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Complete List of Authors:	Lange, Nancy; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine Sordillo, Joanne; Channing Laboratory, Brigham and Women's Hospital, Tarantini, Letizia; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Bollati, Valentina; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Sparrow, David; VA Boston Healthcare System and Boston University School of Medicine, Medicine Vokonas, Pantel; VA Boston Healthcare System and Boston University School of Medicine, Medicine Zanobetti, Antonella; Harvard School of Public Health, Environmental and Occupational Health Schwartz, Joel; Harvard School of Public Health, Environmental Health; Harvard School of Public Health, Environmental and Occupational Health Baccarelli, Andrea; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Litonjua, Augusto; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine DeMeo, Dawn; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine
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Alu and LINE-1 Methylation and Lung Function in the Normative Aging Study

Short Title: Global Methylation and Lung Function

Nancy E. Lange MD MPH,[1-3] Joanne Sordillo ScD[1,3] Letizia Tarantini BS,[4] Valentina Bollati PhD,[4] David Sparrow DSc,[5] Pantel Vokonas MD,[5] Antonella Zanobetti PhD,[6] Joel Schwartz PhD,[6] Andrea Baccarelli MD PhD,[4,6] Augusto A. Litonjua MD MPH[1-3], Dawn L. DeMeo MD MPH[1-3]

[1]Channing Laboratory, [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA; [3]Harvard Medical School, Boston, MA, USA; [4]Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health, Università degli Studi di Milano and IRCCS Maggiore Policlinico Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy; [5]Veterans Administration Boston Healthcare System and Department of Medicine, Boston University School of Medicine, Boston, MA, USA; [6] Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

Corresponding author:

Nancy E. Lange, MD, MPH
Channing Laboratory
Brigham and Women's Hospital
181 Longwood Avenue, room 454
Boston, MA 02115, USA
Phone: 617-525-0874
Fax: 617-525-0958
Email: renal@channing.harvard.edu

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ABSTRACT

Objectives: To investigate the association between methylation of transposable elements *Alu* and LINE-1 and lung function.

Design: Cohort study

Setting: Outpatient Veterans Administration facilities in greater Boston, MA, USA.

Participants: Subjects from the Veterans Administration Normative Aging Study, a longitudinal study of aging, evaluated between 1999 and 2007.

Primary and secondary outcome measures: Primary predictor was methylation, assessed using PCR-pyrosequencing after bisulfite treatment. Primary outcome was lung function as assessed by spirometry, performed according to ATS/ERS guidelines at the same visit as the blood draws.

Results: In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking and race, *Alu* hypomethylation was associated with lower FEV_1 (β=28ml per 1% change in *Alu* methylation, p= .017), FVC (β=27ml, p=.06) and lower FEV_1/FVC (β=0.3%, p=.058). In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking, % lymphocytes, race and baseline lung function, LINE-1 hypomethylation was associated with more rapid decline of FEV_1 (β=6.9ml/yr per 1% change in LINE-1 methylation, p= .005) and of FVC (β=9.6ml/yr, p=.002).

Conclusions: In multiple regression analysis, *Alu* hypomethylation was associated with lower lung function, and LINE-1 hypomethylation was associated with more rapid lung function decline. Future studies should aim to replicate these findings and determine if *Alu* or LINE-1 hypomethylation may be due to specific and modifiable environmental exposures.

Article Summary:

Article Focus:

Association between methylation, an epigenetic marker, and lung function

Key Message(s):

Hypomethylation of transposable elements is associated with lower lung function and more rapid lung function decline in a cohort of elderly North American men.

Strengths and Limitations:

First study to evaluate methylation of transposable elements in relation to lung function.

Difficult to interpret implications of methylation patterns in transposable elements.

INTRODUCTION

Lung function has both environmental and genetic determinants.[1-4] Epigenetic changes, which may influence gene expression patterns without changing DNA sequence, may mediate the effects of environmental exposures on disease outcomes. DNA methylation, one type of epigenetic change, is the reversible addition of a methyl group to cytosine nucleotides. Methylation changes may or may not persist over time in the human genome, as epigenetic marks are highly plastic.

A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as *Alu* and LINE-1 (long interspersed nuclear element) which are among the most common and best characterized repetitive elements.[5-7] *Alu* is the most abundant of the SINEs (short-interspersed nuclear elements) with over one million copies per genome.[8] *Alu* elements compose approximately 11% of the mass of human genome and contain 30% of its methylation sites.[6 9] LINE-1 elements are present at over half a million copies.[8 10] Methylation of repetitive elements such as *Alu* and LINE-1 has been shown to correlate with total genomic methylation content.[10 11] Hypomethylation in transposable elements is associated with higher genomic instability and alterations or deregulation of gene expression.[12 13]

Prior studies have found associations between methylation of *Alu* or LINE-1 elements and various diseases including multiple cancers,[6] cardiovascular disease[14-16] and neurologic disease[17] as well as with markers of inflammation[18] and the inflammatory response.[19] Studies on gene-specific methylation and non-neoplastic lung disease have found associations between GATA4, CDKN2A (p16) and lung function and an interaction with wood smoke exposure.[20] To our knowledge no prior study has investigated associations between methylation of transposable elements and non-neoplastic lung disease. Moreover, case-control studies such as are common in genomic studies are more problematic for epigenetic marks since sampling cases after disease onset makes it impossible to determine whether epigenetic changes preceded the disease. Hence cohort studies or nested case-control studies within cohorts are particularly valuable. Our aim was to examine whether methylation of the repetitive elements *Alu* and LINE-1 was associated with measures of lung function, COPD status, and longitudinal change in lung function in a cohort of men, the Normative Aging Study. Preliminary results from these analyses were previously reported in abstract form.[21]

METHODS

Population:

Study participants were from the Veterans Administration Normative Aging Study, an ongoing longitudinal study of aging established in 1963.[22] This is a cohort of 2,280 healthy male volunteers from the greater Boston, MA, area who were 21–80 years of age at entry and who enrolled after an initial health screening determined that they were free of known chronic medical conditions. Participants were reevaluated every 3–5 years using detailed on-site physical examinations and questionnaires. The study was approved by the Institutional Review Boards of all participating institutions. All participants gave written informed consent.

For this study, individuals evaluated at least once between January 1999 and June 2007 with a blood sample drawn and concomitant spirometry were included. During the study period, this included 663 total subjects, 194 of whom reported for examination two times, for a total of 857 samples collected.

Measures:

Spirometry was performed according to ATS/ERS guidelines.[23] All spirometric values are pre-bronchodilator. Percent predicted values for FEV_1 and FVC were calculated using equations by Crapo *et al*.[24] COPD was defined as GOLD stage II or higher (FEV_1/FVC<70% and FEV_1<80% predicted).[25] Techniques for assessing DNA methylation were previously described in detail.[26 27] Briefly, we performed DNA methylation assessment of *Alu* and LINE-1 repetitive elements on bisulfite-treated blood leukocyte DNA using highly quantitative polymerase chain reaction (PCR)–pyrosequencing technology. The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Each marker was tested in triplicate, and their average was used in the statistical analysis.

Statistical Analysis

Analyses for cross-sectional associations were performed using repeated measures with adjustment for the correlation between measurements in a given individual using mixed effects models (PROC MIXED) for continuous outcomes (FEV_1, FVC, FEV_1/FVC) and generalized estimating equations (PROC GENMOD) for binary outcomes (COPD). Covariates in multivariable models were chosen for their clinical relevance and strong bivariate associations ($p \leq .05$) with lung function or change in effect estimate criterion of >10% after addition to the model and included age, height, race, pack-years of cigarette smoking, smoking status (dichotomized as current vs. ex and never smokers) and body mass index (BMI). We also considered variables previously associated with methylation of repetitive elements[28] such as folate intake, alcohol intake, total white blood cell count and both percent neutrophils and percent lymphocytes. With the exception of percent lymphocytes, which was included in models with LINE-1 only, these covariates were not included in final models because they were not associated with *Alu* or LINE-1 methylation and did not meet the change in estimate criteria. Because Figure 2 depicts bivariate relationships, percent predicted values were used for both FEV_1 and FVC to show an adjusted value; actual values for FEV_1 and FVC were utilized in multivariable models. To examine associations between methylation of *Alu* and LINE-1 and change in lung function over time, a rate was calculated using the change in lung function between the two time points divided by the amount of time elapsed between the two measurements in years. This value was utilized as an outcome and analyzed using multivariate linear regression models. A total of 301 subjects had a second lung function data point subsequent to the initial methylation value. SAS version 9.1 (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Baseline characteristics of the 663 individuals included in this study are shown in **Table 1**. All subjects were male and the majority (640, 97%) of white race. Few subjects were current smokers and 197 (30%) were never smokers. There was wide variation in lung function values. Of the 107 individuals with COPD, 77 (72%) were GOLD stage II, 26 were stage III and 4 were stage IV; overall 20 (20%) of the individuals with COPD were current smokers.

The distribution of percentage methylation of both *Alu* and LINE-1 elements among the population is shown in **Figure 1**.

Bivariate relationships between *Alu* and LINE-1 with outcomes and covariates considered for inclusion in the multivariable model are shown in **Table 2**. *Alu* methylation was associated or showed a trend towards association positively with FEV_1, BMI and FEV_1/FVC and negatively with age and COPD status. LINE-1 was positively associated with current smoking and negatively with percent lymphocytes. Neither *Alu* nor LINE-1 was associated with FVC, pack-years of smoking or ever smoking status. Folate intake, alcohol intake, total white blood cell count and percent neutrophils were not significantly associated with *Alu* or LINE-1 in bivariate analyses. There was no significant relationship between methylation of *Alu* and LINE-1 to each other (p=.23).

In multivariate models that included age, height, race, pack-years of smoking, smoking status, and BMI, *Alu* methylation was positively associated with FEV_1, FEV_1/FVC and showed a trend towards association with FVC. When analyzed excluding current smokers, there was a negative association between *Alu* and COPD (higher *Alu* with lower odds of COPD) that was statistically significant (OR 0.80 [0.64, 0.99] p=.046). There were no significant associations between LINE-1 and any of the outcomes. (**Table 3**). **Figure 2** depicts the bivariate associations of *Alu* methylation with FEV_1 % predicted, FVC % predicted and FEV_1/FVC.

We also analyzed whether methylation of *Alu* and LINE-1 were associated with rate of change in lung function in a subset of participants who had two consecutive lung function measures (N=301). The mean number of years elapsed between measurements was 4.03 (SD 1.23). Models were adjusted for baseline FEV_1, FVC or FEV_1/FVC (respectively for the given outcome) as well as age, pack-years of smoking, BMI, height, race, percent lymphocytes and smoking status. LINE-1 but not *Alu* was associated negatively with rate of change in FEV_1 and FVC (p<.005). Neither measure was associated with rate of change of FEV_1/FVC. (**Table 4**) Including both *Alu* and LINE-1 in the models did not change the results (data not shown).

DISCUSSION

We examined associations between methylation levels of the repetitive elements *Alu* and LINE-1 in a cohort of elderly men in relation to lung function and COPD status. In cross-sectional analyses, we found that *Alu* hypomethylation was associated with lower FEV_1 with a trend towards association with lower FVC and FEV_1/FVC. LINE-1 hypomethylation was associated with more rapid lung function decline (FEV_1 and FVC).

Prior studies have found associations between methylation of repetitive transposable elements such as *Alu* and LINE-1 and several diseases including multiple cancers,[6] cardiovascular disease[14-16] and neurologic disease[17] as well as with markers of inflammation.[18] To our knowledge this is the first study to examine associations between methylation of *Alu* and LINE-1 transposable elements and measures of lung function.

Previous work has shown that in normal subjects, *Alu* hypomethylation is associated with increased age[7 29], greater alcohol use and gender (lower in males).[28] In this same cohort (NAS), hypomethylation has been associated with higher incidence of cancer in general and lung cancer specifically (LINE-1), as well as higher mortality from cancer (*Alu* and LINE-1).[30] A variety of environmental exposures such as lead [31] traffic particles[27] organic pollutants,[32] metals, air pollutants and endocrine disrupting agents[33] may all affect global methylation levels, specifically some that may relate to lung function such as various air pollutants.

Hypomethylation of transposable elements may or may not be causally linked to lower lung function and faster rates of lung function decline. Lower methylation of *Alu* and LINE-1 may increase their activity as retrotransposable sequences, leading to greater genomic instability and more mutations.[12] Furthermore, oxidative damage caused by environmental exposures may cause hypomethylation.[34] This may lead to alteration of gene expression through a variety of mechanisms including disrupting transcription factor binding sites or reading frames, altering regulatory sequences, altering methylation patterns of gene promoters, or introducing new transcription factor binding sites.[35-37] *Alu* elements specifically are preferentially found in gene-rich regions.[38] Black carbon and increased $PM_{2.5}$ exposure[27] as well as PM_{10} exposure[33] have been found to be inversely associated with LINE-1 and both *Alu* and LINE-1 methylation, respectively which may impact lung function or lung function decline.[39] LINE-1 hypomethylation may also increase transcription of genes that have LINE-1 in regulatory regions. It is possible that other specific environmental or dietary exposures previously not known to be associated with lung function may be mediated through epigenetic changes such as *Alu* or LINE-1 hypomethylation. Alternatively, this may be a marker of a specific exposure but not causally linked to lower lung function. Lastly, because *Alu* methylation decreases with increasing age, as does lung function, our findings may represent some other measure of 'aging' or exposures resulting in similar processes beyond just chronological age.[7] As our understanding of epigenetic processes and the exposures that affect these processes increases, the implications of our findings will become clearer.

These data must be interpreted in the context of the study design. Our study was limited to elderly men the majority of whom were white, and our findings may or may not be generalizable to other populations. It is difficult to know how to interpret methylation of retrotransposons, as opposed to gene-specific methylation, in relation to specific outcomes such as lung function and lung function decline. Future studies should include gene-specific methylation analyses to elucidate mechanisms by which methylation changes may relate to these outcomes. We did not control for a variety of environmental exposures that may be associated with both lung function and methylation, however, alteration in methylation patterns may be the pathway through which these changes are mediated and thus including these exposures in multivariate models would be overadjusting. Methylation levels vary in different tissue types and it is possible that assessments of methylation in white blood cells may not reflect alterations seen in lung tissue. However, systemic processes involving white blood cells, such as inflammation, may play a role in the pathophysiology of lung function decline[40] and may nonetheless be markers of specific exposures (such as cigarette smoking) that exert a systemic effect.

In summary, we found that relative hypomethylation of *Alu* was associated with lower lung function measures, and that LINE-1 hypomethylation was associated with more rapid lung function decline. Future studies on both gene-specific methylation as well as exposures related to methylation of retrotransposons will improve our understanding of the relationship between epigenetic changes and lung function, potentially informing new diagnostic and therapeutic approaches to lung function decline.

Table 1: Baseline characteristics of 663 individuals from the Normative Aging Study

	Mean (SD) or N (%)	Range
Age	72.7 (6.7)	(55.3-100.9)
BMI	28.5 (4.2)	(19.4-52.3)
Pack-years*	30.6 (24.8)	(0.1-145.5)
Current smokers	43 (7%)	
Ever smokers	466 (70%)	
FEV_1	2.70 (.64)	(.85-4.69)
FEV_1%pred	81 (17)	(28-125)
FVC	3.56 (0.72)	(1.63-6.32)
FVC%pred	82 (14)	(43-124)
FEV_1/FVC	75 (8)	(36-94)
COPD	107 (16%)	
Alu	26.4 (1.1)	(22.8-32.4)
LINE-1	76.8 (1.8)	(70.1-84.6)

*pack-years in current or ex-smokers only

Table 2: Bivariate associations between *Alu*, LINE-1 and other covariates

	Alu		**LINE-1**	
	β	**p value**	**β**	**p value**
Age	-0.3	0.07	-0.2	0.1
BMI	0.106	0.059	0.054	0.17
Current smoking	0.35	0.14	0.697	0.0002
% Lymphocytes	0.08	0.73	-0.31	0.04
FEV_1	0.024	0.06	-0.006	0.53
FEV_1/FVC	0.31	0.046	-0.05	0.67
COPD	OR .87 [.73, 1.03]	0.1	1.02 [.92, 1.13]	0.76

Table 3: Multivariate models for lung function and both *Alu* and LINE-1 methylation*

	Alu		LINE-1	
	β	p value	β	p value
FEV_1	0.028	0.017	-0.015	0.08
FVC	0.027	0.06	-0.017	0.11
FEV_1/FVC	0.3	0.057	-0.092	0.44
COPD	0.85 [0.71, 1.03]	0.09	1.01 [.89, 1.15]	0.83

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status. Models with LINE-1 also include % lymphocytes.

Table 4: Multivariate models for rate of change in lung function (in liters/yr) and both *Alu* and LINE-1 methylation*

	FEV_1 rate		FVC rate		ratio rate	
	β	p val	β	p val	β	p val
Alu	-0.0028	0.49	-0.00098	0.84	-0.00079	0.17
LINE-1	-0.0069	0.005	-0.0096	0.0021	0.00005	0.89

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status, and baseline FEV_1, FVC or FEV_1/FVC respectively depending on outcome. Models with LINE-1 also adjusted for % lymphocytes.

Figure Legends

Figure 1: Distribution (median, interquartile range) of percentage *Alu* and LINE-1 methylation

Figure 2: ***Alu* Methylation and Lung Function**
Bivariate associations between *Alu* and FEV_1%predicted, FVC%predicted and FEV_1/FVC

* For FEV_1/FVC y axis is percent, not percent predicted

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AUTHOR CONTRIBUTIONS: N.E.L. designed the study, performed the data analysis, and prepared the manuscript. J.S. contributed to the data analysis and provided critical revision of the manuscript. L.T. contributed to data collection and provided critical revision of the manuscript. V.B. contributed to data collection and provided critical revision of the manuscript. D.S. and P.V. were involved in conception of the study and critical revision of the manuscript. A.Z. contributed to data collection and provided critical revision of the manuscript. J.S. contributed to study design and provided critical revision of the manuscript. A.B. contributed to data collection and provided critical revision of the manuscript. A.A.L. contributed to study design, assisted with the data analysis, and provided critical revision of the manuscript. D.L.D. designed the study, assisted with the data analysis, and provided critical revision of the manuscript.

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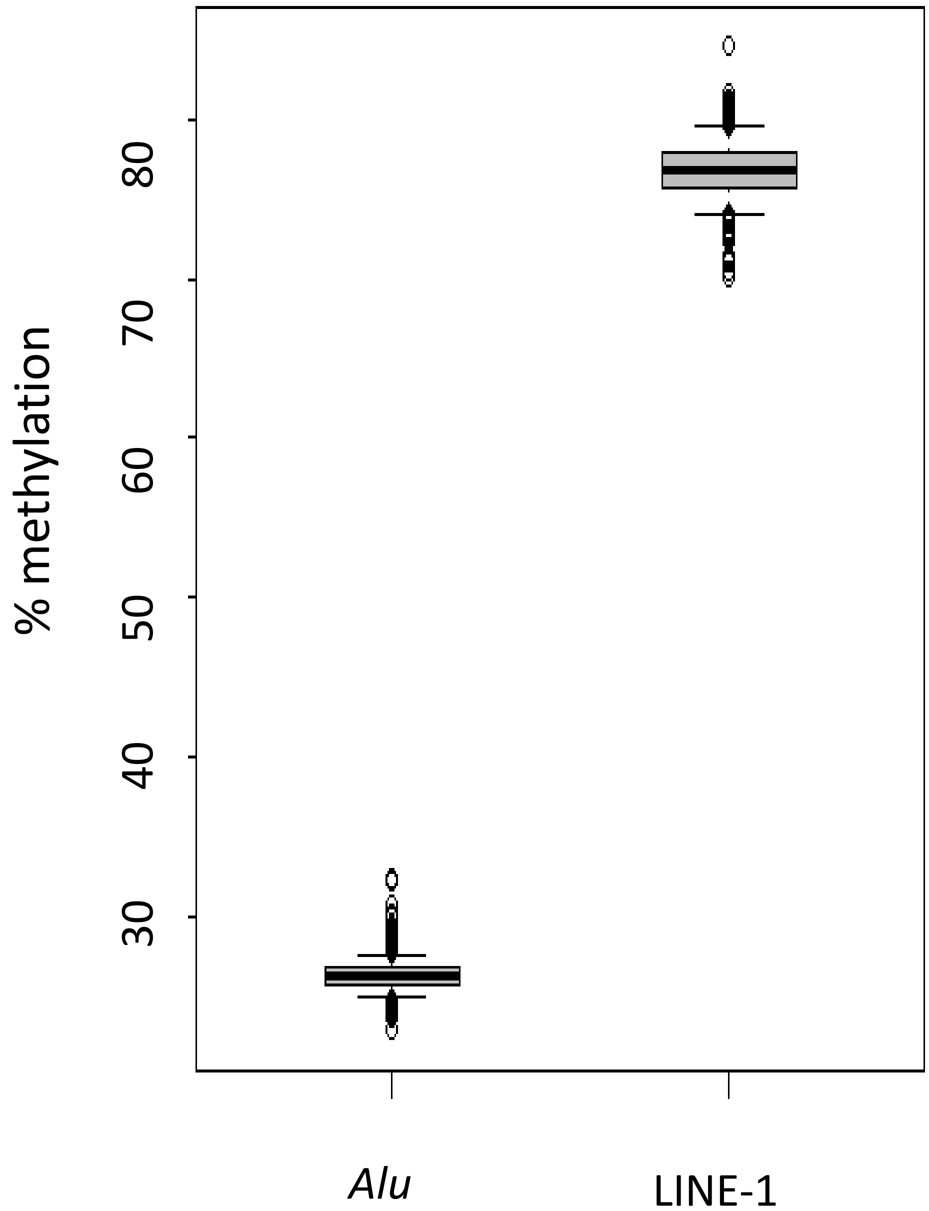
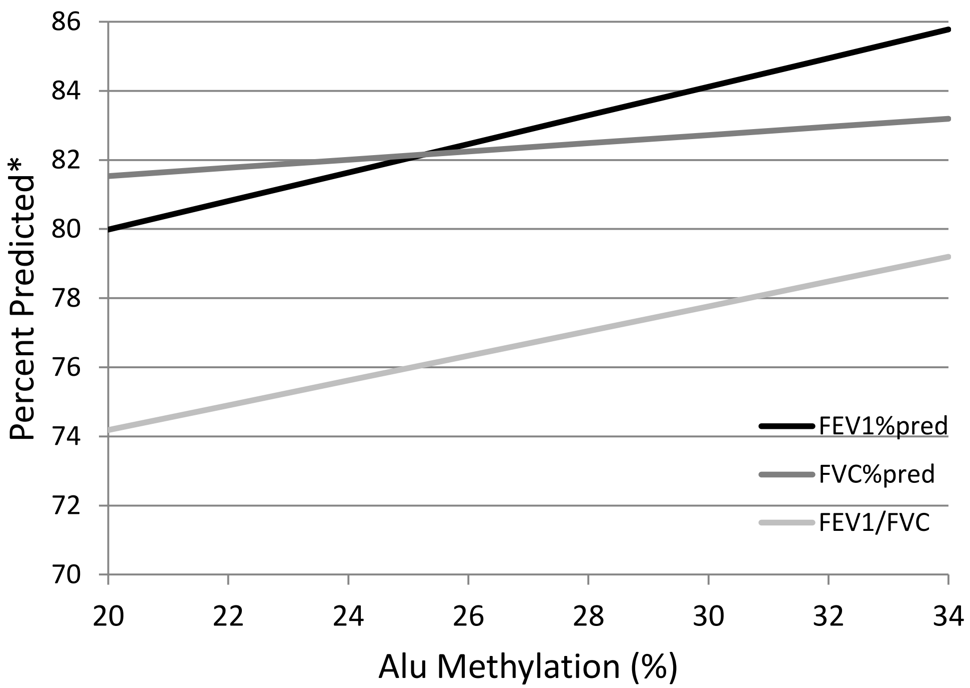

% methylation
30
40
50
60
70
80
Alu
LINE-1


86
84
82
80
78
76
74
72
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22
24
26
28
30
32
34
Percent Predicted*
Alu Methylation (%)
FEV1%pred
FVC%pred
FEV1/FVC

Alu and LINE-1 Methylation and Lung Function in the Normative Aging Study

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Complete List of Authors:	Lange, Nancy; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine Sordillo, Joanne; Channing Laboratory, Brigham and Women's Hospital, Tarantini, Letizia; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Bollati, Valentina; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Sparrow, David; VA Boston Healthcare System and Boston University School of Medicine, Medicine Vokonas, Pantel; VA Boston Healthcare System and Boston University School of Medicine, Medicine Zanobetti, Antonella; Harvard School of Public Health, Environmental and Occupational Health Schwartz, Joel; Harvard School of Public Health, Environmental Health; Harvard School of Public Health, Environmental and Occupational Health Baccarelli, Andrea; Universita degli Studi di Milano and IRCCS MAggiore Policlinico Hospital, Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health Litonjua, Augusto; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine DeMeo, Dawn; Brigham and Women's Hospital, Channing Laboratory/Division of Pulmonary and Critical Care Medicine

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Alu and LINE-1 Methylation and Lung Function in the Normative Aging Study

Short Title: Global Methylation and Lung Function

Nancy E. Lange MD MPH,[1-3] Joanne Sordillo ScD[1,3] Letizia Tarantini BS,[4] Valentina Bollati PhD,[4] David Sparrow DSc,[5] Pantel Vokonas MD,[5] Antonella Zanobetti PhD,[6] Joel Schwartz PhD,[6] Andrea Baccarelli MD PhD,[4,6] Augusto A. Litonjua MD MPH[1-3], Dawn L. DeMeo MD MPH[1-3]

[1]Channing Division of Network Medicine, [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA; [3]Harvard Medical School, Boston, MA, USA; [4]Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health, Università degli Studi di Milano and IRCCS Maggiore Policlinico Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy; [5]Veterans Administration Boston Healthcare System and Department of Medicine, Boston University School of Medicine, Boston, MA, USA; [6] Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

Corresponding author:

Nancy E. Lange, MD, MPH
Channing Laboratory
Brigham and Women's Hospital
181 Longwood Avenue, room 454
Boston, MA 02115, USA
Phone: 617-525-0874
Fax: 617-525-0958
Email: renal@channing.harvard.edu

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ABSTRACT

Objectives: To investigate the association between methylation of transposable elements *Alu* and LINE-1 and lung function.

Design: Cohort study

Setting: Outpatient Veterans Administration facilities in greater Boston, MA, USA.

Participants: Subjects from the Veterans Administration Normative Aging Study, a longitudinal study of aging in men, evaluated between 1999 and 2007. The majority (97%) of subjects were white.

Primary and secondary outcome measures: Primary predictor was methylation, assessed using PCR-pyrosequencing after bisulfite treatment. Primary outcome was lung function as assessed by spirometry, performed according to ATS/ERS guidelines at the same visit as the blood draws.

Results: In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking and race, *Alu* hypomethylation was associated with lower FEV_1 (β=28ml per 1% change in *Alu* methylation, p= .017) and showed a trend towards association with a lower FVC (β=27ml, p=.06) and lower FEV_1/FVC (β=0.3%, p=.058). In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking, % lymphocytes, race and baseline lung function, LINE-1 hypomethylation was associated with more rapid decline of FEV_1 (β=6.9ml/yr per 1% change in LINE-1 methylation, p= .005) and of FVC (β=9.6ml/yr, p=.002).

Conclusions: In multiple regression analysis, *Alu* hypomethylation was associated with lower lung function, and LINE-1 hypomethylation was associated with more rapid lung function decline in a cohort of older and primarily white men from North America. Future studies should aim to replicate these findings

and determine if *Alu* or LINE-1 hypomethylation may be due to specific and modifiable environmental exposures.

Article Summary:

Article Focus:

Association between methylation, an epigenetic marker, and lung function

Key Message(s):

Relative hypomethylation of transposable elements is associated with lower lung function and more rapid lung function decline in a cohort of older North American primarily white men.

Strengths and Limitations:

First study to evaluate methylation of transposable elements in relation to lung function.

Difficult to interpret implications of methylation patterns in transposable elements.

INTRODUCTION

Lung function has both environmental and genetic determinants.[1-5] Epigenetic variation, which may influence gene expression patterns without changing DNA sequence, may mediate the effects of environmental exposures on disease outcomes. DNA methylation, one type of epigenetic change, is the reversible addition of a methyl group to cytosine nucleotides. Methylation changes may or may not persist over time in the human genome, as epigenetic marks are highly plastic.

A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as *Alu* and LINE-1 (long interspersed nuclear element) which are among the most common and best characterized repetitive elements.[6-8] *Alu* is the most abundant of the SINEs (short-interspersed nuclear elements) with over one million copies per genome.[9] *Alu* elements compose approximately 11% of the mass of human genome and contain 30% of its methylation sites.[7 10] LINE-1 elements are present at over half a million copies.[9 11] Methylation of repetitive elements such as *Alu* and LINE-1 has been shown to correlate with total genomic methylation content.[11 12] Hypomethylation in transposable elements is associated with higher genomic instability and alterations or deregulation of gene expression.[13 14]

Prior studies have found associations between methylation of *Alu* or LINE-1 elements and various diseases including multiple cancers,[7] cardiovascular disease[15-17] and neurologic disease[18] as well as with markers of inflammation[19] and the inflammatory response.[20] Studies on gene-specific methylation and non-neoplastic lung disease have found associations between GATA4, CDKN2A (p16) and lung function and an interaction with wood smoke exposure,[21] as well as multiple genes in association with COPD presence and severity.[22] To our knowledge no prior study has investigated associations between methylation of transposable elements and non-neoplastic lung disease. Moreover, case-control studies which are common in genomic studies are more problematic for epigenetic marks since sampling cases after disease onset makes it impossible to determine whether epigenetic changes preceded or resulted from the disease. Hence cohort studies or nested case-control studies within cohorts are particularly valuable. Our aim was to examine whether methylation of the repetitive elements *Alu* and LINE-1 was associated with measures of lung function, COPD status, and longitudinal change in lung function in a cohort of men, the Normative Aging Study. Preliminary results from these analyses were previously reported in abstract form.[23]

METHODS

Population:

Study participants were from the Veterans Administration Normative Aging Study, an ongoing longitudinal study of aging established in 1963.[24] This is a cohort of 2,280 healthy male volunteers from the greater Boston, MA, area who were 21–80 years of age at entry and who enrolled after an initial health screening determined that they were free of known chronic medical conditions. Participants were reevaluated every 3–5 years using detailed on-site physical examinations and questionnaires. The study was approved by the Institutional Review Boards of all participating institutions. All participants gave written informed consent.

Prior to 1999, 706 subjects had died and others were either lost to follow-up, being followed by questionnaire only, or had no blood samples left for analyses (n=792). Seven hundred and eighty two subjects had blood samples that were available for methylation analysis resulting in 704 subjects with unique IDs and methylation data as previously described.[25 26] For this study, individuals evaluated at least once between March 1999 and June 2007 with methylation data and concomitant spirometry were included. During the study period, this included 663 total subjects, 194 of whom reported for blood draw two times, for a total of 857 samples collected. For the analysis of lung function decline, a second spirometric measurement was available on 301 subjects who had had an initial blood draw for methylation measurement.

Measures:

Spirometry was performed as previously described [27] and was repeated up to a maximum of 8 spirograms, so that at least 3 acceptable spirograms were obtained, at least 2 of which were reproducible with FEV_1 and FVC measurements within 5% of each spirogram; the best of these 2 values was selected from a given encounter. Acceptability of spirograms was judged according to ATS standards.[28 29] All spirometric values are pre-bronchodilator. Percent predicted values for FEV_1 and FVC were calculated using equations by Crapo *et al*.[30] COPD was defined as GOLD stage II or higher (FEV_1/FVC<70% and FEV_1<80% predicted).[31] Techniques for assessing DNA methylation were previously described in detail.[32 33] Briefly, we performed DNA methylation assessment of *Alu* and LINE-1 repetitive elements on bisulfite-treated blood leukocyte DNA using highly quantitative polymerase chain reaction (PCR)–pyrosequencing technology. The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Each marker was tested in triplicate, and their average was used in the statistical analysis.

Statistical Analysis

Analyses for cross-sectional associations were performed using repeated measures with adjustment for the correlation between measurements in a given individual using mixed effects models (PROC MIXED) for continuous outcomes (FEV_1, FVC, FEV_1/FVC) and generalized estimating equations (PROC GENMOD) for binary outcomes (COPD). Covariates in multivariable models were chosen for their clinical relevance and strong bivariate associations (p≤.05) with lung function or change in effect estimate criterion of >10% after addition to the model and included age, height, race, pack-years of cigarette smoking, smoking status (dichotomized as current vs. ex and never smokers) and body mass index (BMI). We also considered variables previously associated with methylation of repetitive elements[34] such as folate intake, alcohol intake, total white blood cell count and both percent neutrophils and percent lymphocytes. With the exception of percent lymphocytes, which was included in models with LINE-1 only, these covariates were not included in final models because they were not associated with *Alu* or LINE-1 methylation and did not meet the change in estimate criteria. Because Figure 2 depicts bivariate relationships, percent predicted values were used for both FEV_1 and FVC to show an adjusted value; actual values for FEV_1 and FVC were utilized in multivariable models. To examine associations between methylation of *Alu* and LINE-1 and change in lung function over time, a rate was calculated using the change in lung function between the two time points divided by the amount of time elapsed between the two measurements in years. This value was utilized as an outcome and analyzed using multivariate linear regression models. A total of 301 subjects had a second lung function data point subsequent to the initial methylation value. SAS version 9.1 (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Baseline characteristics of the 663 individuals included in this study as well as of the subset of 301 individuals with two lung function measures are shown in **Table 1**. All subjects were male and the majority (640, 97%) of white race. Forty-three subjects (7%) were current smokers and 197 (30%) were never smokers. There was wide variation in lung function values. Of the 107 individuals with COPD, 77 (72%) were GOLD stage II, 26 were stage III and 4 were stage IV; overall 20 (20%) of the individuals with COPD were current smokers.

The distribution of percentage methylation of both *Alu* and LINE-1 elements among the population and stratified by smoking status is shown in **Figure 1**.

Bivariate relationships between *Alu* and LINE-1 methylation with outcomes and covariates considered for inclusion in the multivariable model are shown in **Table 2**. *Alu* methylation was associated or showed a trend towards association positively with FEV_1, BMI and FEV_1/FVC and negatively with age and COPD status. LINE-1 methylation was positively associated with current smoking and negatively with percent lymphocytes. Neither *Alu* nor LINE-1 methylation was associated with FVC, pack-years of smoking or ever smoking status. Folate intake, alcohol intake, total white blood cell count and percent neutrophils were not significantly associated with *Alu* or LINE-1 methylation in bivariate analyses. There was no significant relationship between methylation of *Alu* and LINE-1 to each other (p=.23).

In multivariate models that included age, height, race, pack-years of smoking, smoking status, and BMI, *Alu* methylation was positively associated with FEV_1, and showed a trend towards association with FVC and FEV_1/FVC. Because of recent data suggesting that current smoking status may have differential effects on methylation[35 36] and because this may relate to disease outcome or risk, we investigated whether our results would change if current smokers were excluded from the analyses. Higher *Alu* methylation was still associated with lower odds of COPD (OR 0.80 [0.64, 0.99] p=.046). In analyses of lung function measures, results were in the same direction but were no longer significant except for FEV_1/FVC (FEV_1 p=0.17, FVC p=0.7, FEV_1/FVC p=.029). There were no significant associations between LINE-1 methylation and any of the cross-sectional outcomes (**Table 3**). **Figure 2** depicts the bivariate associations of *Alu* methylation with FEV_1 % predicted, FVC % predicted and FEV_1/FVC.

We also analyzed whether methylation of *Alu* and LINE-1 were associated with rate of change in lung function in a subset of participants who had two consecutive lung function measures (N=301). The mean number of years elapsed between measurements was 4.03 (SD 1.23). Models were adjusted for baseline FEV_1, FVC or FEV_1/FVC (respectively for the given outcome) as well as age, pack-years of smoking, BMI, height, race, percent lymphocytes and smoking status. Relative hypomethylation in LINE-1 but not *Alu* was associated with faster rate of decline in FEV_1 and FVC ($p<.005$). Neither measure was associated with rate of change of FEV_1/FVC. (**Table 4**) Including both *Alu* and LINE-1 methylation in the models did not change the results (data not shown). Because of prior associations between methylation of repetitive elements and cardiovascular disease[15-17], we repeated both cross-sectional and longitudinal analyses including variables for cardiovascular disease (myocardial infarction, stroke, angina, hypertension, ischemic heart disease) and diabetes and found no difference in the results (data not shown). Analyses were also repeated in whites only to determine whether results might be due to population stratification and results did not change (data not shown). Analyses excluding current smokers remained significant (data not shown).

DISCUSSION

We examined associations between methylation levels of the repetitive elements *Alu* and LINE-1 in a cohort of older men in relation to lung function and COPD status. In cross-sectional analyses, we found that *Alu* hypomethylation was associated with lower FEV_1 with a trend towards association with lower FVC and FEV_1/FVC. LINE-1 hypomethylation was associated with more rapid lung function decline (FEV_1 and FVC).

Prior studies have found associations between methylation of repetitive transposable elements such as *Alu* and LINE-1 and several diseases including multiple cancers,[7] cardiovascular disease[15-17] and neurologic disease[18] as well as with markers of inflammation.[19] To our knowledge this is the first study to examine associations between methylation of *Alu* and LINE-1 transposable elements and measures of lung function.

Previous work has shown that in normal subjects, *Alu* hypomethylation is associated with increased age[8 37], greater alcohol use and gender (lower in males).[34] In this same cohort (NAS), hypomethylation has been associated with higher incidence of cancer in general and lung cancer specifically (LINE-1 methylation), as well as higher mortality from cancer (*Alu* and LINE-1 methylation).[38] A variety of environmental exposures such as lead [39] traffic particles[33] organic pollutants,[40] metals, air pollutants and endocrine disrupting agents[41] may all affect global methylation levels, specifically some that may relate to lung function such as various air pollutants.

Hypomethylation of transposable elements may or may not be causally linked to lower lung function and faster rates of lung function decline. Lower methylation of *Alu* and LINE-1 may increase their activity as retrotransposable sequences, leading to greater genomic instability and more mutations.[13] Furthermore, oxidative damage caused by environmental exposures may cause hypomethylation.[42] This may lead to alteration of gene expression through a variety of mechanisms including disrupting transcription factor binding sites or reading frames, altering regulatory sequences, altering methylation patterns of gene promoters, or introducing new transcription factor binding sites.[43-45] *Alu* elements specifically are preferentially found in gene-rich regions.[46] Black carbon and increased $PM_{2.5}$ exposure[33] as well as PM_{10} exposure[41] have been found to be inversely associated with LINE-1 methylation and both *Alu* and LINE-1 methylation, respectively which may impact lung function or lung function decline.[47] LINE-1 hypomethylation may also increase transcription of genes that have LINE-1 in regulatory regions. It is possible that other specific environmental or dietary exposures previously not known to be associated with lung function may be mediated through epigenetic changes such as *Alu* or LINE-1 hypomethylation. Alternatively, this may be a marker of a specific exposure but not causally linked to lower lung function. Lastly, because *Alu* methylation decreases with increasing age, as does lung function, our findings may represent some other measure of 'aging' or exposures resulting in similar processes beyond just chronological age.[8] As our understanding of epigenetic processes and the exposures that affect these processes increases, the implications of our findings will become clearer.

These data must be interpreted in the context of the study design. Our study was limited to older men the majority of whom were white, and our findings may or may not be generalizable to other populations. It is difficult to know how to interpret methylation of retrotransposons, as opposed to gene-specific methylation, in relation to specific outcomes such as lung function and lung function decline. Future studies in this and other cohorts should include gene-specific methylation analyses similar to Qiu *et al*[22] to elucidate mechanisms by which methylation changes may relate to these outcomes. We did not control for a variety of environmental exposures that may be associated with both lung function and methylation. However, alteration in methylation patterns may be the pathway through which these changes are mediated and thus including these exposures in multivariate models would be overadjusting. Methylation levels vary in different tissue types and it is possible that assessments of methylation in white blood cells may not reflect alterations seen in lung tissue. However, systemic processes involving white blood cells, such as inflammation, may play a role in the pathophysiology of lung function decline[48] and may nonetheless be markers of specific exposures (such as cigarette smoking) that exert a systemic effect.

In summary, we found that relative hypomethylation of *Alu* was associated with lower lung function measures, and that LINE-1 hypomethylation was associated with more rapid lung function decline. Future studies on both gene-specific methylation as well as exposures related to methylation of retrotransposons will improve our understanding of the relationship between epigenetic changes and lung function, potentially informing new diagnostic and therapeutic approaches to lung function decline and diseases such as COPD.

Table 1: Baseline characteristics of 663 individuals from the Normative Aging Study and subset of 301 individuals who had more than one lung function measurement for analysis of lung function decline

	Full data set		301 subset	
	Mean (SD) or N (%)	Range	Mean (SD) or N (%)	Range
Age	72.7 (6.7)	(55.3-100.9)	71.5 (6.4)	(55.3-91.0)
BMI	28.5 (4.2)	(19.4-52.3)	28.7 (4.1)	(20.3-52.3)
Pack-years*	30.6 (24.8)	(0.1-145.5)	28.6 (23.1)	(0.10-120.8)
Current smokers	43 (7%)		23 (8%)	
Ever smokers	466 (70%)		216 (70%)	
Folate intake† (mcg/day)	570 (333)	(0.23-2235.17)	617 (383)	(0.23-2001.75)
Alcohol intake (gm/day)	12.0 (17.8)	(0-217.8)	10.7 (13.8)	(0-73.5)
WBC ($x10^3/mm^3$)	6.7 (1.8)	(2.7-23.8)	6.6 (2.3)	(3.2-36.6)
% lymphocytes	25.6 (8.0)	(5-88)	25.0 (8.3)	(7-85)
% neutrophils	62.1 (8.7)	(5-85)	62.8 (8.8)	(5-83)
Cardiovascular Disease¥	115 (17%)		49 (16%)	
Hypertension	280 (42%)		143 (47%)	
Diabetes	75 (11%)		33 (11%)	
FEV_1	2.70 (.64)	(.85-4.69)	2.76 (0.62)	(1.29-4.69)
FEV_1%pred	81 (17)	(28-125)	81.8 (15.5)	(39.7-122.6)
FVC	3.56 (0.72)	(1.63-6.32)	3.64 (0.71)	(1.63-6.32)
FVC%pred	82 (14)	(43-124)	82.6 (13.1)	(43.8-123.8)
FEV_1/FVC	75 (8)	(36-94)	75.6 (7.0)	(51.6-94.4)
COPD	107 (16%)		45 (15%)	
Alu	26.4 (1.1)	(22.8-32.4)	26.4 (1.10)	(22.8-32.3)
LINE-1	76.8 (1.8)	(70.1-84.6)	77.0 (1.8)	(70.1-81.6)

*pack-years in current or ex-smokers only
†calculated based on supplement intake and fortified foods from food frequency questionnaire
¥ angina, stroke, myocardial infarction, ischemic heart disease

Table 2: Bivariate associations between *Alu*, LINE-1 methylation and other covariates

	Alu		**LINE-1**	
	β	**p value**	**β**	**p value**
Age	-0.3	0.07	-0.2	0.1
BMI	0.106	0.059	0.054	0.17
Current smoking	0.35	0.14	0.697	0.0002
% Lymphocytes	0.08	0.73	-0.31	0.04
FEV_1	0.024	0.06	-0.006	0.53
FVC	0.023	0.22	-0.004	0.73
FEV_1/FVC	0.31	0.046	-0.05	0.67
COPD	OR .87 [.73, 1.03]	0.1	1.02 [.92, 1.13]	0.76

Table 3: Multivariate models for lung function and both *Alu* and LINE-1 methylation*

	Alu		LINE-1	
	β	p value	β	p value
FEV_1	0.028	0.017	-0.015	0.08
FVC	0.027	0.06	-0.017	0.11
FEV_1/FVC	0.3	0.057	-0.092	0.44
COPD	0.85 [0.71, 1.03]	0.09	1.01 [.89, 1.15]	0.83

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status. Models with LINE-1 also include % lymphocytes.

Table 4: Multivariate models for rate of change in lung function (in liters/yr) and both *Alu* and LINE-1 methylation*

	Alu		LINE-1	
	β	p val	β	p val
FEV_1 rate	-0.0028	0.49	-0.0069	0.005
FVC rate	-0.00098	0.84	-0.0096	0.0021
ratio rate	-0.00079	0.17	0.00005	0.89

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status, and baseline FEV_1, FVC or FEV_1/FVC respectively depending on outcome. Models with LINE-1 also adjusted for % lymphocytes.

Figure Legends

Figure 1: Distribution (median, interquartile range) of percentage a) *Alu* and b) LINE-1 methylation in the overall cohort and stratified by smoking status

Figure 2: ***Alu* Methylation and Lung Function**
Bivariate associations between *Alu* methylation and FEV_1 %predicted, FVC %predicted and FEV_1/FVC

* For FEV_1/FVC y axis is percent, not percent predicted

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AUTHOR CONTRIBUTIONS: N.E.L. designed the study, performed the data analysis, and prepared the manuscript. J.S. contributed to the data analysis and provided critical revision of the manuscript. L.T. contributed to data collection and provided critical revision of the manuscript. V.B. contributed to data collection and provided critical revision of the manuscript. D.S. and P.V. were involved in conception of the study and critical revision of the manuscript. A.Z. contributed to data collection and provided critical revision of the manuscript. J.S. contributed to study design and provided critical revision of the manuscript. A.B. contributed to data collection and provided critical revision of the manuscript. A.A.L. contributed to study design, assisted with the data analysis, and provided critical revision of the manuscript. D.L.D. designed the study, assisted with the data analysis, and provided critical revision of the manuscript.

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***Alu* and LINE-1 Methylation and Lung Function in the Normative Aging Study**

Short Title: Global Methylation and Lung Function

Nancy E. Lange MD MPH,[1-3] Joanne Sordillo ScD[1,3] Letizia Tarantini BS,[4] Valentina Bollati PhD,[4] David Sparrow DSc,[5] Pantel Vokonas MD,[5] Antonella Zanobetti PhD,[6] Joel Schwartz PhD,[6] Andrea Baccarelli MD PhD,[4,6] Augusto A. Litonjua MD MPH[1-3], Dawn L. DeMeo MD MPH[1-3]

[1]Channing ~~Laboratory~~Division of Network Medicine, [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA; [3]Harvard Medical School, Boston, MA, USA; [4]Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health, Università degli Studi di Milano and IRCCS Maggiore Policlinico Hospital, Mangiagalli and Regina Elena Foundation, Milan, Italy; [5]Veterans Administration Boston Healthcare System and Department of Medicine, Boston University School of Medicine, Boston, MA, USA; [6] Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

Corresponding author:

Nancy E. Lange, MD, MPH
Channing Laboratory
Brigham and Women's Hospital
181 Longwood Avenue, room 454
Boston, MA 02115, USA
Phone: 617-525-0874
Fax: 617-525-0958
Email: renal@channing.harvard.edu

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ABSTRACT

Objectives: To investigate the association between methylation of transposable elements *Alu* and LINE-1 and lung function.

Design: Cohort study

Setting: Outpatient Veterans Administration facilities in greater Boston, MA, USA.

Participants: Subjects from the Veterans Administration Normative Aging Study, a longitudinal study of aging in men, evaluated between 1999 and 2007. The majority (97%) of subjects were white.

Primary and secondary outcome measures: Primary predictor was methylation, assessed using PCR-pyrosequencing after bisulfite treatment. Primary outcome was lung function as assessed by spirometry, performed according to ATS/ERS guidelines at the same visit as the blood draws.

Results: In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking and race, *Alu* hypomethylation was associated with lower FEV_1 (β=28ml per 1% change in *Alu* methylation, p= .017), and showed a trend towards association with a lower FVC (β=27ml, p=.06) and lower FEV_1/FVC (β=0.3%, p=.058). In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking, % lymphocytes, race and baseline lung function, LINE-1 hypomethylation was associated with more rapid decline of FEV_1 (β=6.9ml/yr per 1% change in LINE-1 methylation, p= .005) and of FVC (β=9.6ml/yr, p=.002).

Conclusions: In multiple regression analysis, *Alu* hypomethylation was associated with lower lung function, and LINE-1 hypomethylation was associated with more rapid lung function decline in a cohort of older and primarily white men from North America. Future studies should aim to replicate these findings

and determine if *Alu* or LINE-1 hypomethylation may be due to specific and modifiable environmental exposures.

Article Summary:

Article Focus:

Association between methylation, an epigenetic marker, and lung function

Key Message(s):

~~Hypomethylation~~ Relative hypomethylation of transposable elements is associated with lower lung function and more rapid lung function decline in a cohort of ~~elderly~~older North American primarily white men.

Strengths and Limitations:

First study to evaluate methylation of transposable elements in relation to lung function.

Difficult to interpret implications of methylation patterns in transposable elements.

INTRODUCTION

Lung function has both environmental and genetic determinants.[1-51-4] Epigenetic ~~changes~~variation, which may influence gene expression patterns without changing DNA sequence, may mediate the effects of environmental exposures on disease outcomes. DNA methylation, one type of epigenetic change, is the reversible addition of a methyl group to cytosine nucleotides. Methylation changes may or may not persist over time in the human genome, as epigenetic marks are highly plastic.

A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as *Alu* and LINE-1 (long interspersed nuclear element) which are among the most common and best characterized repetitive elements.[6-85-7] *Alu* is the most abundant of the SINEs (short-interspersed nuclear elements) with over one million copies per genome.[98] *Alu* elements compose approximately 11% of the mass of human genome and contain 30% of its methylation sites.[7 106-9] LINE-1 elements are present at over half a million copies.[9 118-10] Methylation of repetitive elements such as *Alu* and LINE-1 has been shown to correlate with total genomic methylation content.[11 1210-11] Hypomethylation in transposable elements is associated with higher genomic instability and alterations or deregulation of gene expression.[13 1412-13]

Prior studies have found associations between methylation of *Alu* or LINE-1 elements and various diseases including multiple cancers,[76] cardiovascular disease[15-1714-16] and neurologic disease[1817] as well as with markers of inflammation[1918] and the inflammatory response.[2019] Studies on gene-specific methylation and non-neoplastic lung disease have found associations between GATA4, CDKN2A (p16) and lung function and an interaction with wood smoke exposure,[2120] as well as multiple genes in association with COPD presence and severity.[22] To our knowledge no prior study has investigated associations between methylation of transposable elements and non-neoplastic lung disease. Moreover, case-control studies ~~such as~~which are common in genomic studies are more problematic for epigenetic marks since sampling cases after disease onset makes it impossible to determine whether epigenetic changes preceded or resulted from the disease. Hence cohort studies or nested case-control studies within cohorts are particularly valuable. Our aim was to examine whether methylation of the repetitive elements *Alu* and LINE-1 was associated with measures of lung function, COPD status, and longitudinal

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change in lung function in a cohort of men, the Normative Aging Study. Preliminary results from these analyses were previously reported in abstract form.[23][21]

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METHODS

Population:

Study participants were from the Veterans Administration Normative Aging Study, an ongoing longitudinal study of aging established in 1963.[24][22] This is a cohort of 2,280 healthy male volunteers from the greater Boston, MA, area who were 21–80 years of age at entry and who enrolled after an initial health screening determined that they were free of known chronic medical conditions. Participants were reevaluated every 3–5 years using detailed on-site physical examinations and questionnaires. The study was approved by the Institutional Review Boards of all participating institutions. All participants gave written informed consent.

Prior to 1999, 706 subjects had died and others were either lost to follow-up, being followed by questionnaire only, or had no blood samples left for analyses (n=792). Seven hundred and eighty two subjects had blood samples that were available for methylation analysis resulting in 704 subjects with unique IDs and methylation data as previously described.[25 26] For this study, individuals evaluated at least once between January March 1999 and June 2007 with a blood sample drawn methylation data and concomitant spirometry were included. During the study period, this included 663 total subjects, 194 of whom reported for examination blood draw two times, for a total of 857 samples collected. For the analysis of lung function decline, a second spirometric measurement was available on 301 subjects who had had an initial blood draw for methylation measurement.

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Measures:

Spirometry was performed according to ATS/ERS guidelines.[23] Spirometry was performed as previously described,[27] and was repeated up to a maximum of 8 spirograms, so that at least 3 acceptable spirograms were obtained, at least 2 of which were reproducible with FEV_1 and FVC measurements

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within 5% of each spirogram; the best of these 2 values was selected from a given encounter. Acceptability of spirograms was judged according to ATS standards.[28 29] All spirometric values are pre-bronchodilator. Percent predicted values for FEV_1 and FVC were calculated using equations by Crapo *et al.*[30][24] COPD was defined as GOLD stage II or higher (FEV_1/FVC<70% and FEV_1<80% predicted).[31][25] Techniques for assessing DNA methylation were previously described in detail.[32 33][26 27] Briefly, we performed DNA methylation assessment of *Alu* and LINE-1 repetitive elements on bisulfite-treated blood leukocyte DNA using highly quantitative polymerase chain reaction (PCR)–pyrosequencing technology. The degree of methylation was expressed as the percentage of methylated cytosines over the sum of methylated and unmethylated cytosines. Each marker was tested in triplicate, and their average was used in the statistical analysis.

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Statistical Analysis

Analyses for cross-sectional associations were performed using repeated measures with adjustment for the correlation between measurements in a given individual using mixed effects models (PROC MIXED) for continuous outcomes (FEV_1, FVC, FEV_1/FVC) and generalized estimating equations (PROC GENMOD) for binary outcomes (COPD). Covariates in multivariable models were chosen for their clinical relevance and strong bivariate associations (p≤.05) with lung function or change in effect estimate criterion of >10% after addition to the model and included age, height, race, pack-years of cigarette smoking, smoking status (dichotomized as current vs. ex and never smokers) and body mass index (BMI). We also considered variables previously associated with methylation of repetitive elements[34][28] such as folate intake, alcohol intake, total white blood cell count and both percent neutrophils and percent lymphocytes. With the exception of percent lymphocytes, which was included in models with LINE-1 only, these covariates were not included in final models because they were not associated with *Alu* or LINE-1 methylation and did not meet the change in estimate criteria. Because Figure 2 depicts bivariate relationships, percent predicted values were used for both FEV_1 and FVC to show an adjusted value; actual values for FEV_1 and FVC were utilized in multivariable models. To examine associations between methylation of *Alu* and LINE-1 and change in lung function over time, a rate was calculated using the change in lung function between the two time points divided by the amount of time elapsed between the

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two measurements in years. This value was utilized as an outcome and analyzed using multivariate linear regression models. A total of 301 subjects had a second lung function data point subsequent to the initial methylation value. SAS version 9.1 (SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Baseline characteristics of the 663 individuals included in this study as well as of the subset of 301 individuals with two lung function measures are shown in **Table 1**. All subjects were male and the majority (640, 97%) of white race. ~~Few~~ Forty-three subjects (7%) were current smokers and 197 (30%) were never smokers. There was wide variation in lung function values. Of the 107 individuals with COPD, 77 (72%) were GOLD stage II, 26 were stage III and 4 were stage IV; overall 20 (20%) of the individuals with COPD were current smokers.

The distribution of percentage methylation of both *Alu* and LINE-1 elements among the population and stratified by smoking status is shown in **Figure 1**.

Bivariate relationships between *Alu* and LINE-1 methylation with outcomes and covariates considered for inclusion in the multivariable model are shown in **Table 2**. *Alu* methylation was associated or showed a trend towards association positively with FEV_1, BMI and FEV_1/FVC and negatively with age and COPD status. LINE-1 methylation was positively associated with current smoking and negatively with percent lymphocytes. Neither *Alu* nor LINE-1 methylation was associated with FVC, pack-years of smoking or ever smoking status. Folate intake, alcohol intake, total white blood cell count and percent neutrophils were not significantly associated with *Alu* or LINE-1 methylation in bivariate analyses. There was no significant relationship between methylation of *Alu* and LINE-1 to each other (p=.23).

In multivariate models that included age, height, race, pack-years of smoking, smoking status, and BMI, *Alu* methylation was positively associated with FEV_1, ~~FEV_1/FVC~~ and showed a trend towards association with FVC and FEV_1/FVC. Because of recent data suggesting that current smoking status may have differential effects on methylation,[35 36] and because this may relate to disease outcome or risk, we investigated whether our results would change if current smokers were excluded from the analyses.

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Higher *Alu* methylation was still associated with lower odds of COPD (OR 0.80 [0.64, 0.99] p=.046). In analyses of lung function measures, results were in the same direction but were no longer significant except for FEV_1/FVC (FEV_1 p=0.17, FVC p=0.7, FEV_1/FVC p=.029). When analyzed excluding current smokers, there was a negative association between *Alu* and COPD (higher *Alu* with lower odds of COPD) that was statistically significant (OR 0.80 [0.64, 0.99] p=.046). There were no significant associations between LINE-1 methylation and any of the cross-sectional outcomes. (**Table 3**). **Figure 2** depicts the bivariate associations of *Alu* methylation with FEV_1 % predicted, FVC % predicted and FEV_1/FVC.

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We also analyzed whether methylation of *Alu* and LINE-1 were associated with rate of change in lung function in a subset of participants who had two consecutive lung function measures (N=301). The mean number of years elapsed between measurements was 4.03 (SD 1.23). Models were adjusted for baseline FEV_1, FVC or FEV_1/FVC (respectively for the given outcome) as well as age, pack-years of smoking, BMI, height, race, percent lymphocytes and smoking status. Relative hypomethylation in LINE-1 but not *Alu* was associated negatively with rate of changewith faster rate of decline in FEV_1 and FVC (p<.005). Neither measure was associated with rate of change of FEV_1/FVC. (**Table 4**) Including both *Alu* and LINE-1 methylation in the models did not change the results (data not shown). Because of prior associations between methylation of repetitive elements and cardiovascular disease,[15-17], we repeated both cross-sectional and longitudinal analyses including variables for cardiovascular disease (myocardial infarction, stroke, angina, hypertension, ischemic heart disease) and diabetes and found no difference in the results (data not shown). Analyses were also repeated in whites only to determine whether results might be due to population stratification and results did not change (data not shown). Analyses excluding current smokers remained significant (data not shown).

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DISCUSSION

We examined associations between methylation levels of the repetitive elements *Alu* and LINE-1 in a cohort of elderlyolder men in relation to lung function and COPD status. In cross-sectional analyses, we found that *Alu* hypomethylation was associated with lower FEV_1 with a trend towards association with lower FVC and FEV_1/FVC. LINE-1 hypomethylation was associated with more rapid lung function decline (FEV_1 and FVC).

Prior studies have found associations between methylation of repetitive transposable elements such as *Alu* and LINE-1 and several diseases including multiple cancers,[76] cardiovascular disease[15-1714-16] and neurologic disease[1817] as well as with markers of inflammation.[1918] To our knowledge this is the first study to examine associations between methylation of *Alu* and LINE-1 transposable elements and measures of lung function.

Previous work has shown that in normal subjects, *Alu* hypomethylation is associated with increased age[8 378 34], greater alcohol use and gender (lower in males).[3428] In this same cohort (NAS), hypomethylation has been associated with higher incidence of cancer in general and lung cancer specifically (LINE-1 methylation), as well as higher mortality from cancer (*Alu* and LINE-1 methylation).[3835] A variety of environmental exposures such as lead,[3936] traffic particles,[3327] organic pollutants,[4037] metals, air pollutants and endocrine disrupting agents[4138] may all affect global methylation levels, specifically some that may relate to lung function such as various air pollutants.

Hypomethylation of transposable elements may or may not be causally linked to lower lung function and faster rates of lung function decline. Lower methylation of *Alu* and LINE-1 may increase their activity as retrotransposable sequences, leading to greater genomic instability and more mutations.[1312] Furthermore, oxidative damage caused by environmental exposures may cause hypomethylation.[4239] This may lead to alteration of gene expression through a variety of mechanisms including disrupting transcription factor binding sites or reading frames, altering regulatory sequences, altering methylation patterns of gene promoters, or introducing new transcription factor binding sites.[43-4540-42] *Alu* elements specifically are preferentially found in gene-rich regions.[4643] Black carbon and increased $PM_{2.5}$ exposure[3327] as well as PM_{10} exposure[4138] have been found to be inversely associated with LINE-1 methylation and both *Alu* and LINE-1 methylation, respectively which may impact lung function or lung function decline.[4744] LINE-1 hypomethylation may also increase transcription of genes that have LINE-1 in regulatory regions. It is possible that other specific environmental or dietary exposures previously not known to be associated with lung function may be mediated through epigenetic changes such as *Alu* or LINE-1 hypomethylation. Alternatively, this may be a marker of a specific exposure but not causally linked to lower lung function. Lastly, because *Alu* methylation decreases with increasing age, as does lung function, our findings may represent some other measure of 'aging' or exposures resulting in similar processes beyond just chronological age.[8~~7~~] As our understanding of epigenetic processes and the exposures that affect these processes increases, the implications of our findings will become clearer.

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These data must be interpreted in the context of the study design. Our study was limited to ~~elderly~~older men the majority of whom were white, and our findings may or may not be generalizable to other populations. It is difficult to know how to interpret methylation of retrotransposons, as opposed to gene-specific methylation, in relation to specific outcomes such as lung function and lung function decline. Future studies in this and other cohorts should include gene-specific methylation analyses similar to Qiu *et al*[22] to elucidate mechanisms by which methylation changes may relate to these outcomes. We did not control for a variety of environmental exposures that may be associated with both lung function and methylation. H~~, h~~owever, alteration in methylation patterns may be the pathway through which these changes are mediated and thus including these exposures in multivariate models would be overadjusting. Methylation levels vary in different tissue types and it is possible that assessments of methylation in white blood cells may not reflect alterations seen in lung tissue. However, systemic processes involving white blood cells, such as inflammation, may play a role in the pathophysiology of lung function decline[48~~45~~] and may nonetheless be markers of specific exposures (such as cigarette smoking) that exert a systemic effect.

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In summary, we found that relative hypomethylation of *Alu* was associated with lower lung function measures, and that LINE-1 hypomethylation was associated with more rapid lung function decline. Future studies on both gene-specific methylation as well as exposures related to methylation of retrotransposons will improve our understanding of the relationship between epigenetic changes and lung function, potentially informing new diagnostic and therapeutic approaches to lung function decline and diseases such as COPD.

Table 1: Baseline characteristics of 663 individuals from the Normative Aging Study and subset of 301 individuals who had more than one lung function measurement for analysis of lung function decline

-	Mean (SD) or N (%)	Range
Age	72.7 (6.7)	(55.3-100.9)
BMI	28.5 (4.2)	(19.4-52.3)
Pack-years*	30.6 (24.8)	(0.1-145.5)
Current smokers	43 (7%)	-
Ever smokers	466 (70%)	-
FEV_1	2.70 (.64)	(.85-4.69)
FEV_1%pred	81 (17)	(28-125)
FVC	3.56 (0.72)	(1.63-6.32)
FVC%pred	82 (14)	(43-124)
FEV_1/FVC	75 (8)	(36-94)
COPD	107 (16%)	-
Alu	26.4 (1.1)	(22.8-32.4)
LINE-1	76.8 (1.8)	(70.1-84.6)

	Full data set		301 subset	
	Mean (SD) or N (%)	Range	Mean (SD) or N (%)	Range
Age	72.7 (6.7)	(55.3-100.9)	71.5 (6.4)	(55.3-91.0)
BMI	28.5 (4.2)	(19.4-52.3)	28.7 (4.1)	(20.3-52.3)
Pack-years*	30.6 (24.8)	(0.1-145.5)	28.6 (23.1)	(0.10-120.8)
Current smokers	43 (7%)		23 (8%)	
Ever smokers	466 (70%)		216 (70%)	
Folate intake† (mcg/day)	570 (333)	(0.23-2235.17)	617 (383)	(0.23-2001.75)
Alcohol intake (gm/day)	12.0 (17.8)	(0-217.8)	10.7 (13.8)	(0-73.5)
WBC (x10^3/mm^3)	6.7 (1.8)	(2.7-23.8)	6.6 (2.3)	(3.2-36.6)
% lymphocytes	25.6 (8.0)	(5-88)	25.0 (8.3)	(7-85)
% neutrophils	62.1 (8.7)	(5-85)	62.8 (8.8)	(5-83)
Cardiovascular Disease¥	115 (17%)		49 (16%)	
Hypertension	280 (42%)		143 (47%)	
Diabetes	75 (11%)		33 (11%)	
FEV_1	2.70 (.64)	(.85-4.69)	2.76 (0.62)	(1.29-4.69)
FEV_1%pred	81 (17)	(28-125)	81.8 (15.5)	(39.7-122.6)
FVC	3.56 (0.72)	(1.63-6.32)	3.64 (0.71)	(1.63-6.32)
FVC%pred	82 (14)	(43-124)	82.6 (13.1)	(43.8-123.8)
FEV_1/FVC	75 (8)	(36-94)	75.6 (7.0)	(51.6-94.4)
COPD	107 (16%)		45 (15%)	
Alu	26.4 (1.1)	(22.8-32.4)	26.4 (1.10)	(22.8-32.3)
LINE-1	76.8 (1.8)	(70.1-84.6)	77.0 (1.8)	(70.1-81.6)

*pack-years in current or ex-smokers only
†calculated based on supplement intake and fortified foods from food frequency questionnaire
¥ angina, stroke, myocardial infarction, ischemic heart disease

Table 2: Bivariate associations between *Alu*, LINE-1 methylation and other covariates

-	*Alu*		LINE-1	
-	β	p value	β	p value
Age	-0.3	0.07	-0.2	0.1
BMI	0.106	0.059	0.054	0.17
Current smoking	0.35	0.14	0.697	0.0002
% Lymphocytes	0.08	0.73	-0.31	0.04
FEV_1	0.024	0.06	-0.006	0.53
FEV_1/FVC	0.31	0.046	-0.05	0.67
COPD	OR .87 [.73, 1.03]	0.1	1.02 [.92, 1.13]	0.76

	Alu		LINE-1	
	β	p value	β	p value
Age	-0.3	0.07	-0.2	0.1
BMI	0.106	0.059	0.054	0.17
Current smoking	0.35	0.14	0.697	0.0002
% Lymphocytes	0.08	0.73	-0.31	0.04
FEV_1	0.024	0.06	-0.006	0.53
FVC	0.023	0.22	-0.004	0.73
FEV_1/FVC	0.31	0.046	-0.05	0.67
COPD	OR .87 [.73, 1.03]	0.1	1.02 [.92, 1.13]	0.76

Table 3: Multivariate models for lung function and both *Alu* and LINE-1 methylation*

	Alu		LINE-1	
	β	p value	β	p value
FEV_1	0.028	0.017	-0.015	0.08
FVC	0.027	0.06	-0.017	0.11
FEV_1/FVC	0.3	0.057	-0.092	0.44
COPD	0.85 [0.71, 1.03]	0.09	1.01 [.89, 1.15]	0.83

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status. Models with LINE-1 also include % lymphocytes.

Table 4: Multivariate models for rate of change in lung function (in liters/yr) and both *Alu* and LINE-1 methylation*

~~-~~	~~FEV_1 rate~~		~~FVC rate~~		~~ratio rate~~	
~~-~~	~~β~~	~~p val~~	~~β~~	~~p val~~	~~β~~	~~p val~~
~~*Alu*~~	~~-0.0028~~	~~0.49~~	~~-0.00098~~	~~0.84~~	~~-0.00079~~	~~0.17~~
~~LINE-1~~	~~-0.0069~~	~~0.005~~	~~-0.0096~~	~~0.0021~~	~~0.00005~~	~~0.89~~

	Alu		LINE-1	
	β	p val	β	p val
FEV_1 rate	-0.0028	0.49	-0.0069	0.005
FVC rate	-0.00098	0.84	-0.0096	0.0021
ratio rate	-0.00079	0.17	0.00005	0.89

*adjusted for age, height, race, BMI, pack-years of smoking, smoking status, and baseline FEV_1, FVC or FEV_1/FVC respectively depending on outcome. Models with LINE-1 also adjusted for % lymphocytes.

Figure Legends

Figure 1: Distribution (median, interquartile range) of percentage a) *Alu* and b) LINE-1 methylation in the overall cohort and stratified by smoking status

Figure 2: ***Alu* Methylation and Lung Function**
Bivariate associations between *Alu* and FEV_1%predicted, FVC%predicted and FEV_1/FVC

* For FEV_1/FVC y axis is percent, not percent predicted

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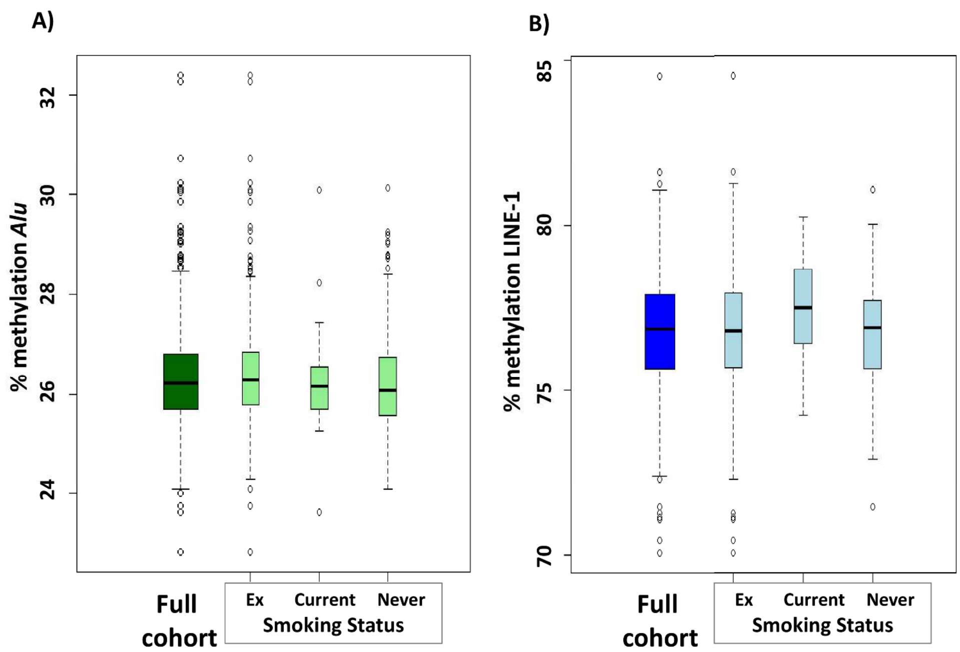

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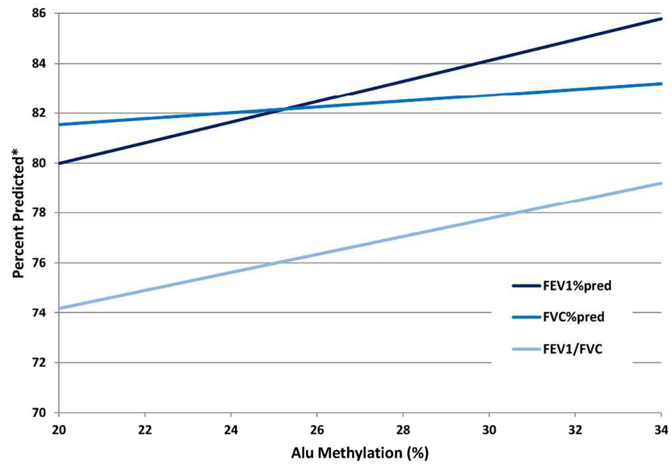

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