CHRONIC EARLY CHILDHOOD EXPOSURE TO INORGANIC ARSENIC IS ASSOCIATED WITH A TNF-MEDIATED PROTEOMIC SIGNALING RESPONSE

Lisa Smeester

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Approved by:

Rebecca C. Fry

Leena Nylander-French

Jill Stewart

ABSTRACT

Lisa Smeester: Chronic Early Childhood Exposure to Inorganic Arsenic is Associated with a TNF-mediated Proteomic Signaling Response
(Under the direction of Rebecca C. Fry)

Exposure to inorganic arsenic (iAs) in drinking water remains a global issue of concern and is associated with a range of health outcomes, including immune dysfunction. Young children have been identified as a particularly sensitive population, yet mechanisms of adverse health outcomes are understudied. Here we set out to examine the effects of iAs exposure on circulating serum proteins in adolescents. To identify proteins as potential indicators of disease, levels of total urinary arsenic (U-tAs) and its methylated metabolites were determined and serum proteins assessed for differences in expression. The results indicate an enrichment of TNF-regulated immune and inflammatory response proteins that display decreased expression levels in relation to increasing U-tAs. Notably, when analyzed in the context of the arsenical proportions, there was minimal overlap between the protein lists, with the most robust response observed in relation to %MMAs. These data represent the first assessment of protein expression in serum in adolescents exposed to inorganic arsenic.

To MFC & MGS, for the countless hours of "bunny duty," shifting birthday and holiday celebrations to more convenient dates, and your determination to find wi-fi in the middle of nowhere so I could connect to Sakai on our road trips. My decision to return to school threw our lives into upheaval and never once was I met with anything but encouragement and understanding.

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LIST OF ABBREVIATIONS

As	Arsenic
iAs	Inorganic arsenic
BMI	Body mass index
DAVID	The Database for Annotation, Visualization, and Integrated Discovery
DMAs	DMA(III) + DMA(V)
DMA^{III}	Dimethylarsinous acid
DMA^{V}	Dimethylarsinic acid
EPA	Environmental Protection Agency
HRP	Horseradish peroxidase
ICP-MS	Inductively coupled plasma mass spectrometry
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
MMAs	MMA(V)+MMA(III)
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
ppb	Parts per billion
TNF	Tumor necrosis factor
U-iAs	Urinary inorganic arsenic
U-DMAs	Urinary DMA(III) + DMA(V)
U-MMAs	Urinary MMA(III) + MMA(V)
U-tAs	Urinary total arsenic
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

Exposure to elevated levels of inorganic arsenic (iAs) in drinking water remains a global issue of concern. Over 100 million people worldwide are exposed to levels of iAs in their drinking water that exceed the World Health Organization's recommended limit of 10 μg/L (WHO 2011). While new populations continue to be identified, those at risk of elevated exposure include, but are not limited to, populations in Bangladesh, Mexico, the United States, and China, among others (Mandal and Suzuki 2002).

Chronic exposure to iAs is associated with a range of health outcomes in adults, including diabetes mellitus, impaired cognition and neurological effects, hypertension, immune dysfunction, and skin, lung, bladder, liver, and kidney cancers (Naujokas, Anderson et al. 2013). Of increasing concern, young children have been identified as a particularly sensitive population (Vahter 2008, Naujokas, Anderson et al. 2013). Specifically, early childhood exposure to iAs has been associated with outcomes manifesting during both adolescence and adulthood, including impaired cognitive development, increased mortality due to bladder, laryngeal, and lung cancers, increased non-cancer mortality due to bronchiectasis, myocardial infarction, and increased infection risk (Hamadani, Tofail et al. 2011, Smith, Marshall et al. 2012, Naujokas, Anderson et al. 2013, Rahman, Sohel et al. 2013). The long-lasting impact of iAs exposure during early childhood suggests that early life represents a critical period during which there is heightened sensitivity to the toxic effects of iAs (Vahter 2008, Naujokas, Anderson et al. 2013).

The mechanisms underlying the health effects of childhood exposure to iAs remain understudied. Previous studies examining immune functioning in iAs-exposed children suggest that iAs can act as an immunosuppressant. Specifically, it has been reported that children with iAs exposure exhibited decreased plasma concentrations of the Th1 cytokines, TNF-α and IL-2, and reduced responsiveness on functional immune tests (Soto-Peña, Luna et al. 2006, Ahmed, Moore et al. 2014). In support of these data, there is also evidence that iAs exposure during childhood impairs monocyte functioning and immune-specific reactive oxygen species (ROS) signaling (Pineda-Zavaleta, García-Vargas et al. 2004, Luna, Acosta-Saavedra et al. 2010). Paradoxically, there is also substantial evidence that iAs acts as a pro-inflammatory agent in children. For instance, *in utero* exposure to arsenic is associated with an activation of inflammation, including the NF-kB signaling cascade (Fry, Navasumrit et al. 2007, Bailey, Laine et al. 2014). Early life exposure has also been linked to iAs-induced chronic inflammation mediated impaired lung function (Olivas-Calderon, Recio-Vega et al. 2015). Taken together, this suggests that iAs can act as an immunomodulatory agent during childhood and development, possibly impacting maturation of the immune system during a critical period of development (Luna, Acosta-Saavedra et al. 2010, Dangleben, Skibola et al. 2013). Such an effect may play a critical role in the development of the diverse adverse health effects associated with iAs exposure.

Arsenic metabolism is a multi-step process (Figure 1), with six major arsenic species that have been identified in human urine. These species include inorganic arsenics (iAs^{III} and iAs^{V}), which are metabolized to the monomethylated arsenic species (MMAs), monomethylarsenous acid (MMA^{III}) and monomethylarsonic acid (MMA^{V}). MMAs are methylated again to become dimethylated (DMAs), forming dimethylarsinous acid (DMA^{III}) and dimethylarsinic acid

(DMA^V) (Tseng 2007). The efficiency of these methylation reactions has been identified as an important factor underlying the effects observed following iAs exposure as iAs, MMAs, and DMAs are differentially associated with As-related outcomes such as hypertension, atherosclerosis, cancer, and chromosomal aberrations (Huang, Tseng et al. 2007, Tseng 2007).

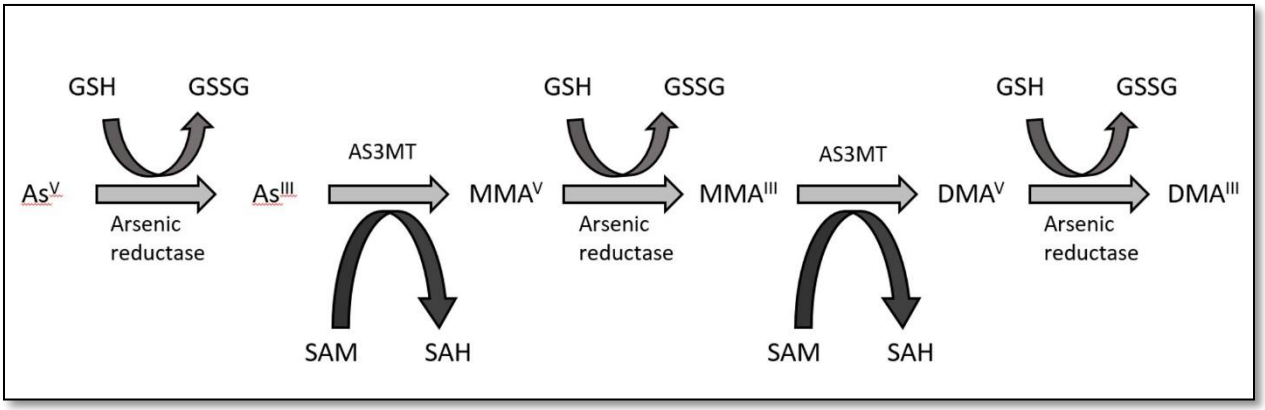

Figure 1. Arsenic metabolism is a multi-step process. Arsenic is reduced from its V to III form by arsenic reductase, using glutathione (GSH) as an essential co-factor. Arsenic is methylated by Arsenic (+3 Oxidation State) Methyltransferase (AS3MT), using S-adenosyl methionine (SAM) as the methyl donor.

Additionally, we have previously demonstrated that urinary iAs is positively associated with lower gestational age and newborn length, while urinary MMAs is associated with lower gestational age and birth weight (Laine, Bailey et al. 2015). Therefore, inter-individual differences in the efficiency of arsenic methylation may play a role in metabolite-specific associated disease risk. The impact that differences in iAs metabolism may have on protein expression during childhood is currently unknown.

Given evidence that exposure to iAs during childhood leads to abnormal immune functioning (Bailey, Laine et al. 2014, Olivas-Calderon, Recio-Vega et al. 2015), we hypothesized that childhood iAs exposure is likely to disrupt expression levels of proteins involved in immune function. Therefore, we conducted a proteomic assessment of serum in iAs-exposed children

selected from a cohort of subjects living in Comarca Lagunera, an area in North-Central Mexico. Moreover, in light of the knowledge that different arsenical species yield different health effects, we included an assessment for the relationship of proteins to %iAs, %MMAs and %DMAs. In the present study, we describe differences in protein expression levels in adolescents' serum associated with concentrations of each class of urinary arsenic metabolites.

CHAPTER 2: METHODS

Study Subjects and Sample Collection

The study sample for the present analysis represents a subset of 40 subjects of a cohort reported previously (Recio-Vega, Gonzalez-Cortes et al. 2014). Participants were children, both male and female, aged 6-12 years living in one of four rural communities in the Comarca Lagunera area, located in north-central Mexico. These communities represent those with the highest arsenic tap water levels (104-360 ppb) detected in the last 20 years in the area. Arsenic is present in the local water supply due to the over-extraction of groundwater. Children included in this study had mothers who remained in these communities for the duration of their pregnancy, and have since remained residents of these same communities.

Questionnaires

Information was collected through in-person interviews and included socio-demographic variables (education, socioeconomic status), lifetime residential history, lifestyle factors (secondhand smoke), parent's occupational history, water source types (municipal tap water, bottled, other), current medications, medical history, and diet. Questionnaires were completed by the mothers at their own residing community. Water consumption habits, including source of drinking water, were ascertained through a standardized questionnaire (Recio-Vega, Gonzalez-Cortes et al. 2014).

Determination of arsenic concentrations in water and in urine

Drinking water samples (well) were collected from each rural community included in the study and analyzed for inorganic arsenic levels. Well water samples from each rural community are representative of the water that participants drank and is provided through the unique local water supply system. Individual exposure was assessed based on U-tAs. A first morning void urine sample was collected in sterile 120-mL screw-topped polypropylene containers. Urine samples were analyzed as described previously (Olivas-Calderon, Recio-Vega et al. 2015). Briefly, samples were analyzed at the Arizona Laboratory for Emerging Contaminants, University of Arizona, Tuscon, Arizona. Urinary As^{V}, As^{III}, MMA^{V}, DMA^{V}, and arsenobetaine were separated by HPLC and concentrations were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Standard Reference Water, SMR 1640 (NIST, Gaithersburg, MD, USA) and the freeze-dried Urine Reference Material for trace elements (Clinchek-control; RECIPE Chemicals instruments GmbH, Munich, Germany) were used as quality controls for urinary arsenic measurement.

Assessment of protein expression in serum

Subjects selected for proteomic assessment are representative of the extremes of exposure (median $\text{U-tAs}_{\text{high}}$ = 399.35 µg/L, median $\text{U-tAs}_{\text{low}}$ = 26.03 µg/L). As detailed in our prior publication (Bailey, Laine et al. 2014), the relative expression levels of 507 proteins were determined using the Biotin Label-based Human Antibody Array I, L series 507 (RayBiotech, Norcross,GA), which includes cellular signaling proteins such as 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 40 µl of

each serum sample. Briefly, primary amines of serum proteins are biotinylated and hybridized to a membrane array containing antibodies specific for each of the 507 protein targets, incubated with a horseradish peroxidase (HRP)-streptavidin conjugate, and detected by chemiluminescence following incubation with an HRP substrate buffer. The protein array contains two types of positive controls: a biotin-labeled protein, independent of the sample, that is spotted on each array in a series of known concentrations enabling signal intensity normalization across arrays, as well as an internal positive control which is an exogenous, nonmammalian protein added to the serum sample prior to biotinylation, serving as a control for the labeling and incubation steps.

Statistical Analyses

Statistical analyses were performed using Partek Genomics Suite software (version 6.6; Partek, Inc., St Louis, MO). All data were analyzed for their distribution patterns and homogeneity, and filtering was used to remove any non-expressed proteins. Multivariable regression models were used to examine relationships between individual arsenic measures (U-tAs, %iAs, %MMAs, %DMAs) and the normalized, background-subtracted signal intensities of the 393 proteins. Age, sex, and body mass index (BMI) were selected as *a priori* covariates due to their potential influence on protein expression levels and were controlled for in the models. Statistical significance was defined as $p < 0.05$, with false discovery (type II error) controlled for using a q-value <0.1.

Functional Analyses of Proteins

Proteins that showed statistically significant association with either U-tAs, %iAs, %MMA, or %DMA were analyzed for biological functions, canonical pathways, upstream

regulatory molecules, and interacting molecular networks using Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA). Proteins were also analyzed for KEGG pathway molecular interactions using The Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/), and immunologic signatures using Gene Set Enrichment Analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp).

CHAPTER 3: RESULTS

Study characteristics

Demographic characteristics of the study sample (n=40), as well as those of the entire cohort (n=358), can be found in Table 1. These subjects were selected as representatives of the extremes of exposure (median U-tAs $_{high}$ = 399.35 µg/L, median U-tAs $_{low}$ = 26.03 µg/L). Anthropometric characteristics recorded include sex, age, (years) weight (kg), and BMI (calculated as weight/height^2). The majority of the subjects were male (n=22, 55%), have lived at their current residence their entire lives (n=36, 90%), and were, on average 9.3 years of age (range 7-12). Other possible sources of arsenic exposure, including diet (seafood, rice and others), agrochemicals, fuels, preservatives or other compounds containing arsenicals, were negligible (Recio-Vega, Gonzalez-Cortes et al. 2014).

Indicators of arsenic exposure examined include total urinary arsenic (U-tAs; µg/L), as well as the percentages of inorganic arsenic (iAs), monomethylated arsenicals (MMAs) and dimethylated arsenicals (DMAs) as indicators of arsenic metabolism. All samples were within detectable limits and U-tAs, defined as the sum of U-iAs, U-MMAs and U-DMAs, ranged from 5.33 µg/L to 664.53 µg/L. The average proportions of U-tAs were 24.4% iAs, 14.9% MMAs, and 60.7% DMAs. Arsenic tap water levels and urinary arsenic levels were highly correlated (R^2 = 0.69).

Table 1. Demographic and exposure characteristics of study population

	Study Sample	***Cohort***
	Mean, Median (Range) or n (n%)	Mean, Median (Range) or n (n%)
Sex		
male	22 (55%)	188 (53%)
female	18 (45%)	170 (47%)
age (years)	9.3, 9.5 (7-12)	8.9, 9.0 (6-12)
height (m)	1.4, 1.38 (1.18-1.63)	1.3, 1.3 (1.1-1.7)
weight (kg)	37.0, 34.5 (17.0-82.0)	34.1, 31.43 (15.9-82.0)
BMI*	18.3, 17.8 (7.3-32.9)	17.9, 16.7 (7.2-33.8)
time current address (yrs)	9.3, 9.0 (5-12)	8.4, 8.0 (5-12)
U-As Total (µg/L)	220.0, 135.11 (5.33-664.53)	141.15, 114.59 (4.72-894.3)
Low exposure	32.94, 26.03 (5.33-109.13)	n/a
High exposure	384.73, 399.35 (159.82-610.51)	n/a
%iAs	21.6, 13.8 (5.44-84.4)	21.4, 20.58 (3.26-91.38)
%MMA	14.5, 12.7 (7.14-68.42)	13.23, 4.0 (2.06-42.80)
%DMA	58.8, 68.9 (1.6-81.9)	61.91, 21.64 (0.99-89.83)

* calculated as weight/height^2

Identification of proteins associated with total urinary arsenic and arsenic metabolites

Multivariable analyses identified 58 proteins that displayed a statistically significant association ($p<0.05$, $q=0.1$) with U-tAs, the vast majority of which are negatively associated (n=56, 96.6%) (Appendix 1). When analyzed in the context of the arsenical proportions, 8 proteins were significantly ($p<0.05$) associated with %iAs, 18 proteins were associated with %MMAs, and 11 proteins displayed significant association with %DMAs. There were no proteins identified in common across all three methylated metabolites (Figure 2, Appendix 1).

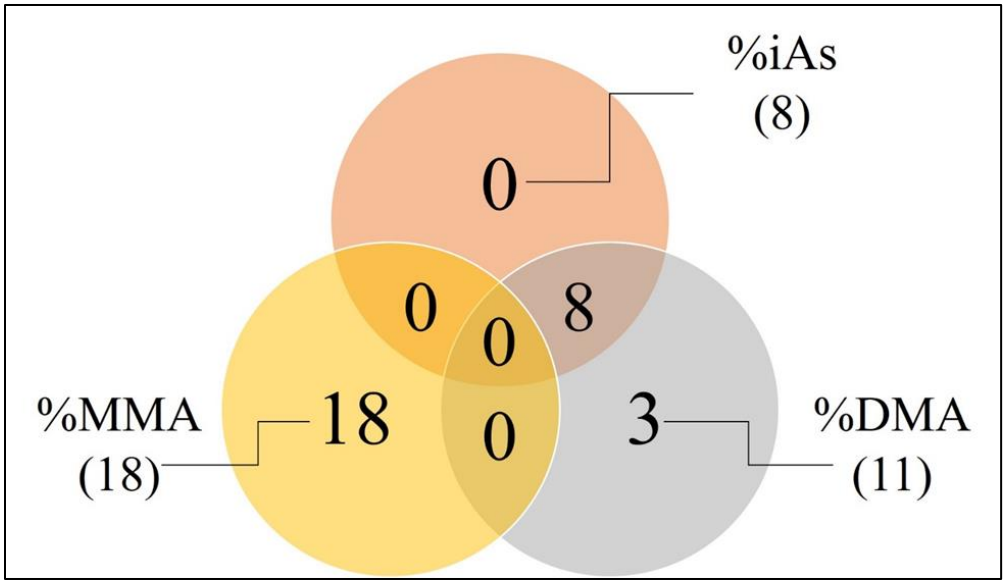

Figure 2. Total number of %iAs, %MMA, and %DMA associated proteins as well as commonality among arsenical associated lists. Venn diagram illustrating the number of serum protein that have a statistically significant association between expression levels and urinary proportions of iAs, MMAs and/or DMAs.

The 58 proteins identified as being significantly associated with U-tAs were analyzed for functional interactions (Appendix 2). Canonical pathway analysis revealed a strong enrichment of cytokine-mediated communication between immune cells (p= 1.12 x 10^{-2}), comprised largely of a family of interleukins, including Interferon, Alpha 13 (IFNA1/IFNA13) and Chemokine (C-X-C Motif) Ligand 8 (CXCL8). Network analysis revealed a protein cluster involved in cellular development, cellular growth and proliferation (p= 1 x 10^{-34}), which interacts with the major histocompatibility complex (MHC), class II (Figure 3).

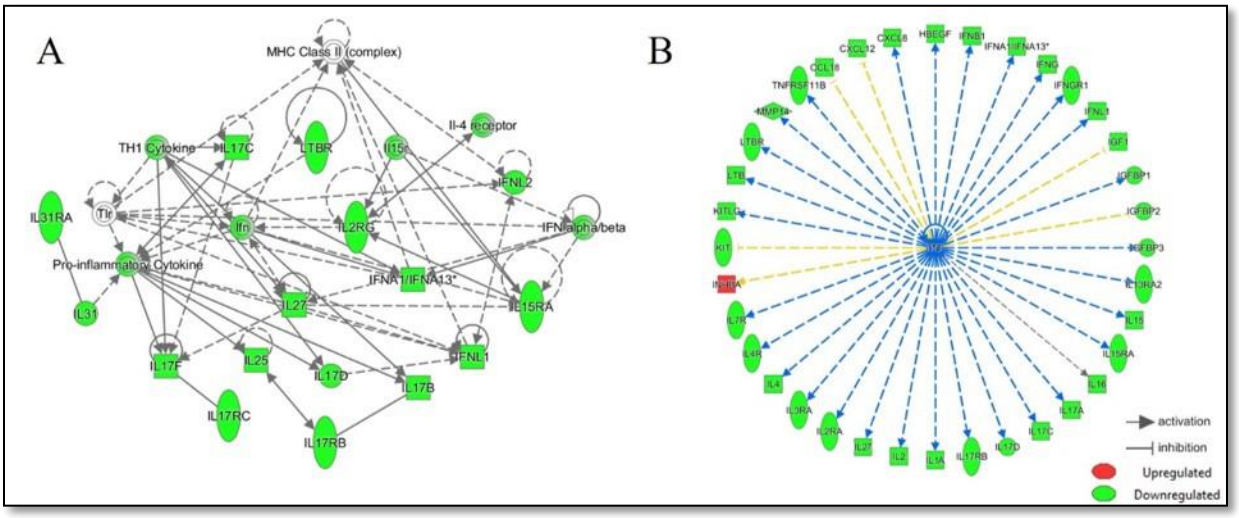

Figure 3. Interacting network of U-tAs associated serum proteins and upstream regulation by TNF. Interacting network of U-tAs-associated serum proteins are identified (A). TNF is predicted to regulate the expression of the majority (n=36, 62%) of the U-tAs associated proteins (B). Proteins are displayed as predicted to be upregulated (red) or downregulated (green).

A similar approach was used to investigate the pathways that were enriched among the proteins associated with the arsenical proportions. In relation to %iAs, canonical pathway analysis revealed several proteins involved in immune surveillance (p= 2.90 x 10^{-4}), and include Platelet-Derived Growth Factor C (PDGFC) and Platelet-Derived Growth Factor D (PDGFD). Network analysis revealed a cellular growth and proliferation component (p=1.00 x 10^{-24}), mediated by ERK 1/2 kinase and containing several members of the PDGF family, which are responsible for cellular proliferation and differentiation.

In relation to %MMAs, altered proteins were associated with an apoptosis signaling pathway (p=1.09 x 10-9), including Tumor Necrosis Factor (Ligand) Superfamily, Member 10 (TNFSF10) and many related TNF receptor superfamily (TNFRSF) members and vascular endothelial growth factors B and C (VEGFB, VEGFC). Network analysis centered on

organismal and cardiovascular system development and connective tissue disorders (p= 1 x 10-47), modulated by ERK 1/2 and NFkB.

In relation to %DMAs, proteins involved in immune surveillance, specifically macropinocytosis signaling (p= 3.36x10^{-8}), were altered and include Platelet Derived Growth Factor B (PDGFB), PDGFC, and PDGFD. Network analysis reveals cellular growth and proliferation (p= 1 x 10^{-33}), and includes many members of the PDGF family modulated by NFkB. Additionally, DAVID was used to confirm all pathway analyses and showed Cytokine-cytokine receptor interaction as the common top KEGG pathway for U-tAs (p= 3.76 x 10^{-21}), %iAs (p=0.015), %MMAs (p= 6.80 x 10^{-09}), and %DMAs (p=0.015).

Transcription factor regulation of arsenic-associated proteins

Analysis was performed for all arsenic-associated protein lists in order to identify key upstream regulators, including cell signaling molecules and transcription factors (Appendix 2). Tumor necrosis factor (TNF) was found to be the top regulator of the U-tAs-associated proteins (p=1.59 x 10^{-27}), and was predicted to be inhibited in relation to U-tAs. For the individual arsenicals, Dual Specificity Phosphatase 5 (DUSP5) was the top regulator of the %iAs-associated proteins (p= 1.98 x 10^{-5}), pyruvic acid was the top regulator of the %MMAs-associated proteins (p= 1.98 x 10^{-10}), and for %DMAs-associated proteins, Fibroblast Growth Factor 2 (Basic) (FGF2) (p= 1.47 x 10^{-5}) was the most significant regulator. Because of the small number of proteins in each arsenical-associated list, predicted effects (i.e., activation or inhibition) could not be established.

CHAPTER 4: DISCUSSION

Early childhood exposure to iAs has been associated with adverse health outcomes manifesting during both adolescence and adulthood (Hamadani, Tofail et al. 2011, Smith, Marshall et al. 2012, Naujokas, Anderson et al. 2013, Rahman, Sohel et al. 2013). Moreover, inter-individual differences in the efficiency of arsenic metabolism has been shown to influence iAs-associated disease, with high urinary proportions of MMAs associated with detrimental outcomes such as cancer of the urinary bladder, skin cancer, and cardiovascular disease (Huang, Tseng et al. 2007, Tseng 2007). Despite this, the effects of childhood exposure to iAs remain understudied, particularly in terms of effects on cellular and molecular functions. In the present study, we set out to identify biological pathways which may be modulated at the protein level by early life arsenic exposure. Using a subset of 40 subjects from a previously reported cohort (Recio-Vega, Gonzalez-Cortes et al. 2014), we have identified proteins with expression levels associated with inorganic arsenic exposure. While we have previously examined prenatal arsenic-associated differences in the expression in the newborn cord serum proteome (Bailey, Laine et al. 2014), to our knowledge this is the first study to examine differences in the expression of proteins in children's serum associated with total urinary arsenic, as well as proportions of each class of urinary arsenic metabolites. The proteins that showed altered expression largely fell into functional categories of inflammation and immune response. Additionally, TNF was identified as a predicted upstream regulator of the U-tAs proteome, as well as a mediator of metabolite-dependent differences in identified proteins and regulators.

We demonstrate a massive repression of immune-associated cytokines as U-tAs levels increase. Interestingly, TNF was identified as a key regulator of these repressed proteins. TNF has a known roll in immune response, apoptosis, inflammation, and cell migration, as well as having been implicated in numerous diseases (Naujokas, Anderson et al. 2013). Our prior research highlight TNF as a regulator of the newborn proteome (Bailey) as well as the newborn transcriptome (Fry, Navasumrit et al. 2007). Here we show that many of the identified suppressed proteins are involved in the major histocompatibility (MCH) complex II, which is responsible for triggering localized inflammation and B cell activation in the adaptive immune response (Andrew, Jewell et al. 2008), and included many interleukins and interferons. These data highlight altered expression of cytokines known to play a role in adaptive and innate immune signaling and could provide a mechanistic hypothesis for increased risk of infection (Farzan, Li et al. 2016).

When analyzed in relation to inter-individual differences in arsenic metabolism in adolescents, we found minimal overlap between the %iAs, %MMAs and %DMAs-associated proteins. These data suggest a urinary arsenic metabolite-specific response to iAs exposure and reflects the strong inter-individual component to the response. The most robust response in terms of differential protein expression was observed in relation to %MMAs. This finding is interesting as inter-individual differences in iAs metabolism, particularly elevation in %MMAs, have been shown to be associated with disease risk (Huang, Tseng et al. 2007, Tseng 2007, Laine, Bailey et al. 2015). In our prior work, we have shown inter-individual differences in iAs metabolism as it relates to epigenetic signatures of disease (Bailey, Laine et al. 2014, Rager, Bailey et al. 2014). Proteins associated with %MMAs include many TNF superfamily (TNFSF) members. Among these are TNFSF10 and TNFSF15 previously shown to play roles in hepatic and cardiovascular

disease (Guha Mazumder 2005, Straub, Stolz et al. 2007, Coulon, Heindryckx et al. 2011, Ghatak, Biswas et al. 2011, Naujokas, Anderson et al. 2013, Mohammed Abdul, Jayasinghe et al. 2015). In addition to the TNFSF, members of the VEGF family also displayed a positive association with %MMAs. The VEGF family is a known regulator of angiogenesis (Detoraki, Staiano et al. 2009). VEGFB has been shown to play a role in cancer metastasis and tumor invasion (Yang, Zhang et al. 2015) and VEGFB expression has been found to be upregulated in ovarian, colorectal, renal, and prostate cancer (Gunningham, Currie et al. 2001, Hanrahan, Currie et al. 2003). VEGFC also plays a role in metastatic spread of certain tumor types (Gunningham, Currie et al. 2001). These data highlight differences in the expression of proteins with known association to arsenic-associated diseases.

While we have identified a large TNF-mediated response in relation to U-tAs with metabolite-specific differences, the study is not without its limitations. Despite being one of the largest proteomic studies to date, we have only assessed a fraction of the thousands of circulating serum proteins and due to the small sample size in the present study, associations seen should be confirmed in a larger cohort. Additionally, it possible that other environmental exposures and/or potential confounding variables not assessed in the present study may contribute to the observed differences. Moreover, while proteins identified have known associations with arsenic-related adverse outcomes it is unknown if the current cohort will present with such diseases. Future studies could examine both genomic and epigenetic mechanisms that may underlie the observed functional differences in protein expression (Bailey, Smith et al. 2016).

In summary, the data from the present study suggest that iAs acts as an immunomodulator, and also that it does so in a metabolite-specific manner. The proteomic differences in expression seen here highlight the role of iAs in possibly impacting maturation of

the immune system during a critical period of development (Luna, Acosta-Saavedra et al. 2010), and as such, has the potential to alter disease risk later in life. Early childhood exposure to iAs has been associated with outcomes manifesting during both adolescence and adulthood, and the long-lasting impact of iAs exposure during early childhood suggests that early life represents a critical period during which there is heightened sensitivity to the toxic effects of iAs (Vahter 2008, Naujokas, Anderson et al. 2013). These data inform potential mechanisms by which early life and childhood exposure to iAs may be linked to detrimental health outcomes.

APPENDIX 1: SUPPLEMENTARY TABLE 1

Supplementary Table 1. Identification of proteins associated with total urinary arsenic (U-tAs) and proportions of arsenic metabolites (%iAs, %MMAs, %DMAs). Bold denotes statistical significance.

Protein Symbol	Protein Name	Genbank ID	U-tAs *p*-val	U-tAs *q*-val	U-tAs Beta	%iAs *p*-val	%iAs Beta	%MMA *p*-val	%MMA Beta	%DMA *p*-val	%DMA Beta
ACVR1	Activin A receptor type 1	NM_001105.4	**0.0097**	**0.0883**	**0.4032**	0.9293	-0.0146	0.7729	-0.0473	0.8996	0.0207
CCL18	Chemokine (C-C Motif) Ligand 18	NM_002988	**0.0131**	**0.0902**	**-0.4097**	0.9918	0.0018	0.5778	0.0961	0.9300	-0.0152
CXCL12	Chemokine (C-X-C Motif) Ligand 12	NM_000609	**0.0198**	**0.0909**	**-0.3834**	0.6177	0.0855	0.0986	0.2775	0.4769	-0.1215
EDA2R	X-linked ectodysplasin-A2 receptor	NM_021783	0.7142	0.4957	0.0639	0.3523	0.1614	**0.0133**	**0.4127**	0.2153	-0.2139
FLT4	Fms-Related Tyrosine Kinase 4	NP_002011.2	0.9024	0.5469	-0.0214	0.5218	-0.1115	**0.0296**	**0.3665**	0.7461	0.0565
GPC3	Glypican 3	NM_00484	**0.0218**	**0.0918**	**-0.3752**	0.6067	0.0875	0.5085	-0.1122	0.6854	-0.0689
GPNMB	Glycoprotein (Transmembrane) Nmb	NM_001005340.1	**0.0196**	**0.0909**	**-0.3854**	0.6265	-0.0836	0.9730	-0.0058	0.6345	0.0817
HBEGF	Heparin-binding Epidermal Growth factor	NM_001945	**0.0241**	**0.0947**	**-0.3635**	0.4124	0.1366	0.8680	-0.0278	0.4419	-0.1282
HGFR	Hepatocyte growth factor receptor	NP_000236.2	0.2710	0.2882	-0.1855	0.0503	0.3233	0.4886	0.1173	**0.0460**	**-0.3291**

IFNAR1	Interferon alpha / beta receptor 1	NM_000629.2	**0.0168**	**0.0909**	**-0.3982**	0.9882	-0.0026	0.4492	-0.1311	0.9046	0.0209
IFNAR2	Interferon alpha / beta receptor 2	NM_000874	**0.0068**	**0.0850**	**-0.4474**	0.7065	0.0658	0.3869	-0.1504	0.8077	-0.0425
IFNB1	Interferon beta 1	NP_002167.1	**0.0087**	**0.0883**	**-0.4324**	0.9817	-0.0040	0.1934	-0.2229	0.8398	0.0351
IFNG	Interferon gamma	NM_000619	**0.0064**	**0.0850**	**-0.4452**	0.9154	0.0183	0.1571	-0.2405	0.9263	0.0160
IFNGR1	Interferon gamma receptor 1	NM_000416	**0.0040**	**0.0645**	**-0.4653**	0.7794	0.0481	0.2235	-0.2070	0.9187	-0.0175
IGFBP1	Insulin-like growth factor binding proteins 1	NM_001013029	**0.0020**	**0.0645**	**-0.4916**	0.6951	0.0670	0.3847	-0.1479	0.7967	-0.0441
IGFBP2	Insulin-like growth factor binding proteins 2	NM_000597	**0.0199**	**0.0909**	**-0.3659**	0.6689	0.0699	0.6864	-0.0660	0.7212	-0.0584
IGFBP3	Insulin-like growth factor binding proteins 3	NM_001013398	**0.0018**	**0.0645**	**-0.4895**	0.5559	0.0990	0.8346	-0.0352	0.5892	-0.0908
IGFBP7	Insulin-Like Growth Factor Binding Protein 7	NM_001253835.1	**0.0028**	**0.0645**	**-0.4450**	0.5445	0.0963	0.6734	0.0671	0.5186	-0.1026
IGFI	Insulin-Like Growth Factor 1 (Somatomedin C)	NM_000618	**0.0036**	**0.0645**	**-0.4616**	0.3632	0.1527	0.8279	0.0367	0.3628	-0.1529
IL11	Interleukin 11	NM_000641	0.2372	0.2655	-0.2002	**0.0429**	**0.3359**	0.4338	0.1334	**0.0381**	**-0.3436**
IL13RA2	Interleukin 13 receptor alpha 2	NM_000640	**0.0246**	**0.0947**	**-0.3772**	0.7466	-0.0563	0.3317	-0.1681	0.6542	0.0780
IL15	Interleukin 15	NM_000585	**0.0171**	**0.0909**	**-0.3984**	0.7216	-0.0621	0.2347	-0.2051	0.6100	0.0888
IL15RA	Interleukin 15 receptor alpha	NM_172200	**0.0193**	**0.0909**	**-0.3904**	0.7686	-0.0511	0.2185	-0.2115	0.6487	0.0791
IL16	Interleukin 16	NM_172217	**0.0111**	**0.0883**	**-0.4190**	0.9827	-0.0038	0.2627	-0.1923	0.8599	0.0306
IL17A	Interleukin 17	NM_002190	**0.0090**	**0.0883**	**-0.4261**	0.8779	-0.0264	0.2832	-0.1828	0.7659	0.0511

IL17B	Interleukin 17B	NM_014443	**0.0095**	**0.0883**	**-0.4179**	0.8588	-0.0302	0.3705	-0.1510	0.7665	0.0504
IL17C	Interleukin 17C	NM_013278	**0.0142**	**0.0902**	**-0.3816**	0.9853	0.0030	0.6759	-0.0680	0.9675	0.0066
IL17D	Interleukin 17D	NM_138284	**0.0032**	**0.0645**	**-0.4502**	0.8869	0.0232	0.6395	-0.0762	0.9426	-0.0117
IL17F	Interleukin 17F	NM_052872	**0.0028**	**0.0645**	**-0.4634**	0.7662	0.0493	0.6039	-0.0858	0.8298	-0.0356
IL17RB	Interleukin 17B receptor	NM_172234	**0.0033**	**0.0645**	**-0.4609**	0.9577	0.0089	0.5335	-0.1040	0.9715	0.0060
IL17RC	Interleukin 17 receptor C	NM_032732	**0.0157**	**0.0909**	**-0.3864**	0.5031	0.1116	0.6901	-0.0666	0.5544	-0.0986
IL1A	Interleukin-1 alpha	NM_000575	**0.0209**	**0.0909**	**-0.3806**	0.2374	0.2006	0.8954	0.0226	0.2456	-0.1972
IL2	Interleukin 2	NM_000586	**0.0105**	**0.0883**	**-0.4252**	0.9783	-0.0047	0.3439	-0.1642	0.8745	0.0276
IL25	Interleukin 17E	NM_172314	**0.0022**	**0.0645**	**-0.4785**	0.6599	0.0736	0.7495	-0.0535	0.7034	-0.0637
IL27	Interleukin-27 subunit alpha	NM_145659	**0.0212**	**0.0909**	**-0.3812**	0.9133	-0.0187	0.3031	-0.1759	0.8038	0.0428
IL28A	Interleukin-28A	NM_172138	**0.0145**	**0.0902**	**-0.3959**	0.6518	-0.0764	0.3054	-0.1723	0.5621	0.0980
IL29	Interleukin-29	NM_172140	**0.0133**	**0.0902**	**-0.4072**	0.5031	-0.1150	0.7844	-0.0471	0.4926	0.1179
IL2RA	Interleukin 2 Receptor, Alpha	NM_000417.2	**0.0075**	**0.0850**	**-0.4442**	0.7537	-0.0550	0.2263	-0.2097	0.6371	0.0826
IL2RB	Interleukin 2 Receptor, Beta	NM_000878	**0.0073**	**0.0850**	**-0.4419**	0.8717	0.0281	0.2472	-0.1993	0.9966	0.0007
IL2RG	Interleukin 2 Receptor, Gamma	NM_000206	**0.0062**	**0.0850**	**-0.4475**	0.8349	0.0361	0.3396	-0.1642	0.9453	-0.0119
IL31	Interleukin-31	NM_001014336	**0.0102**	**0.0883**	**-0.4048**	0.7376	0.0555	0.5097	-0.1089	0.8167	-0.0384
IL31RA	Interleukin-31 Receptor A	NM_139017	**0.0138**	**0.0902**	**-0.3886**	0.6953	0.0647	0.5173	-0.1067	0.7733	-0.0476
IL3RA	Interleukin-3 receptor subunit alpha	NM_002183	**0.0014**	**0.0645**	**-0.5067**	0.6848	0.0696	0.4518	-0.1286	0.7739	-0.0493
IL4	Interleukin 4	NM_172348	**0.0009**	**0.0645**	**-0.5211**	0.6534	0.0764	0.6015	-0.0889	0.7181	-0.0615
IL4R	Interleukin-4 receptor alpha chain	NM_001008699	**0.0021**	**0.0645**	**-0.4955**	0.8377	-0.0354	0.8899	-0.0239	0.8278	0.0376

IL7R	Interleukin-7 receptor subunit alpha	NP_002176.2	**0.0040**	**0.0645**	**-0.4547**	0.4267	0.1329	0.9951	-0.0010	0.4427	-0.1284
IL8	Interleukin 8	NM_000584	**0.0183**	**0.0909**	**-0.3819**	0.4580	0.1248	0.9545	-0.0096	0.4780	-0.1194
INHBA	Inhibin, Beta A	NM_002192.2	**0.0263**	**0.0998**	**0.3398**	0.3749	0.1401	0.8732	0.0253	0.3785	-0.1390
INHBB	Inhibin, Beta B	NP_002184.2	**0.0066**	**0.0850**	**-0.4295**	0.7378	0.0559	0.7646	-0.0500	0.7782	-0.0470
KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog	NP_000213.1	**0.0196**	**0.0909**	**-0.3818**	0.7820	0.0472	0.2073	0.2124	0.6580	-0.0754
LCN1	Lipocalin-1	NM_002297	**0.0228**	**0.0927**	**-0.3763**	0.3310	-0.1660	0.2535	-0.1944	0.2704	0.1878
LRP1	Prolow-density lipoprotein receptor-related protein 1	NM_002332	**0.0147**	**0.0902**	**-0.3986**	0.7725	-0.0494	0.2185	-0.2079	0.6523	0.0769
LTB	Lymphotoxin beta (TNFR Superfamily, Member 3)	NM_009588	**0.0186**	**0.0909**	**-0.3706**	0.8988	-0.0209	0.5577	-0.0960	0.8375	0.0337
LTBR	Lymphotoxin beta receptor (TNFR Superfamily, Member 3)	NM_002342	**0.0143**	**0.0902**	**-0.3906**	0.9040	0.0201	0.7164	-0.0605	0.9476	-0.0110
MMP11	Matrix Metallopeptidase 11	NM_005940	**0.0226**	**0.0927**	**-0.3717**	0.5729	-0.0954	0.3424	-0.1599	0.4974	0.1147
MMP14	Matrix Metallopeptidase 14 (Membrane-Inserted)	NM_004995	**0.0122**	**0.0902**	**-0.4027**	0.9006	-0.0211	0.6989	-0.0651	0.8610	0.0295
MMP-15	Matrix metalloproteinase -15	NM_002428	**0.0173**	**0.0909**	**-0.3828**	0.9275	0.0153	0.9077	-0.0195	0.9428	-0.0120

NRCAM	Neuronal cell adhesion molecule	NP_001032209.1	**0.0243**	**0.0947**	**-0.3800**	0.1244	-0.2645	0.8318	-0.0372	0.1296	0.2611
NRG3	Pro-neuregulin-3, membrane-bound isoform	XM_166086	0.5694	0.4402	-0.0991	**0.0482**	**-0.3347**	0.2681	-0.1915	**0.0380**	**0.3506**
OSTN	Osteocrin	NM_198184	**0.0095**	**0.0883**	**-0.4194**	0.9473	-0.0112	0.7407	0.0562	0.9860	0.0030
PDGFB	Platelet-derived growth factor subunit B	NP_002599.1	0.1536	0.2066	-0.2443	0.0651	0.3126	0.2294	0.2067	**0.0498**	**-0.3313**
PDGFC	Platelet-derived growth factor C	NM_016205	0.1593	0.2092	-0.2418	**0.0268**	**0.3719**	0.1752	0.2331	**0.0190**	**-0.3924**
PDGFD	Platelet-derived growth factor D	NM_025208	0.3386	0.3247	-0.1652	**0.0174**	**0.3963**	0.1510	0.2458	**0.0117**	**-0.4177**
PPBP	Pro-Platelet Basic Protein (Chemokine (C-X-C Motif) Ligand 7)	NM_002704	0.2816	0.2935	-0.1845	**0.0329**	**0.3563**	0.2831	0.1839	**0.0261**	**-0.3704**
SCF	Stem Cell Factor	NP_000890.1	**0.0212**	**0.0909**	**-0.3769**	0.8750	0.0268	0.2152	0.2086	0.7459	-0.0551
Siglec-9	Sialic acid-binding Ig-like lectin 9	NP_055256.1	0.1715	0.2164	-0.2357	0.0669	0.3124	0.1077	0.2758	**0.0445**	**-0.3408**
SLC2A3	Glucose transporter 3	NM_006931	0.0605	0.1285	-0.3073	0.8080	-0.0408	**0.0498**	**-0.3204**	0.6151	0.0844
SLPI	Secretory Leukocyte Peptidase Inhibitor	NM_003064	0.2497	0.2769	-0.1981	**0.0177**	**0.3953**	0.1585	0.2415	**0.0121**	**-0.4161**
TFPI	Tissue factor pathway inhibitor	NM_006287	0.7176	0.4957	0.0612	**0.0302**	**-0.3543**	0.6011	-0.0884	**0.0298**	**0.3550**
TGFBR2	TGF-beta receptor type-2	NM_001024847.2	0.2367	0.2655	-0.2029	0.7096	0.0645	**0.0322**	**0.3585**	0.5145	-0.1126

TGFBR3	TGF-beta receptor type III	NM_003243	0.3210	0.3173	-0.1724	0.5610	0.1015	**0.0194**	**0.3926**	0.3786	-0.1532
TGFR2	TGF-Beta Receptor Type IIB	NM_001024847	0.2066	0.2446	-0.2171	0.5181	0.1121	**0.0202**	**0.3878**	0.3463	-0.1628
THPO	Thrombopoietin	NM_000460	0.3252	0.3185	-0.1713	0.5330	0.1090	**0.0253**	**0.3776**	0.3635	-0.1584
TMPO	Thymopoietin	NP_003267.1	0.3603	0.3282	-0.1581	**0.0404**	**0.3452**	0.0859	0.2921	**0.0251**	**-0.3748**
TNFRSF 10A	Tumor necrosis factor receptor superfamily member 10A	NP_003835.2	0.7093	0.4957	-0.0649	0.7890	0.0466	**0.0231**	**0.3809**	0.5712	-0.0985
TNFRSF 10B	Tumor necrosis factor receptor superfamily member 10B	NP_003833.4	0.9646	0.5665	-0.0078	0.8296	0.0376	**0.0375**	**0.3518**	0.6234	-0.0857
TNFRSF 10C	Tumor Necrosis Factor Receptor Superfamily, Member 10C	NM_003841	0.7995	0.5176	-0.0445	0.4532	0.1309	**0.0178**	**0.3980**	0.2940	-0.1824
TNFRSF 10D	Tumor Necrosis Factor Receptor Superfamily, Member 10D	NM_003840	0.8624	0.5335	-0.0304	0.4184	0.1410	**0.0099**	**0.4295**	0.2570	-0.1966
TNFRSF 11B	Tumor Necrosis Factor Receptor Superfamily, Member 11b	NP_002537.3	**0.0165**	**0.0909**	**-0.3860**	0.9787	-0.0045	0.6172	0.0839	0.9648	-0.0074
TNFSF10	Tumor Necrosis Factor (Ligand) Superfamily, Member 10	NP_003801.1	0.8216	0.5245	-0.0393	0.8321	0.0369	**0.0280**	**0.3689**	0.6151	-0.0874
TNFSF11	Tumor Necrosis Factor (Ligand) Superfamily, Member 11	NM_033012	0.9780	0.5703	-0.0048	0.3451	0.1637	**0.0271**	**0.3717**	0.2228	-0.2104

TNFSF15	Tumor necrosis factor ligand superfamily member 15	NM_005118	0.8792	0.5371	-0.0265	0.5502	0.1037	**0.0143**	**0.4076**	0.3628	-0.1574
TNFSF4	Tumor Necrosis Factor (Ligand) Superfamily, Member 4	NM_003326	**0.0113**	**0.0883**	**-0.4071**	0.8525	0.0314	0.4328	0.1317	0.7723	-0.0488
VEGFB	Vascular endothelial growth factor B	NM_003377	0.7961	0.5169	-0.0450	0.9062	0.0205	**0.0206**	**0.3873**	0.6700	-0.0741
VEGFC	Vascular endothelial growth factor C	NM_005429	0.8025	0.5181	-0.0435	0.8381	0.0355	**0.0146**	**0.4061**	0.5991	-0.0913
VEGFD	Vascular endothelial growth factor D	NM_182925.4	0.9529	0.5644	-0.0103	0.7751	0.0498	**0.0182**	**0.3946**	0.5518	-0.1034
WISP1	WNT1-inducible-signaling pathway protein 1	NP_003873.1	0.9567	0.5644	0.0095	0.2142	0.2140	**0.0170**	**0.3985**	0.1248	-0.2628

APPENDIX 2: SUPPLEMENTARY TABLE 2

Supplementary Table 2. Canonical IPA pathways (A.), canonical KEGG pathways (B.), Upstream regulators (C.), and IPA molecular networks (D.) of the As-and arsenical-associated proteins.

A. IPA pathways

Pathway	Canonical pathway description	*p*-value	Associated proteins in pathway
U-tAs	Role of Cytokines in Mediating Communication between Immune Cells	1.12E-02	CXCL8, IFNA1/IFNA13, IFNB1, IFNG, IFNL1, IL2, IL4, IL15, IL25, IL27, IL17A, IL17F, IL1A
%iAs	Macropinocytosis Signaling	2.90E-04	PDGFD, PDGFC
%MMA	Retinoic acid Mediated Apoptosis Signaling	1.09E-09	TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, TNFSF10
%DMA	Macropinocytosis Signaling	3.36E-08	MET, PDGFB, PDGFC, PDGFD

B. KEGG pathways

Pathway	Canonical pathway description	*p*-value	Associated proteins in pathway
U-tAs	Cytokine-cytokine receptor interaction	3.76E-21	CXCL12, CXCL8, ACVR1, IFNGR1, IFNG, IL1A, IL15RA, IL15, IL17RB, IL17A, IL2RB, IL2RG, IL2, IL25, IL3RA, IL4R, IL4, LTBR, LTB, TNFSF4
%iAs	Cytokine-cytokine receptor interaction	1.50E-02	IL11, PDGFC, CXCL7
%MMA	Cytokine-cytokine receptor interaction	6.80E-09	FIGF, EDA2R, TGFBR2, TNFSF11, TNFSRF10C, TNFSRF1D, VEGFB, VEGFC
%DMA	Cytokine-cytokine receptor interaction	1.50E-02	IL11, PDGFC, CXCL7

C. Upstream regulators

Pathway	Canonical pathway description	*p*-value	Predicted Activation
U-tAs	TNF	1.59E-27	Inhibited
%iAs	DUSP5	1.98E-05	no prediction
%MMA	Pyruvic acid	1.98E-10	no prediction
%DMA	FGF2	1.47E-05	no prediction

D. IPA networks

Network	Most significant associated functions	*p*-value	Number of associated proteins in network	Molecules in network
U-tAs	Cellular Development, Cellular Growth and Proliferation, Hematological System Development and Function	1.00E-34	16	Eotaxin, ERK1/2, Fibrin, HDL, Ifn, IFN alpha/beta, ↓IFNA1/IFNA13, ↓IFNL1, ↓IFNL2, Iga, ↓IL23, ↓IL25, ↓IL27, ↓IL31, Il-4 receptor, Il15r, ↓IL15RA, ↓IL17B, ↓IL17C, ↓IL17D, ↓IL17F, ↓IL17RB, ↓IL17RC, ↓IL2RG, ↓IL31RA, INTERLEUKIN, ↓LTBR, MHC Class II (complex), MHC II, Notch, Oas, Pro-inflammatory Cytokine, SAA, TH1 Cytokine, Tlr
%iAs	Cardiovascular Disease, Hematological Disease, Cellular Growth and Proliferation	1.00E-24	8	Akt, BCL2, CELA2A, CLEC11A, CTSG, DUSP5, ERK1/2, F2, F10, F3-F7, GP5, HABP2, HPSE, ↑IL11, Il8r, LOX, MMP15, NFkB (complex), ↓NRG3, PDGF (family), PDGF-CC, PDGF-DD, ↑PDGFC, ↑PDGFD, PMAIP1, ↑PPBP, PROK1, PROS1, PRSS3, S100a7a, ↑SLPI, ↓TFPI, ↑TMPO, Vegf, VEGFA

%MMA	Organismal Development, Cardiovascular System Development and Function, Connective Tissue Disorders	1.00E-47	16	caspase, Collagen(s), death receptor, DR4/5, ERK1/2, ↑FIGF, ↑FLT4, Focal adhesion kinase, FSH, growth factor receptor, Growth hormone, IL1, Immunoglobulin, Mapk, p70 S6k, Pro-inflammatory Cytokine, ↓SLC2A3, Tgf beta, TGFBR, ↑TGFBR2, ↑TGFBR3, ↑THPO, Tnf (family), ↑TNFRSF10A, ↑TNFRSF10B, ↑TNFRSF10C, ↑TNFRSF10D, ↑TNFSF10, ↑TNFSF11, ↑TNFSF15, Trail-R, Vegf, ↑VEGFB, ↑VEGFC, ↑WISP1
%DMA	Cellular Growth and Proliferation, Organismal Development, Cancer	1.00E-33	11	Akt, Ap1, Collagen(s), ERK, ERK1/2, GP130 dimer, Histone h3, ↓IL11, Jnk, Mapk, ↓MET, Met dimer, NFkB (complex), ↑NRG3, P38 MAPK, p85 (pik3r), Pdgf (complex), PDGF (family), PDGF-CC, PDGF-DD, ↓PDGFB, ↓PDGFC, ↓PDGFD, PI3K (complex), ↓PPBP, Pro-inflammatory Cytokine, PROK1, Ras, S100A12, ↓SIGLEC9, ↓SLPI, ↑TFPI, Tgf beta, ↓TMPO, Vegf

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