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A Pilot Investigation of the Impact of Smoking Cessation on Biological Age

Man-Kit Lei, PhD^{1,2,*}, Steven R.H. Beach, PhD^{1,3}, Meeshanthini V. Dogan, MS⁴, and Robert A. Philibert, MD, PhD^{4,5}

¹ Center for Family Research, University of Georgia, Athens, GA, USA, 30602

² Department of Sociology, University of Georgia, Athens, GA, USA, 30602

³ Department of Psychology, University of Georgia, Athens, GA, USA, 30602

⁴ Department of Psychiatry, University of Iowa, Iowa City, IA, USA, 52242

⁵ Behavioral Diagnostics, 316 East Court Street, Iowa City, IA, USA, 52240

Abstract

Background and objectives—Smoking is known to increase biological age. However, whether this process is reversible through smoking cessation is not known. In this pilot study, we attempt to determine whether smoking cessation reduces biological age.

Methods—We conducted regression analyses of methylation data from 22 subjects, as they entered and exited inpatient substance use treatment, to determine change in biological age, as indicated by the deviation of their methylomic age from chronological age across two time points.

Results—We found that, as compared to those subjects who did not stop smoking, subjects who significantly decreased their smoking consumption over a one month time period exhibited a marked reduction in methylomic age.

Conclusion—The rapid and substantial reversal of accelerated aging associated with successful smoking cessation suggests that it can reverse well-known smoking effects on methylomic aging. This preliminary finding can be readily examined in other, larger data sets, and if replicated, this observation may provide smokers with yet another good reason to quit smoking.

Scientific significance—Successful smoking cessation makes patients appear biologically younger than they were at baseline, and to do so quite rapidly. In today's youth driven society, our observations may serve as a powerful impetus for some to quit smoking.

*Corresponding author, Man-Kit Lei, PhD, Center for Family Research, University of Georgia, 1095 College Station Road, Athens, GA 30605, Tel: 706-425-3305, Fax: 706-425-2985, karlo@uga.edu.

DECLARATION OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Dr. Philibert is the Chief Executive Officer and majority stockholder of Behavioral Diagnostics, Inc. (www.bdmethylation.com). He is also a co-owner of U.S Patent 8,637,652 and 9,273,358 which cover the use of methylation at cg0557921 for the ascertainment of smoking status.

Keywords

DNA Methylation; Smoking; Smoking Cessation; AHRR; Biological Age

INTRODUCTION

Smoking is the leading cause of morbidity and mortality in the United States. Each year, nearly 450,000 Americans die prematurely from tobacco consumption (1). Several evidence-based therapies for smoking cessation are available, but their real world effectiveness is low with only ~6% of patients achieving abstinence in traditional clinical settings (2). The reasons for this abysmal rate of response to treatment are complex, but one recurrently cited reason is a lack of motivation for change in patients. Specifically, despite the overwhelming, often statistically complex evidence for the health benefits of smoking cessation, patients do not often fully embrace the benefits of smoking cessation (3). Therefore, one way to potentially increase motivation for smoking cessation may be to identify outcomes which are conceptually easy to understand and also potentially more motivating for some than are long-term health benefits.

One beneficial effects of smoking cessation may be decreased biological age. The eternal search for youth, as illustrated by Ponce de Leon, has been a recurrent theme in human history, and does not appear to have lost its appeal in modern times. Americans spend over \$80 billion annually for products or treatments that make them appear younger (4). If quitting smoking was shown to result in a change in biological age, providing that information to those contemplating smoking cessation treatments might serve as a powerful additional inducement to change.

Unfortunately, although several studies have shown that smoking increases biological indices of age (5-7), the evidence that quitting smoking “makes one younger” is relatively sparse, which may be secondary to the numerous methodological challenges in conducting studies of the effect of smoking cessation on aging. Since the 1960s, however, researchers have been aware that there is a strong association between age and DNA methylation. The recent introduction of epigenetic “clocks” as a method of determining biological age may present an approach that could surmount many of these challenges (8-10). Specifically, because of the highly quantitative and objective manner in which these indices demarcate age, conducting studies of the effects of treatments on biological aging may become more feasible.

One of the most potentially interesting therapies to examine with regard to its potential impact on biological age may be that of smoking cessation. In recent work which used an epigenetic clock as an indicator of biological age (methylomic aging), we have shown that smoking is associated with accelerated biological aging (5). However, because of the cross sectional samples used in that study, we could not address the hypothesis that the effects of smoking on methylomic aging may be reversible; that is that quitting smoking may decrease ones methylomic age.

To examine the possibility that smoking cessation decreases methylomic age, we analyzed publically available genome wide data from a recent study of the effects of alcohol cessation therapy (11). As a note to those unfamiliar with alcohol cessation treatment, although concurrent smoking cessation is highly encouraged, it is not required in most chemical dependency treatment settings. Therefore, although many patients undergoing inpatient treatment quit smoking as well, many do not. However, it is well known that self-report of smoking can be unreliable in high risk populations (12). Therefore, in order to quantify the success of smoking cessation in these subjects, we used another epigenetic assessment embedded in the array data. In previous work, we and others have shown that the most reliably and robustly differentially methylated CpG residue associated with smoking is referred to as cg05575921, which is found in the aryl hydrocarbon receptor repressor (AHRR) (13). Since its initial association in 2012, over a dozen consecutive genome wide analyses have shown that it is consistently most robustly differentially methylated residue in the genome in response to smoking in all age groups with demethylation of this residue being strongly correlated with a variety of short term and long term consumption measures including serum cotinine, cigarettes per day and pack year measures (13-21). What is more, reversion of this residue is exquisitely sensitive to smoking cessation with the average re-methylation of the locus being between 3-4% after one month of smoking cessation. As a result, by using this measure, one can objectively determine changes in smoking intensity in any dataset containing Illumina HumanMethylation450 Beadchip array data or any other methylation data set containing this specific probe, and this assessment will be available regardless of the availability of self-report data.

Taking advantage of these two methylomic indices; i.e., for aging and for smoking intensity, we re-examined methylation of DNA from blood taken from 22 subjects for whom we had samples from inpatient entry and exit. This allowed us to divide the sample with respect to smoking cessation and examine its impact on methylomic aging.

METHODS

Participants

The data for the present study was taken from the publically available data set from a case and control study of the effects of alcohol consumption on whole blood DNA methylation (GEO accession number, GSE57853, the data are available online in <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57853>) (11). In this study, genome wide DNA methylation data from both entry and exit were available for 26 subjects. Given that the four remaining subjects denied smoking in the past five years but all had smoked at least 5 packs of cigarettes in their lifetime, we excluded these cases from the analyses to avoid potential bias. All protocols and procedures for the study were approved by the University of Iowa Institutional Review Board.

Methylation

Genome wide methylation analyses and data processing of these data were conducted as previously described (11). The Illumina Infinum (Sequenom, Inc., San Diego, CA) HumanMethylation450 Beadchip was used to assess genome-wide DNA methylation. After

quality control analyses, quantile normalization methods were used to derive the methylation indices used for the current investigation (22). Genome-wide methylation data were obtained for all participants with successful measurements for > 99.5% of all probes for all samples including two lymphoblast DNA standards and one internal replicate. The correlation between the independently prepared replicate samples was greater than 0.998. From the processed data set, we extracted the data for the DNA methylation residues used by Hannum and colleagues (9) and Horvath (10) to calculate the methylation clock. We also extracted data for cg055750921 and cg23193759, the first a well-documented indicator of smoking status and more recently, smoking cessation, and the second an indicator of alcohol consumption (13-16, 19, 21).

Measures

Smoking cessation—In previous work, we and others have shown that methylation at AHRH reverts as a function of smoking cessation (16). In particular, in a recent clinical study designed to measure this reversion rate, we found that smoking cessation was associated with a 6% increase in methylation less than six months after quitting smoking with the greatest amount of change noticeable after the first month (23). Therefore we used reversion of methylation at cg05575921 between intake and exit as a marker of the degree of smoking cessation during inpatient stay. In particular, because 2% change in methylation is well outside the range expected for measurement error or changes for reasons unrelated to smoking cessation we used this as our cut-point to indicate successful smoking cessation (23).

Accelerated methylomic aging—Methylomic age was calculated in two ways, following the methods proposed by Hannum et al. (9) and Horvath (10). Hannum's methylomic age is based upon the weighted sum of methylation values at 71 CpG sites. Horvath's methylomic age is calculated using a publicly available R code, which aggregates 353 CpG sites that are scattered throughout the human genome. There is very little overlap in the CpGs used by the two measures (6 CpG sites overlap). However, the relationship between the methylomic ages calculated using the two different approaches was significant ($r = .807, p < .001$), and Hannum's and Horvath's methylomic ages were both strongly associated with chronological age (Hannum: $r = .731, p < .001$; Horvath: $r = .832, p < .001$). Finally, we formulated measures of accelerated methylomic aging using the deviation of predicted methylomic age from chronological age. For smokers this is expected to be a positive number, indicating that smoking is associated with accelerated methylomic aging. In the current sample, the 22 smokers were accelerated by 4.29 years, on average, using Hannum's method and 2.15 years, on average, using Horvath's method, at entry into the study.

Cell-type composition—Although cg055750921 is designed for use with blood samples and assessment of smoking effects at cg055750921 are robust to cell type variation (9, 24), several studies have indicated that cg055750921 may vary depending on cell composition (25). To control for any effects due to cellular heterogeneity, cell-type composition was estimated using the “EstimateCellCounts” function in the minfi Bioconductor package, which is based on the reference-based method developed by Houseman and colleagues (26).

Using this approach we estimated cell-type composition in whole blood for lymphocytes (CD8+T, CD4+T, NK cells and B cells) and monocytes and controlled for this variation in the regression analyses.

Covariates—To isolate the effect of smoking cessation per se, analyses were controlled for gender (1 = males), racial group (1 = white), marijuana use, and cg23193759 (to provide a non-self-report index of alcohol consumption).

RESULTS

The clinical characteristics of the subjects are similar to those of the overall pool of subjects from the original study. Twenty two of the subjects were serum confirmed current smokers at baseline with an average current consumption of 13 cigarettes per day total, and with an average lifetime consumption of 20 pack years. Descriptive statistics are presented in Table 1. Among the 22 subjects used in our analysis, they are largely male ($n = 17$, $M = .773$, $SD = .429$), White ($n = 20$, $M = .909$, $SD = .294$) and in their mid to late forties (46.773 ± 6.689 yrs). About 86 percent of subjects have used marijuana. Drinking is quantified using methylation status at cg23193759 ($M = .156$, $SD = .016$ at Time 1; $M = .160$, $SD = .019$ at Time 2) with methylation decreasing in response to greater consumption (5). On average, the length of time between their intake phlebotomy draw and their exit phlebotomy draw was 25 days.

In total, 11 of the 22 subjects had less than a 2% change in cg05575921, indicating continued smoking, while 11 others had a greater than the 2% increase in methylation at cg05575921 indicating that they quit or significantly reduced their cigarette consumption during the time between their entry (Time 1) and exit (Time 2) phlebotomy draw.

Before testing our hypotheses, we first checked the distributions of cg05575921 and methylomic age. The results showed that the median level of cg05575921 increased between Time 1 (Median = .671, IQR = .151) and 2 (Median = .712, IQR = .194). Moreover, the assumption of normality was met (Shapiro-Wilk test: $W = .976$, *n.s.* at Time 1; $W = .920$, *n.s.* at Time 2). Please change the sentences to:

The distributions of methylomic age at Time 1 (Hannum's median age = 51.360, IQR = 12.246; Horvath's median age = 49.834, IQR = 5.968) and Time 2 (Hannum's median age = 50.871, IQR = 8.115; Horvath's median age = 48.335, IQR = 5.302) indicated a tendency in the sample toward a lower age at Time 2. Further, methylomic age met assumptions for normality.

To examine whether change in cg05575921 (smoking) status triggers change in biological age, we calculated difference scores by subtracting entry (Time 1) scores from exit (Time 2) scores. They reflected the degree to which methylation status at cg05575921 or methylomic age changed from Time 1 to Time 2. As can be seen in Model 1 in Table 2, the results reveal that methylation status at cg05575921 (T_2-T_1) has a strong effect on Hannum's methylomic age (T_2-T_1) ($\beta = -.671$, $p < .001$) and that this association is maintained ($\beta = -.588$, $p = .003$) after controlling for race, gender, cg23193759, and cell-type variation. Similarly, using Horvath's methylomic age, Model 2 shows a similar pattern of findings. The results suggest

that smoking reduction or cessation was robustly associated with decelerated aging. Using G*Power software, a regression model with a sample size of 22 has 80% power to detect R^2 of .450 for Hannum's age and .291 for Horvath's age which suggests that our sample size was indeed adequate to test our theoretical model.

Using the methods of Hannum or Horvath, we then calculated degree of methylomic age acceleration for each of the paired (entry and exit) DNA samples. Then, the relationship between smoking cessation, as indicated by change in cg05575921 methylation, and degree of accelerated methylomic age was analyzed using a linear mixed model with repeated measures. The results from these analyses are shown in Figure 1A and Table 3. As expected, smoking cessation was associated with a significant improvement in Hannum's methylomic age (i.e. reduced acceleration of methylomic aging) from 6.205 ($SD = 3.784$) to .849 ($SD = 6.763$) on average. The simple paired t-test for quitters was significant ($t = 4.099$, $p = .002$), but the simple paired t-test for non-quitters was non-significant and in the opposite direction. The resulting effect capturing the interaction of time of assessment by quit status was highly significant as can be seen in Table 3. Notably, the effect size (f^2) of that change was .365 suggesting that the effects of smoking cessation on biological aging are large in addition to being rapid. There were no changes in pattern of effects when controls were entered for race, gender, cg23193759, and cell-type variation. As shown in Figure 1B and Table 3, we found an identical pattern of results by using Horvath's methylomic age. Finally, to insure robustness of effects, we repeated all analyses excluding outliers. The results showed no change in the pattern of results.

DISCUSSION

It is well known that for those who smoke, smoking cessation is the single most effective health-promoting intervention that a clinician can provide (27). Despite this only a minority of smokers attempt to quit smoking each year. These new preliminary results may give some smokers greater impetus to try, particularly those whose quit attempts are primarily focused on alcohol rather than smoking as was the case for the current sample. The directionality of the effect is consistent with known health effects of quitting smoking. For example, it is well established that if one stops smoking, within five years, the risk for stroke is nearly back to normal (28). Similarly, smoking cessation is also associated with reduced risk for a variety of other adverse health outcomes including heart attacks and cancer (29). Accordingly, effects on aging at the cellular level could fit comfortably with a list of other health benefits of smoking cessation while introducing a novel dimension of potential interest to those more worried about premature aging than specific health complications. Methylomic age is a sensitive marker of cellular-level aging, and many of the CpG sites included in the Hannum's and Horvath's measures are within or near genes with known functions in aging-related conditions, including cancer, DNA damage, tissue degradation, and chronic diseases (9, 10). Thus, the current results fit well with expectations for broad health effects following smoking. However, it may be more compelling to some considering smoking cessation that within one month, smoking cessation showed medium effect sizes on decelerated aging. It is unlikely that there is any other anti-aging remedy that works faster or more robustly. Despite consistencies with other known effects of smoking cessation, replication of the current findings in a larger data set and confirmation of subject smoking status, using both self-

report and biological measures, will be needed before the results of this promising study can be widely disseminated.

One obvious question raised by the current findings is “what are the pathways through which the decrease in age is mediated?” Unfortunately, the answer to that question is as complex as the explanation for smoking's impact on accelerated aging. Even with much larger samples sizes the window on pathways implicated in smoking and smoking cessation will be dependent on the CpG residues included in the particular clock used in the analyses. In our prior work, we showed that smoking had its effect on aging primarily by accelerating demethylation at a number of loci. Pathways identified using other versions of epigenetic clocks, which incorporate data from largely non-overlapping loci, may be different (8, 10). Hence, any answer to the question of pathways will necessarily be dependent on the type of clock being used as well as the sample size involved. A second reason that mechanisms may be hard to identify is that the epigenetic response to smoking is extraordinarily broad. At the nominal level, over one quarter of all CpG residues in the genome of peripheral white blood cell DNA from smokers are differentially methylated as compared to age and sex non-smoking controls (30). As individuals quit smoking, methylation at each of these residues shows a complex response with some residues showing little change while others show a gradual reversion to the population mean (31, 32). As a result, an exact understanding of the mechanisms through which decrease in methylomic age is mediated will not be easily answered.

Still, the current preliminary results are promising and suggest that epigenetic techniques will lead to a rich reservoir of “facts” that can be used to increase motivation for smoking. As such, the current findings, if replicated, may be useful with respect for those engaged in intervention, particularly in the context of motivational interviewing (MI) techniques (33). MI assists individuals in changing their perceptions of the consequences of behavior, increasing the likelihood of sustained change. Meta-analysis of its effects shows that subjects that receive MI are roughly 45% more likely to quit smoking than sham controls and that incorporation of MI into smoking therapy is recommended as part of standard best practices (34, 35). In contrast to many of the reasons given for smoking cessation such as reducing likelihood of death, disability, disease, appearing younger is a non-threatening approach goal that may be an easier point of focus for some patients. As a result, it may be much easier to engage in patient dialogues focused on relatively rapid benefits related to looking and feeling younger compared to the longer-term benefits of disease avoidance.

Although the results are convincing, some limitations must be noted. First our sample was small. Second, we examined smoking cessation in the context of a very broad life style change, treatment for alcohol problems, which may have amplified observed effects. Third, our samples were dominated by middle-aged White men, and so it would be useful to examine the robustness of effects for other racial groups and for females. Accordingly, the current results clearly need to be replicated with larger samples, that are more ethnically diverse and that include females, and that capture those quitting smoking in a range of contexts representative of quit attempts.

In summary, we report that smoking cessation is associated with decreased methylomic age. Current findings should be considered preliminary until replicated with larger and more diverse sets of patients. Power to detect changes was limited in the current study by sample size but enhanced by our use of measures at both intake and exit from treatment. Clinicians counseling patients should consider whether the addition of this information may be useful in persuading patients to quit smoking.

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Fig 1.

Explication of interaction effect reported in Table 3. Among patients who quit or significantly reduced smoking (white bar) there is a significant decrease in methylomic aging, leading to a significant difference at Time 2 (post-test) in accelerated methylomic aging between quitters and those continuing to smoke (black bar). Note: Deviation of Hannum's/Horvath's age from chronological age in years is calculated as Hannum's/Horvath's age minus chronological age; means shown with standard deviations in parentheses.

Table 1Descriptive statistics for variables used in the study ($N = 22$)

	Time 1 (Entry)				Time 2 (Exit)			
	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Chronological age	46.773	6.989	31	59				
Males	.773	.429	0	1				
Ethnicity								
White	.909	.294	0	1				
African American	.091	.294	0	1				
Marijuana use	.863	.351	0	1				
Smoking cessation					.500	.512	0	1
cg23193759	.156	.016	.120	.183	.160	.019	.125	.198
Methylomic age								
Hannum's age	51.828	8.047	37.461	68.850	50.400	8.656	33.976	64.527
Horvath's age	49.241	6.133	36.608	62.634	48.712	6.120	36.137	61.213
Cell types								
Lymphocyte cell	.570	.166	.099	.758	.619	.176	.206	.869
Monocytes cell	.264	.093	.133	.448	.202	.097	.055	.374

Note: smoking cessation: 2% methylation change at cg05575921; cg23193759: CpG is associated with alcohol consumption.

TABLE 2

OLS regression models of the results using methylomic age as the outcome ($N = 22$).

	Hannum's age (T2-T1)				Horvath's age (T2-T1)			
	Model 1A		Model 1B		Model 2A		Model 2B	
	<i>b</i>	β	<i>b</i>	β	<i>b</i>	β	<i>b</i>	β
cg05575921 (T2-T1)	-46.483 ** (11.492)	-.671	-40.766 ** (11.272)	-.588	-20.144 * (7.027)	-.539	-23.019 * (9.117)	-.617
Chronological age			.111 (.088)	.216			-.006 (.071)	-.021
cg23193759 (T2-T1)			-29.387 (35.355)	-.136			-11.467 (28.595)	-.099
Males			-.788 (1.303)	-.094			-.291 (1.054)	-.064
White			-3.963 * (1.765)	-.324			-.069 (1.427)	-.010
Marijuana use			3.516 $\dagger$ (1.687)	.343			.562 (1.364)	.102
Lymphocyte cell			.428 (3.602)	.020			-5.466 $\dagger$ (2.913)	-.468
Monocytes cell			-11.638 (6.949)	-.302			-2.405 (5.621)	-.116
Constant	-.855 (.600)		-2.026 (6.340)		-.280 (.367)		3.619 (5.127)	
<i>R</i> -square	.450		.765		.291		.470	
Statistical power for cg05575921 (T2-T1)	.981				.815			

Note. Unstandardized (*b*) and standardized coefficients (β) are shown with standard errors in parentheses.

$\dagger$ $p < .10$

* $p < .05$

** $p < .01$ (two-tailed tests).

TABLE 3

Linear mixed model with repeated measures analyses predicting accelerated methylomic aging from time, groups, and the interaction between time and group ($N = 22$)

	Hannum's age		Horvath's age	
	Model 1		Model 2	
	<i>b</i>	<i>p</i> -value	<i>b</i>	<i>p</i> -value
Group (1 = Smoking cessation)	−1.515 (1.751)	.387	.256 (1.654)	.877
Time (1 = Time 2)	1.114 (.770)	.148	.440 (.566)	.437
Time × Group	−2.954 ** (1.082)	.006	−2.041 * (.804)	.011
cg23193759	−.883 † (.470)	.061	−.519 (.364)	.153
Males	4.311 * (1.897)	.023	−.604 (1.826)	.741
White	−3.634 (2.703)	.179	−1.252 (2.622)	.633
Marijuana use	−.258 (2.525)	.919	1.640 (2.410)	.496
Lymphocyte cell	−1.644 (.606)	.007	.210 (.486)	.666
Monocytes cell	.798 (.474)	.093	−.007 (.355)	.984
Constant	5.426 (3.911)	.165	2.505 (3.769)	.506
Effect size (f^2) for Time × Group	.365		.190	
Deviance	215.417		199.973	
Random effect				
δ^2	2.475 ** (.757)	.002	1.302 ** (.406)	.002
τ_{constant}^2	11.150 ** (3.816)	.003	11.016 ** (3.659)	.003
τ_{time}^2	1.41e-13 (1.55e-12)	.464	6.00e-13 (5.77e-09)	.500

Note: Unstandardized (*b*) are shown with standard errors in parentheses. cg23193759 and cell-types are standardized by z-transformation (mean = 0 and $SD=1$). cg23193759: CpG is associated with alcohol consumption; δ^2 : within-individual variations in accelerated methylomic aging; τ_{constant}^2 : between-individual variations in initial status; τ_{time}^2 : between-individual variations in time effect. Effect size (Selya et al., 2012):

$$f_{\text{Time} \times \text{Group}}^2 = \left(R_{\text{Full}}^2 - R_{\text{without Time} \times \text{Group}}^2 \right) / \left(1 - R_{\text{Full}}^2 \right)$$

† $p < .10$

* $p < .05$

** $p < .01$ (two-tailed tests).