

1 **Title:** In Utero Arsenic Exposure and Epigenome-Wide Associations in Placenta, Umbilical
2 Artery and Human Umbilical Vein Endothelial Cells

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26 **ABSTRACT**

27 Exposure to arsenic early in life has been associated with increased risk of several
28 chronic diseases and is believed to alter epigenetic programming in utero. In the present study,
29 we evaluate the epigenome-wide association of arsenic exposure in utero and DNA methylation
30 in placenta (n=37), umbilical artery (n=45) and human umbilical vein endothelial cells (HUVEC)
31 (n=52) in a birth cohort using the Infinium HumanMethylation450 BeadChip array. Unadjusted
32 and cell mixture adjusted associations for each tissue were examined along with enrichment
33 analyses relative to CpG island location and omnibus permutation tests of association among
34 biological pathways. One CpG in artery (cg26587014) and four CpGs in placenta (cg12825509;
35 cg20554753; cg23439277; cg21055948) reached a Bonferroni adjusted level of significance.
36 Several CpGs were differentially methylated in artery and placenta when controlling the false
37 discovery rate (q-value<0.05), but none in HUVEC. Enrichment of hypomethylated CpG islands
38 was observed for artery while hypermethylation of open sea regions were present in placenta
39 relative to prenatal arsenic exposure. The melanogenesis pathway was differentially methylated
40 in artery (Max F $P<0.001$), placenta (Max F $P<0.001$) and HUVEC (Max F $P=0.002$). Similarly,
41 the insulin signaling pathway was differentially methylated in artery (Max F $P=0.02$), placenta
42 (Max F $P=0.03$) and HUVEC (Max F $P=0.002$). Our results show that prenatal arsenic exposure
43 can alter DNA methylation in artery and placenta but not in HUVEC. Further studies are needed
44 to determine if these alterations in DNA methylation mediate the effect of prenatal arsenic
45 exposure and health outcomes later in life.

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49 **INTRODUCTION**

50 Over 200 million individuals worldwide are exposed to elevated levels of inorganic
51 arsenic. This is a public health concern because arsenic is a known human carcinogen and
52 chronic exposure is associated with the development of skin, lung, bladder, kidney, liver and
53 potentially prostate cancer.¹ Particularly, early life exposure to arsenic has been associated with
54 the development of many latent health effects including carcinogenesis.² Human ecological
55 studies from the Antofagasta region of Chile have associated prenatal and early childhood
56 exposure to arsenic from contaminated municipal water with increased risk of lung and bladder
57 cancer later in life.³ Increased mortality from acute myocardial infarction and cancers of the
58 bladder, kidney, lung, and liver have also been reported from this population decades after the
59 exposure declined.^{4, 5}

60 Of public health interest is the ability of early life arsenic exposure, particularly exposures
61 occurring *in utero*, to increase disease risk and susceptibility to adverse health conditions later in
62 life. For example, animal models support the involvement of transplacental arsenic exposure in
63 the development and progression of atherosclerosis, consistent with human studies linking early
64 life exposure and cardiovascular disease.^{6, 7} Emerging evidence also indicates that exposure to
65 arsenic can disrupt normal immune function and *in utero* exposure can increase the susceptibility
66 and severity of infections later in life.⁸⁻¹⁰ Furthermore, arsenic exposure during fetal development
67 has been associated with growth restrictions and adverse perinatal health outcomes such as low
68 birth weight, still births, infant mortality, and preterm births.¹¹ Lastly, latent adverse neurological
69 health outcomes have also been documented with maternal exposure to arsenic during
70 pregnancy.^{12, 13}

71 ~~The exact molecular mechanisms of the toxicological effects attributed to arsenic exposure~~

72 ~~remains elusive and no single mechanism has been identified in the development of arsenic~~
73 ~~associated diseases and the observed latency of health effects.~~¹⁴ However, The latency of health
74 effects documented in epidemiological studies and animal models along with the observed
75 susceptibility of prenatal exposures are suggestive of an epigenetic mode of action. Fetal
76 programming events involving DNA methylation occur at critical windows of fetal development
77 in a cell-specific manner shown to be sensitive to environmental exposures.¹⁵ Experimental
78 evidence from animal models demonstrate that transplacental exposure to arsenic leads to
79 epigenetic alterations, changes in gene expression and increased incidence of tumors in the
80 offspring.^{16, 17} Therefore, it is postulated that epigenomic regulation including, but not limited to,
81 DNA methylation is a potential mechanism of arsenic induced carcinogenesis and latent disease
82 risk.^{2, 18, 19} Other likely interacting mechanisms of early life exposure to arsenic and latent
83 disease risk include the development of cancer stem cells and perturbations of immune function.²

84 Several human studies have evaluated the impact of prenatal arsenic exposure on the cord
85 blood and whole blood epigenome..¹ Among these epidemiological studies evaluating cord blood
86 or whole blood DNA methylation no common loci has been identified to be differentially
87 methylated across studies.²⁰⁻²⁵ However, significant DNA methylation disruption of unique loci
88 along with enrichment of key regulatory CpG regions has been documented across different
89 study populations.²¹⁻²⁷ Besides studies that examined cord and whole blood epigenome, only two
90 studies to date have evaluated the association between arsenic exposure and CpG methylation of
91 target tissue by evaluating DNA methylation in urothelial carcinoma samples and CpG
92 methylation of exfoliated urothelial cells, respectively.^{28, 29} These studies found differentially
93 methylated loci associated with arsenic exposure in key regulatory genes potentially involved in
94 development of arsenic induced urothelial carcinoma.

95 Epigenetic reprogramming during fetal development resulting from transplacental exposure
96 is one of the main hypothesized mechanisms of arsenic's associated-disease.² To further our
97 understanding of how prenatal arsenic exposure could alter epigenetic programming it is
98 important to evaluate its effect on different tissues with diverse cellular compositions. Evaluating
99 if exposure to arsenic in utero alters DNA methylation of different tissues could yield insights
100 into the etiology of toxicant-mediated disease and epigenetic modifications of relevant tissues
101 with specific biological functions. Subsequently, we examined the association between maternal
102 drinking water arsenic as a proxy of transplacental exposure during fetal development and the
103 epigenome of placenta, umbilical artery and Human Umbilical Vein Endothelial Cells (HUVEC)
104 from a birth cohort conducted in arsenic affected regions of Bangladesh.

105 **RESULTS**

106 The sample size varied by tissue type with a maximum of 52 samples present for HUVEC
107 followed by 45 samples in umbilical artery and 37 placenta samples. Arsenic concentration in
108 maternal drinking water at study enrollment ranged from below the detection limit of <1µg/L to
109 510 µg/L with a mean exposure concentration of 63.7 µg/L. Selected sample characteristics are
110 shown in Table 1.

111 **Arterial Tissue**

112 **Locus-by-Locus Analysis:** In the analysis that was unadjusted for cellular composition, one
113 CpG loci (cg26587014) located in chromosome 19 and not annotated to any gene was
114 differentially methylated in arterial tissue in relation to arsenic exposure using a Bonferroni
115 threshold for statistical significance ($P < 1.33 \times 10^{-7}$). Controlling for the false discovery rate at 5%
116 (q-value < 0.05) revealed 2,105 CpGs that were differentially methylated relative to log₂-
117 transformed maternal drinking water arsenic. However, after adjusting for cellular composition

118 using the Houseman reference-free method, no loci reached a Bonferroni corrected level of
119 significance or a q -value <0.05 . Unadjusted and adjusted results are shown in Figure 1A and 1B,
120 respectively. The top 100 differentially methylated loci ranked on lowest P -value are
121 summarized in supplementary table S1 and S2 for unadjusted and cell mixture adjusted analyses,
122 respectively. In unadjusted analyses, differentially methylated loci with a q -value <0.05 were
123 disproportionately located in CpG islands (54%) compared to the distribution of CpG island
124 probes in the rest of array (33%) ($P<1\times 10^{-4}$), supplementary Figure S1A. The majority of
125 unadjusted hypomethylated loci with a q -value <0.05 were located in CpG islands (83%), Figure
126 1C. After adjusting for cellular heterogeneity, a similar enrichment of hypomethylated loci in
127 CpG islands was observed among top loci having a nominal p -value $<1\times 10^{-4}$, Supplementary
128 Figure S1B.

129 **Biological Pathway Analysis:** Omnibus permutation based tests revealed significant
130 associations between *in utero* exposure to arsenic and epigenetic disruption of KEGG biological
131 pathways in arterial tissue (Mean F-statistics $P=0.009$ and maximum F-statistic $P=0.006$).
132 Pathways that were observed to have the strongest association based on the lowest mean F-static
133 level of significance ($P=0.004$) were: maturity onset of diabetes of the young (hsa04950),
134 primary immunodeficiency (hsa05340), ABC transporters (hsa02010), allograft rejection
135 (hsa05330) and vibrio cholerae infection (hsa05110). Differentially methylated pathways
136 observed to have a strong association using a maximum F-statistic level of significance
137 ($P<0.001$) included: the Hedgehog signaling pathway (hsa04340), Melanogenesis (hsa04916),
138 Wnt signaling pathway (hsa04310), Basal cell carcinoma (hsa05217), DNA replication
139 (hsa03030) and the p53 signaling pathway (hsa04115). The summary for all associations
140 between maternal drinking water arsenic and epigenetic disruption of KEGG biological

141 pathways are shown in supplementary table S7.

142 **Placenta Tissue**

143 **Locus-by-Locus Analysis:** In the analyses that were unadjusted for cellular composition, no
144 single CpG loci reached Bonferroni adjusted significance in placenta ($P < 1.37 \times 10^{-7}$). However,
145 two CpG loci (cg26390526; cg03857453) annotated to the Epidermal Filaggrin gene (*FLG*) and
146 the nuclear receptor subfamily 3, group C, member 1 glucocorticoid receptor gene (*NR3C1*) were
147 hypermethylated relative to maternal drinking water arsenic after controlling for the false-
148 discovery rate (q-value < 0.05). In analyses that adjusted for cell mixture in the placenta, four
149 CpGs reached Bonferroni adjusted significance: cg12825509 (*TRA2B* gene), cg20554753,
150 cg23439277 (*PLCE1* gene) and cg21055948 (*CD36* gene). Moreover, analyses adjusted for
151 cellular heterogeneity revealed 518 CpG loci that were differentially methylated after controlling
152 for the false discovery rate (q-value < 0.05). Unadjusted and cell mixture adjusted results are
153 shown in Figure 2A and 2B, respectively. The top 100 differentially methylated loci ranked on
154 lowest *p*-value are summarized in supplementary table S3 and S4 for unadjusted and cell mixture
155 adjusted results, respectively. For the top unadjusted differentially methylated loci with a
156 nominal $P < 1 \times 10^{-4}$ a disproportionate amount of CpGs were located within open sea regions of
157 CpG islands (76%) compared with the distribution of open sea loci in the rest of array (33%)
158 ($P < 1 \times 10^{-4}$), supplementary figure 2A. Among these loci, the great majority of hypermethylated
159 CpGs were located within open sea regions (89%) relative to CpG islands, Figure 2C. For the
160 cell mixture adjusted analyses a similar enrichment of hypermethylated loci in open sea regions
161 was observed for loci with a q-value < 0.05, supplementary Figure 2B.

162 **Biological Pathway Analysis:** Omnibus permutation based tests indicated that exposure to
163 arsenic *in utero* disrupts methylation of a small number of CpGs within KEGG biological

164 pathways in the placenta tissue (Omnibus maximum F-statistic $P=0.004$). However, a marginal
165 association among KEGG biological pathways and arsenic exposure was observed using an
166 omnibus Mean F-statistic test for association ($P=0.108$). KEGG biological pathways that were
167 differentially methylated in relationship to arsenic exposure with a maximum F-statistic $P<1\times 10^{-3}$
168 included: Melanogenesis (hsa04916), Neuroactive ligand-receptor interaction (hsa04080),
169 Calcium signaling pathway (hsa04020), GnRH signaling pathway (hsa04912), Dilated
170 cardiomyopathy (hsa05414), Gap junction (hsa04540), Vasopressin-regulated water reabsorption
171 (hsa04962), Vascular smooth muscle contraction (hsa04270), Oocyte meiosis (hsa04114), Vibrio
172 cholerae infection (hsa05110), Progesterone-mediated oocyte maturation (hsa04914) and the
173 Peroxisome pathway (hsa04146). Several other pathways were significantly associated with
174 arsenic exposure using a maximum F-statistic $P<0.05$ and summarized in supplementary table
175 S7.

176 **Umbilical Vein Endothelial Cells (HUVEC)**

177 **Locus-by-Locus Analysis:** In both unadjusted and cell mixture adjusted analyses no single CpG
178 loci was associated with arsenic exposure at a Bonferroni corrected level of significance
179 ($P<1.44\times 10^{-7}$) or a q-value <0.05 after controlling for the false discovery rate, Figure 3A and 3B.
180 Among the top 31 CpG loci with a nominal $P<1\times 10^{-4}$ no significant differences were present for
181 the occurrence of top loci relative to CpG island location compared to the rest of the array for
182 unadjusted analyses, Figure 3C. The top 100 differentially methylated loci ranked by lowest p -
183 value are summarized in supplementary table S5 and S6 for unadjusted and cell mixture adjusted
184 results, respectively.

185 **Biological Pathway Analysis:** Omnibus permutation tests for association among KEGG
186 biological pathways indicated that arsenic exposure was not significantly associated with a large

187 number of changes in DNA methylation across pathways in HUVEC (Mean F-statistic $P=0.129$)
188 and the presence of a small number of strong associations was borderline significant (Max F-
189 statistic $P=0.06$). Few individual biological pathways reached statistical significance using a
190 maximum F-statistic level of significance. The top differentially methylated biological pathways
191 (maximum F-statistic $P=0.002$) in HUVEC included: Melanogenesis (hsa04916), Wnt signaling
192 pathway (hsa04310), Basal cell carcinoma (hsa05217) and the Insulin signaling pathway
193 (hsa04910), all KEGG biological pathway based associations are summarized in supplementary
194 table S7.

195 The overlap among CpGs within each tissue for unadjusted and cell mixture adjusted
196 analyses using the top 100 differentially methylated CpGs was 26 loci in artery, 21 loci in
197 placenta and 33 loci for HUVEC, supplementary figure S3. Among the top 100 differentially
198 methylated loci, only one CpG (cg21002651) located within the body of the *CASP1* gene was
199 differentially methylated across two tissues in unadjusted analyses. This loci was
200 hypomethylated in placenta ($\beta=-0.20$, $P=5.73 \times 10^{-6}$) but hypermethylated in HUVEC ($\beta=0.20$,
201 $P=1.29 \times 10^{-4}$) in relationship to maternal drinking water arsenic. No other CpGs overlapped in
202 unadjusted or adjusted analyses.

203 **DISCUSSION**

204 Our study provides evidence that *in utero* exposure to arsenic can disrupt DNA
205 methylation of artery and placenta tissues but the association with umbilical vein endothelial
206 vein cells was marginal. However, the association of prenatal arsenic exposure on the epigenome
207 on artery and placenta depended on the cell mixture adjustment. For instance, the association in
208 artery was attenuated after controlling for cellular heterogeneity but strengthened in placenta. *In*
209 *utero* exposure to arsenic was also associated with DNA methylation levels of key biological

210 pathways across tissues providing new insights into the potential etiology of arsenic-mediated
211 diseases with a plausible epigenetic reprogramming component.

212 In normal tissue, the majority of CpG islands remain unmethylated and methylation of
213 CpG islands located within promoter regions of genes is usually restricted to genes at which
214 there is long-term stabilization of repressed states such as in gene silencing of imprinted genes.³⁰
215 However, CpG island methylation is not deterministic of gene expression and further studies are
216 needed to determine if the observed alterations in DNA methylation are associated with
217 biological effects. Conversely, we observed an enrichment of hypomethylated loci in CpG
218 islands relative to prenatal arsenic exposure. This is of particular interest because both animal
219 and human studies have demonstrated that DNA hypomethylation occurs in atherosclerotic
220 lesions and that hypomethylation of CpG islands is observed broadly in human atherosclerotic
221 arteries^{31, 32} and in arterial disease pathogenesis.³³ ~~In animal models, *in utero* arsenic exposure~~
222 ~~has been shown to induce the early onset of atherosclerosis along with epidemiological studies~~
223 ~~linking early life exposure with cardiovascular disease.^{6,7} Therefore, we hypothesize that the~~
224 ~~observed hypomethylation of influential genomic regions such as CpG islands could play a role~~
225 ~~in the development of arsenic-associated cardiovascular disease, particularly atherosclerosis of~~
226 ~~arterial tissue. Another early observation from epigenetic cancer studies was the global~~
227 ~~hypomethylation of tumor samples compared to normal tissue mainly at repetitive genomic~~
228 ~~elements and that hypomethylation of these regions can lead to hypermethylation of tumor~~
229 ~~suppressor genes.³⁴ Along with this observation, previous studies of arsenic exposure have~~
230 characterized hypermethylation of the promoter region of the p53 gene a mechanisms
231 hypothesized to contribute to the carcinogenesis of arsenical compounds.^{35, 36} Consistent with
232 these reports, our gene set analysis shows that CpG methylation within the p53 signaling

233 pathway is associated with arsenic exposure during pregnancy suggesting that artery might be a
234 target tissue for the epigenetic toxicity of arsenic, and potentially involved in carcinogenesis.
235 However, this hypothesis needs to be evaluated.

236 The placenta is an important regulator of fetal development and intrauterine growth that
237 plays a crucial role mediating the maternal and fetal environment. Furthermore, the placenta is a
238 unique epigenetic target organ as the majority of imprinted genes in animal models are both
239 expressed and imprinted in the placenta and hypothesized to contribute to fetal
240 neurodevelopment.^{37, 38}In unadjusted analyses a CpG located in the body of the glucocorticoid
241 receptor gene (*NR3C1*) was significantly hypermethylated in the placenta relative to prenatal
242 arsenic exposure. Previous studies have shown that hypermethylation of the *NR3C1* gene
243 influences cortisol response, infant behavior and self-regulation.^{39, 40} Interestingly, a recent
244 experimental study demonstrated that exposure to arsenic *in utero* lowers the activity of the
245 glucocorticoid receptor pathway and these changes were maintained into adolescence of the
246 mouse model.⁴¹ Although further studies are needed to confirm whether these biological findings
247 are connected and related to behavioral outcomes. ~~The placenta has also been characterized as~~
248 ~~one of the hypomethylated tissues as LINE-1 elements have lower levels of methylation when~~
249 ~~compared to other tissues. Furthermore, it has been shown that normal human placenta contains~~
250 ~~partially methylated domains (37%) with the ability to suppress genes and impact tissue specific~~
251 ~~functions independent of the tissue of origin.⁴²The observed hypermethylation of open sea~~
252 ~~regions relative to CpG island location could have implications for normal methylation of LINE-~~
253 ~~1 elements and partially methylated domains, potentially affecting normal biological function~~
254 ~~and development of the placenta.~~

255 A few KEGG biological pathways were differentially methylated in relation to maternal

256 drinking water arsenic in all three tissues. Namely, DNA methylation of the melanogenesis
257 pathway was strongly associated with exposure to arsenic in artery, placenta and HUVEC. An
258 early clinical symptom of arsenicosis include the appearance of hyperpigmentation changes of
259 the skin in the trunk, neck and chest regions of the body eventually progressing to the palmar and
260 plantar regions and eventually leading to hyperkeratosis.⁴³ Consistent with the differential
261 methylation of this pathway in our data, arsenic-associated alterations in DNA methylation of
262 leukocytes has been previously associated with increased risk of developing skin lesions.⁴⁴
263 Lastly, the insulin signaling pathway was observed to be differentially methylated across all
264 three tissues with respect to arsenic exposure. Exposure to arsenic has been consistently
265 associated with Type 2 diabetes and insulin resistance in both animal models and
266 epidemiological studies.⁴⁶ Previous studies have documented the epigenetic disruption of several
267 genes involved in the development of diabetes and insulin resistance for individuals chronically
268 exposed to arsenic.⁴⁷ Although epigenetic distribution was characterized among these biological
269 pathways relative to arsenic exposure, future studies need to evaluate if these changes are
270 associated with changes in gene expression, metabolism and ultimately pathological phenotypes.
271 It is also important to note that the permutation test used in this analysis evaluates the DNA
272 methylation disruption of the biological pathways at a global level and not on a gene by gene
273 basis. Therefore, it is not possible to determine if individual genes are differentially methylated
274 with regards to arsenic exposure.

275 It is crucial to highlight that HUVEC is a homogenous tissue in terms of cellular
276 composition and was not significantly disrupted in the locus-by-locus analysis and marginally
277 associated among some biological pathways. However, artery and placenta, both representing a
278 diverse mixture of cell types, were observed to be differentially methylated relative to prenatal

279 arsenic exposure. DNA methylation is cell specific playing a key role in tissue differentiation
280 and lineage commitment making this process particularly vulnerable to environmental stimuli
281 and exposures during fetal development. The placenta represents the most diverse tissue composed
282 of fetal vascular cells, mesenchymal cells, cytotrophoblast and syncytiotrophoblast that originate
283 from the trophoblast.⁴⁸ Furthermore, it has also been observed that the human placenta contains
284 both hematopoietic stem cells and mesenchymal stem cells.⁴⁹ All tissues derived from the fetus
285 are an extension of the mesoderm that differentiates during embryonic development to form the
286 umbilical cord and placenta. Therefore, it might be possible to arsenic exposure during fetal
287 development could affect cellular differentiation for placenta and artery but not HUVEC as this
288 is a cellular homogenous tissue. Epidemiologic studies often rely on preserved samples and have
289 limited fresh tissue availability making the sorting or isolation of target cell types not feasible.
290 Therefore, future experimental studies should evaluate the development of cancer stem cells
291 (CSCs) and alterations to the immune function as factors or intermediary mechanisms of the
292 observed epigenetic perturbations, as others have also suggested.² Moreover, the interaction
293 between prenatal arsenic exposure and other transplacental contaminants should also be
294 considered, as prenatal exposure to arsenic has been previously shown to interact with other
295 prenatal exposures such as mercury.⁵⁰

296 One of the major strengths for the present study is the epigenome-wide analysis of three
297 different tissues collected from the same maternal-infant pairs yielding insights for the potential
298 biological impact of arsenic exposure during fetal development. Also, the prospective design of
299 this birth cohort along with the exposure assessment early during pregnancy are important
300 qualities that strengthens the temporality of the epigenetic perturbations reported. Although the
301 present study relies on a single water sample during early pregnancy and exposure

302 misclassification cannot be ruled out, previous studies in rural Bangladesh have demonstrated
303 that drinking water arsenic exposure is relatively constant and correlated with biomarkers of
304 internal doses, such as urine and toenails^{51, 52} and that arsenic readily crosses the placenta.⁵³
305 Additionally, the availability of umbilical samples at birth provides one of the few opportunities
306 for examining epigenetic programming in cardiovascular target tissue in a non-invasive and
307 feasible manner. There are a number of important limitations to our current study including the
308 relatively small sample size and the lack of validation using a complementary DNA methylation
309 method due to sample availability. The lack of reference methylomes for placenta, artery and
310 HUVEC also raise an important challenge when interpreting the observed epigenetic
311 perturbations in tissues that might represent a mixture of cell types such as artery or placenta.
312 However, we implemented a complementary bioinformatics method to adjust for cellular
313 heterogeneity to identify potential perturbations in loci hypothesized to be associated with
314 methylation levels independent of cellular heterogeneity. Furthermore, unique tissue samples
315 were analyzed in separate plates raising the possibility that differences across tissue could be
316 potentially attributed to technical plate effects. Finally, gene expression was not measured and
317 the observed changes in DNA methylation need to be further confirmed and evaluated.
318 Particularly significant association between DNA methylation among KEGG biological
319 pathways might not result in functional gene expression or proteomic alterations within
320 pathways.

321 In conclusion, we show that prenatal arsenic exposure is associated with altered DNA
322 methylation of umbilical artery and placenta tissue but evidence of an association for HUVEC is
323 limited. Furthermore, we present evidence of DNA methylation disruption of key biological
324 pathways across different tissues holding the potential to mediate arsenic-associated diseases

325 previously described from exposures *in utero*.

326 **MATERIALS & METHODS**

327 **Study Population**

328 This pilot study was nested within an established birth cohort recruited in Bangladesh
329 (2007-2011) and designed to characterize the potential epigenetic disruption associated with
330 arsenic exposure during pregnancy in different tissues collected at birth. A more detailed
331 explanation of the full birth cohort has been published previously.²¹ Briefly, pregnant women
332 with ≤ 16 weeks of gestation confirmed by ultrasound were enrolled in a prospective
333 reproductive birth cohort in Bangladesh. Trained health care workers at community health clinics
334 in Sirajdikhan and Birahimpur recruited pregnant women 18 years of age or older that used a
335 tube-well as their primary drinking water source, planned to live at their current residency during
336 the duration of the pregnancy and received prenatal health care at Dhaka Community Hospital
337 (DCH) or affiliated community clinic. Study participants agreed to deliver at DCH or at home
338 with a DCH trained midwife. Informed consent was obtained from all participants prior to
339 enrollment. All participants were provided with prenatal care and prenatal vitamins offered by
340 DCH. This study was approved by the Human Research Committees at the Harvard School of
341 Public Health, Oregon State University and Dhaka Community Hospital Trust.

342 Three distinct tissues were collected at the time of delivery including: artery from the
343 umbilical cord, placenta, and endothelial cells isolated from the umbilical vein. Since the goal of
344 this pilot study was to examine the potential exposure-response relationship between arsenic and
345 DNA methylation, specimens were selected based on maternal drinking water arsenic
346 concentrations at study enrollment to cover a wide range of exposures (<1 - 510 $\mu\text{g/L}$). A total of
347 37 placenta samples, 45 artery samples and 52 HUVEC samples were included in the final

348 analysis.

349 **Drinking Water Arsenic**

350 Water samples were collected from the tube-well identified by participants as their main
351 source of drinking water at the time of their enrollment into the study as previously described.²¹
352 Briefly, water samples were collected in a 50-mL polypropylene tubes (BD Falcon, BD
353 Bioscience, Bedford, MA), preserved with Reagent Grade nitric acid (Merck, Germany) to a
354 pH<2 and stored at room temperature. Arsenic concentrations were measured by inductively
355 coupled plasma-mass spectrometry (ICP-MS) using the US EPA method 200.8 to determined
356 metals in water (Environmental Laboratory Services, North Syracuse, New York).⁵⁴ Average
357 percent recovery for Arsenic from plasmaCal multi-element QC standard #1 solution (SCP
358 Science) was 102% ± 7%. The limit of detection (LOD) for arsenic in drinking water was 1
359 µg/L.

360 **Tissue Collection: Umbilical Artery, Placenta & HUVEC**

361 Trained medical workers present at delivery collected a sample of the umbilical cord and
362 placenta immediately after the delivery was completed. Using sterile techniques, approximately
363 5-7 cm of umbilical vein was dissected out of fresh umbilical cord and rinsed with phosphate
364 buffered saline solution to remove external contamination. The vein lumen was then bisected and
365 the interior cavity was flushed with approximately 100 mL of phosphate buffered solution to
366 remove blood. The interior lumen wall was gently rubbed using a sterile cytology brush to
367 collect endothelial cells. The cytology brush was then vortexed in 1 mL of cell lysis solution
368 (Qiagen) to transfer the cells. The cell lysis solution was then stored at 4 °C. Samples were
369 shipped to Harvard School of Public Health where the DNA was extracted using DNeasy Blood
370 & Tissue Kit (Qiagen) following manufacturer's instructions.

371 Approximately 1 cm of umbilical cord artery was dissected out of fresh umbilical cord,
372 the exterior of the artery was scraped to remove Wharton's Jelly, and rinsed with phosphate
373 buffered saline solution to remove blood. The arterial cross section was placed in 2 mL of
374 RNase later and stored at -20 °C. Samples were shipped to Harvard School of Public Health on
375 dry ice. The artery sample was then minced using a sterile scalpel and added to Maxwell Cell
376 DNA Purification kits (Promega) with an additional 20 µL of Proteinase K (Qiagen). Samples
377 were allowed to sit for 30 minutes before being extracted using the Maxwell 16 Research
378 instrument following manufacturer's instructions.

379 For placenta samples, a one centimeter tissue plug was excised from fresh placenta. The
380 tissue plug was placed into a sterile vial and covered with Tissue-Tek O.C.T. gel (Electron
381 Microscopy Sciences) and frozen at -20 °C. Samples were then shipped to Harvard School of
382 Public Health on dry ice. Next, approximately 10 grams of placenta tissue was removed from the
383 plug and minced using a sterile scalpel and added to Maxwell Cell DNA Purification kits
384 (Promega) with an additional 20 µL of Proteinase K (Qiagen). Samples were allowed to sit for
385 30 minutes before being extracted using the Maxwell 16 Research instrument following
386 manufacturer's instructions.

387 **DNA Methylation Assessment and Quality Control**

388 DNA was shipped to the University of Minnesota's Biomedical Genomic Center that
389 quantified DNA methylation using the Illumina Infinium HumanMethylation450 BeadChip
390 (Illumina, San Diego, CA) following standard manufacturer's protocols. The
391 HumanMethylation450 BeadChip measures DNA methylation at > 485,000 CpG sites at single
392 nucleotide resolution, covering 99% of the RefSeq genes.

393 Tissues were analyzed in separate plates and randomly allocated to different chips. Data
394 were obtained and processed from raw methylation image files and normalized using internal
395 control probes via the functional normalization method with two principal components to
396 account for technical variation between samples using the *minfi* package of R.⁵⁵ DNA
397 methylation was estimated at each CpG as the fraction of DNA molecules whose target CpG loci
398 is methylated and referred to as β -values. Measurements at CpG loci on X and Y chromosomes
399 were excluded from the analysis to avoid gender-specific methylation bias. Previously identified
400 non-specific and cross-reactive probes within the array along with polymorphic CpG loci ($\geq 5\%$
401 of the minor allele frequency) were removed for the analysis.⁵⁶ Furthermore, a detection *P-value*
402 was computed for all CpGs and probes with non-significant detection ($P > 0.01$) in greater than
403 10% of the samples were removed from the analysis. After quality control, the total number of
404 autosomal CpGs left in the analysis were 374,320 loci for artery, 365,994 loci for placenta and
405 347,650 loci in HUVEC samples. Finally, a beta-mixture quantile intra sample normalization
406 procedure (BMIQ) was further applied to the data to reduce the potential bias that can arise from
407 type 2 probes as previously described.⁵⁷ Strip plots and signal intensities of control probes were
408 visually examined for bisulfite conversion, probe hybridization and single base extension.
409 Density plots for the β -values were examined for all samples at each normalization step
410 described above.

411 **Statistical Analysis**

412 **Unadjusted and Cell-adjusted Locus-by-Locus Analysis:** We first aimed to identify
413 differentially methylated CpG loci in relationship to prenatal arsenic exposure from maternal
414 drinking water. Maternal arsenic concentration in water was right skewed and subsequently log₂-
415 transformed. In order to evaluate linear associations between prenatal exposure to arsenic and

416 differentially methylated CpG loci, β -values were logit-transformed to M-values previously
417 described to be more appropriate for differential analysis of DNA methylation.⁵⁸ In the locus-by-
418 locus approach, two different but complementary methodologies were implemented. First, the
419 linear association between individual CpG methylation on the M-value scale and \log_2 -
420 transformed arsenic was evaluated adjusting for infant sex using the *limma* function found in the
421 *minfi* package of R. Second, due to the lack of reference methylomes of isolated cell types in
422 placenta, artery or HUVEC, a novel reference-free method of adjusting for cellular heterogeneity
423 was implemented using the *RefFreeEWAS* package of R. The reference-free method is an
424 extension of the original Houseman method that utilizes a deconvolution approach similar to
425 surrogate variable analysis (SVA) that is data driven to identify latent variables or dimensions as
426 surrogates of cellular composition.⁵⁹ Using this method, the sex adjusted linear association
427 between individual CpG methylation on the β -value scale and \log_2 -transformed maternal
428 drinking water arsenic was evaluated using 1000 bootstrap samples for estimating the standard
429 errors of association in placenta, umbilical artery and HUVEC. Results from the unadjusted
430 *limma* models and the reference-free cell mixture adjusted analyses were compared within
431 tissues and across tissues. Enrichment analyses for the distribution of CpGs relative to CpG
432 island location of the top differentially methylated loci based on a q -value <0.05 or a nominal
433 $P<1\times 10^{-4}$, were compared to the distribution of probes on the rest of the array.

434 **Biological Pathway Analysis:** Omnibus permutation based tests and p -values were obtained by
435 mapping subsets of CpGs to their associated genes in specific KEGG biological pathways. Gene
436 sets were compiled from the Kyoto Encyclopedia of Genes and Genomes (KEGG) corresponding
437 to specific biological pathways using the Entrez IDs matched to KEGG biological pathways
438 using the Bioconductor library *org.Hs.eg.db*. The permutation distribution was obtained from

439 unadjusted cell mixture models by permuting the exposure with respect to measured DNA
440 methylation over subgroups of CpGs defined by biological pathways (1000 permutations).
441 Unadjusted cell mixture methylation analyses were used for the KEGG pathways as the
442 reference-free Houseman method is unable to accommodate for the permutation test of CpGs
443 across individual biological pathways. Pathway based associations of DNA methylation with
444 prenatal arsenic exposure as a continuous variable were summarized using a maximum nominal
445 F-statistics *p*-value (akin to a minimum *p*-value) and an average nominal F-statistic *p*-value. The
446 maximum and minimum F-statistic *p*-values are better suited for detecting a small number of
447 strong associations and a large number of more variable associations, respectively, as previously
448 described.²¹ This approach allowed us to test for significant DNA methylation disruption across
449 single KEGG pathways and not over individual genes.

450 All statistical analyses were performed using the R statistical package version 3.2.0
451 (<http://www.R-project.org>).

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663 **Figure Legends**

664 **Figure 1.** Locus-by-locus epigenome-wide analysis for umbilical artery: volcano plots for the
665 association between log₂-transformed maternal drinking water arsenic (A) unadjusted for cellular

666 heterogeneity and (B) adjusting for cellular heterogeneity using the Houseman reference-free
667 method. (C) Distribution of differentially methylated loci (q -value <0.05) relative to CpG islands
668 for the unadjusted cell mixture analysis.

669
670 **Figure 2.** Locus-by-locus epigenome-wide analysis for placenta: volcano plots for the
671 association between \log_2 -transformed maternal drinking water arsenic (A) unadjusted for cellular
672 heterogeneity and (B) adjusting for cellular heterogeneity using the Houseman reference-free
673 method. (C) Distribution of differentially methylated loci (nominal $P<1\times 10^{-4}$) relative to CpG
674 islands for the unadjusted cell mixture analysis.

675
676 **Figure 3.** Locus-by-locus epigenome-wide analysis for HUVEC: volcano plots for the
677 association between \log_2 -transformed maternal drinking water arsenic: (A) unadjusted for
678 cellular heterogeneity and (B) adjusting for cellular heterogeneity using the Houseman reference-
679 free method. (C) Distribution of differentially methylated loci (nominal p -value $<1\times 10^{-4}$) relative
680 to CpG islands for the unadjusted cell mixture analysis.

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686 **Tables**

687 **Table 1.** Sample characteristics for the 52 mother-infant pairs eligible for the analysis

Sample characteristics	Mean \pm SD	Range
Drinking water arsenic at recruitment ($\mu\text{g/L}$)	63.7 \pm 116.5	<1 - 510

Gestational age at recruitment (weeks)	12.2±2.5	6 - 16
Gestational age at delivery (weeks)	37.6±2.1	33 - 41
Birth weight (grams)	2923±372	2080 - 4050
Gender	N (%)	
Male	33 (63.5 %)	
Female	19 (36.5%)	
Number of samples available by tissue	n	CpG loci analyzed
HUVEC	52	347,650
Artery	45	374,320
Placenta	37	365,994

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