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Sex-specific patterns and deregulation of endocrine pathways in the gene expression profiles of Bangladeshi adults exposed to arsenic contaminated drinking water¹

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Abstract

Arsenic contamination of drinking water occurs globally and is associated with numerous diseases including skin, lung and bladder cancers, and cardiovascular disease. Recent research indicates that arsenic may be an endocrine disruptor. This study was conducted to evaluate the nature of gene expression changes among males and females exposed to arsenic contaminated water in Bangladesh at high and low doses. Twenty-nine (55% male) Bangladeshi adults with water arsenic exposure ranging from 50–1000 µg/L were selected from the Folic Acid Creatinine Trial. RNA was extracted from peripheral blood mononuclear cells for gene expression profiling using Affymetrix 1.0 ST arrays. Differentially expressed genes were assessed between high and low exposure groups for males and females separately and findings were validated using quantitative real-time PCR. There were 534 and 645 differentially expressed genes ($p < 0.05$) in the peripheral blood mononuclear cells of males and females, respectively, when high and low water arsenic exposure groups were compared. Only 43 genes overlapped between the two sexes, with 29 changing in opposite directions. Despite the difference in gene sets both males and females exhibited common biological changes including deregulation of 17 β -hydroxysteroid

¹Abbreviations: androgen receptor (AR), Arsenic (As), Cardiovascular disease (CVD), estrogen receptor (ER), Folic Acid Creatinine Trial (FACT), glucocorticoid receptor (GR), Health Effects of Arsenic Longitudinal Study (HEALS), 17 β -Hydroxysteroid dehydrogenase (HSD17B), hydroxysteroid dehydrogenase (HSD), single channel array normalization (SCAN), specificity protein 1 (Sp1), steroid receptors (SR), urinary As (uAs), water As (wAs)

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dehydrogenase enzymes, deregulation of genes downstream of Sp1 (specificity protein 1) transcription factor, and prediction of estrogen receptor alpha as a key hub in cardiovascular networks. Arsenic-exposed adults exhibit sex-specific gene expression profiles that implicate involvement of the endocrine system. Due to arsenic's possible role as an endocrine disruptor, exposure thresholds for arsenic may require different parameters for males and females.

Keywords

arsenic; humans; HSD17B-1; cardiovascular disease

Introduction

Inorganic arsenic (iAs), a known human carcinogen, naturally contaminates drinking water and currently affects approximately 150 million people in at least 70 countries throughout the world (IARC, 2004; Ravenscroft et al., 2009). Exposure to As is associated with skin, lung, bladder, liver and prostate cancers (Benbrahim-Tallaa and Waalkes, 2008; Rahman et al., 2009), cardiovascular disease (Chen et al., 2013; Tseng et al., 2003), diabetes (Pan et al., 2013), and hypertension (Chen et al., 1995). The mechanisms that underlie As's diverse pathogenesis remain unclear and are thought to include oxidative stress, endocrine disruption, alteration of cell signaling and proliferation processes, and impairment of DNA damage responses (Andrew et al., 2003; Davey et al., 2007; Flora, 2011; Schuhmacher-Wolz et al., 2009). In addition, there is a growing body of evidence to indicate that As acts through epigenetic mechanisms as part of its pathogenesis by altering the abundance of histone modifications and the level of DNA methylation (Reichard and Puga, 2010; Ren et al., 2011).

The evidence to suggest that As acts as an endocrine disruptor includes *in vitro* activation of steroid hormone receptors (Bodwell et al., 2006; Davey et al., 2007), animal models with altered fertility (Chang et al., 2007; Jana et al., 2006) and sex-specific disease distributions (Waalkes et al., 2003), as well as human studies indicating altered fertility (Hsieh et al., 2008; Xu et al., 2012), sex-specific disease distributions (Aballay et al., 2012), and sex-linked changes in methylation profiles (Pilsner et al., 2012). Previous research from our lab supports this trend with the observed sex-specific changes in the abundance of histone modifications in Bangladeshi adults exposed to As contaminated drinking water (Chervona et al., 2012). Microarray studies that have assessed gene expression changes in response to As contaminated water have evaluated changes with males and females pooled together (Andrew et al., 2008; Lu et al., 2001; Wu et al., 2003) or with only females (Wu et al., 2003). However, given the potential endocrine activity of As, in particular its possible role as a xenoestrogen (Jana et al., 2006; Waalkes et al., 2004), division of sexes in assessing its impact may be necessary to understand its pathogenesis. Indeed a recent study conducted among Flemish adults exposed to an array of toxins, including xenoestrogens, exhibited sex-specific transcriptomic responses (De Coster et al., 2013) that were revealed when sexes were evaluated separately.

Here we evaluate the gene expression profiles of RNA from the peripheral blood mononuclear cells (PBMC) of Bangladeshi adults (n=29) chronically exposed to As contaminated drinking water. Subjects with high water As exposure are compared to those with low water As exposure among males and females separately. To evaluate the sex-specific profiles we compare the areas of biological relevance for each sex and evaluate to what extent the same genes are found among biologically relevant areas that are common to males and females.

Materials and Methods

Study Site and Subject Recruitment

The Health Effects of Arsenic Longitudinal Study (HEALS) cohort is part of Columbia University's Superfund Research Program and currently includes approximately 30,000 participants (Ahsan et al., 2006). The present study utilizes samples from a subset of 600 HEALS participants who were recruited for enrollment into the Folic Acid and Creatine Trial (FACT), a clinical trial that aimed to lower blood As concentrations with nutritional supplementation and therefore required that participants be drinking from wells with water As concentrations greater than or equal to 50 µg/L. Fieldwork was completed in June 2011. Study participants were given a water filtration system for removal of As at the time of enrollment. Participants with renal or gastrointestinal diseases, those taking nutritional supplements, or who were pregnant and/or planning to become pregnant were not included in the study. This study evaluates the gene expression patterns of 29 study participant from the FACT clinical trial. Our study population is comprised of adults ranging in age from 26–53. Here the effects of relatively low exposure (50–200 µg/L) to water arsenic are compared to high exposure (250–1000 µg/L) for males (n=16) and females (n=13) separately. Among the males there were 9 males with low exposure and 7 with high exposure. Among the females there were 6 females with low exposure and 7 with high exposure. Please see table 1 of the results for other information regarding the demographic features of the groups.

Sample collection and handling

Blood samples were obtained by venipuncture, and collected into EDTA-containing vacutainer tubes, which were then placed in IsoRack/IsoPack cool packs (Brinkmann Instruments). Samples were transported in hand-carried coolers to the local laboratory at the field clinic in Araihaazar, within four hours of collection. Samples were centrifuged for 10 minutes at $3,000 \times g$ at 4°C, and the plasma fraction was stored at –80°C. PBS was added to the remaining cells followed by a ficoll-hypaque gradient extraction of PBMCs using standard techniques. PBMCs were stored at –80°C. Urine samples were collected with 50-mL acid-washed polypropylene tubes. Within 4 hours samples were transported from portable coolers to freezers (–20°C) in the Araihaazar laboratory, and were then hand carried on dry ice to New York.

Water and urinary As

Water sample collection, sample handling, and analysis for this study were performed as previously described (Chervona et al., 2012). The water As samples were analyzed at Columbia University's Lamont Doherty Earth Observatory by inductively coupled mass

spectrometry (ICP-MS), with a detection limit of 0.1 µg/L. Total urinary As (uAs) concentrations were measured at Columbia University Trace Metals Core Lab by GFAA spectrometry using an AAnalyst 600 graphite furnace system (PerkinElmer), as described (Nixon et al., 1991). This laboratory is part of a quality control program run by the Institut de Sante Publique du Quebec, Canada. Intraclass correlation coefficients (ICCs) were 0.99 between the Columbia laboratory's values and samples calibrated at the Quebec laboratory. To correct for hydration status, urinary creatinine was analyzed using a method based on the Jaffe reaction.

RNA isolation, amplification, and hybridization

Total RNA was extracted from each sample according to the TRIzol (Invitrogen) manufacturer's protocol, and purified using RNeasy Plus Micro Kit (Qiagen). Sense strand cDNA probes were synthesized (amplified) using Ambion Whole Transcript Expression Kit. Amplified single stranded cDNA was fragmented and labeled using GeneChip WT Terminal Labeling Kit (Affymetrix). The fragmented and labeled DNA underwent hybridization with Affymetrix GeneChip Human Gene 1.0 ST Array that contains 28,869 well-annotated genes. Samples were processed in two batches with representatives from each exposure category in each batch and were randomized during processing.

Data analysis to identify differentially expressed genes

Gene expression analyses were performed using R. Gene expression data were imported and normalized using a single-sample method, single channel array normalization (SCAN) (Piccolo et al., 2012), which summarizes probes to Entrez IDs, in R 2.15.0 GUI 1.53 64-bit. Data were processed to expression sets using the Affymetrix package version 1.30.0. in R 2.15.1 GUI 1.42 Leopard build 64-bit, and were filtered to remove negative expression values as per SCAN protocol. Unsupervised hierarchical clustering using the Pearson correlation method was used to evaluate potential batch effects. Surrogate variable analysis was used to estimate seven surrogate variables and performed in order to remove batch effects and other sources of expression heterogeneity (Leek and Storey, 2007). Seven surrogate variables were incorporated into the analysis and such incorporation was used in lieu of correcting for demographic features of the population, which was not possible due to the small sample size. Significance of gene expression changes for males and females were assessed separately between high and low water As exposure groups using a gene-wise linear model approach with LIMMA 3.14.4 (Smyth, 2004). LIMMA utilizes an empirical Bayes approach to generate moderated t-statistics by taking into account the standard errors and estimated log-fold changes. Probe summarization and filtration occurred prior to LIMMA analysis and therefore 11623 genes were tested for significance using moderated t-statistics and a p-value threshold of $p < 0.05$.

Heat maps were generated using the heatmap.2 function in R and unsupervised hierarchical clustering was governed by Pearson correlation method. Networks and functional analysis were generated using IPA (Ingenuity® Systems, www.ingenuity.com). Gene set enrichment analysis was performed using the molecular signatures database (Broad Institute Gene Set Enrichment Analysis, <http://www.broadinstitute.org/gsea>). To evaluate the influence of cigarette smoking, lists of differentially expressed genes were evaluated for the presence of

known biomarkers of cigarette smoking (van Leeuwen et al., 2005) and statistical significance of biomarker representation was assessed using a hypergeometric distribution as previously described (Draghici et al., 2003). All microarray data is MIAME compliant and the raw data has been deposited in NCBI's Gene Expression Omnibus (GEO), and assigned series accession number GSE57711.

Quantitative real-time PCR

Gene expression changes were validated using quantitative real-time polymerase chain reaction (q-RT-PCR). Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen), and converted to single stranded cDNA using Superscript III (Invitrogen). q-RT-PCR analysis was performed using SYBR green PCR system (Applied Biosystems) on ABI prism 7900HT system (Applied Biosystems). All q-RT-PCR reactions were performed in triplicate. Relative gene expression level, normalized to 18s rRNA expression, was presented as median relative mRNA values $\pm$ SD (n=9) for participants in low and high exposure groups. Statistical significance was tested using two-tailed, unpaired t-test. The expression of the target genes was first normalized to the expression of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (*GADPH*).

Results

Demographic data

Study population characteristics are presented in Table 1 for the study population by water As exposure category (low vs. high). Briefly, a total of 29 participants were selected for this study with 16 males and 13 females. Among the males the average age was 42.6 ± 6.4 for the males with low exposure and 41.7 ± 6.8 for the males with low exposure. Among the females the average age was 36.7 ± 9.6 for females with low exposure and 37.7 ± 5.6 for the females with high exposure. The median water As concentration for the low exposure group was 103 $\mu\text{g/L}$ for males and 117 $\mu\text{g/L}$ for females (range 50–200 $\mu\text{g/L}$). For the high exposure group, the median water As concentration was 355 $\mu\text{g/L}$ for males (range 250–500 $\mu\text{g/L}$) and 434 $\mu\text{g/L}$ for females (range 232–1000 $\mu\text{g/L}$).

Gene expression analysis

In the PBMC's of highly exposed males, there were 534 differentially expressed genes ($p < 0.05$), when compared to males with low As exposure, with 271 up-regulated and 264 down-regulated. For the highly exposed females there were 645 differentially expressed genes ($p < 0.05$) when compared to females with low water As exposure, with 303 up-regulated and 342 down-regulated. The unsupervised hierarchical clustering analysis for differentially expressed genes of males (Figure 1a) and females indicates clear clustering based on water As exposure group (low vs. high) for males and females (Figure 1b), with the exception of one outlier for females, indicating that dose is driving clustering in our lists. Only 43 genes overlapped between the two sexes, with 29 out of 43 genes changing in opposite directions (Figure 1C; Table 2). Biological relevance of gene expression was assessed via network analysis using IPA and top ranked networks and enrichment scores are presented in Table 3.

Differentially expressed genes were also evaluated for highly ranked gene signatures using GSEA database and the top ranked signatures from all possible signatures to query are presented in Supplementary Material, Table S1. Cardiovascular disease was a primary factor in a top-ranked network for both males and females, and each network was comprised of unique gene lists that are presented in Supplemental Material, Tables S2 and S3, with only one gene in common (*RBBP8* (retinoblastoma-binding protein 8)) between the sexes. Genes responsive to Sp1 (specificity protein 1) transcription factor comprised a top-ranked gene signature for both males and females. Sp1 responsive genes were evaluated for their similarity between the sexes. Males had 110 Sp1 regulated genes and females had 91, both with a 50/50 split between up and down-regulated genes. There was little overlap between the lists with only 9 genes overlapping, which are presented in Supplementary Material, Table S4. Due to the fact that the majority of males were smokers and no females were reported as smokers (Table 1), we screened the male and female gene sets for 26 genes that exhibited 90% sensitivity in predicting smokers versus nonsmokers in order to evaluate whether smoking status was confounding the sex-effect (Lampe et al., 2004). For the males, three of the smoking biomarker genes appeared on our gene lists, which was statistically significant ($p < 0.0295$); and there were no smoking genes in the female gene lists. The smoking genes found in the male gene set included (*CYP1B1* (cytochrome P450, family 1, subfamily B, polypeptide 1), *EPB4IL3* (erythrocyte membrane protein band 4.1-like 3), and *BRD3* (bromodomain containing 3)). To further assess the relationship, we probed differentially expressed genes of high males vs. high females, and 3 smoking genes were present in this gene set. While a comparison of low males vs. low females, contained no smoking biomarker genes. Collectively these findings indicate that smoking status is primarily an influence in the high males, but its signature is not dominant as only three smoking biomarker genes are present (*CYP1B1*, *EPB4IL3*, *IL1B*) and clustering in heatmaps is driven by As dose not smoking status.

Real-time PCR Validation

To validate the results obtained from the microarray study, quantitative real-time PCR was performed on a subset of genes with biological relevance. *POLR2E* (polymerase (RNA) II (DNA directed) polypeptide E, 25kDa), *CKS2* (CDC28 protein kinase regulatory subunit 2), and *TGM2* (transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)) were validated from the male differentially expressed genes. *E2F5* (E2F transcription factor 5, p130-binding) was validated from the female differentially expressed genes. As shown in Table 4 the up- or down-regulated patterns for all genes obtained from real-time PCR were consistent with those obtained in the microarray study.

POLR2E (polymerase (RNA) II (DNA directed) polypeptide E, 25kDa), *CKS2* (CDC28 protein kinase regulatory subunit 2), and *TGM2* (transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)) were validated in the males. *E2F5* (E2F transcription factor 5, p130-binding) was validated in the females. Patterns of fold change found in quantitative realtime PCR exhibit consistent directionality to those obtained by microarray.

Discussion

Our findings suggest that As exposure deregulates expression of genes involved in the immune system, heat shock response and DNA repair processes, which is consistent with previous human studies on this topic. The novelty of our paper is that we find evidence to support the notion that As acts through endocrine associated pathways in both sexes, and that such pathways involve mediation of responses through sex-specific mechanisms as evidenced by the distinct gene sets for males and females.

Given that the exposure inclusion criteria of the FACT study design required that As in drinking water exceed 50 µg/L, we have conducted a comparison of high and low exposure groups. This study design is consistent with previous studies of this cohort that detected changes in the abundance of histone marks in response to high and low levels of As contaminated drinking water (Chervona et al., 2012). Due to the comparison between high and low groups, the range of As exposure represented within each group, and the compression of expression values that occurs when the SCAN normalization protocol is employed, our fold change values are tightly expressed with maximum values in the range of ± 1.3 . In turn we focus on evaluating the biological significance of the differentially expressed genes, highlighting genes and areas of biological activity that are “deregulated” across exposure categories, and evaluating how such deregulation manifests differently in males and females.

As’s ability to impact gene expression changes is consistent with previous studies which demonstrated that As deregulates the expression of heat shock, DNA repair, and immunoregulatory genes. We briefly mention such changes here to biologically validate our gene lists and contextualize our findings with other similar studies. Heat shock proteins are consistently found to be aberrantly expressed in response to As. Here, *HSP1AB*, *HSPBP1*, and *HSFS* are deregulated in males, and *HSPA13* is deregulated in females (Table 5). Our findings are consistent with Andrew et al. and Argos et al. who find *HSP1AB* is deregulated in their male dominant and female never smoker cohorts, respectively (Andrew et al., 2008; Argos et al., 2006). As’s ability to interfere with DNA repair and promote carcinogenesis as a co-mutagen has been firmly established in experimental systems (Hartwig et al., 1997; Rossman et al., 2004). We find that drinking water As exposure deregulates expression of DNA repair genes in both males and females and includes genes that are involved in excision repair (*LIG3*, *SMUG1*), DNA repair regulation (*RBBP8*, *MDC1*), and mismatch repair (*MSH2*) (Table 5). Previous drinking water exposure studies have found that As interferes with nucleotide excision repair (Andrew et al., 2006; Andrew et al., 2003) as well with base excision repair and strand breaks (Wu et al., 2003). With respect to the immune system we find As deregulates a number of interleukins (IL) in both sexes (Table 5), that there was a viral-response like signature in the males (*RSAD2*, *IFIT1*, *IFIT3*, *IFIT5*), and deregulation of major histocompatibility (MHC) class genes (*HLA-DRB6*, *HLA-B*, *HLA-G*) in the females. Our findings are consistent with previous studies, as Andrew et al. also found deregulation of MHC II class genes in their male dominant cohort (Andrew et al., 2006) and Wu et al. and Argos et al. report deregulation of interleukins. Of those IL deregulated, *IL16* (females) was previously reported by Wu et al. in females exposed to As contaminated water in Taiwan where it exhibited dose-responsiveness (Wu et al., 2003) and *IL1RN* (males) was

reported by Argos et al. to be down-regulated in women with Asal induced skin lesions (Argos et al., 2006).

In addition to the areas discussed above, we observe that As deregulates expression of 17 β -hydroxysteroid dehydrogenases (17 β -HSDs), which are a family of enzymes involved in regulating steroid hormones throughout the body. Both males and females exhibited deregulation of a HSD family member. In females 17 β -hydroxysteroid dehydrogenase type 4 (*HSD17B4*) was deregulated. This enzyme can oxidize estradiol to estrone and has widespread constitutive expression with the highest levels in the liver where it is involved in fatty acid metabolism and synthesis of bile acids (Moeller and Adamski, 2006). In males *HSD17B10* was deregulated, and it exhibits broad substrate specificity for biomolecules including fatty acids, estrogens, androgens, corticosteroids and progestins and is constitutively expressed in the liver, gonads, and brain, where its regional expression levels may play a critical role in neurosteroidogenesis (Moeller and Adamski, 2006; Yang et al., 2005). *HSD17B10* has been implicated in the development of Alzheimer's disease (Moeller and Adamski, 2006), which is a highly ranked gene signature for males and females (Supplementary Material, Table S1). *HSD17B10* is also utilized in the alternative pathway for biosynthesis of 5 α -dihydrotestosterone (5 α -DHT) from andiol (Yang et al., 2005). Deregulation of *HSD17B10* may help to explain why males exposed to As exhibit reduced semen quality (Xu et al., 2012) and increased risk of erectile dysfunction (Hsieh et al., 2008). Animal models support this notion and consistently yield decreased levels of testicular enzymes including 17 β -HSD in conjunction with declined fertility parameters in both rats and mice exposed to As contaminated drinking water (Chang et al., 2007; Jana et al., 2006; Pant et al., 2004).

For both males and females cardiovascular disease was a top-ranked network, and this finding is consistent with strong epidemiological data implicating As in the progression and development of cardiovascular disease (Argos et al., 2010; Chen et al., 2009). The specific nature of the male and female cardiovascular disease networks was distinct as represented by their unique gene lists (Supplementary Material, Tables S2 and S3), which had only one gene in common (*RBBP8*), and different secondary network features. Such differences may arise via sex-specific actions of sterol pathways as both networks share the involvement of the estrogen receptor (ER) and luteinizing hormone (LH) as key hubs (Supplementary Material, Figures S2 and S3). Our findings suggest that while cardiovascular disease is a common outcome of long-term As exposure, males and females may have different mechanisms that dominate in the pathogenesis leading to cardiovascular disease. Sex-specific pathogenesis of As-associated cardiovascular disease is also evident in recent work from the HEALS cohort study, which finds a positive association in women only between long-term As exposure and QT prolongation time, a measurement of the electrocardiogram that indicates increased risk for severe cardiac events (Chen et al., 2013).

The top ranked gene signature for both males and females was genes containing transcriptional binding sites for specificity protein 1 (Sp1) transcription factors. As mentioned in the results there were about 100 genes regulated by Sp1 in gene sets of males and females with only 9 genes overlapping between the genders (Supplemental Material, Table S4). Sp1 can positively or negatively regulate gene expression (Li et al., 2004), is

deregulated in numerous cancers and neurodegenerative disorders, and is involved in cell cycle regulation, angiogenesis, and senescence (Chang and Hung, 2012), which are all events consistent with As's pathogenesis. It is unlikely that Sp1 deregulation occurs through direct interaction of As and Sp1 zinc fingers, because recent research indicates that trivalent As only binds zinc finger motifs with more than two cysteine residues, and does not bind to Sp1 which has two (Zhou et al., 2014). However, Sp1 activity is modulated by post-translational modifications including acetylation, methylation, sumoylation, and ubiquitination that regulate Sp1 protein level, transactivation activity, and DNA binding affinity (Chang and Hung, 2012). As may influence Sp1 modifications as it has been shown to alter acetylation of histones (Chervona et al., 2012), methylation of DNA (Ren et al., 2011), and ubiquitination of proteins (Bredfeldt et al., 2004; Kirkpatrick et al., 2003). Such modifications can alter Sp1's ability to partner with steroid receptors (SR) including ER α , the mineralocorticoid receptor (MR), the glucocorticoid receptor (GR), and the androgen receptor (AR), and thereby alter the synergistic couplings involved in transcription of SP1 or SR responsive genes (Meinel et al., 2013; Ou et al., 2006; Porter et al., 1997; Yuan et al., 2005). *In vitro* models indicate that As influences transcription of genes downstream of ER, MR, and GR, but the mechanism driving these changes is unknown (Bodwell et al., 2006), and we suggest that post-translational modification of the Sp1 pool may be involved.

The gene expression profiles observed in males and females indicate that similar channels mediate As's impact in both sexes. However, the striking difference in gene sets for the males and females, suggests that As may exert its influence through sex-specific endocrine interactions, which could lead to sex-specific treatment or prevention strategies. Our data support the growing trend that As can be considered an endocrine disruptor and further study is needed to elucidate how this interaction may occur (Bodwell et al., 2006; Davey et al., 2007). In addition, our data support the perspective that toxic actions may be sex-specific and that evaluation of biomarkers and determination of safety thresholds may require separate analyses for males and females responses (De Coster et al., 2013).

Given the nature of working with a human population, there are a number of study limitations arising from demographic features of the study population that must be addressed before concluding. The smoking status of the male cohort is one demographic feature that may be influencing the presentation of our data. Due to the prevalence of smoking among males in this region of Bangladesh, the majority of males were self-reported smokers while none of the females were reported as smokers. In order to evaluate the effect of smoking status on our results, we probed for detection of genes that are biomarkers for smoking among multiple datasets and found limited detection of such genes. To further evaluate the influence of smoking, we assessed the biological validity of our gene lists to insure that our lists are representative of a response to As exposure rather smoking status. As discussed we found that deregulation of heat shock, DNA repair, and immunoregulatory responses were consistent with changes observed in these areas in similar studies (Andrew et al., 2008; Andrew et al., 2006; Andrew et al., 2003; Argos et al., 2006; Wu et al., 2003), indicating that the lists generated were a reflection of As exposure. The relevance of our gene lists is further supported by the fact that the unsupervised clustering in the heatmap (Figure 1a) is driven by As dose and not smoking status. Collectively, this analysis suggests that the sex-

specific profiles exhibited in our data are primarily a reflection of As exposure, which may to a limited extent be influenced by smoking status of males. Another constraint of this study was the limited sample size, which in combination with the fold-change compression previously discussed, effectively inhibited our ability to detect differentially expressed genes using multiple hypothesis correction (i.e. FDR). Other epidemiologic studies conducting gene expression analysis in human populations have also generated uncorrected gene lists, which were utilized for pathway analysis in a similar manner (De Coster et al., 2013; Hebel et al., 2011; Wang et al., 2005). The biological validation performed on our datasets again suggests that the gene lists generated here reflect As exposure rather than randomly selected genes, given their consistency with previous gene expression studies as well as their prediction of diseases well-known to be associated with As exposure such as cardiovascular disease.

Conclusion

Our data indicate that As is influencing gene expression profiles in a manner consistent with previous human gene expression studies in which heat shock proteins, DNA repair processes, and immune responses were deregulated. In addition, we observe sex-specific profiles that may be driven by an interaction between As and the endocrine system. The observed deregulation of HSD enzymes, the presence of the ER α and LH as predicted hubs in the cardiovascular disease networks, and the deregulated Sp1 genes all implicate possible mechanisms through which As may be exerting its multifactorial influence. The involvement of steroid regulating enzymes, the prediction of steroid responsive hubs, and the cooperation between Sp1 and steroid receptors all indicate that As may be acting through endocrine mediated channels and that such effects require separate evaluation in males and females. These findings suggest that exposure thresholds may require different parameters for males and females and that further study is needed to fully understand the impact and nature of sex differences in As pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Males and females exhibit unique gene expression changes in response to arsenic
- Only 23 genes are common among the differentially expressed genes for the sexes
- Male and female gene lists exhibit common biological implications
- Both sexes exhibit deregulation of cardiovascular and endocrine pathways

Figure 1. Gene Expression Profiles of As Exposed Adults

Unsupervised hierarchical cluster analysis and heatmap of differentially expressed genes ($p < 0.05$) for males (A) and females (B) with chronic exposure to As contaminated drinking water at high (H) and low (L) doses. C) Unsupervised hierarchical cluster analysis and heatmap of differentially expressed genes represented among males and females ($p < 0.05$). Each row represents a statistically significant gene determined via LIMMA. Samples (columns) are labeled according to the following codes with appended numbers corresponding to unique individuals: ML - males with low water As exposure, MH - males

with high water As exposure, FL- females with low water As exposure, FH - females with high water As exposure.

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Table 1

General characteristics for the total study sample by sex and water As group^a

	Total sample (n=29)	Males, Low water As ^b (n=9)	Males, High water As ^c (n=7)	Females, Low water As ^d (n=6)	Females, High water As ^e (n=7)
Age	40.0 ± 7.2	42.6 ± 6.4	41.7 ± 6.8	36.7 ± 9.6	37.7 ± 5.6
BMI, kg/m ²	20.4 ± 2.9	19.7 ± 2.1	18.2 ± 1.1	23.8 ± 2.1	20.6 ± 3.0
Education, years	2.9 ± 3.7	2.8 ± 3.4	0	5.0 ± 4.5	4.0 ± 3.8
water As µg/L	246.4 ± 207.4	103.0 ± 33.7	354.5 ± 136.2	116.7 ± 61.9	433.9 ± 274.2
Urinary As, µg/L	264.1 ± 349.3	116.8 ± 76.4	456.3 ± 597.3	113.3 ± 99.7	390.6 ± 281.7
Urinary creatinine (mg/dL)	75.0 ± 41.5	75.3 ± 40.9	58.0 ± 38.8	74.5 ± 54.7	91.9 ± 33.9
Urinary As/gm creatinine	345.8 ± 324.9	165.5 ± 74.9	650.2 ± 450.7	178.9 ± 186.7	416.3 ± 250.1
Blood As (µg/L)	11.4 ± 10.3	6.8 ± 3.1	20.7 ± 15.3	7.1 ± 7.2	11.7 ± 7.3
Male, %	55.2	-	-	-	-
Land Ownership, %	37.9	44.4	42.9	33.3	28.6
Television Ownership, %	24.1	11.1	14.3	33.3	42.9
Current cigarette smoking, %	44.8	77.8	85.7	0	0
Current betel nut Use, %	34.5	55.6	42.9	16.7	14.3

^a mean ± SD unless otherwise noted,

^b water As 50 to 150 µg/L,

^c water As 232 to 500 µg/L,

^d water As 50 to 200 µg/L,

^e water As 250 to 1000 µg/L

Table 2Differentially expressed genes common to both males^a and females^b

Gene Symbol	Description	Absolute fold change	
		Males ^a	Females ^b
<i>SLC37A2</i>	solute carrier family 37 (glycerol-3- phosphate transporter), member 2	1.12987	-1.05461
<i>ZNF429</i>	zinc finger protein 429	1.11314	-1.09785
<i>ABCA1</i>	ATP-binding cassette, sub-family A (ABC1), member 1	1.09686	-1.11128
<i>KMO</i>	kynurenine 3- monooxygenase (kynurenine 3-hydroxylase)	1.09616	-1.11952
<i>CISH</i>	cytokine inducible SH2-containing protein	1.08392	1.10739
<i>TUBA1B</i>	tubulin, alpha 1b	1.07894	1.06552
<i>PKIG</i>	protein kinase (cAMP-dependent, catalytic) inhibitor gamma	1.07413	-1.06864
<i>SEMA4A</i>	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	1.06561	1.08562
<i>CLCN4</i>	chloride channel, voltage-sensitive 4	1.06541	-1.08737
<i>RBBP8</i>	retinoblastoma binding protein 8	1.06036	-1.07956
<i>TMEM68</i>	transmembrane protein 68	1.05458	-1.08187
<i>IFIH1</i>	interferon induced with helicase C domain 1	1.05346	-1.10904
<i>GLB1</i>	galactosidase, beta 1	1.04594	1.04002
<i>BTF3P11</i>	basic transcription factor 3 pseudogene 11	1.04533	1.04236
<i>VKORC1L1</i>	vitamin K epoxide reductase complex, subunit 1-like 1	1.04435	-1.04563
<i>NOL11</i>	nucleolar protein 11	1.04418	-1.05934
<i>MRPS18B</i>	mitochondrial ribosomal protein S18B	1.04346	-1.03351
<i>SNORA29</i>	small nucleolar RNA, H/ACA box 29	1.04276	1.04468
<i>PRMT10</i>	protein arginine methyltransferase 10 (putative)	1.04159	-1.04801
<i>HSF5</i>	heat shock transcription factor family member 5	1.03604	1.04256
<i>PQLC1</i>	PQ loop repeat containing 1	1.03523	1.04041
<i>KRTAP5-1</i>	keratin associated protein 5-1	1.03504	1.03366
<i>MRPS22</i>	mitochondrial ribosomal protein S22	1.03337	-1.04741
<i>MAD2L1BP</i>	MAD2L1 binding protein	1.03253	-1.04613
<i>SMAP1</i>	small ArfGAP 1	1.02688	1.03809

Gene Symbol	Description	Absolute fold change	
		Males ^a	Females ^b
<i>MAP3K6</i>	mitogen-activated protein kinase kinase kinase 6	−1.02623	1.03868
<i>ZFPL1</i>	zinc finger protein-like 1	−1.02681	−1.036
<i>KBTBD3</i>	kelch repeat and BTB (POZ) domain containing 3	−1.03002	−1.03796
<i>RNF126</i>	ring finger protein 126	−1.03488	1.04559
<i>PPP1R12B</i>	protein phosphatase 1, regulatory subunit 12B	−1.03503	1.0613
<i>RORA</i>	RAR-related orphan receptor A	−1.0373	1.05826
<i>SLC11A2</i>	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	−1.04141	1.05051
<i>SNTB1</i>	syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	−1.04204	1.06052
<i>HERC2</i>	HECT and RLD domain containing E3 ubiquitin protein ligase 2	−1.04328	1.04903
<i>TNIK</i>	TRAF2 and NCK interacting kinase	−1.04349	1.04896
<i>ATIC</i>	5-aminoimidazole-4- carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	−1.04567	1.04872
<i>IL11RA</i>	interleukin 11 receptor, alpha	−1.04633	1.1028
<i>ETS2</i>	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	−1.04934	1.06649
<i>ACER2</i>	alkaline ceramidase 2	−1.05118	−1.06698
<i>CLYBL</i>	citrate lyase beta like	−1.06358	1.07364
<i>CCDC144NL</i>	coiled-coil domain containing 144 family, N-terminal like	−1.065	−1.05019
<i>PRDM1</i>	PR domain containing 1, with ZNF domain	−1.06919	1.08734
<i>KIAA1324</i>	KIAA1324	−1.09944	1.14391

^a 534 differentially expressed genes (p<0.05) for males high water As vs. males low water As

^b 645 differentially expressed genes (p<0.05) for females high water As vs. females low water As

^c expression values for males high water As relative to males low water As

^d expression values for females high water As relative to females low water As

Table 3

Top IPA networks for differentially expressed genes of males (males high water As vs. males low water As) and females (females high water As vs. females low water As)

Network	Score	Network	Score
Males		Females	
Post-Translational Modification, Protein Folding, Antimicrobial Response	75	Cardiovascular Disease, Cellular Compromise, Neurological Disease	67
Cardiovascular Disease, Congenital Heart Anomaly, Developmental Disorder	66	Cell Death and Survival, Hematological System Development and Function, Tissue Morphology	42
Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry	62	Molecular Transport, RNA Trafficking, Cellular Development	35
Lipid Metabolism, Small Molecule Biochemistry, Cell Morphology	48	DNA Replication, Recombination, Repair, Cell Morphology, Cellular Function and Maintenance	25
Cellular Development, Cellular Growth and Proliferation, Hematological System Development	48	Cellular Function and Maintenance, Cell Death and Survival, Tissue Morphology	24

Table 4

Validation of microarray results by quantitative real-time PCR

Gene	Affymetrix fold change	Q-RT-PCR fold change
<i>POLRE2</i>	1.12	4.31
<i>CKS2</i>	−1.06	−1.96
<i>TGM2</i>	1.06	1.87
<i>E2F5</i>	−1.10	−1.44

Table 5

Biological validation of gene lists. Differentially expressed genes involved in heatshock response, DNA repair, and immune response for males and females.

	Gene symbol	Description	Fold change
Heat shock			
Males			
	<i>HSP1AB</i>	heat shock 70kDa protein 1B	1.055
	<i>HSPBP1</i>	heat shock 70kDa biding protein, cytoplasmic chaperone 1	1.038
	<i>HSF5</i>	heat shock transcription family member 5	1.036
Females			
	<i>HSPA13</i>	heat shock 70kDa protein 13	−1.054
DNA repair			
Males			
	<i>RBBP8</i>	retinoblastoma binding protein 8	1.060
	<i>CDK7</i>	cyclin-dependent kinase 7	1.058
	<i>POLD3</i>	polymerase (DNA-directed), delta 3, accessory subunit	1.036
	<i>POLI</i>	polymerase (DNA directed) iota), <i>RPA3</i> (replication protein A3, 14kDa	−1.054
	<i>RPA3</i>	replication protein A3, 14kDa	1.030
	<i>LIG3</i>	ligase III, DNA, ATP-dependent	−1.027
Females			
	<i>RBBP8</i>	retinoblastoma binding protein 8	−1.080
	<i>MDC1</i>	mediator of DNA-damage checkpoint 1	1.041
	<i>LIG4</i>	ligase IV, DNA, ATP-dependent	−1.060
	<i>MSH2</i>	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	−1.059
	<i>SMUG1</i>	single-strand-selective monofunctional uracil-DNA glycosylase 1	−1.069
Immune system			
Males			
	<i>RSAD2</i>	S-adenosyl methionine domain containing protein 2	1.186
	<i>IFIT1</i>	interferon-induced protein with tetratricopeptide repeats 1	1.207
	<i>IFIT3</i>	interferon-induced protein with tetratricopeptide repeats 3	1.123

Gene symbol	Description	Fold change
<i>IFIT5</i>	interferon-induced protein with tetratricopeptide repeats 5	1.095
<i>IFI44L</i>	interferon-induced protein 44-like	1.323
<i>IFI6</i>	interferon, alpha-inducible protein 6	1.201
<i>MX1</i>	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	1.180
<i>IL23A</i>	interleukin 23, alpha subunit p19	-1.045
<i>IL11RA</i>	interleukin 11 receptor, alpha	-1.046
<i>IL15RA</i>	interleukin 5 receptor, alpha	-1.047
<i>IL1RL1</i>	interleukin 1 receptor-like 1	-1.102
<i>IL1RN</i>	interleukin 1 receptor antagonist	-1.084
<i>IL17C</i>	interleukin 17C	-1.037
Females		
<i>IL11RA</i>	interleukin 11 receptor, alpha	1.103
<i>IL13RA1</i>	interleukin 13 receptor, alpha 1	1.089
<i>IL18BP</i>	IL18 binding protein	1.070
<i>IL16</i>	interleukin 16	-1.035
<i>HLA-DRB6</i>	major histocompatibility complex, class II, DR beta 6 (psuedogene)	-1.083
<i>HLA-B</i>	major histocompatibility complex, class I, B	-1.083
<i>HLA-G</i>	major histocompatibility complex, class I, G	-1.129
<i>IFIH1</i>	interferon induced with helicase C domain 1	-1.109