

# A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs

Robert A Philibert<sup>1,2,\*</sup>, Brandan Penaluna<sup>2</sup>, Teresa White<sup>2</sup>, Sarah Shires<sup>2</sup>, Tracy Gunter<sup>3</sup>, Jill Liesveld<sup>2</sup>, Cheryl Erwin<sup>4</sup>, Nancy Hollenbeck<sup>2</sup>, and Terry Osborn<sup>1</sup>

<sup>1</sup>Behavioral Diagnostics; Iowa City, IA USA; <sup>2</sup>Department of Psychiatry; University of Iowa; Iowa City, IA USA; <sup>3</sup>Department of Psychiatry; Indiana University School of Medicine; Indianapolis, IN USA; <sup>4</sup>Departments of Medical Education and Psychiatry; Texas Tech University Health Sciences Center; Lubbock, TX USA

**Keywords:** Alcohol, DNA Methylation, Smoking, Abstinence

Alcoholism has a profound impact on millions of people throughout the world. However, the ability to determine if a patient needs treatment is hindered by reliance on self-reporting and the clinician's capability to monitor the patient's response to treatment is challenged by the lack of reliable biomarkers. Using a genome-wide approach, we have previously shown that chronic alcohol use is associated with methylation changes in DNA from human cell lines. In this pilot study, we now examine DNA methylation in peripheral mononuclear cell DNA gathered from subjects as they enter and leave short-term alcohol treatment. When compared with abstinent controls, subjects with heavy alcohol use show widespread changes in DNA methylation that have a tendency to reverse with abstinence. Pathway analysis demonstrates that these changes map to gene networks involved in apoptosis. There is no significant overlap of the alcohol signature with the methylation signature previously derived for smoking. We conclude that DNA methylation may have future clinical utility in assessing acute alcohol use status and monitoring treatment response.

## Introduction

Excessive alcohol consumption is the fourth leading preventable cause of death in the United States, causing 88,000 deaths and costing 223 billion dollars annually.<sup>1,2</sup> Consumption is widespread, with over 90% of Americans drinking alcoholic beverages at some point during their life. In small to moderate doses, the use of alcohol is not generally considered harmful. However, heavier consumption (i.e., > 2 drinks per day), particularly when chronic, can have severe medical and socioeconomic consequences. To minimize those consequences, state and federal governments have implemented policies designed to promote the assessment and treatment of substance use disorders,<sup>3</sup> while the treatment community has developed behavioral and pharmacological interventions.<sup>4-6</sup> To date, these efforts have only been modestly effective.

Among the greatest barriers to improving alcohol prevention and treatment efforts are: 1) our inability to reliably detect chronic heavy alcohol consumption and; 2) the relative lack of reliable biomarkers to accurately monitor abstinence in those undergoing treatment. The former is problematic because many of the most severely affected persons present only after a sentinel

event, such as an arrest, job loss, or divorce. Because intact personal and financial resources are essential to the recovery process, individuals presenting for alcohol treatment after a sentinel event are less able to recover healthy behavioral habits.<sup>7</sup> A sensitive, easily implementable biomarker could aid in the prevention of these psychosocial tragedies and prevent alcohol induced medical comorbidities. The lack of biomarkers to monitor treatment success is equally challenging because relapse is common yet often unrecognized.<sup>8</sup> The availability of a more effective test for detecting alcohol use in those undergoing treatment for alcohol dependence could go a long way in helping to avert the often tragic results of relapse.

In the absence of observational data or positive self-report, the most widely used methods through which chronic alcohol consumption can be inferred consist of algorithms that incorporate levels of liver proteins (e.g., alanine aminotransferase,  $\gamma$ -glutamyl transferase, aspartate aminotransferase or carbohydrate-deficient transferrin) or metabolites such as ethyl glucuronide (EtG).<sup>8,9</sup> Serious deficiencies in the sensitivity and specificity of these assays, as well as their high cost, have limited their clinical impact. Hence, there continues to be a substantial need for the further identification of potential biomarkers

\*Correspondence to: Robert A Philibert; Email: robert-philibert@uiowa.edu, behavioral.diagnostics@gmail.com.  
Submitted: 05/17/2014; Revised: 07/30/2014; Accepted: 08/04/2014; Published Online: 08/11/2014  
<http://dx.doi.org/10.4161/epi.32252>

**Table 1.** Clinical and demographic characteristics of study subjects

	Case	Control
n =	33	33
Age	45.5 ± 7.8	46.7 ± 7.8
Ethnicity		
White	31	31
African American	2	1
Hispanic	0	1
Gender		
Male	25	25
Female	8	8
Days since last drink	4.0 ± 1.8	
Average daily drink consumption prior to admission by self report		
Past Week	13 ± 13	0
Past Month	13 ± 11	0
Smoking Status		
Current Daily	27	1
Positive Cotinine	28	1
Cannabis Use Status		
Use in past Year by self report?	11	0
Positive Hydroxy-THC	9	1

whose predictive value or clinical implementation characteristics could aid current efforts to diagnose and treat alcohol abuse and dependence.

The use of DNA methylation to quantify the consumption of other certain other substances has already been demonstrated and it is possible that a similar approach may fill this critical clinical need for new biomarkers of alcohol consumption. For example, three years ago, using data from human lymphoblast cell line and pulmonary macrophage DNA, our group demonstrated that methylation status at the aryl hydrocarbon receptor repressor (AHRR) was a sensitive index of smoking.<sup>10</sup> Since our initial work, at least seven other studies have confirmed those findings and a quantitative PCR assay that assesses methylation status at that locus has entered Phase I testing.<sup>11-17</sup> Critically, analysis of the initial data used to demonstrate the effects of smoking also showed that alcohol consumption patterns over the six months prior to cell line immortalization were associated with broad, low-level changes in genome-wide methylation patterns.<sup>18</sup>

Unfortunately, there were important limitations of that prior analysis of alcohol associated methylation that included the use of a non-primary source of DNA. In an attempt to address those shortcomings and better understand the effects of high levels of alcohol consumption, we now examine the genome-wide methylation signatures of peripheral mononuclear cell (a.k.a. lymphocyte) DNA from subjects with heavy alcohol consumption as they enter and exit a 30 day inpatient treatment program.

## Results

The clinical and demographic characteristics of case and control subjects are described in **Table 1**. Overall, the subjects were well matched with respect to age, ethnicity, and sex. In keeping with the population of Iowa and the overall characteristics of patients referred for alcohol treatment, the subjects were mostly in their 40s and were mostly male and Caucasian. Case subjects were heavy alcohol users with an average consumption of 13 drinks per day in the week prior to admission. The delay between the time of the first phlebotomy (Time 1 or T1) and their last drink was a mean of four days but varied from one to eight days. Not surprisingly, 85% of the case subjects were daily smokers. These subjects also tended to have a higher rate of THC use; 10 subjects had a marginal or markedly positive hydroxy-THC levels. Other substance use reported in the six months prior to admission by the subjects including opiates, cocaine and amphetamines, was virtually absent.

The self-reports of the case subjects were checked by comparing them with objective measures. In each case, the results of the serum cotinine and THC ELISA tests were consistent with self-report data.

Only 26 of the 33 case subjects who participated in the T1 portion of the study completed the Time 2 (T2) assessment. The T2 DNA from one of those subjects was of insufficient quality to allow genome wide analysis leaving only 25 samples with both T1 and T2 data.

In contrast, the 33 control subjects had lower rates of both tobacco and cannabis use. In fact, only one control subject was a daily smoker; all control subjects denied cannabis use in the past year. In contrast to the results from the case subjects, one discrepancy between self-report and serum ELISA testing was observed (positive test for THC).

Genome-wide methylation data were obtained (measurements for > 99.5% of all probes) for all samples including two lymphoblast DNA standards and one internal replicate. This included 33 case subjects and 33 controls at T1 and 25 case subjects at T2. The correlation between the independently prepared replicate samples was greater than 0.998. The average  $\beta$  values, which is an estimate of the fractional methylation (between 0 and 1), for the controls, case subjects at T1 and case subjects at T2, were 0.4788, 0.4800 and 0.4833, respectively.

**Table 2** lists the 30 most significant results from the comparison of methylation status of the DNA from the 33 T1 subjects with that from the 33 healthy controls. After genome-wide correction using the FDR (false discovery rate) method, a total of 8636 were differentially methylated while when using more conservative Bonferroni correction, 56 comparisons were statistically significant (see **Table S1** for a complete list of all 8636 probes, average methylation levels with accompany gene annotation files). Examination of the QQ plot for the comparison reveals the basis for these observed differences in significant with positive skewing (greater numbers of more significant p values) being markedly prominent (**Figure S1**).

A frequent concern about studies of biomarkers of alcohol use in high-risk cohorts is the effect of the use of other substances.

**Table 2.** The thirty most significantly associated probes in case and control analyses

Average Beta Values							
Probe ID	Gene	Placement	Island Status	Case	Control	T-test	Corrected P value
cg23193759	C10orf35	TSS200	Island	0.128	0.168	4.66E-12	2.26E-06
cg02583484	HNRNPA1	Body	S_Shelf	0.250	0.319	1.42E-11	3.46E-06
cg23779890	GDAP1	TSS200	Island	0.194	0.243	1.30E-10	2.10E-05
cg13415831	SEMA4D*			0.073	0.098	3.18E-10	3.87E-05
cg09935388	GFI1	Body	Island	0.647	0.799	1.35E-09	0.0001
cg01432120	LOC010927881*	Island		0.660	0.720	1.86E-09	0.0002
cg12655542	SCD1*			0.225	0.282	2.18E-09	0.0002
cg11832281	CUGBP2	Body	S Shelf	0.070	0.097	4.20E-09	0.0003
cg06126421	CYP21A2*			0.643	0.750	5.34E-09	0.0003
cg12895631	C11orf75	5'UTR		0.161	0.192	7.07E-09	0.0003
cg25998745	PTK2*			0.588	0.666	8.43E-09	0.0003
cg08352774	TMEM181	Body	S Shelf	0.110	0.149	8.46E-09	0.0003
cg19939077	PPIF	Body	S Shore	0.147	0.184	8.85E-09	0.0003
cg13126206	TNFRSF10B*		S Shelf	0.468	0.521	1.11E-08	0.0004
cg22888484	SNHG11	TSS200	N Shore	0.043	0.056	1.21E-08	0.0004
cg00159243	SELPLG	5'UTR		0.397	0.474	1.76E-08	0.0005
cg24046474	RPL12	Body	N Shore	0.221	0.296	1.89E-08	0.0005
cg00957665	TRIM8	Body	S Shore	0.097	0.125	1.93E-08	0.0005
cg06093152	NENF*			0.572	0.654	2.03E-08	0.0005
cg12126344	TNFRSF8*			0.795	0.835	2.13E-08	0.0005
cg17485265	FAM50B	TSS1500	N Shore	0.677	0.744	2.29E-08	0.0005
cg16854826	ZMIZ1	5'UTR		0.603	0.654	2.42E-08	0.0005
cg23028436	STK38L	TSS200	Island	0.064	0.094	2.89E-08	0.0006
cg00690082	STAT5A	TSS1500	N Shore	0.309	0.376	3.00E-08	0.0006
cg06285727	ATG16L2	TSS1500	N Shore	0.152	0.216	3.68E-08	0.0007
cg09267773	LOC102467146*		N Shore	0.517	0.433	4.00E-08	0.0007
cg21475150	RPL31	TSS1500	Island	0.794	0.849	4.30E-08	0.0007
cg21416692	PHC2	5'UTR		0.742	0.792	4.41E-08	0.0007
cg02348119	TBC1D16	5'UTR		0.598	0.647	4.48E-08	0.0007
cg10691866	TPST1	Body		0.419	0.491	4.55E-08	0.0007

All average methylation values are non-log transformed  $\beta$ -values. Island status refers to the position of the probe relative to the island. Classes include: 1) Island, 2) N (north) shore, 3) S (south) shore, 4) N (north) shelf, 5) S (south) shelf and 6) blank denoting that the probe does not map to an island. \* denotes that the nearest gene was obtained via use of the UCSC Genome Browser. TSS200 and TSS1500 denote distance (either 200 or 1500 bp) upstream from the transcription start site (TSS).

This is of special concern with the current results because smoking also has profound effects on DNA methylation, and 27 of the 33 cases were active smokers. In this regard, cg05575921 was the 31st ranked probe. However, there was no significant overlap between the signal for alcohol consumption and the signal for smoking. Only 22 of the 910 probes (19 would be expected by random chance) that attained genome wide significance with respect to smoking in Dogan et al.<sup>13</sup> were ranked among the top 10000 most highly associated probes in the current study. Conversely, the overall rank of the 10000 most highly associated

probes for smoking in the Dogan et al. in the current data set was 302264th (of 485577) with the median of the distribution being 318258th. Hence, there appears to be scant overlap in the signature of alcohol and tobacco smoke exposure.

Next, we analyzed the differential distribution of the 1000 most significantly associated probes using the GoMiner™ algorithm. As shown in Table 3, there was a marked enrichment of the most highly associated probes for pathways involved in programmed cell death (apoptosis) or GTPase signaling.

**Table 3.** The top 30 most differentially regulated gene ontology pathways

Genes Log10					
GO Category	Category Name	Total Changed	P-Value	FDR	
GO:0005515	protein binding	6815	286	-7.37	0
GO:0005737	cytoplasm	7845	312	-5.75	0
GO:0008219	cell death	1392	78	-5.69	0
GO:0016265	death	1395	78	-5.66	0
GO:0005829	cytosol	1884	98	-5.63	0
GO:0012501	programmed cell death	1278	72	-5.36	0
GO:0007264	small GTPase mediated signal transduction	566	40	-5.33	0
GO:0043067	regulation of programmed cell death	981	59	-5.3	0
GO:0010941	regulation of cell death	989	59	-5.2	0
GO:0006915	apoptosis	1271	71	-5.16	0
GO:0042981	regulation of apoptosis	974	57	-4.79	0
GO:0023033	signaling pathway	2812	130	-4.73	0
GO:0019899	enzyme binding	671	43	-4.63	0
GO:0048523	negative regulation of cellular process	2069	101	-4.61	0
GO:0002376	immune system process	1256	68	-4.52	0
GO:0023034	intracellular signaling pathway	1707	86	-4.44	0.01
GO:0023052	signaling	3787	164	-4.36	0.01
GO:0065007	biological regulation	7226	283	-4.33	0.01
GO:0048519	negative regulation of biological process	2235	106	-4.31	0.01
GO:0035556	intracellular signal transduction	1454	75	-4.24	0.01
GO:0005622	intracellular	11231	411	-4.22	0.01
GO:0060548	negative regulation of cell death	441	31	-4.21	0.01
GO:0044464	cell part	14663	511	-4.14	0.02
GO:0005623	cell	14664	511	-4.13	0.01
GO:0035466	regulation of signaling pathway	1158	62	-4.02	0.02
GO:0009987	cellular process	11702	424	-3.98	0.02
GO:0043069	negative regulation of cell death	434	30	-3.96	0.02
GO:0007265	Ras protein signal transduction	335	25	-3.91	0.02
GO:0051056	regulation of GTPase signal transduction	339	25	-3.83	0.03
GO:0050794	regulation of cellular process	6319	249	-3.81	0.03

FDR, false discovery rate.

Whereas the primary goal of many biomarker studies is to determine whether a given marker can be used to differentiate disease states from others, a secondary goal of our study was to determine if DNA methylation could be used to monitor alcohol abstinence. As a first step, we compared the genome-wide DNA methylation patterns of 25 subjects for whom we successfully obtained T1 and T2 data. The average length of time between the T1 and T2 DNA sampling for the 25 individuals was 25 d. No single probe crossed the threshold of genome-wide significance with the best observed uncorrected  $P$  value being only  $5 \times 10^{-6}$  with QQ plots of the analysis showing prominent negative skewing (Fig. S2).

Secondary analysis of the T1 and T2 data proved highly interesting. Since exposure to ethanol is stressful to cells and biological systems tend to revert to their homeostatic means after perturbation, we next investigated which methylation assessment (T1 or T2), of the alcohol using subjects was more similar to that of the controls for the 8636 FDR significant probes identified in the case and control analysis. The average methylation at T2 time point was more similar to that of the controls at 7360 of 8636 probes (Chi Square  $P < 0.0001$ ) including 49 of the 56 Bonferroni significant probes (Table S2). Unfortunately, the average reversion at these 7360 CpG residues to the mean of the controls was rather modest with the overall change in the  $\beta$  value being approximately 0.005 (i.e., 0.5%).

## Discussion

In this pilot study, we show that recent, heavy alcohol use is associated with significant changes in DNA methylation as compared with controls, and that the degree of these methylation changes tends to diminish after approximately one month of abstinence. Strengths of the study include a unique subject sample, rigorousness of the substance use classification, and the internal consistency of the findings. Limitations of the study include the small sample size that precludes more complex analyses, the exclusion of subjects with medical comorbidities or other forms of substance use disorders, and lack of sequencing confirmation of the most highly associated probes.

In some respects, the current study is an extension of prior single, multi locus and genome-wide studies of the effect of alcohol on DNA methylation.<sup>10,19-21</sup> Most relevantly, using lymphoblast DNA from subjects participating in the Iowa Adoption Studies (IAS), we have previously shown that alcohol use results in widespread changes in DNA methylation.<sup>10</sup> Yet, there were significant limitations in that earlier study and there are clear differences between the designs of the former and current study. First, a major limitation of the earlier study is that it used lymphoblast DNA whose differential methylation signal can be reliable but is influenced by the effects of cell transformation and length of time in culture.<sup>22</sup> Second, in contrast to the current study, many of the subjects in the IAS study were chronically ill and on medications that could potentially interfere with DNA methylation.<sup>18</sup> Third, all the subjects in that study were female while the majority of the subjects in the current study are male. Finally, “heavy users” in the prior study were defined as those who consumed at least one drink of alcohol in each of the prior 26 wk to phlebotomy. The magnitude of alcohol consumption by subjects in the present study is much greater.

The current results cannot be directly compared with those of Zhang and colleagues who used a more focused 384 probe array because they used whole blood DNA and subjects who were not necessarily actively drinking but had previously met diagnostic criteria for alcohol dependence.<sup>21</sup> Because prolonged abstinence after long periods of alcohol use may allow for remodeling of the epigenome combined with the fact that multiple comparisons are typically made in most papers, direct comparison of the prior studies to the current study of severe active, but otherwise healthy, alcoholics may not be valid. As a result, we invite others to directly examine the results given in the supplementary tables and draw their own conclusions. A quick assessment of our prior results demonstrates an enrichment between the genes identified in our prior work with those in the current work while a more limited examination of the more specific work of Zhang and colleagues does not show any of their 8 most significant probes being within our 10 000 most highly ranked probes.<sup>18,21,23,24</sup>

The major finding of the study is the significant and widespread change in DNA methylation associated with acute sustained consumption of high amounts of alcohol. If the results can be replicated, this suggests that the DNA methylation signature can be used to infer recent alcohol use status. Before these findings can be translated into a set of useful clinical tools, there are

several important considerations that should be noted. First, the clinical translation value is dependent on the assumption that other medical conditions cannot mimic the signature associated with alcohol. Indeed, many speculated that some of the adverse consequences of alcoholism are not mediated by direct effects of alcohol but rather the deprivation of vital nutrients.<sup>25</sup> If this is the case, it is reasonable to suspect that diseases associated with impaired absorption may mimic certain aspects of the observed methylation signature. Second, in general, the magnitude of the changes observed in the current study is not high. In contrast to that of smoking where the differences of methylation at cg05575921 between chronic smokers (~60%) and non-smokers (92%) can exceed 30%, the average differences at the more highly significant probes in the current study tend to be in the 5 to 10% range. Because the levels of alcohol consumption by the subjects in the current study are relatively extreme, it is likely that the changes observed in less intensive settings would be markedly less. This poses a challenge to current DNA methylation analyses because the standard deviation of most DNA methylation assessment measures, such as pyrosequencing, is in that range.<sup>26</sup> It is conceivable that a panel of methylation markers could detect more modest consumption levels.

The greatest positive impact of these findings may be the demonstration that with abstinence, the differential methylation signature between cases and controls tends to diminish over time. This could have strong impact on the choice of settings in which alcohol treatment is conducted and monitored. Before this possibility can become a reality, the relationship of other illnesses, gender and age to variation at the loci identified in the study must be explored to determine if the effects observed in the study are specific to alcohol.

Given prior findings by others,<sup>27,28</sup> it is not surprising to find that the GoMiner™ analyses highlight the differential mapping of the differentially methylated probes to pathways involved in apoptosis. Alcohol is a toxin whose mean maximum concentration in a series of uncomplicated alcohol poisoning deaths in humans was approximately 460 mg/dl.<sup>29</sup> In the days prior to admission, the blood level of alcohol in many of the individuals in the study either approached or exceeded that level. In many ways, the insights provided by the pathway analyses effectively summarize some of the cellular responses as evidenced by the methylation signatures. The lack of other commonality in the genes affected in the current study suggests a more global mechanism behind the observed effects. A first global mechanism may be oxidation; alcohol induces the general reactive oxidative modification of proteins.<sup>30</sup> A second global mechanism may be denaturation. Alcohol is also a less polar solvent than water and though freely miscible with water, preferentially segregates to lipid membranes where it increases oligomerization of membrane proteins.<sup>31</sup> This is a general solvent effect and at higher concentration, ethanol precipitates virtually all cellular proteins. The skewing of the Q-Q plots (Figs. S1 and S2) is supportive of a generalized effect and we have observed similar effects on our unpublished studies of the effects of 0.08% alcohol on lymphoblast methylation.



The relative lack of overlap between the signature associated with alcohol observed in this study with the signatures observed in prior genome-wide studies of smoking is consistent with our prior analyses and our understanding of the cellular role of AHRR.<sup>32,33</sup> The well replicated effects of smoking on AHRR methylation are secondary to the key role of AHRR in moderating the activity of the xenobiotic or aryl hydrocarbon receptor (AHR) pathway. Tobacco smoke is an extraordinarily large source of dioxins and polyaromatic hydrocarbons. Catabolically competent cells in the liver and the blood degrade these toxins via this pathway which includes the key cytochrome enzymes CYP1A1 and CYP1A2.<sup>32</sup> Because unchecked activity of the AHR pathway also is extremely deleterious, activation of this pathway is also accompanied by increased AHRR expression, which is associated with both demethylation and transcription factor binding to the cg05575921 locus.<sup>14</sup> The increased transcription and subsequent translation of AHRR then moderates xenobiotic pathway activity through direct and indirect competition with AHR.<sup>34</sup> In addition, chronic heavy smoking (> 10 pack years) has strong impact on immune system activation that is probably mediated by the intense inflammatory processes in the lung associated with chronic (> 10 pack years) of smoking.<sup>35</sup> Because our subjects were relatively healthy, alcohol is not metabolized by the xenobiotic pathway, and there was a significant admixture of smokers and non-smokers in our group of 33 heavy drinkers, a strong relationship between alcohol use status with the loci previously associated with smoking was not observed. When we re-ran the analyses excluding the non-smoking alcohol cases ( $n = 5$ ), many of the loci associated with smoking became much more significantly associated.

The extent to which these findings will extend to all individuals entering alcohol detoxification and treatment is not clear. Substance use comorbidity is more the rule than the exception in substance use treatment. In fact, because inclusion and exclusion criteria for the current study mandated good overall health and excluded those with significant substance use comorbidities (except for tobacco), the vast majority of subjects admitted to the two treatment centers participating in the study were ineligible for inclusion in the study. Therefore, future replication attempts should include a broader range of subjects.

If replicated, it is possible that DNA methylation could be used as a clinical biomarker for heavy alcohol consumption. At the current time, there are several liver proteins (alanine aminotransferase (ALT),  $\gamma$ -glutamyl transferase (GGT), aspartate aminotransferase (AST) or carbohydrate-deficient transferrin (CDT)) and one ethanol metabolite, ethyl glucuronide (EtG), that can be used to detect heavy alcohol use.<sup>8</sup> Unfortunately, each of these tests have limitations in either sensitivity, specificity or ease of implementation, that has constrained their value in alcohol assessment.<sup>8</sup> If the current findings can be transformed into an easy to use assessment (e.g., commercial qPCR battery), it is conceivable that DNA methylation assessments of alcohol consumption, alone or together with these other measures, could find their way into routine clinical use.

In conclusion, in a pilot study of relatively healthy subjects entering and exiting 30 day inpatient alcohol treatment, we show

that high levels of recent alcohol consumption are associated with broad genome-wide changes in DNA methylation and that these changes tend to partially revert after several weeks of abstinence. We believe that large-scale, longer-term studies are needed to determine the utility of DNA methylation for the assessment and monitoring of persons with alcohol use disorders.

## Subjects and Methods

The protocols and procedures used in this study were approved by the University of Iowa Institutional Review Board. Subjects were recruited from either the University of Iowa Hospitals and Clinics or two local alcohol treatment centers. Following admission to a program potential subjects were informed by facility personnel about the study. The research team was notified when a person expressed interest. Subjects were then contacted by a researcher and screened to ensure the person met study criteria. Subjects were required to have the capacity to consent and could have no additional significant active substance abuse (except tobacco), They could not be taking medications hypothesized to affect DNA methylation (e.g., valproic acid). They could not have significant medical problems such as cancer, gastrointestinal disorders, diabetes, chronic obstructive pulmonary disease or severe cardiac disease. Those who met the study criteria and were willing to participate gave written informed consent.

At the index intake (Time 1 or T1), which could occur up to seven days after admission, all subjects were interviewed with a modified version of the Semi-Structured Assessment for the Genetics of Alcoholism, version 2 (SSAGA-II) which included specific items to assess substance use over the past six months.<sup>36</sup> Blood samples for DNA were obtained from the subjects. Approximately 4 wk later (Time 2 or T2), the same subjects were assessed for changes in medical status and a second blood sample was obtained.

Control subjects were recruited from the Iowa City community. Control subjects were of good overall health, not taking medications hypothesized to influence DNA methylation, abstinent from alcohol for six months, and had no significant current substance use (except tobacco). Controls were interviewed using the same assessments as the case subjects. Blood samples were taken to provide biomaterial for the current study.

Sera and mononuclear cell (i.e., lymphocyte) pellets were prepared as described.<sup>12,13</sup> As part of our efforts to assess the reliability of self-reported data, cotinine and hydroxy-tetrahydrocannabinol levels in sera were assessed using an enzyme linked immunoassay (ELISA) kits supplied by Abnova (Taiwan) which were used according to manufacturer's directions and our previous protocols.<sup>12,13</sup> DNA was prepared from the lymphocyte cell pellets using a QiaAmp kit (Qiagen, Germany) according to manufacturer's directions.

Genome-wide DNA methylation was assessed by the University of Minnesota Genome Center under subcontract using the Illumina HumanMethylation450 BeadChip (Illumina, USA).<sup>12,13</sup> Resulting data were inspected for complete bisulfite conversion, average  $\beta$  values determined using the GenomeStudio

(Illumina USA) suite of programs with all data being transmitted to Behavioral Diagnostics via secure server. The raw data files (which include data from one additional T2 DNA pellet for which there is no matching T1 data) have been deposited to GEO (Accession number GSE57853).

The resulting data were then analyzed using our standard procedures.<sup>12,13</sup> In brief, data were cleaned to remove unreliable  $\beta$  values, which are formally defined as the ratios of the methylated probe fluorescence to that of total probe fluorescence (methylated probe/(methylated + unmethylated probe +  $\alpha$ )), using a PERL-based algorithm. Then, information from an array with < 99.5% complete data was removed. Surviving data were imported into MethLAB and analyzed using a standard linear model algorithm specifying chip and batch variables as co-factors.<sup>37</sup> The resulting data were corrected for genome wide comparisons using either Bonferroni or False Discovery Rate algorithms as indicated in the text.<sup>38</sup> Because of the small sample size and the need to preserve statistical power, genome-wide analyses were not corrected for age, gender, and ethnicity, though we note that the groups were similar with regard to these variables.

Pathway analysis of differentially methylated genes was conducted using the GoMiner™ package and the default settings.<sup>39</sup> This suite of programs uses the resources of the Gene Ontology Database<sup>40</sup> and conducts standard Student's T-tests to map differentially affected genes to known cellular pathways. Comparisons of clinical and demographic data (e.g., age, sex) were conducted

using the indicated tests and the JMP suite of programs (SAS Institute, USA).

#### Acknowledgments and Disclosures

This work was supported by R43AA022041 (Osborn). The use of DNA methylation to assess alcohol use status is covered by pending property claims. The use of DNA methylation to assess smoking status is covered by US patent 8,637,652 and other pending claims. Dr. Philibert is a potential royalty recipient on those intellectual right claims. Both Drs. Osborn and Philibert are officers and stockholders of Behavioral Diagnostics (www.bdmethylation.com). Drs. Gunter, Erwin and Liesveld are also consultants with Behavioral Diagnostics. The authors thank Dr. Donald Black for editorial assistance.

The authors would like to thank the staff and patients of the Center for Drug and Alcohol Services (Davenport, IA) and MECCA Services of Iowa City for their participation in this research. We would also like to thank the staff of the University of Minnesota Genome Center for their conduct of the genome wide assessments. Finally, we thank Drs. Donald Black and Steven R. Beach, for editorial assistance.

#### Supplemental Materials:

Supplemental materials may be found here:  
www.landesbioscience.com/journals/epigenetics/article/32252

#### References

- Bouchery EE, Harwood HJ, Sacks JJ, Simon CJ, Brewer RD. Economic costs of excessive alcohol consumption in the U.S., 2006. *Am J Prev Med* 2011; 41:516-24; PMID:22011424; <http://dx.doi.org/10.1016/j.amepre.2011.06.045>
- Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. *JAMA* 2004; 291:1238-45; PMID:15010446; <http://dx.doi.org/10.1001/jama.291.10.1238>
- Confidentiality of alcohol and drug abuse patient records. Code of Federal Regulations. United States, 1992.
- Treno AJ, Marzell M, Gruenewald PJ, Holder H. A review of alcohol and other drug control policy research. *J Stud Alcohol Drugs Suppl* 2014; 75(Suppl 17):98-107; PMID:24565316
- Martin GW, Rehm J. The effectiveness of psychosocial modalities in the treatment of alcohol problems in adults: a review of the evidence. *Can J Psychiatry* 2012; 57:350-8; PMID:22682572
- Edwards S, Kenna GA, Swift RM, Leggio L. Current and promising pharmacotherapies, and novel research target areas in the treatment of alcohol dependence: a review. *Curr Pharm Des* 2011; 17:1323-32; PMID:21524263; <http://dx.doi.org/10.2174/138161211796150765>
- Walter M, Gerhard U, Duersteler-MacFarland KM, Weijers H-G, Boening J, Wiesbeck GA. Social factors but not stress-coping styles predict relapse in detoxified alcoholics. *Neuropsychobiology* 2006; 54:100-6; PMID:17108710; <http://dx.doi.org/10.1159/000096991>
- Tavakoli HR, Hull M, Michael Okasinski L. Review of current clinical biomarkers for the detection of alcohol dependence. *Innov Clin Neurosci* 2011; 8:26-33; PMID:21487543
- Kharbouche H, Faouzi M, Sanchez N, Daeppen JB, Augsburger M, Mangin P, Staub C, Sporkert F. Diagnostic performance of ethyl glucuronide in hair for the investigation of alcohol drinking behavior: a comparison with traditional biomarkers. *Int J Legal Med* 2012; 126:243-50; PMID:21910015; <http://dx.doi.org/10.1007/s00414-011-0619-9>
- Monick MM, Beach SR, Plume J, Sears R, Gerrard M, Brody GH, Philibert RA. Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers. *Am J Med Genet B Neuropsychiatr Genet* 2012; 159B:141-51; PMID:22232023; <http://dx.doi.org/10.1002/ajmg.b.32021>
- Philibert RA, Beach SR, Brody GH. Demethylation of the aryl hydrocarbon receptor repressor as a biomarker for nascent smokers. *Epigenetics* 2012; 7:1331-8; PMID:23070629; <http://dx.doi.org/10.4161/epi.22520>
- Philibert RA, Beach SR, Lei MK, Brody GH. Changes in DNA methylation at the aryl hydrocarbon receptor repressor may be a new biomarker for smoking. *Clin Epigenetics* 2013; 5:19-26; PMID:24120260; <http://dx.doi.org/10.1186/1868-7083-5-19>
- Dogan MV, Shields B, Cutrona C, Gao L, Gibbons FX, Simons R, Monick M, Brody GH, Tan K, Beach SR, et al. The effect of smoking on DNA methylation of peripheral blood mononuclear cells from African American women. *BMC Genomics* 2014; 15:151; PMID:24559495; <http://dx.doi.org/10.1186/1471-2164-15-151>
- Zeilinger S, Kühnel B, Klopp N, Baurecht H, Kleinschmidt A, Gieger C, Weidinger S, Lattka E, Adamski J, Peters A, et al. Tobacco smoking leads to extensive genome-wide changes in DNA methylation. *PLoS One* 2013; 8:e63812; PMID:23691101; <http://dx.doi.org/10.1371/journal.pone.0063812>
- Besingi W, Johansson Å. Smoke related DNA methylation changes in the etiology of human disease. *Hum Mol Genet* 2014; 23:2290-7; PMID:24334605
- Joubert BR, Håberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, Huang Z, Hoyo C, Midttun Ø, Cupul-Uicab LA, et al. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* 2012; 120:1425-31; PMID:22851337; <http://dx.doi.org/10.1289/ehp.1205412>
- Shenker NS, Polidoro S, van Veldhoven K, Sacerdote C, Ricceri F, Birrell MA, et al. Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. *Hum Mol Genet* 2013; 22:843-51; PMID:23175441
- Philibert RA, Plume JM, Gibbons FX, Brody GH, Beach SR. The impact of recent alcohol use on genome wide DNA methylation signatures. *Front Genet* 2012; 3:54; PMID:22514556; <http://dx.doi.org/10.3389/fgene.2012.00054>
- Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *Am J Med Genet B Neuropsychiatr Genet* 2008; 147B:543-9; PMID:17987668; <http://dx.doi.org/10.1002/ajmg.b.30657>
- Philibert RA, Gunter TD, Beach SR, Brody GH, Madan A. MAOA methylation is associated with nicotine and alcohol dependence in women. *Am J Med Genet B Neuropsychiatr Genet* 2008; 147B:565-70; PMID:18454435; <http://dx.doi.org/10.1002/ajmg.b.30778>
- Zhang H, Herman AI, Kranzler HR, Anton RF, Zhao H, Zheng W, Gelernter J. Array-based profiling of DNA methylation changes associated with alcohol dependence. *Alcohol Clin Exp Res* 2013; 37(Suppl 1):E108-15; PMID:22924764; <http://dx.doi.org/10.1111/j.1530-0277.2012.01928.x>

22. Grafodatskaya D, Choufani S, Ferreira JC, Butcher DT, Lou Y, Zhao C, Scherer SW, Weksberg R. EBV transformation and cell culturing destabilizes DNA methylation in human lymphoblastoid cell lines. *Genomics* 2010; 95:73-83; PMID:20005943; <http://dx.doi.org/10.1016/j.ygeno.2009.12.001>
23. Bönsch D, Lenz B, Kornhuber J, Bleich S. DNA hypermethylation of the alpha synuclein promoter in patients with alcoholism. *Neuroreport* 2005; 16:167-70; PMID:15671870; <http://dx.doi.org/10.1097/00001756-200502080-00020>
24. Chang HW, Ling GS, Wei WI, Yuen AP. Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma. *Cancer* 2004; 101:125-32; PMID:15221997; <http://dx.doi.org/10.1002/cncr.20323>
25. Halsted CH, Villanueva JA, Devlin AM, Chandler CJ. Metabolic interactions of alcohol and folate. *J Nutr* 2002; 132(Suppl):2367S-72S; PMID:12163694
26. Bock C. Analysing and interpreting DNA methylation data. *Nat Rev Genet* 2012; 13:705-19; PMID:22986265; <http://dx.doi.org/10.1038/nrg3273>
27. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 2003; 124:1488-99; PMID:12730887; [http://dx.doi.org/10.1016/S0016-5085\(03\)00276-2](http://dx.doi.org/10.1016/S0016-5085(03)00276-2)
28. Sosne G, Siddiqi A, Kurpakus-Wheater M. Thymosin-β4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. *Invest Ophthalmol Vis Sci* 2004; 45:1095-100; PMID:15037574; <http://dx.doi.org/10.1167/iovs.03-1002>
29. Poikolainen K. Estimated lethal ethanol concentrations in relation to age, aspiration, and drugs. *Alcohol Clin Exp Res* 1984; 8:223-5; PMID:6375435; <http://dx.doi.org/10.1111/j.1530-0277.1984.tb05843.x>
30. Bailey SM, Patel VB, Young TA, Asayama K, Cunningham CC. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. *Alcohol Clin Exp Res* 2001; 25:726-33; PMID:11371722; <http://dx.doi.org/10.1111/j.1530-0277.2001.tb02273.x>
31. Otzen DE, Sehgal P, Nesgaard LW. Alternative membrane protein conformations in alcohols. *Biochemistry* 2007; 46:4348-59; PMID:17371052; <http://dx.doi.org/10.1021/bi700091r>
32. Nguyen LP, Bradfield CA. The search for endogenous activators of the aryl hydrocarbon receptor. *Chem Res Toxicol* 2008; 21:102-16; PMID:18076143; <http://dx.doi.org/10.1021/tx7001965>
33. Haarmann-Stemmann T, Bothe H, Kohli A, Sydlik U, Abel J, Fritsche E. Analysis of the transcriptional regulation and molecular function of the aryl hydrocarbon receptor repressor in human cell lines. *Drug Metab Dispos* 2007; 35:2262-9; PMID:17890447; <http://dx.doi.org/10.1124/dmd.107.016253>
34. Esser C. Biology and function of the aryl hydrocarbon receptor: report of an international and interdisciplinary conference. *Arch Toxicol* 2012; 86:1323-9; PMID:22371237; <http://dx.doi.org/10.1007/s00204-012-0818-2>
35. Sinden NJ, Stockley RA. Systemic inflammation and comorbidity in COPD: a result of 'overspill' of inflammatory mediators from the lungs? Review of the evidence. *Thorax* 2010; 65:930-6; PMID:20627907; <http://dx.doi.org/10.1136/thx.2009.130260>
36. Buchholz KK, Cadoret R, Cloninger CR, Dinwiddie SH, Hesselbrock VM, Nurnberger JI Jr., Reich T, Schmidt I, Schuckit MA. A new, semi-structured psychiatric interview for use in genetic linkage studies: a report on the reliability of the SSAGA. *J Stud Alcohol* 1994; 55:149-58; PMID:8189735
37. Kilaru V, Barfield RT, Schroeder JW, Smith AK, Conneely KN. MethLAB: a graphical user interface package for the analysis of array-based DNA methylation data. *Epigenetics* 2012; 7:225-9; PMID:22430798; <http://dx.doi.org/10.4161/epi.7.3.19284>
38. Benjamini Y, Hochberg H. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc, B* 1995; 57:289-300
39. Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S, et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol* 2003; 4:R28; PMID:12702209; <http://dx.doi.org/10.1186/gb-2003-4-4-r28>
40. Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, et al.; Gene Ontology Consortium. The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* 2004; 32:D258-61; PMID:14681407; <http://dx.doi.org/10.1093/nar/gkh036>