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LABORATORY TESTING

A Four Marker Digital PCR Toolkit for Detecting Heavy Alcohol Consumption and the Effectiveness of Its Treatment

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Background.—Heavy alcohol consumption (HAC) is a shared concern of the forensic, medical and insurance underwriting communities. Unfortunately, there is a relative lack of clinically employable tools for detecting HAC and monitoring treatment response. Building on the results of 3 genome wide methylation studies, we have previously shown in a small group of samples that methylation sensitive digital PCR assays (MSdPCR) have the potential to accurately classify individuals with respect to HAC in a small set of individuals.
Objective.—We now expand on those earlier findings using data and biomaterials from 143 participants with current HAC and 200 abstinent controls.
Results.—We show that a set of 4 digital PCR assays that have a receiver operating characteristic (ROC) area under the curve (AUC) of 0.96 for detecting those with HAC. After a mean of 21 days of inpatient enforced abstinence, methylation status at one of these markers, cg04987734, began to revert to baseline values. Re-examination of methylation data from our smaller 2014 study with respect to this locus demonstrated a similarly significant reversion pattern at cg04987734 in association with treatment enforced abstinence.
Conclusions.—We conclude that clinically implementable dPCR tools can sensitively detect the presence of HAC and that they show promise for monitoring alcohol treatment results. These dPCR tools could be useful to clinicians and researchers in monitoring those enrolled in substance use disorder treatment, employee wellness programs and insurance underwriting.

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Alcohol use is nearly universal, particularly in the United States, with approximately 90% of the adult population reporting lifetime use.[1] For most consumers, alcohol is safe. For example, there is ample evidence to suggest that those who consume approximately one drink per day enjoy greater longevity and better health than those who abstain, or those who drink excessively.[2,3] Moreover, the use of alcohol is important to many cultural and religious practices.[4] For these and other reasons, there is little interest in the medical or public health community to insist on complete abstinence from alcohol.

In contrast to the data supporting the health benefits of more modest amounts of alcohol consumption, excessive consumption is clearly a major societal and economic problem.[5] Worldwide, more than 3 million people die each year as a result of the excessive alcohol use.[6] This accounts for 1 in every 20 deaths and 5% of the overall global disease burden.[6] Thus, there is unanimity in the medical, legal and political circles for curtailing the excessive consumption of alcohol.

Detecting and quantifying heavy alcohol consumption (HAC), particularly in the absence of acute intoxication is problematic. To the clinician, the self-report of heavy alcohol use is unreliable.[7] Often, patients do not present for treatment unless personal situations such as pending divorce, arrest, or job loss, force the issue. Those circumstances are unfortunately associated with the loss of psychosocial support, which is critical to the success of treatment.[8] Conceivably, a test or set of tests that could unambiguously identify HAC could improve medical outcomes by allowing clinicians to more confidently address the HAC before adverse psychosocial events decrease the resources of a patient and their family to address the addiction.

Detecting HAC is also of great importance to the insurance underwriting community. HAC markedly elevates risk of loss from fire and falls.[9–12] HAC is also a major cause of sudden loss of life from motor vehicle accidents or pre-mature mortality alcohol-induced medical illness.[13–15] HAC is responsible for approximately 79,000 deaths and 2.3 million years of preventable life lost annually in the United States alone.[16] However, insurance clients often do not accurately report their non-illicit substance use status. For example, in 2013, approximately 18% of Iowa adults regularly smoked.[17] Unfortunately, that same year, only 7% of Iowans reported regular smoking to Wellmark Blue Cross and Blue Shield, the largest insurer in Iowa.[18] Whether the same level of unreliable self-report for HAC exists is not well known. But a test that detects those with high consumption rates could allow insurers to more accurately assess risk and potentially work with clients to implement programs to reduce HAC and achieve outcomes beneficial to both the insurer and the insured.

To circumvent the inherent problem of self-report, clinicians and insurers can use a combination of serum liver enzyme levels, phosphatidylethanolamine levels in either urine or serum, or serum carbohydrate deficient transferrin assessments to detect HAC.[19] However, each of these measures has its limitations with regard to sensitivity or specificity that have restricted their utilization in screening or monitoring excessive alcohol consumption.[19] Conceivably, a measure that overcomes the inherent limitations of existing biomarkers could be used alone or in tandem with the prior methods to detect HAC before adverse medical consequences or loss of life have occurred.

Epigenetic approaches could provide a partial solution to this problem. Over the past several years, we and others have shown that HAC has a distinctive impact on genome wide DNA methylation patterns.[20–23] Using DNA from a small group of cases (n = 46) and controls (n = 46), we showed that, in principle, it is possible to distill the signal from these genome wide arrays into methylation sensitive digital PCR assays, which could then be used to reliably classify individuals with respect to HAC.[23]

However, a limitation of our earlier results was our reliance on difficult to clinically implement genome wide methylation arrays, the small sample size used in the study, and the absence of information as to whether the methylation signals at these loci could also be used to assess abstinence following the initiation of treatment. In this communication, we extend our work by including additional participants, additional loci and methylation data at both intake and discharge from inpatient treatment.

METHODS

The protocols for collecting the cases and controls have been previously described.[23]

The heavy alcohol consuming individuals were ascertained from 1 of 3 inpatient chemical dependency treatment providers, Prelude Behavioral Services (Iowa City and Des Moines), Alcohol and Drug Dependency Services (Burlington) and the Center for Alcohol and Drug Services (Davenport). Each of these facilities offers detoxification services followed by a 21-28 day inpatient alcohol treatment program. Any individual who is: 1) over the age of 18, 2) capable of giving consent in English, 3) admitted to one of these facilities for treatment of current sustained alcohol dependence in the context of current alcohol intoxication, and 4) expressed interest in our protocol to a member of their healthcare team was eligible for the study. Participants were not approached for intake into the study if they were still intoxicated or judged to be under the influence of other substances. After written informed consent for the study was obtained, each participant was interviewed with a series of instruments including a modified Form of the Semi Structured Assessment for Genetic Studies (Version II) and our Substance Use Questionnaire. After the interview process was complete, each participant was then given a contact card and urged to contact the facility staff or the research assistant if they were interested in completing the exit interview that was held as close to the discharge date as possible. Fifty-four of the 143 individuals who participated in the intake interview also completed the discharge interview, which consisted of an updated Substance Use Questionnaire and phlebotomy.

The control participants were recruited from the University of Iowa community through e-mail and word-of-mouth advertisements. Participation in the control arm of the study was open to all adults who denied any use of alcohol or illegal substances, including cannabis, in the past year and denied a history of past substance abuse. After informed consent was obtained from each of these individuals, they were also interviewed with the Semi Structured Assessment for Genetic Studies (Version II) [24] and our Substance Use Questionnaire,[23] then phlebotomized to provide biomaterials for the study. A total of 210 participants enrolled in the control arm of the study successfully provided DNA and sera for this study. The data from 9 participants (6 males, 3 females) was excluded for having serum cotinine values inconsistent with their self-report of nicotine containing products. The data from one additional participant was excluded for reporting recent cannabis use during the structured interview. This study uses clinical data and biomaterials from a set of protocols approved by the Western Institutional Review Board (www.wirb.com). All participants gave written informed consent.

DNA and sera were prepared from the blood specimens as described elsewhere.[23] Serum cotinine and cannabinoid levels were then determined using enzyme linked immunoassays (ELISA) from AbNova (Taiwan) as we have previously described using manufacturer's directions.[25]

Methylation status at the 4 loci was determined using our previously described procedures using proprietary assays from Behavioral Diagnostics (www.bdmethylation).[26] Briefly, 1 ug of DNA was bisulfite converted using a Fast DNA kit from Qiagen (Germany) according to manufacturer's directions. An aliquot of each of these modified DNA samples was pre-amped, diluted 1:3000 with molecular grade water, and then PCR amplified using proprietary primer probe sets for each locus from Behavioral Diagnostics (Coralville, IA) and universal digital PCR reagents from Bio-Rad (Carlsbad, CA). The number of droplets containing amplicons with at least one "C" allele, one "T" allele or neither allele was then determined using a Bio-Rad QX-200 droplet reader. Percent methylation was calculated using Quantisoft software by fitting the observed ratios to a Poisson distribution.

Standard linear regression was used to examine the relationship of methylation status to age and gender. Boxplots were constructed to display the distribution of methylation status by gender. The primary analyses were

conducted using logistic regression where the outcome was HAC status (ie, case/control), and each model was adjusted for age and gender. The relationship between methylation status at each of the 4 loci and HAC status was investigated, first individually and then together in a combined model. Relative strength among the models was assessed using area under the curve (AUC) and Akaike's Information Criterion (AIC).[27–29] To better understand how our 4 markers capture the genome wide signal associated with heavy alcohol use, we conducted principal components analysis (PCA) of the data from the 4 markers.[30] Pearson correlations were also calculated to show relationships between the methylation markers. Finally, for the HAC participants, we used linear mixed effects regression (LMER) to compare their entry (T1) to their exit (T2) methylation values to investigate possible reversion to control values.[31] Data were analyzed using R Version 3.5.1. The genome wide data described in the text is publically available through the Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo/) via accession GSE57853.

Table 1. Clinical and Demographic Variables of Case and Control Subjects at Intake

	Case N = 143	Control N = 200
Age at Intake†	41.2 ± 11.0 years	43.5 ± 15.1 years
Gender‡		
Female	42 (29.4)	110 (55.0)
Male	101 (70.6)	90 (45.0)
Race		
White, Non-Hispanic	120 (83.9)	178 (89.0)
African American	18 (12.6)	3 (1.5)
White, Hispanic	4 (2.8)	6 (3.0)
Asian	0 (0.0)	11 (5.5)
Other	1 (0.7)	2 (1.0)
Smoking Status		
Positive Self-Report	119 (83.2)	12 (6.0)
Positive Cotinine	123 (86.0)	6 (3.0)
Cannabis		
Positive Self-Report	74 (51.7)	0 (0.0)
Positive THC	47 (32.9)	0 (0.0)
Drinks per Day		
Prior Week	15.2 ± 9.9 drinks	–
Prior Month	15.9 ± 10.4 drinks	–
Prior Six Months	15.6 ± 11.0 drinks	–
Prior Year	15.6 ± 11.0 drinks	
Days Since Last Drink	3.7 ± 1.9 days	–
Average Methylation		
cg02583484	16.8 ± 5.5%	24.8 ± 4.7%
cg04987734	43.7 ± 10.0%	32.1 ± 6.8%
cg09935388	59.0 ± 19.2%	81.3 ± 11.7%
cg04583842	45.8 ± 7.3%	37.4 ± 5.6%

† Mean ± Standard Deviation for Continuous Measures.

‡ N (%) for Categorical Measures.

RESULTS

Key clinical and demographic data for the participants with HAC (case) and control participants is shown in Table 1. HAC and control participants were mostly White and in their early to mid-40s. HAC participants were predominately male (71%) while controls were mostly female (55%). Consistent with prior studies, the vast majority of alcoholic participants (83%) reported smoking cigarettes in the past year with many of other HAC participants who did not smoke cigarettes reporting the smoking of other forms of tobacco, the use of e-cigarettes or chewing tobacco. Four participants who denied any use of nicotine containing products had detectable (>2 ng/ml) cotinine levels at the time of phlebotomy. Approximately half of HAC participants also reported the use of cannabis in the past year.

Individuals with HAC reported an average consumption of approximately 15 standard drinks per day in the 1 week, 1 month, 6-month and 1-year time frames prior to admission to the facility. Overall, the female HAC participants reported slightly less alcohol intake than their male peers (average yearly intake 13.7± 9.7 vs 16.3± 11.4 standard drinks per day).

To better understand the relationship of DNA methylation to gender in the absence of alcohol intake, we examined the relationship of methylation at each of the 4 marker loci to gender while controlling for HAC status. Methylation status at cg02583484 was higher in men ($t = 2.85$, $p = 0.005$). However, methylation status at the other 3 loci, cg04987734, cg09935388 and cg04583842 did not significantly differ between men and women (data

Table 2. AUC and AIC for HAC Logistic Regression Models

Model†	Predictors	AUC	AIC
1	Gender + Age	0.649	421.24
2	Gender + Age + cg02583484	0.910	251.79
3	Gender + Age+ cg04987734	0.876	296.35
4	Gender + Age + cg09935388	0.887	285.41
5	Gender + Age + cg04583842	0.853	307.56
6	Gender + Age + cg02583484 + cg04987734 + cg09935388 + cg04583842	0.964	171.54

† Missing values for predictors result in analysis sample size of 326 participants for all models.

Table 3. AUC and AIC for HAC Logistic Regression Models, Female Participants

Model†	Predictors	AUC	AIC
1	cg02583484	0.861	115.51
2	cg04987734	0.850	134.89
3	cg09935388	0.851	125.35
4	cg04583842	0.861	125.73
5	cg02583484 + cg04987734 + cg09935388 + cg04583842	0.949	89.30

† Missing values for predictors result in analysis sample size of 146 participants for all models.

not shown) after controlling for HAC. It is important to understand if genetic variation could be affecting marker performance. In prior work, we have shown the value of visually inspecting simple boxplots in determining whether genetic variation may be affecting methylation status at CpG residues.[32] Figure 1 illustrates the distribution of methylation at each of the markers separately with respect to HAC status and gender. Visual inspection of the boxplots for, cg04987734 and cg09935388 shows higher variability for males and females than is evident in the cg02583484 and cg04583842 distributions.

We next used linear regression to examine the relationship of methylation status to age. There appeared to be an association between methylation status and age for cg04987734 and cg04583842, with both markers increasing as age increased. There was no evidence of association with age for cg02583484 or cg09935388.

As a next step, we analyzed the relationship of each of the 4 markers to alcohol use status (ie, case/control), in a series of logistic regression models adjusting for age and gender Table 2. By themselves, each of the markers distinguished HAC from controls with Receiver Operating Characteristic (ROC) AUC values cg02583484 (AUC = 0.91, AIC = 252) cg04987734 (AUC = 0.88, AIC = 296), cg09935388 (AUC = 0.89, AIC = 285) and cg04583842 (AUC = 0.85, AIC = 308).

We then considered a final logistic regression model to determine whether the combination of markers would better predict HAC status than the single markers alone Higher AUC and lower AIC values (AUC = 0.96, AIC = 172) show that the combined model provides improved predictive power over the single marker models. The predictive power of the combined model is illustrated by a density plot of the predicted probabilities of HAC classification by group (Figure 2).

Since the HAC cohort was disproportionately male, we also conducted gender specific analyses (Tables 3 and 4) to ensure adequate performance in both male and female subject groups. In both the male and female subject groups, the information from just the

Table 4. AUC and AIC for HAC Logistic Regression Models, Male Participants

Model†	Predictors	AUC	AIC
1	cg02583484	0.903	144.71
2	cg04987734	0.847	176.73
3	cg09935388	0.902	167.39
4	cg04583842	0.814	194.15
5	cg02583484 + cg04987734 + cg09935388 + cg04583842	0.952	110.92

† Missing values for predictors result in analysis sample size of 180 participants for all models.

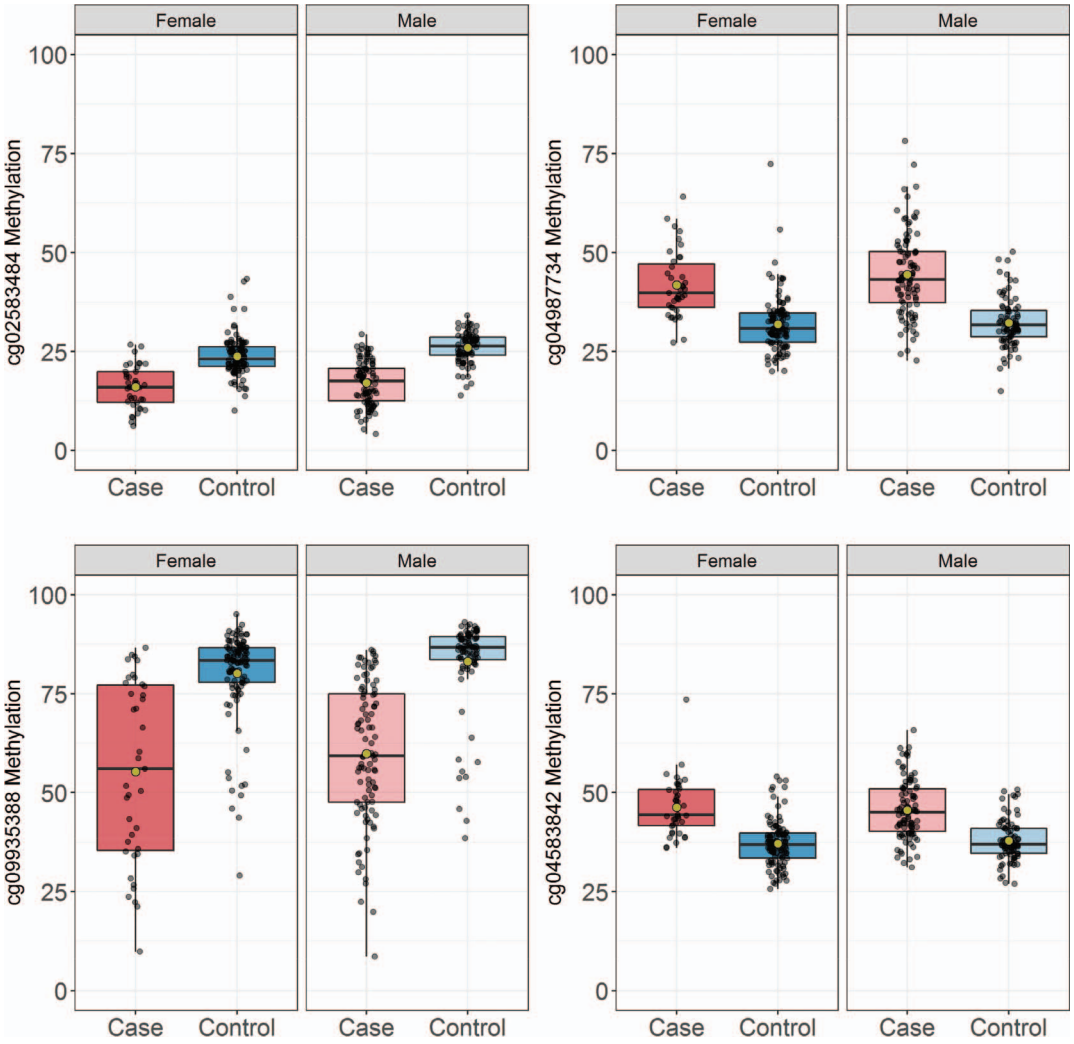

Figure 1. *Distribution of the methylation values for each marker for the cases (red) and controls (blue) split by gender. Mean values are indicated by the gold-colored symbol.*

4 markers predicted case status very well with AUCs of 0.949 and 0.952 for female and male subjects, respectively.

In two earlier studies, we presented PCA results showing that the alcohol associated changes map to one major principle component that accounts for ~2/3 of the variance with the remainder mapping to a handful of more minor components.[20,23] Similar to the prior findings, approximately 63% (eigenvalue 2.5) of the signal from those 4 markers mapped to the first component with no other component having an eigen value greater than 1. Correlations between pairs of markers range from a magnitude of 0.39 to 0.62 (Table 5).

Finally, using LMER we examined the change in methylation for the HAC participants to see if the values moved in the direction of the average control values, adjusting for age and gender. One marker, cg04987734, had evidence of reversion to the average control value (estimated slope = -0.13; t = -3.87, p = 0.0003) during the follow-up period. Two other markers, cg02583484 and cg04583842, had signs for their slopes consistent with reversion to control values, but did not reach statistical significance. And one marker, cg09935388, had a sign for its slope inconsistent with reversion to the average control value, though it also failed to reach statistical significance.

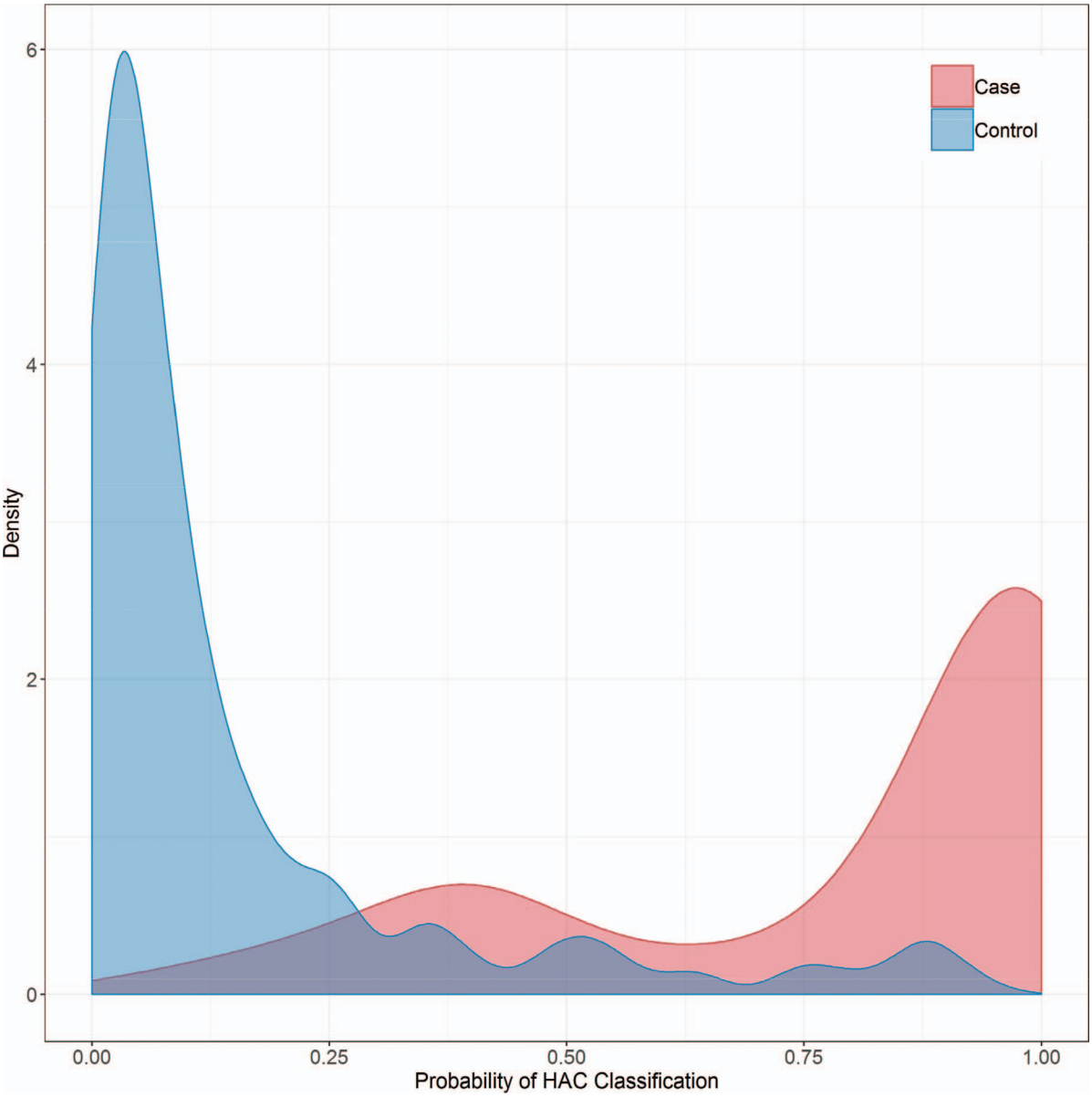

Figure 2. *Density plot of the predicted probabilities of HAC classification from the combined model (Model 6) for cases (red) and controls (blue).*

Table 5. Pearson Correlation Coefficients and p-values (N = 326)

	cg02583484	cg04987734	cg09935388	cg04583842
cg02583484	1.00000			
cg04987734	−0.51150 <.0001	1.00000		
cg09935388	0.62409 <.0001	−0.43367 <.0001	1.00000	
cg04583842	−0.46168 <.0001	0.61621 <.0001	−0.38892 <.0001	1.00000

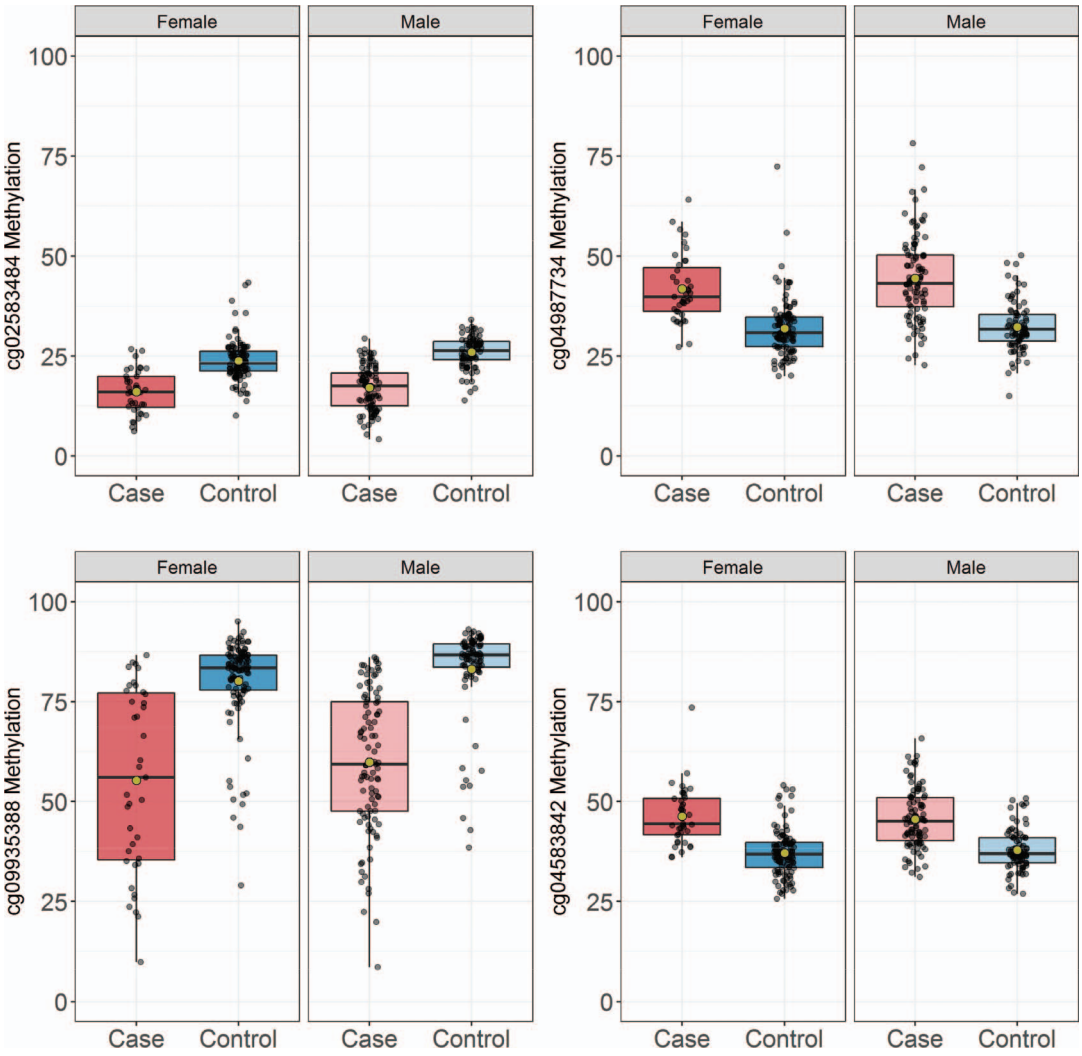

Figure 3. *Change in cg04987734 methylation for HAC participants during the follow-up period. The average baseline cg04987734 methylation value for controls is shown by the dashed line.*

In addition, we also examined these values at these 4 loci from the 26 participants who completed both visits in our similarly structured 2014 study.[20] The average period between the T1 (entry) and T2 (study exit) assessments for the participants from the current (39 male and 15 female) and prior (21 male and 5 female) studies was 22 ± 5 days, and 29 ± 3 days, respectively. Because the dynamic ranges of the ddPCR and array based measurements used in the 2014 study differ, direct comparisons of degree of methylation changes are not possible. However, we again used LMER to investigate reversion to the control average at each of these 4 loci. In the 2014 study, methylation status at cg02583484 and cg04987734 showed evidence of reversion (estimated slope = 0.03; t = 1.98, p = 0.059 and estimated slope = -0.14; t = -6.59, p <.0001, respectively) during the follow-up period. One other marker, cg09935388, had a sign for its slope consistent with reversion to the control average, but did not reach statistical significance. And one marker, cg04583842, had a sign for its slope inconsistent with reversion to the average control value, also failing to reach statistical significance.

DISCUSSION

The study produced potentially useful results. We showed that the results of MSddPCR can be used to accurately classify nondrinking participants from HAC participants, and that methylation status at least two of those loci demonstrates significant amounts of reversion after less than 30 days of abstinence. We observed that alcohol use status of the controls is based solely on self-report and that the MSddPCR data for 92 of the participants (46 cases and 46 controls) with respect to cg02583484 and cg04987734 was reported in our prior report of the genome wide comparisons. A potential caveat of the findings is that the HAC group did not only have much higher alcohol use, but also had much higher rates of smoking and cannabis use. However, these markers were specifically selected because our prior genome-wide studies have shown that the methylation markers considered here are unaffected by smoking and cannabis use, indicating that the markers are distinguishing the groups based on their alcohol use.[23]

DNA methylation assessments have become accepted as a mechanism for measuring environmental exposures. The best case for this can be made with respect to cigarette smoking. Beginning with our 2012 study, dozens of genome wide methylation studies have demonstrated that DNA methylation status at cg05575921 can be used as an indicator of cigarette smoking.[33,34] Building on that body of work, we have developed methylation sensitive quantitative and MSddPCR assays capable of not only robustly ascertaining smoking status, but also imputing daily cigarette consumption.[25] In contrast, the number of genome wide case and control analyses with respect to alcohol consumption is more limited.[20,22,23] Still, all 4 of the loci used in this study were reported as associated with alcohol use in the 3 studies. Therefore, given the strength of the current findings, we are not only optimistic that this method will gain widespread acceptance as a method of detecting heavy alcohol consumption, but that additional methylation assays for other environmental variables, such as toxic metal exposures, will join their ranks.

At present, we are not able to accurately infer the threshold of alcohol consumption necessary to trigger a change in DNA methylation. To accomplish this, we will need to collect a large cohort of individuals reliably characterized for a diverse range of alcohol consumption levels. But it may not be practical to collect such a cohort for several reasons. The first reason is that all our alcohol consumption parameters are based on self-report. These assessments, as we have shown, are inadequate. While we believe that the majority of our HAC participants are reporting their alcohol consumption as best as they can, it is evident that many cannot accurately recall large periods of time prior to their admission. As a result, we believe that the alcohol consumption metrics reported by HAC participants in Table 1 should be viewed as a general approximation. Collecting accurate self-report from moderate or light drinkers or even abstinent controls also might prove equally challenging. Indeed, we note that data from 10 prospective "clean" controls for this study was excluded because their self-report of cigarette smoking was not consistent with serum cotinine values (please note that the participants in question denied any use of a nicotine containing product, not just use of cigarettes). This 5% rate of unreliable self-report for smoking is very similar to that of a separate control cohort gathered previously.[35] It is also likely that data for many other controls would have been excluded if we were able to employ a reliable measure, such as urinary phosphatidylethanolamine, to detect recent short-term alcohol consumption. Conceivably, using a combination of monitoring technologies including transdermal bracelet monitoring of alcohol consumption levels of a large cohort over an extended period, we could circumvent many of the problems that we have encountered to gather a group of "clean" controls. But the financial, legal and

ethical considerations surrounding the conduct of such a study design make this unlikely and such study designs may lead to the oversimplification of the likely complex relationship between alcohol consumption and methylation changes for any given locus. As a result, we do not believe that is likely that an accurate inference of a minimal response threshold will be forthcoming.

We believe that it is likely that the ability of the markers to accurately classify individuals will increase as we better constrain factors, which may alter their methylation. It will be important to understand the effects of confounding genetic variation and diet. We are quite aware from studies by ourselves and others that background genetic variation, some of which sorts with ethnicity, alters DNA methylation at various loci.[36,37] To address this potential problem, we are actively genotyping each of those loci in the hopes of not only improving their individual predictive powers but also decreasing any possibility of ethnic bias even though the current study does not show any significant differences of ethnicity or gender on DNA methylation. Still, even with perfect control for trans and cis genetic modification, additional bias from dietary factors may exist. For example with respect to nutrient levels, we have previously shown that methylation status at the methylene tetrahydrofolate reductase (MTHFR) moderates the methylomic response to smoking.[38] Because activity of this gene is linked to folate availability and folate levels are strongly influenced by diet,[39] it is possible that folate availability or any of a host of variables that co-sort with age, medical illness, ethnicity or region of the world may influence the methylomic response at these or other loci.

A natural topic of curiosity in those involved in biomarker development or testing is the nature of the loci being targeted in each assay or set of assays. For the record, these assays target the following genes: cg02583484, *Heterogeneous Nuclear Ribonucleoprotein A1* (HNRNPA1); cg04987734, *CDC42 binding protein kinase beta* (CDC42BPB); cg09935388, *Growth Factor Independent 1 Transcriptional Repressor* (GFI1), and cg04583842, *BTG3 Associated Nuclear Protein* (BANP). It is important to realize that our genome wide studies show that the methylation status of tens of thousands of CpG residues is changed in association with heavy alcohol consumption. For that reason, we believe that expounding on the characteristics of these 4 loci at the expense of the rest of the genomic response in not warranted and note that we have conducted those analyses previously.[23] Indeed, we selected these 4 loci because: 1) smoking status does not affect the methylation response at these loci, 2) the delta beta (the difference between cases and controls) for each of the loci was relatively large, and 3) each locus had sequence features amenable to MSddPCR assays. Should we have the need to develop additional assays, we have many other loci to potentially target. Still, we believe that once we fully define the other factors, discussed above, influences on DNA methylation at these loci, we will have an even more robust assay for heavy alcohol consumption.

The most obvious applications of this relatively quick to perform MSddPCR technology for the readers of this journal will be for the civil (eg, insurance underwriting) market. HAC significantly increases accidents at work and decreases expected longevity.[13–16] Currently, the underwriting process uses a combination of the previously discussed alcohol biomarkers, most prominently the CDT, to evaluate alcohol use status. However, the effectiveness of these tests in detecting and quantifying surreptitious HAC in the underwriting process is not well constrained. In contrast, the rapid proliferation of genome wide methylation characterizations of large population cohorts, such as the Framingham Heart Study (FHS) and the ESTHER cohort, has created a new potential resource for the understanding the relationship of DNA methylation indices to survival outcomes.[36,40] Most relevant to the current study, in two separate studies using the FHS data, we have

shown that DNA methylation at just one of the markers in our panel, cg04987734, is a strong predictor of mortality. When combined with the quantitative marker for cigarette consumption (cg05575921),[25] and our recently described epigenetically informed tool for predicting cardiovascular disease,[41] methylation approaches could improve the speed and reliability of certain segments of underwriting process.

The implementation of this technology as a screening test for HAC could be highly significant. Despite the best clinical efforts, the first sign of heavy alcohol consumption is often a sentinel event, such as a divorce or arrest, that strips away many of the psychosocial factors that increase the likelihood of a clinical response.[8] Conceivably, a readily employable clinical screening test that can robustly detect heavy alcohol consumption could facilitate a therapeutic dialogue before such events occur. But before that clinical translation can occur, many additional steps, including extension of the current findings in diverse medical settings and scaling the technology so that it can be readily conducted daily in small to medium sized clinical pathology laboratories will be necessary. Fortunately, for these types of applications, digital PCR platforms are becoming increasingly more common in clinical settings.[42] In fact, the QX-200 platform used in this study recently received FDA clearance.[43]

An encouraging finding is that methylation at cg04987734 appears to revert as a function of treatment induced sobriety. This could suggest that methylation status at this or other loci could be used as a quantifiable biomarker of the effectiveness of alcohol cessation treatment that could detect relapse to moderate to heavy alcohol usage in the absence of acute intoxication in those receiving outpatient treatment. Though consistent between the 2014 and 2018 studies, these data cover only one time point in the recovery process. What is more, the magnitude of the estimated average change is small (-0.13% per day). Still, these results are a good start and suggest the possibility that a panel of 2 or 3 other markers whose dynamic response to alcohol cessation is relatively rapid could be useful to clinicians interested in using quantitative biomarkers of harm reduction to assess the effectiveness of their interventions. Indeed, the prospect of having an accurate blood, or better still yet, a saliva-based DNA test for monitoring alcohol abstinence could allow greater numbers of patients to have outpatient treatment first before resorting to inpatient treatment while improving the quality of clinical assessments for those undergoing long-term outpatient treatment.

In conclusion, we show that a panel of MS-ddPCR assays can reliably classify individuals with respect to HAC and the potential of this approach for monitoring alcohol abstinence. Refinement of the assays to increase the power of prediction and integration of these alcohol tests with methylation assays of other health conditions may lead to a set of useful tools for insurance underwriting and medical care.

STATEMENT OF CONFLICT

Dr Philibert is the Chief Executive Officer of Behavioral Diagnostics and the inventor of pending patent applications covering the use of DNA methylation to assess alcohol consumption.

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