Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles

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Accepted 30 January 2012

Background Patterns of DNA methylation change with age and these changes are believed to be associated with the development of common complex diseases. The hypothesis that Long Interspersed Nucleotide Element 1 (LINE-1) DNA methylation (an index of global DNA methylation) is associated with biomarkers of metabolic health was investigated in this study.

Methods Global LINE-1 DNA methylation was quantified by pyrosequencing in blood-derived DNA samples from 228 individuals, aged 49–51 years, from the Newcastle Thousand Families Study (NTFS). Associations between log-transformed LINE-1 DNA methylation levels and anthropometric and blood biochemical measurements, including triglycerides, total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, fasting glucose and insulin secretion and resistance were examined.

Results Linear regression, after adjustment for sex, demonstrated positive associations between log-transformed LINE-1 DNA methylation and fasting glucose (coefficient 2.80 [95% confidence interval (CI) 0.39–5.22]), total cholesterol [4.76 (95% CI 1.43–8.10)], triglycerides [3.83 (95% CI 1.30–6.37)] and LDL-cholesterol [5.38 (95% CI 2.12–8.64)] concentrations. A negative association was observed between log-transformed LINE-1 methylation and both HDL cholesterol concentration [−1.43 (95% CI −2.38 to −0.48)] and HDL:LDL ratio [−1.06 (95% CI −1.76 to −0.36)]. These coefficients reflect the millimoles per litre change in biochemical measurements per unit increase in log-transformed LINE-1 methylation.

Conclusions These novel associations between global LINE-1 DNA methylation and blood glycaemic and lipid profiles highlight a potential role for epigenetic biomarkers as predictors of metabolic disease and may be relevant to future diagnosis, prevention and treatment of this group of disorders. Further work is required to establish the role of confounding and reverse causation in the observed associations.

Keywords Global DNA methylation, LINE-1, cohort study, glucose, HDL/LDL cholesterol, insulin, triglyceride
Introduction

DNA methylation is the covalent modification of cytosine residues in the DNA sequence through the addition of a methyl group that converts cytosine to 5-methyl cytosine (5mC). In the human genome, this covalent modification largely takes place on cytosine residues that are located 5' adjacent to guanine residues. These sequences of nucleotides are known as CpG sites, and in some regions of the genome they cluster together forming motifs known as CpG islands (CGIs). These CGIs make up a maximum of 2% of the genome, and are for the most part unmethylated. Some CGIs, however, are more highly methylated and these tend to be proximal to imprinted genes or transposons. At a global level, DNA methylation can be assessed by utilizing repeat interspersed regions such as Alu or Long Interspersed Nucleotide Element 1 (LINE-1). Comprising ~17% of the human genome, LINE-1 is the most abundant family of non-long terminal repeat retrotransposons found in the genome. Such elements have served as a useful proxy for global DNA methylation as they are commonly heavily methylated in normal tissue (although hypomethylated in tumour tissue), and are spread ubiquitously throughout the genome. The level of correlation of LINE-1 with gene-specific methylation is not well documented, although LINE-1 methylation does correlate with global methylation measured using a variety of different methods including Alu, Sat2 and LUMA. Global hypomethylation is a common event in ageing cells. This has been shown in relation to some interspersed repeat regions such as Alu, but the evidence is less clear with regard to LINE-1. Indeed, a recent study of age-dependent changes in DNA methylation of interspersed repeat regions showed a weak positive correlation between age and LINE-1 methylation.

Limited evidence exists to link LINE-1 methylation with disease, and this has almost exclusively been observed in the cancer field. For example, LINE-1 methylation levels modulate the effects of exposure to dietary folate and alcohol on colon cancer risk, and have prognostic value when analysed in colon tumour tissue. In a recent study of LINE-1 methylation and cardiovascular health in the Boston-based Normative Aging Study, people with prevalent ischaemic heart disease (IHD) and stroke had lower LINE-1 methylation, and, in longitudinal analyses, those with lower LINE-1 methylation were at higher risk for incident IHD, stroke and total mortality. Thus, there is little empirical evidence to date linking LINE-1 methylation with common complex diseases other than cancer and limited evidence for an association with neural tube defects. However, studies both in experimental animals and in human cohorts have shown that environmental insults can influence DNA methylation (reviewed by Mathers et al.). LINE-1 methylation is also susceptible to a wide range of environmental exposures including perfluorooctane sulfonate, prenatal tobacco smoke exposure, polycyclic aromatic hydrocarbons, biomarkers of lead levels in both adults and cord blood, traffic particulates and plasma homocysteine. The present study addresses the hypothesis that global LINE-1 DNA methylation, measured at age 49–51 years, is associated with traits indicative of early-stage metabolic disease.

Methods

Study participants

The Newcastle Thousand Families Study (NTFSS) prospective birth cohort consists of all 1142 individuals born in May and June 1947 to mothers resident within the city of Newcastle upon Tyne in northern England. Two-thirds of these children were followed up regularly until age 15 years, with detailed information collected prospectively on their health, growth and socio-economic circumstances. Follow-up was re-established during the 1990s with participants being traced via media publicity or through the UK National Health Service Central Register. Between October 1996 and December 1998, 412 participants (~50 years of age) attended clinical examinations which included blood collection for DNA analysis and completed questionnaires detailing their family history and lifestyle. DNA from 228 individuals was analysed in the current study, based upon samples of sufficient quality and quantity for LINE-1 DNA methylation analysis. Excluding sex, these 228 individuals were representative of the initial 1142 participants as well as the 412 follow-up participants (data not shown).

Clinical assessments of outcomes and adult height and weight at age 49–51 years

Assessments were performed in the morning following an overnight fast. All lipid analyses were performed on a DAX analyser (Bayer, Basingstoke). Total cholesterol was measured using a cholesterol oxidase/peroxidase method with calibrants traceable to the Centres for Disease Control definitive method. Serum high-density lipoprotein (HDL) cholesterol was measured using a cholesterol oxidase method after precipitation of apolipoprotein B containing lipoproteins with phosphotungstic acid and magnesium chloride (interassay coefficient of variation 2.2%). Low-density lipoprotein (LDL) cholesterol levels were derived by the Friedewald method and the HDL:LDL ratio was calculated. Triglyceride concentrations, excluding glycerol, were estimated by a lipase-glycerol kinase method. Plasma glucose concentrations at 0, 30 and 120 min (after a 75-g oral glucose load) were measured on a Yellow Springs Analyser (YSI Stat Plus 2300; Yellow Springs Instruments, Farnborough, UK) and serum insulin at the same time-points.
was determined by ELISA (Dako Ltd, Ely, UK) (interassay coefficients of variation 3.1 and 3.3%, respectively). Insulin resistance was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR) of Matthews et al. Insulin secretion was estimated as the ratio of the 30-min increment in insulin concentration to the 30-min increment in glucose concentration following oral glucose loading, relative to the baseline concentrations. Height and weight were measured and body mass index (BMI) was calculated. Waist and hip circumferences were measured according to the protocol of the World Health Organization Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) project. Percent body fat was estimated from impedance measurements using a Holtain body composition analyser (Holtain Ltd, Crymych, Wales, UK).

Measurement of global LINE-1 DNA methylation

DNA was extracted from peripheral blood samples using a Nucleon BACC2 kit (Tepnel Life Sciences, UK). One microgram of DNA sample was bisulphite modified using the Zymo EZ DNA Methylation Gold kit (Cambridge Bioscience, Cambridge) using the manufacturer’s standard protocol. One microgram of bisulphite modified DNA was PCR amplified using 2 x HotstarTAQ Mastermix (Qiagen, UK), 2 mM MgCl₂ (Qiagen, UK) and 0.2 μM of each primer (LINE-1 forward primer—5’-TTT TGA GTT AGG TGT GGG ATA TA-3’ and LINE-1 reverse primer—BIO-5’-AAA ATC AAA AAA TTC CCT TTC-3’). PCR conditions were as follows: 95°C for 15 min, 50 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 15 s and finally 72°C for 5 min. Five microlitres of amplicons were utilized for downstream single-strand preparation and hybridization of 0.5 μM sequencing primer (5’-GGG TGG GAG TGA T-3’), using a vacuum prep tool and workstation according to manufacturer’s instructions (Qiagen, UK). LINE-1 methylation was quantified using a PyroMark MD Pyrosequencer (Qiagen, UK) in which the analysis sequence for LINE-1 was: TC/TGATTTTTAGGTC/TGTTCTGGTTA. Zero and 100% methylated controls were generated by carrying out a nested PCR reaction on genomic DNA to generate an unmethylated control, followed by in vitro methylation (SssI treatment) of an aliquot of the PCR product to generate a methylated control. These controls were used to rule out any amplification bias of primers for methylated DNA and to assess assay reproducibility. LINE-1 primer sets were found to be unbiased and were reproducible. 0 and 100% methylated controls were routinely run alongside samples as internal controls. Samples were analysed in duplicate with appropriate quality control measures in place (bisulphite conversion and PCR controls and random repeats). The mean (standard deviation) difference between duplicate samples was 3.0 (3.4%). This assay arbitrarily amplifies LINE-1 sequences from multiple genomic locations, providing a representative measure of methylation that is not site-specific i.e. a global assessment.

Statistical analysis

Pyrosequencing generated estimates of LINE-1 DNA methylation at each of three CpG sites which were expressed as a percentage, i.e. the proportion of methylated residues in the total DNA sample assayed. Correlation between methylation at all three CpG sites was high ($P < 0.001$), therefore a mean of all three sites was calculated and tested for association with the anthropometric, glycaemic and lipid-related outcome variables. Mean methylation levels were comparable with those previously published using a similar (but not identical) assay design. DNA methylation showed a skewed distribution (tested using a Shapiro-Wilk test) so values were log-transformed before further analysis. Linear regression was used to examine relationships between anthropometric measures, glycaemic and lipid-related blood biomarkers (the dependent variables) and the independent variable, log-transformed global LINE-1 DNA methylation at age 50 years. Regression coefficients and corresponding 95% confidence intervals (CIs) are reported showing the level of change in outcome measures per unit increase in log-transformed LINE-1 DNA methylation, after adjustment for sex. Overall $R^2$ values for the models including both log-transformed LINE-1 DNA methylation and sex are given as percentages. Direct $R^2$ values for methylation were estimated by subtracting the $R^2$ value for the models including sex only from the corresponding models including both log-transformed LINE-1 DNA methylation and sex. This gives an estimate of the direct association between outcome and methylation after adjustment for any potential mediation through sex. Finally, the potential influence of covariates known to be associated with global DNA methylation (namely, age, alcohol consumption and smoking status) upon the observed associations was assessed within the linear regression models.

Ethical approval for the study was obtained from the appropriate local research ethics committees and all participants provided their written consent.

Results

LINE-1 DNA methylation measurements were available for 228 study members of the 412 who attended the clinical assessment, with no significant difference in the distribution of mean methylation values between males ($n=85$, 37%) and females ($n=143$, 63%) (Mann–Whitney $z=0.60$, $P=0.55$). Descriptive data for all variables used in this study are given in Table 1. Increased LINE-1 DNA methylation was associated with increasing fasting glucose [regression
coefficient (95% CI) = 2.80 (0.39–5.22) \( P = 0.02 \), total cholesterol = 4.76 (1.43–8.10), \( P = 0.005 \), total triglycerides = 3.83 (1.30–6.37), \( P = 0.003 \) and LDL cholesterol = 5.38 (2.12–8.64), \( P = 0.001 \) and with decreasing HDL cholesterol = 1.43 (2.38 to –0.48), \( P = 0.003 \) and HDL:LDL ratio = 1.06 (–1.76 to –0.36), \( P = 0.003 \) (Table 2). For these analyses, LINE-1 DNA methylation (predictor variable) was log-transformed. Hence, these coefficients reflect the millimoles per litre change in outcome measure per unit increase in log-transformed methylation. Alternatively, following a 10% increase in LINE-1 DNA methylation, fasting glucose, total cholesterol, total triglycerides and LDL cholesterol increase by 0.28, 0.48, 0.38 and 0.54 mmol/l, respectively; and HDL cholesterol and HDL:LDL ratio decrease by 0.14 mmol/l and 0.11 units, respectively. The combined contributions of sex and LINE-1 DNA methylation explained between 4.98 and 9.65% of the variation in outcome measures. LINE-1 DNA methylation alone accounted for 2.12–4.37% of this variability (Table 2). Additional variables thought to influence global methylation (namely, age, alcohol consumption and smoking status) were not associated with LINE-1 DNA methylation in this study cohort and hence did not alter any of the associations observed (data not shown).

**Discussion**

The data presented demonstrate that increased LINE-1 DNA methylation is associated with a number of blood-based biomarkers of metabolic health and provide evidence of an association between LINE-1 DNA methylation and phenotypic traits other than cancer. There is substantial evidence that LINE-1 DNA methylation is modulated by a wide range of environmental exposures, \([12–19]\) and several of these environmental exposures are associated with risk of complex diseases. It is therefore attractive to postulate that LINE-1 DNA methylation may provide a mechanistic link between such environmental exposures and the development of disease-related traits, although the current data suggest no such link with alcohol consumption and smoking status. However, it is also possible that LINE-1 DNA methylation patterns are confounded and are not causally

| Outcome variable | Coefficient (95% CI) | \( P \)-value | \( R^2 \) (%) | Direct \( R^2 \) (%) |
|------------------|---------------------|---------------|---------------|------------------|
| BMI (kg/m\(^2\)) | 2.30 (–9.99 to 14.59) | 0.71 | 0.90 | 0.06 |
| Waist/hip ratio | 0.14 (–0.01 to 0.30) | 0.07 | 65.42 | 0.51 |
| Body fat (%) | 5.14 (–19.01 to 29.30) | 0.68 | 5.65 | 0.08 |
| Fasting glucose (mmol/l) | 2.80 (0.39 to 5.22) | 0.02 | 9.05 | 2.12 |
| Total cholesterol (mmol/l) | 4.76 (1.43 to 8.10) | 0.005 | 4.98 | 3.34 |
| Total triglycerides (mmol/l) | 3.83 (1.30 to 6.37) | 0.003 | 9.65 | 3.57 |
| HDL cholesterol (mmol/l) | –1.43 (–2.38 to –0.48) | 0.003 | 8.96 | 3.54 |
| LDL cholesterol (mmol/l) | 5.38 (2.12 to 8.64) | 0.001 | 7.02 | 4.37 |
| HDL:LDL ratio | –1.06 (–1.76 to –0.36) | 0.003 | 7.00 | 3.67 |
| Insulin secretion | 90.24 (–8.04 to 188.53) | 0.07 | 1.57 | 1.54 |
| HOMA-IR | 2.34 (–2.68 to 7.36) | 0.36 | 5.83 | 0.37 |

Coefficients and corresponding 95% CIs indicate the change in outcome measure per unit increase in log-transformed LINE-1 methylation, after adjustment for sex. \( R^2 \) reflects the variance (%) in outcome measures accounted for by both sex and log-transformed LINE-1 methylation (i.e. the combined effect of both covariates). Direct \( R^2 \) reflects the variance (%) in outcome measures accounted for by log-transformed LINE-1 methylation alone (i.e. the direct effect of methylation).
(or mechanistically) related to disease-related traits. Either way, there is considerable interest in the role of epigenetic mechanisms in common complex disease, given their potential to act as both informative diagnostic and prognostic biomarkers. It is postulated that, in those diseases with a prominent environmental component, it is possible that epigenetic factors contribute to the inter-individual differences in responses to environmental exposures and to the pathogenesis of such diseases.

We observed associations between LINE-1 DNA methylation and fasting concentrations of glucose, triglycerides and total, LDL and HDL cholesterol and also HDL:LDL ratio, all of which are blood-based biomarkers of increased risk of cardiovascular disease (CVD) and/or type 2 diabetes. High concentrations of fasting glucose are associated with the development of both CVD and diabetes. We observed a positive association between LINE-1 DNA methylation and total cholesterol and triglycerides concentrations. Elevated concentrations of these blood lipid markers have been shown previously to be strongly associated with an increased risk of CVD. Furthermore, our results show LINE-1 DNA methylation is associated with both increased LDL cholesterol and decreased HDL cholesterol concentrations. These opposing directional changes are those expected in individuals at increased risk of CVD. Given the high levels of collinearity of many of these measures, we chose however not to take a multivariable approach to the analysis.

As this study was conducted in individuals at age 50 years with no evidence of overt CVD, it was not possible to explore a potential association between LINE-1 DNA methylation and subsequent disease phenotype. However, this is a potential strength of this study as it removes the possible confounding effect of disease status on LINE-1 DNA methylation patterns. The NTFS birth cohort is being followed longitudinally, which will provide the opportunity to ascertain the predictive utility of LINE-1 DNA methylation at age 50 years in respect of later disease risk.

Nonetheless, given that both LINE-1 DNA methylation and blood biochemical measures were assessed at the same time-point, it is not possible to determine the direction of effect between these factors, if indeed, a direct causal (or mechanistic) link exists. Furthermore, given the small effect sizes observed, the contribution of one factor upon the other remains modest.

These findings are among the first observations to link LINE-1 DNA methylation levels with disease-related traits other than cancer. In the Boston-based Normative Ageing Study, persons with prevalent IHD and stroke had, in contrast to our findings, lower LINE-1 DNA methylation and, in longitudinal analyses, those with lower LINE-1 DNA methylation were at higher risk for incident IHD, stroke and total mortality. In a further study of the Boston-based cohort, an association was seen between LINE-1 hypomethylation and vascular cell adhesion molecule-1 for disease-free individuals, but not for those with prevalent IHD or stroke. However, this all-male cohort is considerably older than the NTFS, with a mean age of 74 years at DNA sampling, which may offer some explanation as to the discordance in observations between the cohorts. The widely observed hypomethylation of LINE-1 DNA associated with cancer and the observations reported in the Normative Ageing Study could suggest that LINE-1 DNA methylation would be inversely associated with blood-based biomarkers of metabolic health, whereas our observations demonstrate the opposite association. Given the limited empirical data in this area and the lack of clear association between advancing age and decreased LINE-1 DNA methylation, the current findings warrant further attention. There is some evidence that gene-specific DNA methylation is positively correlated with older age. Ronn et al. showed that elderly, compared with young, non-diabetic twins had both higher DNA methylation and lower gene expression of COX7A1 (a gene associated with peripheral insulin sensitivity, measured in 10 individuals using bisulphite sequencing). Hernandez et al. also recently reported extensive evidence of genome-wide gene-specific hypermethylation with advancing age. The relationship between these gene-specific observations and global LINE-1 DNA methylation remains to be clarified.

Our observations suggest that LINE-1 DNA methylation and hence potentially other forms of epigenetic modification, might be useful in predicting risk of common complex diseases such as type 2 diabetes and CVD. The issues of confounding and reverse causation are fundamental to pursuing this further. DNA methylation is in essence a phenotype and is therefore vulnerable to multiple confounding influences including age, sex, smoking and socio-economic position to name only a few. Although our statistical appraisal of potential confounders did not highlight any obvious culprits, the issue cannot be dismissed. Indeed, it may transpire that DNA methylation provides nothing more than an indirect measure of confounding influences. In addition, it will be crucial to understand the causal relationship between LINE-1 DNA methylation and the blood-based biomarkers associated with this epigenetic signature, whether LINE-1 DNA methylation is causal in altering blood-based biomarkers such as fasting glucose and lipid concentrations or whether the reverse applies. There is limited evidence to suggest that altering glucose levels changes DNA methylation patterns, but to our knowledge there is no direct evidence to link lipid levels to perturbed DNA methylation, or vice versa. A recent study of genome-wide methylation in cord-blood DNA highlighted numerous methylation-variable loci whose biological roles were related to lipid metabolism, suggesting a causal influence of altered methylation on...
lipid levels. A previous study of patients with coronary artery disease and controls showed that global DNA methylation was associated with coronary artery disease risk, and that this association was accentuated by increased plasma homocysteine concentration. Further insight into the direction of causality may be obtained by adoption of a Mendelian randomization approach, as proposed recently by Relton and Davey Smith. This approach involves the use of genetic variants as proxies for specific exposures, such that an association between genotype and DNA methylation would be indicative of a causal relationship (as lipid levels could not plausibly influence genotype and thus the possibility of reverse causation is removed). Numerous genetic variants have recently been reported to influence blood lipid profiles and these could be used collectively as a proxy for lipid concentrations to investigate the association between lipid levels and DNA methylation.

In this study we estimated global DNA methylation using the LINE-1 assay, which measures cytosine methylation in common non-coding sequences that occur widely across the genome. The functional consequences of altered DNA methylation at these CpG sites within LINE-1 for the development of CVD and diabetes-related risk markers is not known and indeed may not be easily decipherable through the analysis of non-target tissues such as peripheral blood. A limitation of this, and many similar studies, is the reliance upon epigenetic profiling of peripheral blood DNA, with the assumption that it will be informative about target tissues. Interrogation of methylation status of promoters in genes implicated directly in pathways of lipid metabolism and glucose homeostasis may provide greater insight. Methylation has been reported to change with both actual age (serial sampling) and chronological age (cross-sectional sampling). As the study members were all born within a 2-month period in 1947 and assessed within an 18-month period at age 49–51 years, this minimizes the likelihood of confounding effects of chronological age.

In summary, we have presented evidence which supports the hypothesis that global LINE-1 DNA methylation at age 50 years is associated with biomarkers of metabolic health. Although these cross-sectional associations do not allow conclusions to be drawn with respect to the direction of causation, and the potential for confounding cannot be dismissed, the findings may have important implications for prediction, early diagnosis, prevention and treatment of common complex diseases such as CVD and type 2 diabetes.

**Funding**

MRC studentship (to J.C.Mc.); the Centre for Brain Ageing & Vitality (which is funded through the Lifelong Health and Wellbeing cross-council initiative by the MRC, BBSRC, EPSRC and ESRC); Biomedical Research Centre in Ageing and Age-Related Disease grant funded by the NHS Newcastle Hospitals Trust and the UK National Institute of Health Research.

**Acknowledgements**

The authors thank all Newcastle Thousand Family Study members for taking part in this study, study teams past and present and the various funding bodies that have contributed to funding this study since its inception. C.L.R. conceived and, with M.S.P., oversaw the project. L.P. oversaw the initial collection of samples and the clinical assessment. M.S.P. is the overall Director of the Newcastle Thousand Families Study. J.C.Mc. and L.M.B. analysed the samples, M.S.P. and C.P. analysed the data. M.S.P., J.C.M., J.C.Mc., C.P. and C.L.R. wrote the paper. M.S.P. and C.L.R. have primary responsibility for final content and act as guarantors for this article. All authors read and approved the final manuscript.

**Conflict of interest:** None declared.

**KEY MESSAGES**

- Patterns of both global and gene-specific DNA methylation change with age and these changes are believed to be associated with the development of common complex diseases.
- Associations were seen between global LINE-1 DNA methylation and a number of blood glucose and lipid markers (positive for fasting glucose, total cholesterol and triglycerides; negative for HDL cholesterol and the HDL:LDL ratio).
- Confounding and reverse causation represent major problems in epigenetic association studies and require careful consideration in studies of this type.
- These novel associations between global LINE-1 DNA methylation and blood glucose and lipid profiles highlight a potential role for epigenetic biomarkers as predictors of metabolic disease and may be relevant to future diagnosis, prevention and treatment of this group of disorders.
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