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# Prospective changes in global DNA methylation and cancer incidence and mortality

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**Background:** Methylation of repetitive elements Alu and LINE-1 in humans is considered a surrogate for global DNA methylation. Previous studies of blood-measured Alu/LINE-1 and cancer risk are inconsistent.

**Methods:** We studied 1259 prospective methylation measurements from blood drawn 1–4 times from 583 participants from 1999 to 2012. We used Cox regression to evaluate time-dependent methylation as a biomarker for cancer risk and mortality, and linear regression to compare mean differences in methylation over time by cancer status and analyse associations between rate of methylation change and cancer.

**Results:** Time-dependent LINE-1 methylation was associated with prostate cancer incidence (HR: 1.38, 95% CI: 1.01–1.88) and all-cancer mortality (HR: 1.41, 95% CI: 1.03–1.92). The first measurement of Alu methylation (HR: 1.39, 95% CI: 1.08–1.79) was associated with all-cancer mortality. Participants who ultimately developed cancer had lower mean LINE-1 methylation than cancer-free participants 10+ years pre-diagnosis ( $P < 0.01$ ). Rate of Alu methylation change was associated with all-cancer incidence (HR: 3.62, 95% CI: 1.09–12.10).

**Conclusions:** Our results add longitudinal data on blood Alu and LINE-1 methylation and cancer, and potentially contribute to their use as early-detection biomarkers. Future larger studies are needed and should account for the interval between blood sample collection and cancer diagnosis.

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DNA methylation aberrations are ubiquitous across various cancers, and are considered a hallmark of cancer (Laird, 2005). In general, cancer is thought to be characterised by global hypomethylation (resulting in increased oncogene expression and genomic instability), and gene-specific promoter hypermethylation (resulting in suppressed DNA repair and other tumour-suppressive functions; Wilson *et al*, 2007; Ohgane *et al*, 2008; Hansen *et al*, 2011). Not only do these methylation changes occur in tumour tissues, but they are frequently mirrored in blood leucocytes (Brennan and Flanagan, 2012; Joyce *et al*, 2015). Because of the time- and cost-effectiveness and non-invasiveness of blood sample collection, methylation detectable in white blood cells (WBCs) can be collected from healthy individuals for screening before their cancer diagnosis, and has long been a target for researchers attempting to develop new early-detection biomarkers of cancer. As an important, if not essential, part of cancer development, global DNA methylation is one such potential biomarker (Das and Singal, 2004).

Current estimates suggest that over one-third of DNA methylation occurs in repetitive elements (Kochanek *et al*, 1993; Schmid, 1998). Among these repetitive elements Alu and LINE-1 are the most plentiful families, collectively representing 30% of the human genome (Kazazian and Goodier, 2002; Grover *et al*, 2004; Babushok and Kazazian, 2007). Because of this high representation, Alu and LINE-1 are often used as surrogates for global DNA methylation (Yang *et al*, 2004; Weisenberger *et al*, 2005). Although tissue-specific studies of global DNA methylation have found associations between hypomethylation and increased cancer risk (Cravo *et al*, 1996; Soares *et al*, 1999; Bariol *et al*, 2003; Ehrlich *et al*, 2006; Van Hoesel *et al*, 2012), studies involving methylation measured from blood leucocytes have had far more mixed results. Case-control studies found that both hypomethylation (Pufulete *et al*, 2003; Lim *et al*, 2008; Moore *et al*, 2008; Hou *et al*, 2010; Di *et al*, 2011) and hypermethylation (Liao *et al*, 2011; Walters *et al*, 2013; Neale *et al*, 2014) were associated with increased cancer risk across multiple tissue types. Prospective studies of global DNA methylation measured at a single time point in blood leucocytes have also found increased cancer risk associated with both global hypomethylation (Gao *et al*, 2012) and hypermethylation (Andreotti *et al*, 2014). Furthermore, to our knowledge no prospective study has examined the longitudinal relationship between repeated measures of global DNA methylation and cancer risk over time. There is evidence to suggest a complex biological relationship, with one study suggesting that the timing of blood draws relative to cancer diagnosis matters (Barry *et al*, 2015), and another finding a 'U-shaped' association (Tajuddin *et al*, 2014).

Faced with these inconsistencies, we set out to use our recently generated multiple measures of Alu and LINE-1 DNA methylation, and perform an update of our previous investigation of global DNA methylation and cancer risk in the Normative Aging Study (NAS; Zhu *et al*, 2011). The updates to our data set allowed us to increase our sample size, which allows a more thorough analysis of cancer incidence and cancer mortality. Furthermore, the increased follow-up time allows a greater analysis of temporal factors affecting the relationship between global DNA methylation markers and cancer (i.e., the interval between sample collection and cancer diagnosis). Finally, our larger sample allows us to exclude Alu and LINE-1 methylation measures in blood collected after cancer diagnosis, meaning that our new analysis can rule out potential 'reverse causality' effects of cancer development on Alu and LINE-1 methylation. Our objective is to leverage additional longitudinal data (and new statistical methods for longitudinal analysis developed by our group) to update our previously published findings regarding Alu and LINE-1 methylation and hazards of cancer incidence and mortality. Our goal is to test the hypothesis that these global

DNA methylation surrogates measured in blood leucocytes can be used as biomarkers of cancer risk.

## MATERIALS AND METHODS

**Study population.** The NAS was established by the US Department of Veterans Affairs in 1963 with an initial study cohort of 2280 healthy men (aged 21–80 years at enrolment) living in the greater Boston area, of primarily (96%) white race. Since then, participants have been recalled periodically for clinical exams every 3–5 years; starting in 1999 these exams included a 7-ml blood sample for genetic and epigenetic analysis. Between 1 January 1999 and 31 December 2012; 802 of 829 active subjects (96.7%) agreed to donate blood for DNA analysis. In total, LINE-1 or Alu methylation was measured at least once in 796 participants. Of these, we excluded 213 participants diagnosed with cancer prior to first blood sample collection, leaving 583 participants cancer-free at the first blood draw for analysis. This population included our previously published study of LINE-1 and Alu methylation and cancer, with an additional 108 cancer cases and 9 new cancer deaths with NAS data collected from 2007 to 2012. This study was approved by the institutional review boards of all participating institutions, and written consent forms were obtained from all participants.

In addition to blood samples, the NAS collected data including anthropometric measurements, standardised medical exams, and questionnaires about medical and smoking history. Our analysis considered the following potential covariates: race (dichotomized as white or non-white), education (<13 years, 13–16 years, >16 years), two cigarette smoking variables (status of never/former/current and pack-years), whether the respondent reported taking two or more alcoholic drinks per day on average, body mass index (BMI) calculated from weight and height measurements, and age. Our multivariable models were also adjusted for WBC count and per cent neutrophils taken from the blood sample, as per previous analyses of blood leucocyte methylation in the NAS (Joyce *et al*, 2015).

Information on cancer diagnoses was obtained from questionnaires and confirmed via review of medical records and histological reports. In contrast to our previous study (Zhu *et al*, 2011) of Alu and LINE-1 methylation in the NAS, we excluded participants who had been diagnosed with cancer prior to their first blood draw from all analyses. Among the 583 participants free of cancer at first visit, 138 (23.7%) developed cancer during a median of 10.54 (IQR: 6.02–12.10) years of follow-up, including 46 prostate cancers and 92 other cancers. For participants who died, death certificates were obtained from the appropriate state health department and reviewed to ensure accurate classification of primary cause of death. These included 37 (6.3%) subjects free of cancer at the first blood draw who later died of cancer during median of 11.41 (IQR: 8.43–12.34) years of follow-up, including 5 subjects who died of prostate cancer.

**Methylation measurement.** The full procedure for blood leucocyte DNA extraction and measurement has been reported previously (Hou *et al*, 2011). In order to maximise methylation measurement accuracy, we used a pyrosequencing-based assay to measure CpG sites at three positions each for LINE-1 and Alu. All assays used built-in controls. Methylation measurements from each position were averaged via a simple mean, and then standardised by batch number to a mean value of 0 and a standard deviation of 1. Figure 1 shows scatter plots of LINE-1 and Alu methylation over time, after batch standardisation. We also show box plots of LINE-1 and Alu methylation over time before (Supplementary Figure 1) and after (Supplementary Figure 2) batch standardisation. Similar

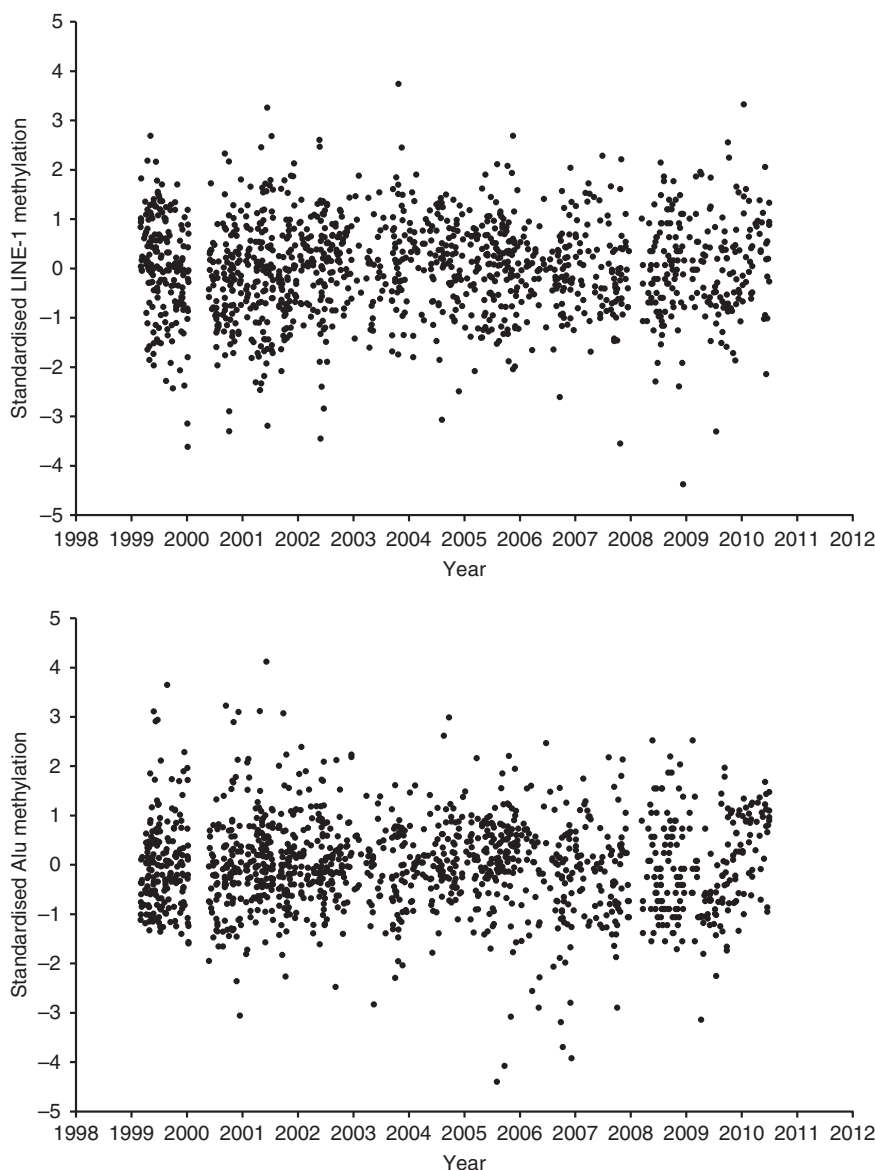


Figure 1. Scatter plots of LINE-1 (Top) and Alu methylation (bottom) over time.

to previous reports (Zhu *et al*, 2011), the within-sample coefficients of variation were 1.6% for Alu and 0.7% for LINE-1.

**Statistical analysis.** We first examined associations between cancer risk factors at the first visit and global DNA methylation using simple, unadjusted linear regression. Next, we used multiple Cox proportional hazards regression models to estimate associations between both first visit and time-dependent global DNA methylation (calculated as the change in methylation between time points for those subjects with multiple measurements), and cancer incidence and mortality. Time-dependent methylation was calculated using all available methylation data, with each subject's follow-up time divided into intervals by methylation measurement, ensuring that each methylation measurement had its own follow-up period. This included a sensitivity analysis adjusting for batch as a covariate in our models (rather than batch-standardising our methylation measurements) to ensure that this decision in our approach did not meaningfully alter our results. We also examined associations between methylation trajectory and cancer incidence. This was done by comparing the mean difference in methylation between cancer cases and cancer-free individuals each year prior to cancer diagnosis. Owing to low sample size, intervals were

collapsed into 5-year increments (<5 years, 5 to <10 years, and >10 years). Logistic regression models corresponding to these categories were run to generate odds ratios stratified by time interval between methylation measurement and cancer diagnosis. We then analysed the relationship between methylation rate of change (in units per year) and cancer incidence and mortality using a linear regression model, to estimate the change in methylation measurement over time for all subjects with multiple measurements, and subsequently treating the  $\beta$ -coefficient from this model as an independent variable in additional Cox regression models. We conducted two additional sensitivity analyses. The first was to repeat the above analyses, but using an exposure created by averaging Alu and LINE-1 methylation measurements weighted by their fractions of genome mass (11% and 17%, respectively, i.e., Alu methylation measurements would be weighted by 11 out of 28 and LINE-1 by 17 out of 28). Qualitatively the results were the same, with most statistical associations attenuated. Therefore, we opted not to report those results (data available upon request). For our second sensitivity analysis, we repeated the above regression models for Alu and LINE-1 measured at each individual position. As these results were not substantively different than those generated using the mean of these positions, we report the mean

Alu and LINE-1 results only (data available upon request). All models were adjusted for the covariates listed above. All analyses were performed using SAS (version 9.3, SAS Institute, Cary, NC, USA). Two-sided tests were used when comparing means and  $\beta$ -coefficients, and  $P \leq 0.05$  was set as the threshold for statistical significance.

## RESULTS

Subject characteristics by cancer status have been reported previously (Zhu *et al*, 2011). Briefly, subjects were male, with a mean age of 72 years at first visit (range 55–100 years), and majority were white (95.5%). Characteristics by LINE-1 and Alu methylation at the first visit are listed in Table 1. Overall, there were no differences in subject characteristics by LINE-1 methylation. However, subjects with higher Alu methylation tended to have a higher BMI ( $P=0.022$ ) than subjects with lower Alu methylation. The details of our LINE-1 and Alu methylation measurements by batch are provided in Table 2.

Table 3 shows the results of our first visit and time-dependent analyses. For cancer incidence, we found a positive association between risk of developing prostate cancer and LINE-1 methylation, which was significant in the time-dependent analysis (HR: 1.38, 95% CI: 1.01–1.88). Alu methylation at the first measurement was directly associated with risk of developing respiratory cancer (HR: 2.03, 95% CI: 1.09–3.78). For cancer mortality we found a positive association between risk of cancer mortality and LINE-1 methylation in time-dependent analysis (HR: 1.41, 95% CI: 1.03–1.92), as well as Alu methylation at the first visit (HR: 1.39, 95% CI: 1.08–1.79). In addition, Alu methylation at the first measurement was directly associated with risks of dying from digestive (HR: 1.55, 95% CI: 1.06–2.29) and respiratory cancers (HR: 2.34, 95% CI: 1.10–5.00). Results of our sensitivity analysis adjusting for batch as a covariate, rather than via standardisation, are presented in Supplementary Table 1.

Figure 2 shows graphs of Alu and LINE-1 methylation by time interval between methylation measurement and cancer diagnosis. On average, subjects who ultimately developed cancer had LINE-1 methylation 0.54 units lower than cancer-free subjects, 10 or more

years before diagnosis ( $P=0.0056$ ). Neither the differences in mean LINE-1 methylation between cancer cases and cancer-free subjects at other time points were not significant, nor were the other differences in mean Alu methylation. The corresponding regression model results can be found in Supplementary Table 2.

Table 4 shows the results of our rate of change analysis. We found significant associations between all-cancer incidence and the rate of change of Alu methylation (in standardised units per year; HR: 3.62, 95% CI: 1.09–12.10). We observed no significant associations between rate of change of LINE-1 methylation and cancer incidence. Neither Alu nor LINE-1 rate of change was associated with cancer mortality (data available upon request). Analysis of the rate of change of other cancers can be found in Supplementary Table 3.

## DISCUSSION

In this study we found higher Alu methylation among participants who were younger and had lower BMI. For cancer incidence, time-dependent LINE-1 methylation was positively associated with risk of prostate cancer only. For cancer mortality, Alu methylation at the first blood draw as well as time-dependent LINE-1 methylation were associated with increased risk of all-cancer mortality. We identified associations between Alu methylation, and respiratory and digestive cancers, however, because of the small number of cases in these analyses our results should be interpreted cautiously. Interestingly, LINE-1 methylation was significantly lower in participants who ultimately developed cancer >10 years prior to conventional diagnosis, whereas a more rapid rate of increase in Alu methylation was significantly associated with risk of developing cancer. The updated results are different from our previous report (Zhu *et al*, 2011), which might be owing to the much larger number of incident cancer cases in our analysis (138 in our analysis vs 30 in the previous report), as well as our exclusion of prevalent cancers from the mortality analysis (leaving us with 37 deaths due to an incident cancer vs 11 in the previous report).

Although most studies of cancer, and Alu and LINE-1 methylation have found inverse relationships between these methylation measures and cancer, the vast majority of these findings have used tumour tissue, or blood leucocytes collected after cancer diagnosis (Brennan and Flanagan, 2012). Furthermore, two recent meta-analyses found that different measures of global DNA methylation in blood produce different associations with cancer risk, and that the methodological heterogeneity of studies makes drawing conclusions as to its use as a cancer biomarker difficult (Brennan and Flanagan, 2012; Woo and Kim, 2012). The findings of the handful of prospective studies of blood leucocyte Alu or LINE-1 methylation have thus far been less consistent. Three studies of gastric, liver, and breast cancer found no prospective associations between cancer risk and LINE-1 methylation (Balassiano *et al*, 2011; Brennan *et al*, 2012; Wu *et al*, 2012). In another prospective study, LINE-1 hypomethylation was associated with breast cancer risk (Deroo *et al*, 2014), whereas two others found marginally significant associations between LINE-1 hypermethylation, and kidney (Karami *et al*, 2015) and bladder cancer (Andreotti *et al*, 2014). A prospective study of Alu methylation found an inverse association between Alu methylation and gastric cancer risk, but only with latencies of >1 year (Gao *et al*, 2012). These discrepant results are likely owing to high variation in study designs and populations (Brennan and Flanagan, 2012), such as the intervals between blood sample collection and cancer diagnosis, which ranged from 1 to 16 years in those cited above. A recent prospective analysis of prostate cancer patients found that the relationship between Alu methylation and prostate cancer risk varied by length of this interval, with associations between Alu

**Table 1. Subject characteristics and global DNA methylation among subjects cancer-free at first visit**

	LINE-1		Alu	
	$\beta$	P-value	$\beta$	P-value
Age (years)	-0.002	0.76	-0.010	0.08
Body mass index (kg m <sup>-2</sup> )	0.001	0.94	0.022	0.022 <sup>a</sup>
Race	Ref.		Ref.	
Non-white	0.158	0.45	-0.0002	0.99
White				
Education (years)	Ref.		Ref.	
< 13	-0.177	0.08	-0.010	0.92
13–16	-0.001	0.99	-0.023	0.84
> 16				
Smoking status	Ref.		Ref.	
Never	0.378	0.09	-0.032	0.87
Current	0.130	0.19	0.097	0.28
Former				
Cumulative smoking (pack-years)	0.003	0.09	0.001	0.74
Alcohol consumption	Ref.		Ref.	
0–1 average drinks per day	0.104	0.36	-0.05	0.61
2+ average drinks per day				
White blood cell count	-0.010	0.62	-0.001	0.97
Neutrophils proportion	0.004	0.42	0.002	0.65

<sup>a</sup>Statistically significant at  $P < 0.05$ .

**Table 2. LINE-1 and Alu measurement data by batch**

Batch	LINE-1				Alu			
	Median	S.d.	Minimum	Maximum	Median	S.d.	Minimum	Maximum
1	77.45	1.59	69.83	81.63	26.29	1.18	22.83	31.47
2	84.38	1.09	80.79	87.99	28.64	1.36	24.41	31.34
3	77.03	1.89	70.05	81.03	26.33	1.09	23.42	29.37
4	76.47	1.68	71.13	84.55	26.17	0.93	24.00	32.40
5	77.68	2.14	70.53	83.00	24.62	1.42	20.45	28.88
6	82.36	1.56	73.41	88.07	25.12	1.18	17.80	28.15
7	83.20	1.06	78.60	85.70	26.40	0.61	25.10	28.00

**Table 3. Association between global DNA methylation with cancer incidence and mortality**

	First methylation measurement				Time-dependent methylation measurement	
	Cancer-free n	Cancer n	HR (95% CI)	P-value	HR (95% CI)	P-value
<b>Incidence</b>						
<b>LINE-1</b>						
All cancer	422	125	1.05 (0.88–1.24)	0.60	1.11 (0.94–1.31)	0.22
Prostate cancer		42	1.32 (0.97–1.80)	0.08	1.38 (1.01–1.88)	0.045 <sup>a</sup>
Skin cancer		41	0.95 (0.70–1.30)	0.76	1.01 (0.74–1.37)	0.97
Digestive cancer		11	0.65 (0.37–1.15)	0.14	0.88 (0.50–1.54)	0.65
Respiratory cancer		9	1.35 (0.64–2.87)	0.43	1.40 (0.66–2.94)	0.38
Blood cancer		7	0.75 (0.34–1.66)	0.48	1.04 (0.54–1.98)	0.91
<b>Alu</b>						
All cancer	432	129	0.97 (0.81–1.17)	0.76	0.95 (0.80–1.13)	0.61
Prostate cancer		43	0.83 (0.58–1.20)	0.33	0.86 (0.62–1.19)	0.38
Skin cancer		41	0.86 (0.61–1.23)	0.41	1.08 (0.79–1.48)	0.62
Digestive cancer		13	1.35 (0.88–2.07)	0.17	1.07 (0.65–1.76)	0.80
Respiratory cancer		9	2.03 (1.09–3.78)	0.026 <sup>a</sup>	0.69 (0.40–1.19)	0.18
Blood cancer		7	0.49 (0.18–1.32)	0.16	0.86 (0.36–2.05)	0.73
<b>Mortality</b>						
<b>LINE-1</b>						
All cancer	513	34	1.24 (0.91–1.68)	0.18	1.41 (1.03–1.92)	0.03 <sup>a</sup>
Prostate cancer		5	2.52 (0.84–7.56)	0.10	1.51 (0.68–3.33)	0.31
Skin cancer		1	NA		NA	
Digestive cancer		9	0.79 (0.42–1.49)	0.46	1.11 (0.57–2.16)	0.77
Respiratory cancer		7	2.44 (0.94–6.34)	0.066	1.91 (0.76–4.84)	0.17
Blood cancer		5	1.65 (0.38–7.22)	0.50	1.27 (0.47–3.48)	0.63
<b>Alu</b>						
All cancer	524	37	1.39 (1.08–1.79)	0.01 <sup>a</sup>	0.91 (0.67–1.24)	0.56
Prostate cancer		5	1.64 (0.62–4.32)	0.32	0.69 (0.27–1.78)	0.45
Skin cancer		1	NA		NA	
Digestive cancer		11	1.55 (1.06–2.29)	0.026 <sup>a</sup>	1.24 (0.72–2.14)	0.44
Respiratory cancer		7	2.34 (1.10–5.0)	0.028 <sup>a</sup>	0.68 (0.40–1.17)	0.17
Blood cancer		5	0.44 (0.09–2.23)	0.32	0.32 (0.11–0.94)	0.04

Abbreviations: CI = confidence interval; HR = hazard ratio; NA = not applicable.

<sup>a</sup>Statistically significant at  $P < 0.05$ . All LINE-1 and Alu measurements were standardised by batch number.

hypermethylation and increased risk appearing only among subjects diagnosed 4 or more years after their blood draw (Barry *et al.*, 2015). Although the effects of various intervals between blood sample collection and cancer diagnosis have not been studied in LINE-1 hypermethylation specifically, this effect may also explain our findings of increased prostate cancer risk with time-dependent LINE-1 methylation.

To our knowledge, few studies have examined associations between global DNA methylation of blood leucocytes collected pre-diagnostically and cancer mortality. Two studies found associations between serum LINE-1 hypomethylation and all-cause mortality in populations composed largely of cancer patients (Tangkijvanich *et al.*, 2007; Ramzy *et al.*, 2011), and our prior analysis of NAS data found associations between both LINE-1 and Alu hypomethylation and all-cancer mortality (Zhu *et al.*, 2011). That our results represent a marked shift from the prior

publication is cause for concern. We feel that this is primarily because of the fact that approximately half (59%) of cancer deaths in the previous study were due to cancers diagnosed prior to the first blood draw, potentially obscuring the temporal (and thus causal) relationship. The fact that our mortality analysis was conducted solely on participants who were free of cancer at the first blood draw may explain the discrepancy between our findings and those reported elsewhere in the literature examining cancer mortality among methylation measures obtained pre- and post-diagnosis. Future studies should further examine Alu and LINE-1 methylation as potential prognostic biomarkers for cancer mortality, and the differences in these relationships between methylation measures pre and post diagnosis.

Recent research suggests a dynamic role of Alu methylation during normal development and aging (Luo *et al.*, 2014). Our analysis found a significant association between faster Alu

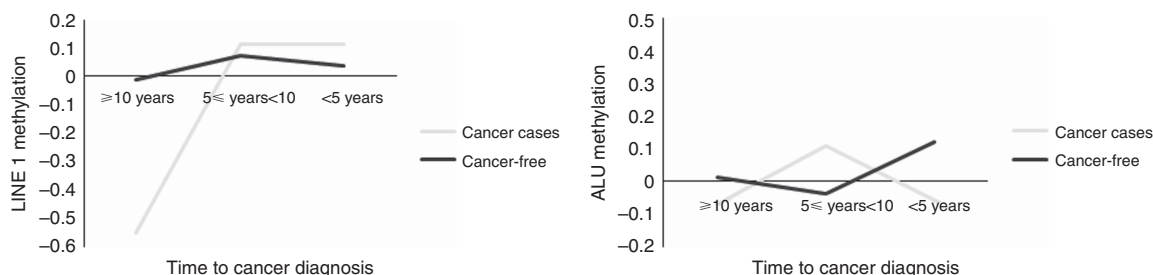


Figure 2. Trajectory of global DNA methylation in cancer and cancer-free subjects by time interval between methylation measurement and cancer diagnosis.

Table 4. Associations between rate of methylation change and cancer

	Cancer-free		All cancer		Prostate cancer		
	n	n	HR (95% CI)	P-value	n	HR (95% CI)	P-value
<b>Incidence</b>							
LINE-1	365	63	1.23 (0.29–5.11)	0.77	13	0.75 (0.03–19.1)	0.86
Alu			3.62 (1.09–12.1)	0.036 <sup>a</sup>		1.58 (0.11–22.2)	0.73
<b>Mortality</b>							
LINE-1	389	23	3.88 (0.44–34.1)	0.22	4	0.75 (0.03–19.1)	0.86
Alu			1.96 (0.37–10.2)	0.43		9.97 (0.14–700)	0.29

Abbreviations: CI = confidence interval; HR = hazard ratio.  
<sup>a</sup>Statistically significant at P<0.05.

methylation and higher cancer incidence, coupled with what is already known about the involvement of Alu methylation in tumorigenesis, this raises the possibility that Alu may have a dynamic role in cancer development as well. Similarly, we found that on average participants who would ultimately develop cancer had much lower LINE-1 methylation compared with cancer-free participants, but only at ~10 years before diagnosis. Although both these findings are not unprecedented in this data set (Joyce *et al*, 2015), the low sample size of the NAS means that these results should be interpreted with caution. Further validation of these longitudinal biomarkers of cancer is required, but, if successful, could yield potent new tools for cancer early detection.

Although methylation of LINE-1 and Alu elements have both been widely used as surrogates for global DNA methylation, growing evidence shows that they each have a distinct functional role potentially affecting cancer development and progression via different mechanisms in methylation regulation, responses to cellular stressors and environmental exposures, and methylation levels (Li and Schmid, 2001; Jones and Baylin, 2002; Biemont and Vieira, 2006; Rusiecki *et al*, 2008). Even specific LINE-1 loci may exert different effects on disease risk (Nusgen *et al*, 2015; though there was no evidence for this in our particular data set). Thus, our finding different relationships between Alu and LINE-1 methylation and cancer is unsurprising, despite both being surrogates for global DNA methylation.

Our use of samples collected and stored before cancer diagnosis is a particular strength of this study as it avoids a number of biases inherent in case-control designs, particularly disease- or treatment-induced epigenetic changes. Nonetheless, this study has several limitations. The trade-off to collecting a large quantity of data across multiple follow-up measurements is a relatively low sample size. These factors limited our ability to examine specific subtypes of cancer beyond prostate cancer. In addition, as noted above, our sample was not representative of the general population. Being older, male, white, educated, and/or having a history of military service may all influence both global DNA methylation and cancer. Therefore, our results will need to be confirmed in other, more representative populations. Finally, it should be noted (as it has in

both recent meta-analyses (Brennan and Flanagan, 2012; Barchitta *et al*, 2014) on the subject) that publication bias is a serious and unresolved concern for studies of Alu and LINE-1 methylation, and that care should be taken in the future to report null results.

In conclusion, this prospective study of global DNA methylation and cancer found a series of complex relationships underlying Alu and LINE-1 methylation, cancer incidence and mortality, and time. Alu and LINE-1 hypermethylation of blood leucocytes may be a predictor of cancer incidence, in particular prostate cancer. Furthermore, Alu and LINE-1 methylation may serve as useful prognostic indicators of cancer mortality. The possible use of these two measures as cancer early-detection and prognostic biomarkers, and whether such relationships are consistent with other measures of global DNA methylation, should be validated in larger prospective studies. In addition, the rate of change of Alu methylation and average LINE-1 methylation 10 years pre-diagnostically are interesting (if speculative) findings that need to be confirmed in future research. Taken together, these findings suggest that global DNA methylation has a dynamic role in tumorigenesis and cancer progression, and identify several new avenues of research for the study of Alu and LINE-1 as biomarkers of cancer.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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