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DNA methylation modifies urine biomarker levels in 1,6-hexamethylene diisocyanate exposed workers: A pilot study

Leena A. Nylander-French[a,*], **Michael C. Wu**[b,d], **John E. French**[c], **Jayne C. Boyer**[a], **Lisa Smeester**[a], **Alison P. Sanders**[a,e], **Rebecca C. Fry**[a]

[a]CIH Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, CB #7431, Chapel Hill, NC 27599-7431, USA

[b]Department of Biostatistics, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

[c]TOXGEN/Toxicogenetics, Chapel Hill, NC, USA

[d]Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

[e]Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

DNA methylation may mediate inter-individual responses to chemical exposure and, thus, modify biomarker levels of exposure and effects. We analyzed inter-individual differences in inhalation and skin exposure to 1,6-hexamethylene diisocyanate (HDI) and urine biomarker 1,6-hexamethylene diamine (HDA) levels in 20 automotive spray-painters. Genome-wide 5-methyl cytosine (CpG) DNA methylation was assessed in each individual's peripheral blood mononuclear cells (PBMC) DNA using the Illumina 450K CpG array. Mediation analysis using linear regression models adjusted for age, ethnicity, and smoking was conducted to identify and assess the association between HDI exposure, CpG methylation, and urine HDA biomarker levels. We did not identify any CpGs common to HDI exposure and biomarker level suggesting that CpG methylation is a mediator that only partially explains the phenotype. Functional significance of genic- and intergenic-CpG methylation status was tested using protein-protein or protein-DNA interactions and gene-ontology enrichment to infer networks. Combined, the results suggest that methylation has the potential to affect HDI mass transport, permeation, and HDI metabolism. We demonstrate the potential use of PBMC methylation along with quantitative exposure and biomarker data to guide further investigation into the mediators of occupational exposure and biomarkers and its role in risk assessment.

[*]Corresponding author. Tel.: +1 919 966 3826; fax: +1 919 966 7911. leena_french@unc.edu (L.A. Nylander-French).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox-let.2014.10.024.

Transparency document

The Transparency document associated with this article can be found in the online version.

Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

DNA methylation; Exposure assessment; Genome-wide analysis; 1,6-Hexamethylene diisocyanate; 1,6-Hexamethylene diamine; Urine biomarker

1. Introduction

Inter-individual genetic differences (*e.g.*, *n*-acetyl transferase fast *vs.* slow acetylator and glutathione *S*-transferase activities) have been linked with adverse health effects and asthma risk in isocyanate-exposed workers (Piirila et al., 2000; Wikman et al., 2002). More recent studies suggest that the interactions between genetic, epigenetic, and environmental factors are critical to toxicity and disease risk (Christiani et al., 2008; Vineis et al., 2009; Vlaanderen et al., 2010). The source of inter-individual variation in toxicity or disease risk may be dependent upon single nucleotide polymorphisms (SNP) [1] (Hinds et al., 2006; McCarroll et al., 2008), copy number variants (CNV) (Conrad et al., 2010), and/or altered patterns of DNA methylation (Smeester et al., 2011) in the highly conserved coding region of genes or in their regulatory control sequences that may influence protein expression and post-transcriptional modifications (Boks et al., 2009; Gibbs et al., 2010). Specifically, individual genetic and epigenetic differences may lead to altered gene function critical to xenobiotic metabolism and toxicokinetics as well as heritable differences in physiological functions and maintenance of homeostasis (blood pressure, heart rate, respiratory rate, *etc.*) (Adeyemo et al., 2009; Baranzini et al., 2009; de Geus et al., 2005; Ehret, 2010; Glinskii et al., 2009). Thus, both genetic and epigenetic variants may directly or indirectly contribute to health effects associated with diisocyanate exposure (*e.g.*, asthma). Individual epigenetic alterations (*e.g.*, DNA methylation, histone modification, and/or non-coding RNA) may play an important role in the toxicokinetics of diisocyanates and explain a significant component of the variability that we have observed in biomarker levels in urine and blood in HDI exposed spray-painters (Flack et al., 2010, 2011; Gaines et al., 2010, 2011). However, only limited work has been carried out to understand the role of epigenetics within the context of exposure assessment. In addition, there is currently a lack of understanding of how epigenetic alterations may modify HDI biomarkers or be modified by exposure. Thus, the current exposure assessment models are limited in their approach to predict interactions between environment (extrinsic), individual epigenetic alterations (intrinsic), and the biological outcome (phenotype) in exposure and risk assessment.

Inter-individual differences in epigenetic profiles, that likely result in significant differences in systemic response to HDI exposure, are not considered as predictors of outcome in current exposure assessment models. In this pilot project, we have developed a novel and innovative research framework to identify epigenetic changes that are influenced by exposure to HDI and that are associated with levels of the urine biomarker, 1,6-hexamethylene diamine (HDA), in a worker population of automotive spray-painters (Fent et al., 2009a,b,b; Flack et al., 2010, 2011; Gaines et al., 2010, 2011). Biomarkers of exposure

[1]APF, assigned protection factor; Chr, chromosome; CNV, copy number variant; CpG, genome-wide 5-methyl cytosine; DMR, differentially methylated genic and intergenic regions; FDR, false discovery rate; HDI, 1,6-hexamethylene diisocyanate; LOD, limit of detection; PBMC, peripheral blood mononuclear cell; SNP, single nucleotide polymorphisms; STD, standard deviation.

are observable endpoints in a continuum of events leading from exposure to disease (Ward and Henderson, 1996). The goal of this research was to investigate the use of 5-methyl cytosine (CpG) DNA methylation levels in peripheral blood mononuclear cells (PBMCs) as epigenetic changes that modify spray-painters' HDI exposure-dose relationship and indicate disparities in toxicokinetics and urine biomarker levels. Thus, based on significant differences in CpG methylation for each model, we identified sets of genes with altered methylation and assessed potential interactions by using large data sets of the corresponding protein-protein or protein-DNA interactions to infer a potential biological response using gene-ontology enrichment analysis. We demonstrate the use of PBMC methylation along with quantitative exposure and biomarker data to guide further investigation in exposure and risk assessment.

2. Materials and methods

2.1 Study population and sample collection

For this pilot study, 20 automotive spray-painters were selected from our study population of 46 (Fent et al., 2009a,b,b; Flack et al., 2010, 2011; Gaines et al., 2010, 2011) based on stratified HDI exposure and acceptable quality of DNA. Workers' low/high exposure status was classified based on each worker's aggregate inhalation, skin, and urine levels and then rank ordering them. The subjects were all male, ranging in age from 21 to 51 years with an average age of 35 years, and six were smokers. Smokers were evenly distributed across the rank-ordered exposure status. Sixteen subjects identified themselves as Caucasians, one as African-American, one as Asian, and two as mixed ethnicity. Information on workers' age, weight, height, ethnicity, and medical history in regards to susceptibility to occupational asthma (*i.e.*, had allergies, asthma, or medical problems after painting) was collected using a questionnaire. No one reported diisocyanate-induced asthma. Information on the type of personal protective equipment typically worn during painting was also obtained. This study was approved by the Institutional Review Board in the Office of Human Research Ethics at the University of North Carolina at Chapel Hill and by the Washington State Institutional Review Board at the Washington State Department of Social and Health Services. Demographics and the measured exposure levels for the 20 spray-painters are provided in Table 1.

2.1.1 Breathing-zone air and skin samples—During the study, we quantified the worker's respiratory and skin exposure to HDI. The collection and analyses of the personal breathing-zone and skin tape-strip samples for quantification of HDI exposure during every spray-application have been published (Fent et al., 2009a,b,b). Briefly, on each sampling visit, breathing-zone air samples were collected during each HDI-containing painting task, and tape-strip samples were collected on the right and left volar and dorsal arm, the right and left dorsal hand, and right and left neck immediately following each task. The painter was observed during the paint tasks to note the duration of exposure and the type of respirator worn. The assigned protection factor (APF) designated by the Occupational Safety and Health Administration (OSHA, 2006) for the respirator worn by a worker (none, APF = 1; air purifying half-face, APF = 10; air-purifying full-face, APF = 50; supplied air full-face or hood, APF = 1000; powered air-purifying, full-face or hood, APF = 1000) was used to adjust

the measured breathing-zone concentrations in order to account for the respiratory protection in inhalation exposure levels used in the analyses. The median number of paint tasks performed by the spray-painters was 3 (range: 1–4) during the sampling day (Table 1). The measured HDI breathing-zone concentration adjusted for respirator type varied from below limit of detection (LOD) to 42.0 μg/m^3 with an average of 11.8 μg/m^3 (Table 1). HDI skin concentrations varied from ≤LOD to 6989.6 μg/mm^3 with an average of 723.9 μg/mm^3 (Table 1).

2.1.2 Urine samples—The collection of the urine samples and analysis of HDA and creatinine levels in urine have been published (Gaines et al., 2010). During each sampling visit, urine samples were obtained from the worker each time he urinated. On average, 2.7 (range 1–4) urine samples were obtained per worker during the sampling day (Table 1). The average HDA concentration and standard deviation measured in the urine samples was 0.18 ± 0.33 μg/g creatinine (Table 1).

2.1.3 Blood samples—One whole blood sample was collected in an EDTA tube from each worker at the end of the workday and peripheral blood mononuclear blood cells (PBMC) were isolated by Ficoll™ separation via centrifugation. DNA was purified from PBMC pellets using QiaAmp Blood mini kit (Qiagen, Germantown, MD) and stored in elution buffer at −20 ○C until analysis of epigenetic marks. DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and diluted with 10 mM Tris, pH 7.4 to 50 ng/μL. Prior to methylation analysis, the DNA concentration and quality (A 260/280 = 1.8–1.9) were confirmed using PicoGreen fluorescence quantification by Expression Analysis (Durham, NC).

2.1.4 Analysis of DNA methylation—DNA methylation in PBMC was analyzed using the Illumina HumanMethylation450 BeadChip arrays (Illumina, Inc., San Diego, CA) at Expression Analysis, Inc. (Durham, NC; www.expressionanalysis.com). These arrays contain probes for approximately 485,000 CpG sites. Target was prepared and hybridized according to manufacturer's specification (Illumina Part #15019522 Rev. A) (Illumina, 2012). Details of the protocol are described below.

2.1.4.1 Bisulfite conversion.: A bisulfite conversion reaction was employed using 500 ng of genomic DNA according to the manufacturer's protocol for the Zymo EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA). DNA was added to Zymo M-Dilution buffer and incubated for 15 min at 37 °C. CT-conversion reagent was then added and the mixture was denatured by heating to 95 °C for 30 s followed by incubation for 1 h at 50 °C. This denature/incubation cycle was repeated for a total of 16 h. After bisulfite conversion, the DNA was bound to a Zymo spin column and desulfonated on the column using desulfonation reagent per manufacturer's protocol. The bisulfite-converted DNA was eluted from the column in 10 μl of elution buffer.

2.1.4.2. Infinium methylation assay.: Bisulfite converted product (4 μl) was transferred to a new plate with an equal amount of 0.1 N NaOH and 20 μl of MA1 reagent (Illumina) then allowed to incubate at room temperature for 10 min. Immediately following incubation, 68 μl of MA2 reagent and 75 μl of MSM reagent (both Illumina) were added and the plate was

incubated at 37 ºC overnight for amplification. After amplification, the DNA was fragmented enzymatically, precipitated and resuspended in RA1 hybridization buffer.

2.1.4.3. Hybridization and scanning.: Fragmented DNA was dispensed onto the multichannel HumanMethylation450K BeadChips and hybridization performed in an Illumina Hybridization oven for 20 h. BeadChips were washed, primer extended, and stained per manufacturer protocols. BeadChips were coated and then imaged on an Illumina iScan Reader and images were processed with GenomeStudio software methylation module (v. 1.8 or later; Illumina).

2.2. Statistical analyses

2.2.1 Data preprocessing—GenomeStudio (Illumina) was used to calculate the raw methylation level at each CpG locus as well as the detection *p*-value. Raw methylation levels were expressed as beta-values [β = intensity of the methylated allele (M)/(100 + intensity of the unmethylated allele (U) + intensity of the methylated allele (M)] (Bibikova et al., 2011), which represent approximate percent methylation at each CpG locus. All subsequent statistical analyses were conducted within the R statistical computing environment (R Development Core Team, 2005). For each sample, individual probes with detection *p*-values > 10^{-5} were considered to have failed and were set to missing. The BMIQ normalization method (Teschendorff et al., 2013) was applied to reduce technical variability and to reduce variation that results from differences in probe design type (Marabita et al., 2013; Wu et al., 2014).

2.2.2 Association analysis—For our primary analysis, we assessed the association between urine biomarker levels and CpG methylation status while adjusting for potential confounders. We considered models with and without adjustment for individual covariates and the results were qualitatively similar (data not shown). Urine biomarker levels below the LOD were assigned nominal values determined by dividing the LOD value by the square root of two. Natural log-transformation was applied to improve normality, reduce influential points and stabilize the variance. Using individual CpG analysis wherein methylation at each CpG on the array was tested for association with the urine biomarker level, one-by-one. Specifically, for each CpG, we regressed the log-transformed urine HDA level on the normalized beta-value (as well as continuous age and indicators of non-white ethnicity and smoking status) using the model

$$P_{\mathrm{HDA}_i} = \beta_0 + \beta'_{\mathrm{cov}} C_{\mathrm{i}} + \beta_{\mathrm{M}} M_{\mathrm{i}} \quad (1)$$

where $P_{\mathrm{HDA}_{\mathrm{i}}}$ is the P_{HDA} biomarker level, C_i is the vector of additional covariates (age, ethnicity and smoking status), and M_i is the normalized beta-value for person i. We tested for the effect of methylation on the biomarker level by testing the null hypothesis H_0:βM = 0 using a 1-df likelihood ratio test. Statistical significance was determined by controlling the false discovery rate (FDR) at 0.05 (Benjamini and Hochberg, 1995).

2.2.3 Mediation analysis—Following identification of individual CpGs that modify urine biomarker levels independent of exposure, we conducted a mediation analysis to

determine whether the CpGs identified mediated the relationship between HDI exposure and urine HDA biomarker levels. Since all measured quantities were continuous, we directly used the standard causal-steps approach to mediation (Baron and Kenny, 1986; MacKinnon and Dwyer, 1993). Specifically, we identified CpGs that were associated with HDI exposure using the model

$$M_{\mathrm{i}} = \beta_0 + \beta'_{\mathrm{cov}} C_{\mathrm{i}} + \beta_{\mathrm{air}} \mathrm{Air}_i + \beta_{\mathrm{skin}} \mathrm{Skin}_i \quad (2)$$

where M_i is again the methylation status at a particular CpG for each individual i, and the C_i are the covariates (ethnicity, smoking, and age), as before. Here, Air_i and Skin_i are the natural log-transformed HDI exposure values for air and skin, respectively, for person i. Exposure levels below the LOD were assigned nominal values determined by dividing the LOD value by the square root of two before natural log-transformation for analysis. To compute a p-value for the association between CpG methylation levels and exposure, we tested the null hypothesis $H_0{:}\beta_{\mathrm{skin}} = \beta_{\mathrm{air}} = 0$ using a 2-df likelihood ratio test. Significance, defined as FDR < 0.05, was used to establish a relationship between the HDI inhalation and skin exposure and the mediator (CpG methylation). Because we have already established that HDI exposure alters urine HDI biomarker levels (Gaines et al., 2010, 2011), then any CpG that is associated with the biomarker [main analysis, Model (1)] and also associated with HDI exposure [Model (2)] is, at least, considered to be a partial mediator of the effect of HDI exposure on urine HDA biomarker levels.

Association of a CpG with both biomarker and with exposure implies that the particular CpG sequence is a mediator. A further step would be to establish complete mediation and determine whether the effect of exposure is fully mediated through methylation. This is potentially accomplished by cautiously examining whether the HDI exposure is no longer associated with HDA biomarker level after adjusting for the methylation levels in the model

$$P_{\mathrm{HDA}_i} = \beta_0 + \beta'_{\mathrm{cov}} C_{\mathrm{i}} + \beta_{\mathrm{M}} M_{\mathrm{i}} + \beta_{\mathrm{air}} \mathrm{Air}_i + \beta_{\mathrm{skin}} \mathrm{Skin}_i \quad (3)$$

and testing the null hypothesis $H_0{:}\beta_{\mathrm{skin}} = \beta_{\mathrm{air}} = 0$. If HDI exposure is no longer significant after controlling for methylation, then we have evidence of complete mediation. Importantly, this analysis is only meaningful if a particular CpG is already determined to be a partial mediator, *i.e.*, significant under Models (1) and (2). We further note that such an analysis should be conducted with extreme caution and that the sample size should be large, since the interpretation of a failure to detect significance does not necessarily guarantee that there is no association. Interpretation is highly dependent upon the statistical power. Although our analyses did not identify any full mediators, purely for illustration of our approach, we also analyzed the data using Model (3) as though we expected to identify full mediators common to both exposure and biomarker phenotype.

2.3 Bioinformatics and gene-ontology enrichment analyses

CpG loci for differentially methylated genic and intergenic regions (DMR) associated with urine HDA biomarker levels and/or HDI inhalation and skin exposures were identified by genome wide-association linear regression models as described above. The Illumina 450K array provides coverage of approximately 99% of known human genes by targeting CpG

probes across gene (5'UTR, promoter, first exon, gene body, and 3'UTR) and intergenic regions (CpG islands, shores, DNAase hypersensitive sites, miRNA promoters, *etc.*). Genes (including ncRNA) and intergenic sequence flanking gene lists based upon statistically significant (FDR < 0.05) CpG locus identifiers (chromosome, genome coordinate, and build) were compiled for each model and examined for potential network interactions by gene ontology enrichment analysis using GeneMANIA (http://gene-mania.org) online accessed databases (Montojo et al., 2014a,b,b; Mostafavi et al., 2008; Warde-Farley et al., 2010). GeneMANIA's algorithms and functional interface enables interrogation of large datasets of validated protein–protein and protein–DNA interactions and biological pathways to establish predicted networks of interactions and their biological processes or molecular function (GO terms) enrichment using the gene lists based on significant statistical associations of DMR associated genes. These computational tools are used to assess the relative biological plausibility of the models and predicted outcome to guide research for functional validation of the effects of an individual's HDI exposure and methylation on urine HDA biomarker levels. The same gene lists were used to search Ingenuity Pathway Analysis (http://www.ingenuity.com) and MetaCore™ (http://www.GeneGo.com) databases to compare and ascertain predicted networks/pathways and associated biological/molecular processes based on proprietary manually curated published literature (Brennan et al., 2009; Chang, 2009). These tools enable the computational prediction and association with networks and pathways that may be associated with environmental exposures and other risk factors (Benton et al., 2011; Liu et al., 2010; Rager et al., 2011a,b; Smeester et al., 2011). All CpG-marker associated genes significant in Models (1) and (2) were further investigated and their curated sequence identification for genomic and epigenomic features, location, and gene ontology were confirmed using NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/snp), Ensembl BioMart (http://www.biomart.org), and/or UCSC gene browser (http://genome.ucsc.edu).

3. Results

3.1 Methylation and urine HDA biomarker levels

Using Model (1), we identified CpG loci that were associated with average creatinine adjusted urine HDA levels and calculated a significant association between DNAmethylation status and biomarker levels with *LPHN3* (latrophilin 3) (*p*-value 1.06×10^{-8}; FDR 0.005) while *SCARA5* (scavenger receptor class A, member 5) was borderline significant (*p*-value 2.97×10^{-7}; FDR 0.072) (Table 2). Plots for the DNA methylation *vs.* creatinine adjusted urinary HDA levels (further adjusted for age, ethnicity, and smoking) for the two significant CpGs are presented in the Supplementary materials (Supplementary materials, Fig. 1). Average beta-values and standard deviations for *LPHN3* and *SCARA5* in the sampled population were 0.507 ± 0.051 and 0.642 ± 0.067, respectively. $LPHN_3$ belongs to a family of proteins that function in both cell adhesion and signal transduction (G-protein coupled receptor-signaling pathway) (Boucard et al., 2014; Promel et al., 2012). Together, both *LPHN3* and *SCARA5* were interconnected with more than 20 other genes with reported connections (36.5% co-expression; 6.3% co-localization, 2.2% genetic interaction, 12.4% pathway associated, 28.7% physical interaction, 8.4% predicted, and 4.2% shared protein domains) to 3 validated pathways (G-protein coupled receptor extracellular domain,

microinhibitory RNAs with TGTTTAC shared domain, and scavenger receptor) (Fig. 1). The percentages are based on the number of categorized interaction of the total number of observations, which demonstrates the relative weight of the evidence (Fig.1). $LPHN_3$ and UBC (ubiquitin C) are known binding partners, which suggests a role in protein ubiquitination. Ubiquitination has been associated with a number of biological functions including endocytosis and regulation of cell-signaling pathways. $SCARA_5$ pattern recognition functions may be critical to host defense by initiating immune responses and aiding endocytosis of epithelial cells (Jiang et al., 2006; Pluddemann et al., 2007). The $LPHN_3$ and $SCARA_5$ predicted network involving lysyloxidases (Lugassyetal., 2012;Rimaretal.,2014;Smith-MungoandKagan,1998) suggests the potential to influence urine biomarker levels through fibrosis (epithelial to mesenchymal transition) (Szauter et al., 2005, 2010) affecting HDI skin permeation in addition to oxido reductase activity affecting amine (—CH-NH_2) metabolism (Table 2 and Fig. 1). The predicted functions identified in Model (1) are plausible and independentof HDIexposure.

3.2 HDI inhalation and skin exposure and methylation

Thirty significant CpG loci lying within 28 linked genes ($p < 3.55 \times 10^{-6}$) had altered methylation associated with individual HDI inhalation and skin exposure at FDR < 0.05 [Model (2); Supplementary materials, Table 1]. Plots of the DNA methylation (adjusted for age, ethnicity, and smoking) *vs.* HDI levels measured in the breathing zone and the skin for the two most significant CpGs are presented in Supplementary materials, Fig. 2. Four of the 30 CpG loci were intergenic and may represent undefined regulatory sequences that were not further investigated. Of the 30 significant CpG loci, almost half (12CpGs) were highly methylated (beta-value > 0.8) and half exhibited a very low level of methylation (beta-value ≤ 0.12). Neither *LPHN3* nor *SCARA5* were associated with HDI exposure. Thus, the results suggest only partial mediation between HDI exposure and DNA methylation mediated effects on HDA urine biomarker levels. Absence of *LPHN3* and *SCARA5* may indicate absence of HDI induced methylation effects of these loci or possible false positive association. However, 27 out of the 30 HDI-exposure associated genes containing the significant CpG loci were predicted to interconnect with 20 additional genes (40 total; 84.1% co-expression, 0.9% genetic interaction, 1.7% consolidated pathways, 4.2% transcriptional-factor targets, and 9% shared protein domains) (Fig. 2). Consolidated pathways included ATPase related ion and lipid transport and shared transcript target sequences without defined function. *DSCAML1* (Down syndrome cell adhesion molecule like 1) showed the most significant gene association (p-value 1.33×10^{-11}; FDR 1.08×10^{-6}) (Table 3 and Supplementary materials, Table 1). $DSML_1$ has been reported to negatively regulate cell adhesion in neuro development (Agarwala et al., 2001), but not for skin and/or dermal fibroblasts. Other highly significant associations were observed with *ATOX1* (antioxidant 1 copper chaperone) that protects against superoxide and hydrogen peroxide radicals (Hamza et al., 2001), as well as *FARP1* [FERM, RhoGEF(ARHGEF), and pleckstrin domain protein 1] and *FAM210A* (family with sequence similarity 210, member A), which are predicted to be integral components of cell membranes. These and other critical molecules show conservation and enrichment for function for mass transport of substances involving increased turnover of membranes, lipids, phospholipids, and energy (Table 3, Fig. 2, and Supplementary materials, Table 1).

3.3 HDI inhalation and skin exposure, urine HDA levels, and methylation

Because no common single CpG or set of CpGs are common to Models (1) and (2), a test of Model (3) is not necessary to test for complete mediation. Here, we show Model (3) only for illustration of all steps in mediation analysis. For a CpG significantly associated with exposure and also associated with biomarker levels tested by linear regression in Model (3), no significance would indicate complete mediation by the methylation status of that CpG. Although no CpGs were identified as partial mediators in Models (1) and (2), we, nevertheless, assessed the association between HDI exposure and average creatinine normalized urine HDA levels to illustrate the final step in this approach. We observed 114 CpG loci significantly associated with the HDI exposure and urine HDA biomarker levels at a FDR < 0.001 (data not shown). If these CpGs had been significant and common to both under Models (1) and (2), then these results would indicate that they are only partial mediators of the exposure–methylation–biomarker continuum. However, Model (3) results confirm that no single or a set of CpGs function as a complete mediator and, therefore, CpG methylation is only a partial mediator of the relationship between HDI exposure and urine HDA biomarker levels.

4. Discussion

To effectively use biomarkers of exposure, we must quantitatively factor and understand the contribution of intrinsic (age, coincident disease, genetic and epigenetic variation, gender, smoking, weight, *etc.*) and extrinsic factors (environmental and occupational exposures, nutrition, psychosocial, environmental conditions, *etc.*). Genetic (DNA, SNP, and CNV) and epigenetic variation (differentially methylated regions, and chromatin modification) are interrelated primary factors that are poorly understood and used in exposure and risk assessment. Here, we show the results of a pilot study that demonstrates the potential for differential DNA methylation to modify and contribute to quantitative measures of exposure and biomarker of exposure.

We correlated spray-painters' HDI exposure to differential methylation and modulation of HDI biomarker of exposure using computational tools with large data sets of protein–protein and/or protein–DNA interactions based on statistically significant correlations and predicted functional interactions. We were able to define and identify differentially methylated genes (*LPHN3* and *SCARA5*) that are likely defined by HDI exposure that show potential for functional interactions affecting biological and molecular processes based on conserved gene ontology (Liu et al., 2014; Mostafavi and Morris, 2012). The predicted functional interactions are likely related to HDI permeation of the skin through mass transport based upon individual differences in cell adhesion, lipid and membrane turnover, and metabolism of amines, induction of host immune mechanisms and responses, and toxicity that may lead to fibrosis in the skin (National Toxicology Program, 2005).

The evidence for diisocyanate exposure and gene-phenotype association is limited to candidate-gene studies. Only *GSTP1* (Broberg et al., 2010), *NAT2* (Berode, 1991; Wikman et al., 2002), and *CTNNA3* (Kim et al., 2009) have been identified to be significantly associated with diisocyanate exposure, metabolism, and/or increased risk for asthma. Most, if not all, of these and other genes identified have SNPs, CNV, and CpG islands in regulatory

sites in coding and noncoding regions of the gene body that modulate gene and protein expression and influence outcome. In the context of the results presented here, the differential CpG methylation association for *SCARA5* (Table 2 and Fig. 1) predicts an oxidoreductase activity involving CH-NH_2 substrates that may interact with NAT_2. . Both *SCARA5* and *NAT2* areco-located with a number of other genes on chromosome 8q21–22 that show significant CNV (gain and loss) (Lappalainen et al., 2013) that might affect HDI metabolism. Allele specific expression (functional SNPs and CNV) and an increase ordecrease in CpG in regulatory sequences may illustrate the relationship between a genetic (*NAT* CNV) and epigenetic interaction (*SCARA5*).

The strength of the statistical associations provide both confidence in the predicted outcomes and the effect-sizes provide guidance for further hypothesis based bench research and replicate studies using proteomics and/or metabolomics with biomarkers of exposure. Strikingly, each of the gene sets for each of the models described and tested and selected on the basis of statistical probability and FDR were shown to be likely interacting partners with conserved molecular functions consistent with the current knowledge on HDI exposure and toxicity (immune response associated with occupational asthma). No differentially methylated genes identified in this approach were without potential functionally related partners, which reduces false discovery and provides additional confidence in the approach described.

Potential effects and outcomes are deduced and inferred by statistical correlations with DNA methylation affected by HDI exposures that are associated with HDI biomarker levels using an mediation approach (Baron and Kenny, 1986; MacKinnon and Dwyer, 1993). In this pilot study with a limited number of individuals, we used a chemical induced intermediate phenotype based upon individual differences in exposure, DNA methylation, and urine biomarker levels, and tested for differentially methylated CpG locus as a mediator between HDI exposure and urine biomarker levels. The outcome from the linear regression models appears to be dependent on induced exposure effects and presence of DNA sequences subject to differential methylation that produce qualitative changes. This approach is different from that of large genome-wide association studies based on case-control comparisons where control of genetic variation for rare and common SNP and CNV is difficult. In SNP-based genome-wide association studies for disease associations, a large number (hundreds to thousands) of individuals are required to increase power to be able to detect rare (≤1%) variants with large size effects versus common (≥1%) variants with smaller size effects (Manolio et al., 2009). Further power is derived from *in silico* analysis using predicted network and pathway analysis employing very large genome-wide data sets and powerful search algorithms. An issue of concern involves reliance on the use of a surrogate tissue to investigate a biological outcome in other tissues (skin, lung, *etc.*) and correlation with biological media (*e.g.*, plasma or urine). Here, we describe the outcome from HDI exposure based upon methylated CpG loci from PBMC DNA. HDI is a site-of-contact toxicant and likely directly affects both epithelial and tissue immune cells, which may be reflected in circulating PMBC DNA. A direct correlation between DNA methylation in target tissues versus surrogate tissues like PBMCs has not been adequately demonstrated (Heijmans and Mill, 2012). We show the potential to use PBMC as a surrogate tissue to assess biomarkers of exposure based on the data presented here and as has been shown for

the use of PBMC to discriminate methylation patterns in case-control studies for soft and solid cancers (Langevin et al., 2012; Terry et al., 2011; Teschendorff et al., 2013; Widschwendter et al., 2008; Wu et al., 2011). This process of discovery anchored by statistical association and strength of likely size-effects may reduce hypothesis-based research by guiding research toward the most likely interactions and outcomes that can be functionally validated through reverse genetics and molecular biology studies. In addition, these results may guide candidate-gene studies in epidemiology databases with requisite qualitative or quantitative data that will have to be replicated in independent studies, through meta-analysis, bench research, or relevant *in vitro* or *in vivo* targeted testing in cell lines or animal models, respectively.

Environmental contaminants have been shown to be related to epigenetic changes in non-occupational settings, and under a larger, non-pilot sample, it would be ideal to adjust for these exposures in order to better elucidate the specific mechanisms underlying associations. In particular, it is possible that the association between DNA methylation and HDI is driven by common association with an unmeasured exposure. We only measured workers' exposure to diisocyanates but adjusted the association analyses for potential confounders (*e.g.*, smoking). Although demethylation of the *AHHR* gene is strongly associated with tobacco smoking (Shenker et al., 2013; Zeilinger et al., 2013), we did not observe an association between HDI exposure and aryl hydrocarbon receptor repressor CpG methylation status. This was not unexpected because we have shown that urine HDA levels are not affected by smoking (Gaines et al., 2011). Because we did not detect individual mediators, the interpretation of the results presented here is not affected. Further, we emphasize that the inter-individual differences in HDI quantitative exposure levels correlated strongly with individual differences in specific CpG methylation status and the urine HDA biomarker of exposure. This indirect evidence (discovery) described in this report will ultimately have to be proven using hypothesis based research methods. Here, we can account for a limited number of variables and chemical exposures, but the statistical evidence for the role of HDI exposure on DNA methylation in a sentinel tissue (PBMC) and the urine HDA biomarker levels strongly supports the observation reported.

We have developed a novel and innovative research framework with statistical tools to investigate the potential role of epigenetics in the assessment of factors that can modify biomarker of exposure and may affect individual differences in response to HDI exposure in a small well-characterized worker population of automotive spray painters. Although we could not resolve and identify specific mediators, we show the relevance of these significant epigenetic alterations using pathway and network analysis along with gene ontology enrichment tools to understand the impact of individual differences on the observed biomarker level. This research is critical to identify individual differences in DNA CpG epigenetic alterations for HDI exposure and biomarker levels that we observed in our previous studies (Flack et al., 2011; Flack et al., 2010; Gaines et al., 2010, 2011) and may indicate genes and their products that influence disparities in toxicity and toxicokinetics of HDI. This may be related to inter-individual differences in activity of the enzyme(s) involved in the toxicokinetics of HDI *via* epigenetic modulation. Therefore, we may be able to account for individual differences in HDI toxicokinetics that affect systemic exposure levels that in turn may affect individual differences in exposure-dose relationships without

having to perform case-control studies. Case-control studies may be confounded by not being able to control for genetic and epigenetic variation between individuals. The approach described here requires only exposed individuals and linear regression or mixed models with repeat quantitative measures of exposure over time in order to investigate causally related mediators and functional genetic and epigenetic interactions. A larger population study will be required to corroborate these observations, further describe mediators between exposure and biomarkers of exposure, and further deconstruct the models to substantiate the predicted interactions.

5. Conclusion

We have shown in this pilot study that HDI exposure modifies differentially methylated regions (CpG loci) genome-wide and that changes in methylation status of specific CpG loci significantly influence the quantification of urine biomarker of exposure. Results demonstrate that epigenetic modifications are, at least, a partial mediator of the HDI exposure and biomarker of exposure relationship. Other variables, genetic, confounding individual exposures, or other unknown variables may also partially explain inter-individual variation between exposure and the biomarker of exposure. The investigation of the interactions between environment, individual epigenetic alterations, and the biological outcome in occupational and environmental exposure assessment studies can provide an effective approach to identify human epigenetic–environment interactions. This research strategy has the potential to reduce uncertainty and increase confidence in biomarkers of exposure and/or early biological effect classification and, thus, improve exposure classification in occupational epidemiology studies and significantly contribute to the development of our understanding of exposure dose-effect relationships.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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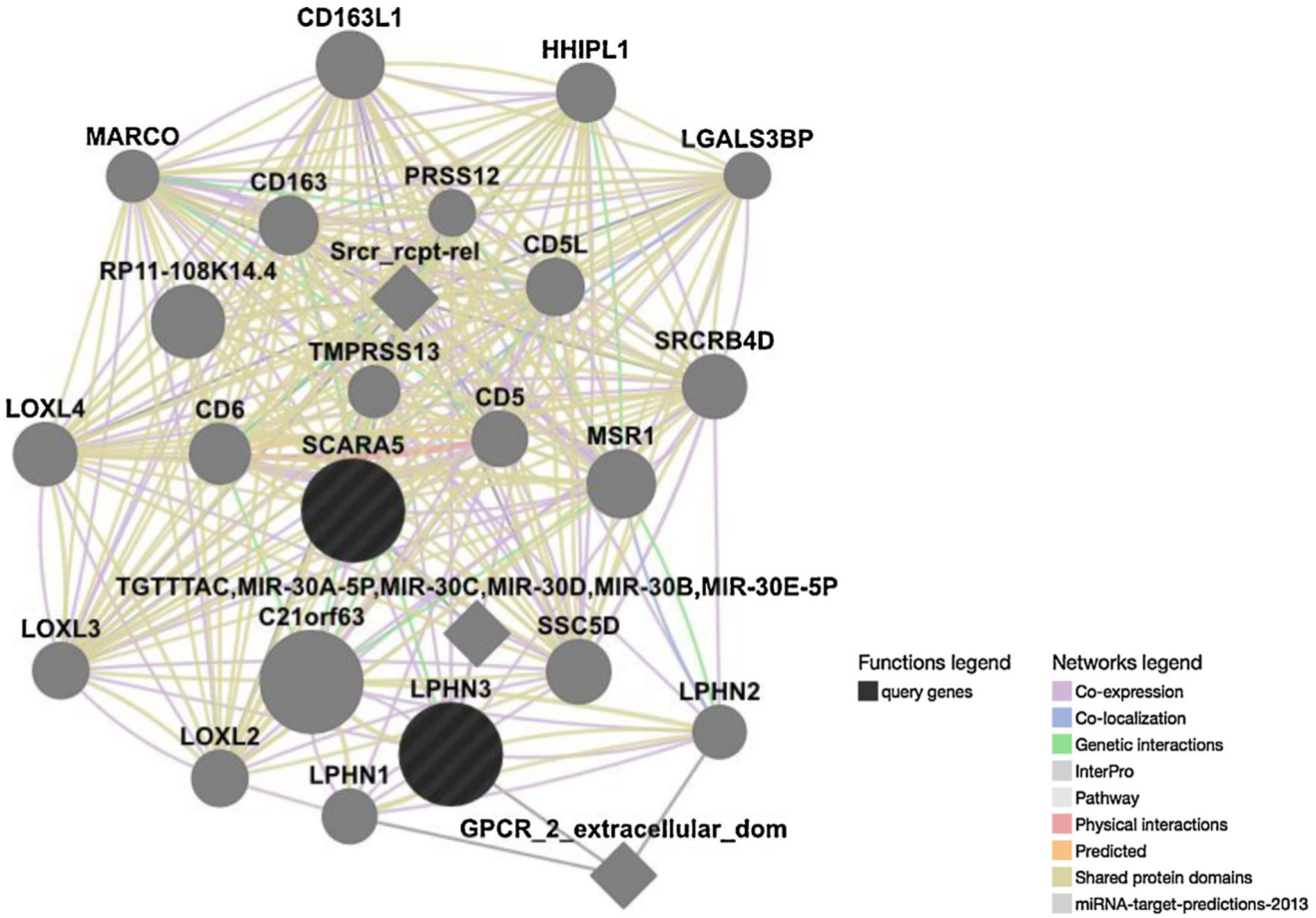
HIGHLIGHTS

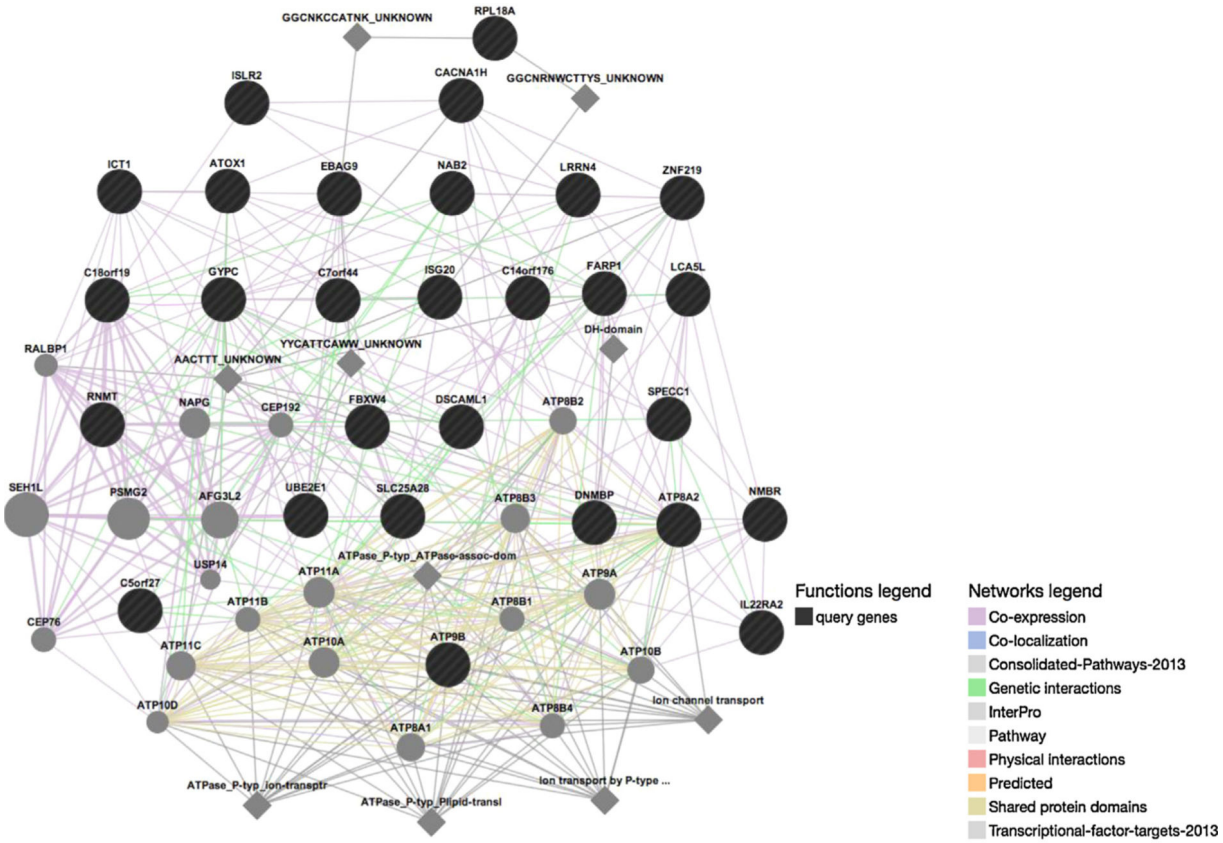
- HDI exposure modifies differentially methylated regions (CpG loci) genome-wide.
- Epigenetic modifications partially mediate the HDI exposure and biomarker relationship.
- HDI mass transport, permeation, and metabolism are associated with epigenetic modifications.
- The developed tools can be used to identify epigenetic marks affecting exposure and outcome.



Molecular functions	FDR	Coverage
Epithelial to mesenchymal transition	0.57	3 / 67
Oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor	0.57	2 / 10
Oxidoreductase activity, acting on the CH-NH2 group of donors	0.57	2 / 12

Fig. 1.

Enrichment of molecular functions derived from predicted network interactions based on CpG loci associated genes identified by Model (1).



Molecular functions	FDR	Coverage
Lipid translocation	2.99×10^{-23}	10 / 10
Regulation of membrane lipid distribution	5.93×10^{-20}	10 / 15
Phospholipid transport	1.90×10^{-16}	10 / 28
Organophosphate ester transport	9.60×10^{-15}	10 / 40
Lipid transport	3.92×10^{-9}	10 / 138
Phospholipid transporter activity	1.20×10^{-8}	6 / 19
Lipid localization	1.38×10^{-8}	10 / 161
ATPase activity, coupled to movement of substances	4.45×10^{-8}	7 / 47
Lipid transporter activity	3.16×10^{-6}	6 / 47
ATPase activity, coupled	1.28×10^{-4}	7 / 148
Ion transmembrane transport	2.17×10^{-4}	8 / 244
ATPase activity	7.36×10^{-4}	7 / 196

Fig. 2.

Enrichment of molecular functions derived from predicted network interactions based on CpG loci associated genes identified by Model (2).

Table 1

Summary of the study population characteristics ($n = 20$).

Variable	Mean ± STD	Median	Range
Age (years)	35.3 ± 8.9	35.5	21.0 – 51.0
Years in spray painting job	13.8 ± 11.0	13.5	1.5 – 34.0
Number of tasks during the sampling day	2.7 ± 1.1	3.0	1.0 – 4.0
HDI breathing-zone concentration adjusted for respirator type (μg/m^3)	11.8 ± 13.2	7.7	≤LOD – 42.0
HDI skin concentration (μg/mm^3)	723.9 ± 1874	14.7	≤LOD – 6989.6
Average urine HDA concentration normalized with creatinine concentration (μg/g)	0.18 ± 0.33	0.06	≤LOD – 1.35

LOD: limit of detection.

Table 2

Significant differentially methylated CpGs associated with average creatinine normalized urine HAD levels in 20 automotive spray-painters exposed to HDI [Model (1)].

Name	Coordinate	Beta[a] ± STD	Gene	Chr	CPG Islands	Relation	Enhancer	Regulatory	*p*-value	FDR
cg21814870	62065835	0.507 ± 0.051	*LPHN3*[b]	4	62383013–62383248	Gene body	No		1.06×10^{-8}	0.00513
cg24554944	27832807	0.642 ± 0.067	*SCARA5*[c]	8	27779081–27779767	Gene body	No		2.97×10^{-7}	0.07206

STD: standard deviation.

Chr: chromosome.

FDR: false discovery rate.

[a]Beta-value = the average percent methylation measured for the CpG and the standard deviation in the 20 spray-painters.

[b]*LPHN3* and a number of other genes are included in the same region on Chr 4 that are reported to be associated with cognitive disabilities.

[c]*SCARA5* and *NAT2* along with 200 other genes are included in a region on Chr 8 that shows frequent copy number variation but have not been linked to specific pathologies.

Table 3

Fifteen most significant differentially methylated CpGs associated with HDI inhalation and skin exposure in 20 automotive spray-painters [Model (2); see Supplementary materials, Table 1 for complete list].

Name	Coordinate	Beta[a] ± STD	Gene	Chr	CPG islands	Relation	Enhancer	Regulatory	p-value	FDR
cg01877524	117156569	0.983 ± 0.009	DSCAML1	11	117651216–117651429	Island	No		1.33×10^{-11}	1.08×10^{-6}
cg18321631	151118586	0.052 ± 0.009	ATOX1	5	151137992–151138418	Island	No	Promoter	1.31×10^{-8}	0.00091
cg10115148	2862768	0.648 ±0.050		1	2876545–2876808	N_Shelf	No		1.26×10^{-7}	0.00762
cg27369325	97760076	0.961 ± 0.009	FARP1	13			Yes		2.31×10^{-7}	0.01204
cg12813323	13716491	0.011 ±0.005	FAM210A; RNMT	18	13725979–13727370	Island	No	Promoter	2.48×10^{-7}	0.01204
cg16411204	101369803	0.064 ±0.008	SLC25A28	10	101379708–101381445	Island	No		3.85×10^{-7}	0.01550
cg27484662	75227180	0.973 ±0.015	ATP9B	18	77125676–77125885	S_Shore	Yes		4.25×10^{-7}	0.01550
cg23576507	20641684	0.046 ± 0.010	ZNF219; TMEM253	14	21571835–21572520	Island	No	Promoter	4.27×10^{-7}	0.01550
cg17345081	142451632	0.109 ±0.025	NMBR	6	142409473–142410112	Island	No		4.82×10^{-7}	0.01550
cg19951424	86983212	0.073 ± 0.008	ISG20	15			No	Promoter	4.95×10^{-7}	0.01550
cg03895392	72213387	0.124 ±0.014	ISLR2	15	74425091–74428821	Island	No		5.11×10^{-7}	0.01550
cg09949666	43645491	0.882 ± 0.031	COA1	7			No		5.61×10^{-7}	0.01603
cg25090499	24941165	0.097 ± 0.012	ATP8A2	13	26042693–26043486	Island	No		7.12×10^{-7}	0.01877
cg14931122	19999698	0.070 ±0.010	CYTSB	17	20059028–20060060	Island	No	Unclassified	7.40×10^{-7}	0.01877
cg15966253	23822484	0.039 ±0.007	UBE2E1	3	23847871–23848633	N_Shore	No	Promoter	7.73×10^{-7}	0.01877

STD: standard deviation.

Chr: chromosome.

FDR: false discovery rate.

[a]Beta-value = the average percent methylation measured for the CpG and the standard deviation in the 20 spray-painters.