of these proteins were among the 111 U-tAs-associated proteins identified here. Ahmed *et al.* (2011) identified four cord blood proteins, namely interleukin 1 beta (IL1B), interleukin 8 (IL8), interferon gamma (IFNG), and TNF that had expression levels that formed U-shaped curves across quartiles of maternal U-tAs. TNF, INFG and IL1B are components of the upstream regulatory network of U-tAs-associated cord blood proteins described in the current study (Fig. 2).

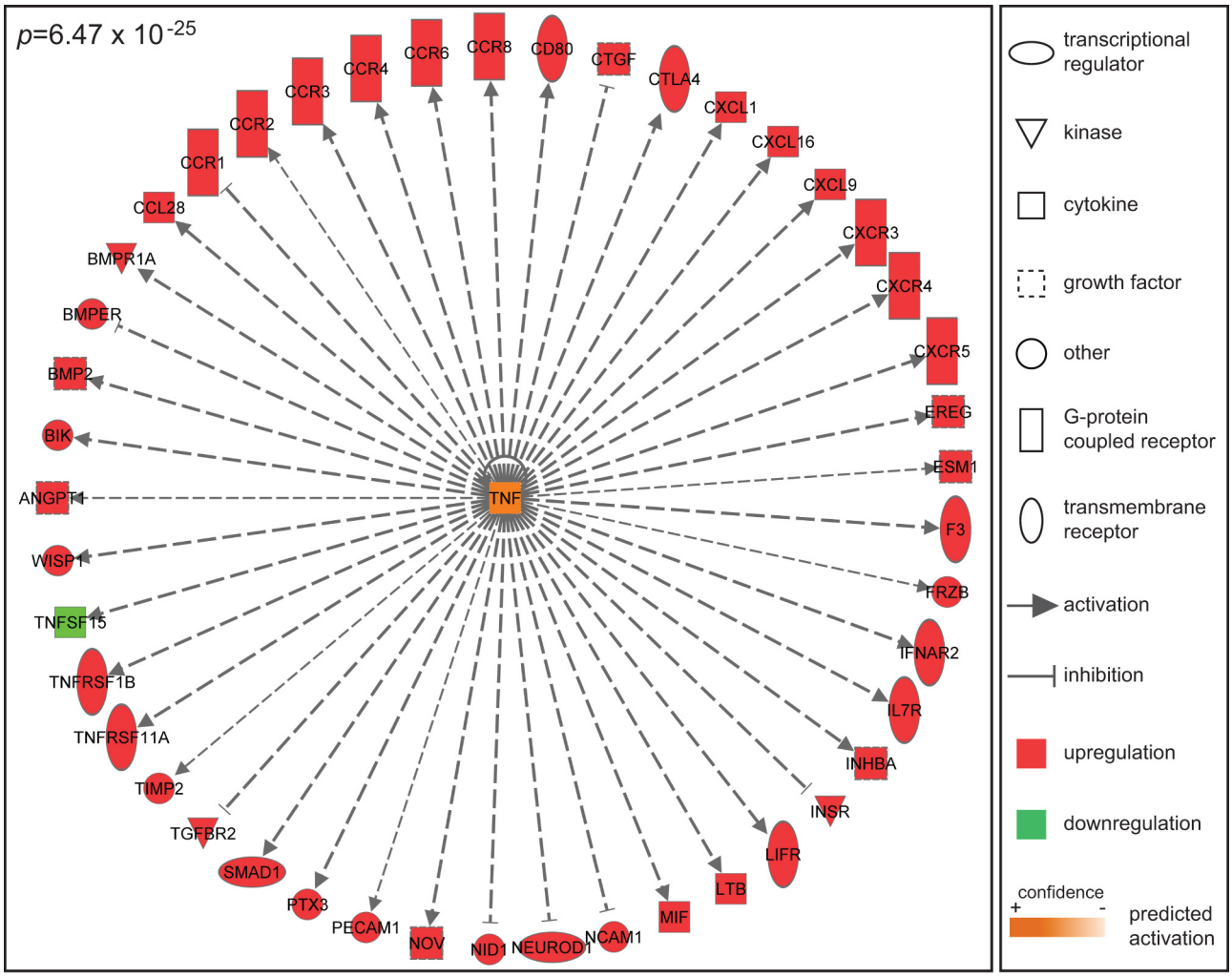

FIG. 3. TNF-associated cord blood proteins. TNF is predicted to regulated the expression of the majority ($n = 45$) of the U-tAs-associated proteins. Proteins are displayed as predicted to be activated (orange), up-regulated (red), or down-regulated (green) with increasing maternal U-tAs, a trend representative of the activator newborn response. TNF: tumor necrosis factor. Complete names of the U-tAs-associated proteins are provided in Supplementary table 1.

DISCUSSION

Prenatal iAs exposure is clearly linked to adverse health effects such as poor birth outcomes, increased susceptibility to opportunistic infections in infancy and childhood, and altered disease risk later in life (Farzan *et al.*, 2013; Vahter, 2009). In the present study, we set out to identify biological pathways that may be altered at the protein level by *in utero* iAs exposure using a proteomics assessment of more than 500 proteins in newborn cord blood. The results presented here represent the most comprehensive proteomic analysis of the effects of *in utero* iAs exposure in human populations to date and may help identify proteins that may be used as biomarkers of exposure and/or predictors of disease risk.

Using samples from 50 mother-child pairs from the recently-established BEAR prospective pregnancy cohort, a total of 111 cord blood proteins were identified with expression levels associated with maternal U-tAs. When analyzed in the context of their proteomic responses, the newborns could be divided into two distinct groups based on the relationship between the expression levels of these 111 proteins and maternal U-tAs. Most newborns ($n = 30$; 60%) were activators, who in general demonstrated a positive association between U-tAs and protein expression, whereas the remainder ($n = 20$; 40%) were repressors, in which cord blood protein expression levels and U-tAs had an inverse relationship. It is important to note that the identification of the activator and repressor newborns, with potentially high biological relevance, required an analysis of each newborn's proteomic response relative to the average newborn proteomic response. A relationship was identified between this activator/repressor status, maternal indicators of arsenic exposure (i.e., U-tAs and U-DMAs), and head circumference in

newborn males. Unexpectedly, we observed this relationship at lower doses of U-tAs within the cohort, as activator males were associated with reduced average U-tAs, U-DMAs, and head circumference relative to repressor males. Few studies have examined the effects of prenatal arsenic exposure on newborn head circumference, but two separate studies reported negative associations between newborn head circumference and arsenic levels in cord blood (Guan *et al.*, 2012) and between U-tAs and newborn head circumference, chest circumference, and size at birth at low levels of maternal arsenic exposure (U-tAs < 100 μg/l) (Rahman *et al.*, 2009b). These relationships between prenatal arsenic exposure and head circumference may be significant for health effects later in life as in general, there is a positive relationship between head circumference at birth and childhood cognitive ability (Heinonen *et al.*, 2008; Veena *et al.*, 2010). Furthermore, inverse associations between iAs exposure and cognitive function have been reported in children (Rosado *et al.*, 2007; von Ehrenstein *et al.*, 2007; Wasserman *et al.*, 2007) but little is known in terms of the impact of prenatal exposure on intellectual function and must be addressed in future studies.

It is important to highlight that the relationship between U-tAs and newborn head circumference in activator newborn boys was not only dependent on activator/repressor status but was also sex-dependent. Various other sex-specific effects of iAs exposure have been reported in the literature. For instance, observations in exposed populations suggest women are more likely to develop several internal cancers whereas men are more likely to develop skin lesions, and different carcinogenic effects are observed between adult male and female mice transplacentally exposed to iAs *in utero* (as reviewed in Vahter *et al.*, 2007). Of potential interest to the current study, research based in Torreón, Mexico demonstrated that boys and girls differed in terms of cognitive deficiencies associated with iAs exposure (Rosado *et al.*, 2007). More cognitive deficiencies were observed in boys than girls and in some cases, cognitive deficits were only noted at low levels of exposure (U-tAs < 50 μg/l). The identification of specific cognitive effects at low levels of exposure is especially interesting considering the association between lower maternal U-tAs and reduced head circumference in activator boys in the present study. Altogether, these data suggest the relationship between activator status, maternal U-tAs and sex-specific trends in birth outcomes warrant further study in terms of later-life health effects.

Many of the identified U-tAs-associated proteins have known links to development, inflammation/immune response, inflammatory diseases, and chronic diseases associated with arsenic exposure. In addition, this analysis revealed that many of the U-tAs-associated proteins are regulated by TNF, which is known to be involved in diverse processes in the cell including apoptosis, necrosis, inflammation, cell survival, angiogenesis, and cell migration (Wajant, 2009). Of potential interest to the birth outcomes cited above, proteins with known links to neurological, brain, and/or skull development were among the TNF-associated and/or ERK1/2-associated proteins such as TGFBR2, BMP2, BMPR2, and NRG1 (Britsch, 2007; Monuki *et al.*, 2001; Seo and Serra, 2009). Therefore, these proteins may serve as novel targets for understanding arsenic-associated impacts on fetal growth and neurological development and disease.

The identification of inflammation/immune response as a major function associated with the U-tAs-associated proteins and their predicted upstream regulatory proteins/complexes is of particular interest. In relationship with maternal U-tAs, these proteins were largely predicted to be activated or up-regulated in the majority of newborns (i.e., activator newborns), which support the proinflammatory responses observed at the transcriptional level (Fry *et al.*, 2007) and protein level (Ahmed *et al.*, 2011) in the cord blood of arsenic-exposed newborns in other populations. Taken together, these data suggest that proinflammatory signaling is a conserved response to prenatal arsenic exposure in newborns across populations. Importantly, long-term proinflammatory signaling is implicated as a critical mechanism by which arsenic induces carcinogenic effects (as reviewed in Valko *et al.*, 2006). In the current study, several U-tAs-associated pathways, proteins and upstream regulators of these proteins have known links to inflammatory diseases associated with arsenic exposure. These included pathways associated with various cancers, hepatic fibrosis, and atherosclerosis. TNF, identified here as a putative master regulator of the proteomic response to prenatal arsenic exposure, plays a key role in the regulation of immune/inflammatory responses and is strongly implicated in chronic inflammatory disease development (Holtmann and Neurath, 2004). Polymorphisms within the TNF promoter are associated with increased levels of TNF and a higher incidence of skin lesions, respiratory diseases, and conjunctivitis in iAs-exposed individuals in West Bengal (Banerjee *et al.*, 2011). TNF is also implicated as a player in inflammatory reactions in the liver leading to tissue injury and fibrosis in arsenic-exposed mice (Das *et al.*, 2005). The proinflammatory, proangiogenic growth factor CXCL1 is a potential biomarker of prenatal arsenic exposure (Fry *et al.*, 2007) that has been associated with an invasive phenotype and higher pathological stage in bladder cancers (Kawanishi *et al.*, 2008) and is associated with hepatocellular carcinoma (Wu *et al.*, 2009). MIF is a regulator of immune/inflammatory responses and is implicated in angiogenesis in all stages of cancer development (Babu *et al.*, 2012). It is important to note that although the expression of these inflammation-associated proteins is generally up-regulated in relationship to U-tAs in activator newborns, these proteins are generally down-regulated in relationship to U-tAs in repressor newborns. Although a persistent inflammatory state can be detrimental to human health, impaired immune function, which has also been associated with prenatal arsenic exposure in human populations (Ahmed *et al.*, 2012; Rahman *et al.*, 2010; Raqib *et al.*, 2009), can also play a role in the development of acute disease as well as increase the susceptibility to the development of chronic diseases later in life. Because markers of immunosuppression and inflammation have been observed simultaneously in arsenic-exposed populations (Soto-Pena *et al.*,

2006), it is likely that these two seemingly divergent effects both play a role in the development of the diverse adverse health effects associated with iAs exposure. In support of this, the results of the current study suggest there may be a relationship between activator/repressor newborn status, differential modulation of proteins involved in host defense, and variation in disease susceptibility.

Although the current study identified 111 cord blood proteins that were associated with maternal U-tAs and thus vastly increased the knowledge of the proteomic response to arsenic exposure *in utero*, it is not without limitations. The sample size in the present pilot study was limited based on proteomic assay cost. Thus, the dose-responsive and sex-dependent trends reported here should clearly be replicated in larger cohorts. In addition, the relationship between maternal U-tAs, cord blood levels of arsenic (BA), and health outcomes will be examined, as there are indications that the relationship between U-tAs and BA may be sex-dependent (Hall *et al.*, 2006). Moreover, it is not possible in the present study to determine correlative versus causal relationships between U-tAs, proteomic responses, and birth outcomes, which will require controlled toxicological studies in animal models.

The relevance of alterations in the expression of the identified proteins in exposed newborns to the susceptibility of chronic diseases that develop later in life is currently unknown. These altered profiles may be linked to epigenetic reprogramming that occurs in the fetal environment in association with iAs exposure. Through the altered, persistent expression of key genes, epigenetic reprogramming has been implicated as a plausible link between prenatal exposure to environmental toxicants such as iAs and altered disease risk in adulthood (Bollati and Baccarelli, 2010; Jirtle and Skinner, 2007). Therefore, the stability of these altered proteomic profiles and their relationship with alterations to the epigenome and disease susceptibility must be investigated in future studies. The results observed here will serve as an important foundation in efforts to elucidate the complex relationship between prenatal arsenic exposure, interindividual proteomic responsiveness, and differences in disease susceptibility.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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