PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Alu and LINE-1 Methylation and Lung Function in the Normative Aging Study
AUTHORS	Lange, Nancy ; Sordillo, Joanne; Tarantini, Letizia; Bollati, Valentina; Sparrow, David; Vokonas, Pantel; Zanobetti, Antonella; Schwartz, Joel; Baccarelli, Andrea; Litonjua, Augusto; DeMeo, Dawn

VERSION 1 - REVIEW

REVIEWER	Dana Hancock Genetic Epidemiologist Research Triangle Institute International
REVIEW RETURNED	04-Jun-2012

GENERAL COMMENTS	I commend the authors on a well-written manuscript detailing the associations between methylation of repetitive elements and lung function in a cohort of nearly 700 elderly men from the Normative Aging Study. The findings implicated Alu hypomethylation with lower cross-section lung function and LINE-1 hypomethylation with more rapid decline in lung function. As outlined below, I recommend a few further clarifications and some additional analyses to more fully integrate these associations. (1) In the Participants section of the abstract, please specify that the cohort included only males. (2) In the first sentence in the introduction, the authors might also want to include the most recent GWAS of pulmonary function (Soler Artigas et al. Nat Genet 2011). (3) Introduction: Please clarify the statement on pg. 4 (lines 45-50), which is confusing as currently written. (4) Methods: What other ethnicities were included? The authors might want to consider repeating the analyses in whites only to confirm that the associations are not reflective of any population stratification. (5) In Table 1, it would be helpful to include the characteristics for the factors that are later evaluated for association with methylation of repetitive elements but found to be not significant. Of particular note, folate intake was evaluated. It has strong implications for methylation patterns, but how common was folate intake in a cohort of elderly men? I suspect that folate intake is not very common, so the lack of association might be due to limited statistical power. (6) Methods: Pg. 5, line 35 indicates that 194 subjects had repeated lung function measurements available, but throughout the rest of the manuscript, the longitudinal analyses are based on 301 subjects.

	Please clarify this discrepancy. (7) Methods and Results: The threshold used to declare statistical significance is not clear. The last line on pg. 7 suggests a threshold of P<0.005, but discussion of earlier results implies a less stringent threshold. (8) Methods, pg. 7, lines 33-38: How would the FEV1, FVC, and FEV1/FVC results change after the exclusion of current smokers? (9) Discussion, pg. 8, line 35: Age is known to be associated with hypomethylation, and the authors appropriately include age as a covariate in their adjusted multivariate models. How would the results change if age^2 were included as an additional covariate in order to fully saturate the model for an age effect? See the Soler Artigas et al. reference and Jackson B et al. (Int J Epidemiol 2004) for examples. (10) Discussion: Alu hypomethylation was associated with lower lung function but was not associated with lung function decline. The reverse is true for LINE-1 hypomethylation. Is there any biological plausibility to explaining these seemingly discrepant patterns? (11) Table 4: For consistency, it would be helpful to show the results in the same orientation as Table 3.

REVIEWER	Guy Brusselle Ghent University hospital Ghent B-9000 BELGIUM
REVIEW RETURNED	12-Jun-2012

THE STUDY	The authors demonstrate an association between Alu and LINE-1 hypomethylation and lung function, but this association is probably due to confounders (and especially cardiovascular comorbidities), which are not described in the paper.
REPORTING & ETHICS	Please add flowchart of participants in the NAS (according to STROBE guidelines).
GENERAL COMMENTS	In the Normative Aging Study, the authors demonstrate association of Alu hypomethylation with lower lung function (cross-sectional analysis), and that LINE-1 hypomethylation is associated with more rapid lung function decline (longitudinal analysis). Although these observations are novel and interesting, the following comments need to be addressed. Major comments 1) As the authors acknowledge in the discussion, the authors only studied Caucasian males. The fact that they did not investigate females nor other ethnicities than Caucasians, should be emphasized in the abstract, conclusion and article summary. 2) In the abstract, the authors write that Alu hypomethylation was associated with lower FVC, whereas in the results (page 7) they state that Alu was not associated with FVC. Please clarify. 3) Throughout the manuscript, the authors use the terms Alu and LINE-1, where they intend Alu methylation (or hypomethylation) and LINE-1 methylation. Please make a clear difference between the transposable elements (Alu or LINE-1) and its methylation status (Alu or LINE-1 methylation): this difference between “genetics” and

	"epigenetics" is crucial. 4) Results: why did the authors exclude current smokers to investigate the association between Alu and COPD? To obtain statistically significance in a post-hoc subgroup analysis? 5) Table 1: add also the baseline characteristics of the 301 individuals from the Normative Aging Study who had two lung function measurements (the longitudinal analysis of lung function decline). Are there significant differences between the 663 (all) and the 301 (decline) participants? 6) Table 1: the mean FVC of all participants was only 82%, suggesting the following possibilities: a) a large subgroup with restrictive spirometries, suggesting comorbidities such as heart faiure and/or diabetes, obesity …; these comorbidities should be added to the table. b) too short expiration time during the spirometries, inducing a pseudo-restrictive syndrome: please mention the applied quality control measures. 7) Interpretation of the data: since Alu and LINE-1 methylation have been associated not only with increased age, but also with several (co)morbidities such as cardiovascular disease, the authors should take these confounders into account in the statistical analysis/adjustment. Moreover, the LINE-1 hypomethylation was associated with more rapid decline of FVC (more than FEV1), implicating no association with the FEV1/FVC ratio (and thus COPD, as illustrated in table 2 and 4). In table 2,the data for FVC should be added. Therefore, the association of LINE-1 hypomethylation with decline of both FVC and FEV1 is probably most due to aging ("NAS"), inflammation and (co)morbidities, and less due to specific respiratory disease such as COPD. This distinction should be made clear. 8) Figure 1: please stratify according to smoking status and smoking history (current smokers, ex-smokers and never-smokers).

VERSION 1 – AUTHOR RESPONSE

Reviewer: Dana Hancock
Genetic Epidemiologist
Research Triangle Institute International

I commend the authors on a well-written manuscript detailing the associations between methylation of repetitive elements and lung function in a cohort of nearly 700 elderly men from the Normative Aging Study. The findings implicated Alu hypomethylation with lower cross-section lung function and LINE-1 hypomethylation with more rapid decline in lung function. As outlined below, I recommend a few further clarifications and some additional analyses to more fully integrate these associations.

(1) In the Participants section of the abstract, please specify that the cohort included only males.
This has been changed in the abstract.

(2) In the first sentence in the introduction, the authors might also want to include the most recent GWAS of pulmonary function (Soler Artigas et al. Nat Genet 2011).
This reference has been added.

(3) Introduction: Please clarify the statement on pg. 4 (lines 45-50), which is confusing as currently written.

The statement has been changed as follows:

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.

(4) Methods: What other ethnicities were included? The authors might want to consider repeating the analyses in whites only to confirm that the associations are not reflective of any population stratification.

In our cohort, 640 (96.5%) of the subjects were white (as noted in table 1), 11 (1.7%) black, 4 (0.6%) Hispanic, and 8 (1.2%) unknown. We repeated the analyses including whites only and associations did not change substantially implying that population stratification was not the cause of prior significance (see tables below). We have added the following text in the manuscript on page XXX line XXX:
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).

Cross-sectional analyses:

Lung function decline:

(5) In Table 1, it would be helpful to include the characteristics for the factors that are later evaluated for association with methylation of repetitive elements but found to be not significant. Of particular note, folate intake was evaluated. It has strong implications for methylation patterns, but how common was folate intake in a cohort of elderly men? I suspect that folate intake is not very common, so the lack of association might be due to limited statistical power.

Mean folate intake in the overall cohort (accounting for both supplements and fortified foods) was 570mcg/day (SD 333mcg). This information as well as alcohol intake, WBC count, % neutrophils and % lymphocytes have all been added to table 1.

(6) Methods: Pg. 5, line 35 indicates that 194 subjects had repeated lung function measurements available, but throughout the rest of the manuscript, the longitudinal analyses are based on 301 subjects. Please clarify this discrepancy.

We apologize that the numbers for this portion of the analysis were confusing. We have added an explanation in the methods section as follows.

On page XXX line XXX we have added:
For the longitudinal analysis, a second spirometric measurement was available on 301 subjects who had had an initial blood draw for methylation measurement.

And as previously stated on page XXX line XXX
A total of 301 subjects had a second lung function data point subsequent to the initial methylation

value.

(7) Methods and Results: The threshold used to declare statistical significance is not clear. The last line on pg. 7 suggests a threshold of P<0.005, but discussion of earlier results implies a less stringent threshold.

This statement, now on page XXX of the manuscript, summarizes that all p values referenced in that sentence were <.005. Overall , as is conventional, a p value of < .05 was considered to be statistically significant. Some statements state that there was statistical significance or a trend towards association, specifically in reference to the cross-sectional findings (p values including .017, .05, .06 and .09—see table 2 in original manuscript).

(8) Methods, pg. 7, lines 33-38: How would the FEV1, FVC, and FEV1/FVC results change after the exclusion of current smokers?

Results with lung function were in the same direction but less significant for the cross-sectional analyses and were not significantly changed in relation to lung function decline (see below). We have added text to the manuscript as follows on page XXX line XXX:

Because of recent data suggesting that current smoking status may have differential effects on methylation1 2 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 FEV1/FVC (FEV1 p=0.17, FVC p=0.7, FEV1/FVC p=.029).

Cross sectional:

Lung function decline:

(9) Discussion, pg. 8, line 35: Age is known to be associated with hypomethylation, and the authors appropriately include age as a covariate in their adjusted multivariate models. How would the results change if age^2 were included as an additional covariate in order to fully saturate the model for an age effect? See the Soler Artigas et al. reference and Jackson B et al. (Int J Epidemiol 2004) for examples.

We re-ran the models using age2 as an additional covariate and found no substantial difference. In fact, once both age and age2 were covariates in the model, neither one was statistically significant. Cross-sectional

Lung function decline

(10) Discussion: Alu hypomethylation was associated with lower lung function but was not associated with lung function decline. The reverse is true for LINE-1 hypomethylation. Is there any biological plausibility to explaining these seemingly discrepant patterns?

We agree with the reviewer that these results may be difficult to interpret, as we acknowledge in the discussion section on page XXX line XXX. Determinants of methylation are still being elucidated and

it is likely that different exposures as well as genetic factors play a role in both gene-specific methylation as well as methylation of repetitive elements such as Alu and LINE-1. One could hypothesize that exposures related to LINE-1 methylation might be more likely to be causally related to lung function given that this was associated with subsequent decline, whereas exposures related to Alu methylation may be more likely to be simply associated with the same things that low lung function is associated with. However, any explanation for these findings at this point would be purely speculative and thus beyond the scope of the current study.

(11) Table 4: For consistency, it would be helpful to show the results in the same orientation as Table 3.
The orientation of table 4 has been changed accordingly.

Reviewer: Guy Brusselle
Ghent University hospital
Ghent B-9000
BELGIUM

The authors demonstrate an association between Alu and LINE-1 hypomethylation and lung function, but this association is probably due to confounders (and especially cardiovascular comorbidities), which are not described in the paper.

Please add flowchart of participants in the NAS (according to STROBE guidelines).

We have created a flow chart below and have added detailed information in text form on page XXX line XXX as follows:

Prior to 1999, 706 subjects had died and others were either lost to follow-up, being followed by questionnaires 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.3 4 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.

In the Normative Aging Study, the authors demonstrate association of Alu hypomethylation with lower lung function (cross-sectional analysis), and that LINE-1 hypomethylation is associated with more rapid lung function decline (longitudinal analysis). Although these observations are novel and interesting, the following comments need to be addressed.

Major comments
1) As the authors acknowledge in the discussion, the authors only studied Caucasian males. The fact that they did not investigate females nor other ethnicities than Caucasians, should be emphasized in the abstract, conclusion and article summary.

We have added to the abstract that the participants were all males and majority white. We have also added this to the article summary. As noted in the discussion, we have stated this as a limitation of the study.

2) In the abstract, the authors write that Alu hypomethylation was associated with lower FVC, whereas in the results (page 7) they state that Alu was not associated with FVC. Please clarify.

We have added more specific wording in the abstract as follows:

In multivariable models adjusted for age, height, BMI, pack-years of smoking, current smoking and race, Alu hypomethylation was associated with lower FEV1 (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 FEV1/FVC (β=0.3%, p=.058).

3) Throughout the manuscript, the authors use the terms Alu and LINE-1, where they intend Alu methylation (or hypomethylation) and LINE-1 methylation. Please make a clear difference between the transposable elements (Alu or LINE-1) and its methylation status (Alu or LINE-1 methylation): this difference between “genetics” and “epigenetics” is crucial.

Methylation has been added to locations where previously the word Alu or LINE-1 appeared alone in order to clarify that all analyses were examining methylation of these elements and not other data.

4) Results: why did the authors exclude current smokers to investigate the association between Alu and COPD? To obtain statistically significance in a post-hoc subgroup analysis?

We investigated this as a secondary analysis because of recent data from our group and others suggesting that current smoking status may have a differential effect on methylation,1 2 and this could be associated with disease outcome including COPD. We have added to the manuscript on page XXX line XXX as follows:

Because of recent data suggesting that current smoking status may have differential effects on methylation1 2 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 FEV1/FVC (FEV1 p=0.17, FVC p=0.7, FEV1/FVC p=.029).

5) Table 1: add also the baseline characteristics of the 301 individuals from the Normative Aging Study who had two lung function measurements (the longitudinal analysis of lung function decline). Are there significant differences between the 663 (all) and the 301 (decline) participants?

We have revised table 1 to include baseline characteristics of the subset of individuals included in the longitudinal analysis. We have also included additional information in Table 1 as requested by reviewer 1 (folate, alcohol, WBC etc.) and reviewer 2 (cardiovascular disease, HTN, DM). As can be seen the subset of subjects included in the analyses of lung function decline appear comparable to the larger cohort.

*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

6) Table 1: the mean FVC of all participants was only 82%, suggesting the following possibilities:
a) a large subgroup with restrictive spirometries, suggesting comorbidities such as heart faiure and/or diabetes, obesity …; these comorbidities should be added to the table.
b) too short expiration time during the spirometries, inducing a pseudo-restrictive syndrome: please mention the applied quality control measures.

We have added data to Table 1 (see above in response to comment #5) including the prevalence of

these diagnoses. As can be seen, with the exception of hypertension, the prevalences of these comorbidities was not particularly high. We also re-ran our analyses accounting for these comorbidities and our original models controlled for BMI. We found no significant differences in our results (please see response to comment 7 below for details) therefore our findings are unlikely to be due to these comorbidities. In addition, our models utilized the raw data for lung function, not percent predicted values as these values are less reliable in an aging population. Typically a range of 80-120% predicted is generally considered to be within the normal range,5 6 and though on the lower end of the normal range, in this cohort the mean value of 82% predicted is likely due to aging7 and less likely to the aforementioned comorbidities. In an older population such as our cohort, because lung function decreases with age, the assumption that >80% predicted is normal would be more likely to overdiagnose rather than underdiagnose abnormality.8 Thus we disagree that this implies a large subgroup with restrictive lung disease.
As stated in the methods section, spirometry was performed according to ATS/ERS guidelines, which includes standard quality control measures such as minimum exhalation time of 6 seconds. We have added more information to this paragraph detailing these as follows on page XXX line XXX:

Spirometry was performed as previously described 9 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 FEV1 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.10 11

7) Interpretation of the data: since Alu and LINE-1 methylation have been associated not only with increased age, but also with several (co)morbidities such as cardiovascular disease, the authors should take these confounders into account in the statistical analysis/adjustment. Moreover, the LINE-1 hypomethylation was associated with more rapid decline of FVC (more than FEV1), implicating no association with the FEV1/FVC ratio (and thus COPD, as illustrated in table 2 and 4). In table 2,the data for FVC should be added. Therefore, the association of LINE-1 hypomethylation with decline of both FVC and FEV1 is probably most due to aging ("NAS"), inflammation and (co)morbidities, and less due to specific respiratory disease such as COPD. This distinction should be made clear.

We agree with the reviewer that these findings appear to be some sort of age effect, as we state in our discussion section on page XXX line XXX:

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.12
Additionally, we re-ran the models using age and age2 (see above, response to Reviewer 1 comment 9) to "saturate" the model for an age effect and found no difference in our results and no significance with this new covariate. Therefore these findings are likely not due to chronological age, and are most likely attributable to some kind of environmental exposure or measure of 'aging' that is not chronological and has yet to be determined.
We also agree with the reviewer's important point that because methylation of repetitive elements, specifically LINE-1 methylation, has been associated with cardiovascular outcomes that this could be an important confounder in our analyses. Therefore, we re-ran the analyses including these covariates in the model and found our results did not change substantially, and even became more significant in some cases. We included cardiovascular disease as a covariate in two different ways, one as a composite of MI, stroke, angina, ischemic heart disease and hypertension (HTN), and a second version with all of these except HTN (because HTN may be less likely to be related to lung function). We also analyzed the model with the inclusion of diabetes (DM). See all results for these models below.

Cross sectional models:

Lung function decline models:

We have included this information in the manuscript on page XXX line XXX where we state:

Because of prior associations between methylation of repetitive elements and cardiovascular disease13-15, we re-ran all of our analyses including variables for cardiovascular disease (myocardial infarction, stroke, angina, hypertension, ischemic heart disease) and including diabetes and found no difference in the results (data not shown).

8) Figure 1: please stratify according to smoking status and smoking history (current smokers, ex-smokers and never-smokers).

Figure 1 has been revised to show distribution of Alu and LINE-1 methylation in the overall cohort and stratified by smoking status as below.

References:

1. Breitling LP, Yang R, Korn B, Burwinkel B, Brenner H. Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. Am J Hum Genet 2011;88(4):450-7.
2. Wan ES, Qiu W, Baccarelli A, Carey VJ, Bacherman H, Rennard SI, et al. Cigarette smoking behaviors and time since quitting are associated with differential DNA methylation across the human genome. Hum Mol Genet 2012;21(13):3073-82.
3. Madrigano J, Baccarelli A, Mittleman MA, Wright RO, Sparrow D, Vokonas PS, et al. Prolonged exposure to particulate pollution, genes associated with glutathione pathways, and DNA methylation in a cohort of older men. Environ Health Perspect 2011;119(7):977-82.
4. Wright RO, Schwartz J, Wright RJ, Bollati V, Tarantini L, Park SK, et al. Biomarkers of lead exposure and DNA methylation within retrotransposons. Environ Health Perspect 2010;118(6):790-5.
5. Marini JJ. Respiratory Medicine. 2nd ed. Baltimore: Williams & Wilkins Co., 1997.
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7. Pakhale S, Bshouty Z, Marras TK. Comparison of per cent predicted and percentile values for pulmonary function test interpretation. Can Respir J 2009;16(6):189-93.
8. Lung function testing: selection of reference values and interpretative strategies. American Thoracic Society. Am Rev Respir Dis 1991;144(5):1202-18.
9. Sparrow D, O'Connor G, Colton T, Barry CL, Weiss ST. The relationship of nonspecific bronchial responsiveness to the occurrence of respiratory symptoms and decreased levels of pulmonary function. The Normative Aging Study. Am Rev Respir Dis 1987;135(6):1255-60.
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11. Standardization of Spirometry, 1994 Update. American Thoracic Society. Am J Respir Crit Care Med 1995;152(3):1107-36.
12. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, et al. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 2009;130(4):234-9.
13. Baccarelli A, Wright R, Bollati V, Litonjua A, Zanobetti A, Tarantini L, et al. Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology 2010;21(6):819-28.

14. Castro R, Rivera I, Struys EA, Jansen EE, Ravasco P, Camilo ME, et al. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin Chem 2003;49(8):1292-6.
15. Kim M, Long TI, Arakawa K, Wang R, Yu MC, Laird PW. DNA methylation as a biomarker for cardiovascular disease risk. PLoS One 2010;5(3):e9692.

VERSION 2 – REVIEW

REVIEWER	Dana Hancock Genetic Epidemiologist Research Triangle Institute International I declare that I have no competing interests to disclose.
REVIEW RETURNED	10-Aug-2012

GENERAL COMMENTS	The authors have satisfactorily addressed my comments in the response to reviewers, but I encourage them to include a statement in the revised manuscript on the results from the age2 adjusted models, even if simply stated with results not shown. The importance of methylation in the context of aging is explicitly stated in the manuscript and pointed out by the other reviewer as well. It may be important for readers to know that your models show evidence of adequately capturing the age effect.