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Biogerontology 2014, 15(4), 317-328.

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The final publication is available at Springer via <http://dx.doi.org/10.1007/s10522-014-9500-9>

Date deposited: 26th November 2014 [made available 26th April 2015]

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Biogerontology

Acquisition of aberrant DNA methylation is associated with frailty in the very old: findings from the Newcastle 85+ Study --Manuscript Draft--

Manuscript Number:	
Full Title:	Acquisition of aberrant DNA methylation is associated with frailty in the very old: findings from the Newcastle 85+ Study
Article Type:	Research Article
Section/Category:	Research Article
Keywords:	Ageing; Frailty; DNA methylation; CpG island; epigenetics
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Acquisition of aberrant DNA methylation is associated with frailty in the very old: findings from the Newcastle 85+ Study

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Abstract

Frailty is a major health problem in older people and, as the population ages, identification of its underlying biological mechanisms will be increasingly important. DNA methylation patterns within genomic DNA change during ageing and alterations in DNA methylation, particularly at gene promoter regions, can lead to altered gene expression. However the importance of altered DNA methylation in frailty is largely unknown. Using cross-sectional data from the Newcastle 85+ Study (all participants aged 85 years) frailty was operationalized by the Fried model. DNA methylation levels were assessed by highly quantitative pyrosequencing at the gene promoter associated CpG islands from a panel of five age-related methylation marker loci and at LINE-1 repetitive elements (as a surrogate for genome-wide methylation). While genome-wide methylation (as assessed at LINE-1 elements) showed no association with frailty status, there was a clear association between CpG island methylation and frailty. When compared to participants with CpG island methylation levels in the combined middle 2 (referent) quartiles, those in the lowest quartile had significantly decreased odds of frailty (odds ratio 0.47 (95% CI 0.26-0.85); n=321, p=0.013). Overall this study suggests a potential role for age-related changes in CpG island methylation in the development of frailty.

Key words: Ageing, Frailty, DNA methylation, CpG island, epigenetics

Introduction

Frailty is a major health problem in older people, with growing emphasis on identifying its underlying mechanisms and prospects for intervention. Frailty is characterised by an increased vulnerability to stress (Bortz 2002; Fried et al. 2001; Lipsitz 2002; Walston et al. 2006) which results in an increased risk of adverse health outcomes such as disability, hospitalisation, institutionalisation and death (Bande-en-Roche et al. 2006; Fried et al. 2001; Kulminski et al. 2007; Mitnitski et al. 2005; Rockwood et al. 2011; Romero-Ortuno et al. 2011). This vulnerability results from a decline in homeostatic reserve, secondary to dysregulation in multiple inter-related systems (Bortz 2002; Fried et al. 2001; Lipsitz 2002; Walston et al. 2006). However, knowledge of the mechanisms underlying frailty remains limited, particularly at the molecular and cellular levels. Whilst there is no universally accepted method to identify frailty, the Fried approach is one of the most widely accepted. Frailty is viewed here as a clinical syndrome - a cluster of specific symptoms and signs - including shrinking/weight loss, poor endurance/exhaustion, low physical activity, muscle weakness and slow walking speed (Fried et al. 2001).

DNA methylation is the addition of a methyl group to the carbon-5 position of cytosine residues. This produces 5-methylcytosine, which is often referred to as the 5th base in DNA and constitutes about 1% of the human genome (Bird 1996). DNA methylation occurs almost exclusively at CpG dinucleotides and is an important epigenetic mechanism for controlling gene expression (Costello and Plass 2001). In particular, hypermethylation of gene promoters which have high densities of CpG sites, known as CpG islands, leads to chromatin compaction and consequent loss of gene expression (Bird and Wolffe 1999). This is a critical mechanism for control of expression of imprinted genes, for X-chromosome inactivation and for regulation of

1 many germ cell specific genes (Gopalakrishnan et al. 2008; Weber et al. 2007).
2 Although the majority of human genes possess a promoter associated CpG island,
3 these generally remain methylation free, even in tissues where the associated gene
4 is not expressed (Bird 1996). In contrast, CpG sites outside of promoter associated
5 CpG islands are usually methylated. These normal patterns of DNA methylation have
6 been found to break down during ageing (Fraga and Esteller 2007). This primarily
7 involves a reduction in genome-wide levels of DNA methylation (Bollati et al. 2009),
8 found in association with localised increases in DNA methylation, centring on
9 promoter associated CpG island sequences (Fraga and Esteller 2007). This has led
10 to the hypothesis that age associated changes in DNA methylation may have an
11 important role in age-related pathologies. However the importance of altered DNA
12 methylation in frailty is largely unknown.

13 In this study we investigated the potential link between frailty and alterations
14 in DNA methylation. We used data from the Newcastle 85+ Study, a population-
15 based cohort study capturing detailed information on the health of a large,
16 representative sample who were all aged around 85 at baseline. We examined two
17 aspects of DNA methylation. Firstly we quantified methylation at specific cytosine
18 residues within CpG islands associated with gene transcriptional start sites, using a
19 panel of genes which we have found to exhibit extensive, age-related increases in
20 methylation in peripheral blood (Gautrey et al. unpublished). Secondly we quantified
21 methylation at LINE-1 repetitive elements, which is widely used as a surrogate for
22 genome-wide DNA methylation levels (Weisenberger et al. 2005).

Materials and Methods

Study population

The methodology for the Newcastle 85+ Study has been reported (Collerton et al. 2007; Collerton et al. 2009; Davies et al. 2010). In brief, members of the 1921 birth cohort living in Newcastle or North Tyneside (North-East England) were recruited at around age 85 through general practice patient lists. People living in institutions and those with cognitive impairment were included. At baseline the Newcastle 85+ cohort was socio-demographically representative of the local population and of England and Wales (Collerton et al. 2009). The research complied with the requirements of the Declaration of Helsinki. Ethical approval was obtained from the Newcastle and North Tyneside 1 Research Ethics Committee (reference number 06/Q0905/2). Written informed consent was obtained from participants and where people lacked capacity to consent, for example because of dementia, a formal written opinion was sought from a relative or carer.

Data sources

A multidimensional health assessment - comprising questionnaires, measurements and a fasting blood sample - was carried out in the participant's usual residence by a research nurse. Data on pre-existing diseases and prescribed medication were obtained from general practice medical records.

Frailty Status

Fried frailty status – categorised as robust, pre-frail or frail - was derived using a modified version of the Cardiovascular Health Study methodology (Collerton et al.

2012; Fried et al. 2001). In line with the stipulated methodology, participants with stroke, Parkinson's Disease, a mini-mental state examination score of less than 18, or taking drugs for dementia, Parkinson's Disease or depression were excluded on the basis that they might score as frail as a result of that disease alone. Full details of criteria used are reported in supplementary methods.

Derivation of frailty status required data from both the health assessment and general practice records which was available for 845 participants (Collerton et al. 2009). Of these 845 participants, Fried frailty status could be assigned for 552; applying the Fried exclusion criteria resulted in loss of 252 participants and an additional 41 could not be assigned due to missing values on individual Fried criteria. In comparison to those without Fried status, those with Fried status were less likely to be resident in a care home, less likely to have impaired cognitive function, had a lower disease count and were less disabled (Collerton et al. 2012).

DNA modification and PCR

DNA was extracted from peripheral blood leukocytes. 200ng of genomic DNA was modified with sodium bisulfite using the Methylamp™ One-Step DNA Modification Kit (Epigentek, Brooklyn, NY, USA) as per the manufacturer's instructions. All samples were resuspended in 15µl of TE and 1µl of this was used for subsequent PCR reactions. The samples were amplified in 25µl volumes containing 1X manufacturer's buffer, 1 unit of FastStart taq polymerase (Roche, Welwyn Garden City, UK), 1-4mM MgCl₂, 10mM dNTPs, and 75ng of each primer. PCR was performed with one cycle of 95°C for 6 minutes, 40 cycles of 95°C for 30 seconds, 57-63°C for 30 seconds and 72°C for 30 seconds, followed by one cycle of 72°C for 5 minutes. For each set of primers (see Supplementary Table 1) either the forward or

reverse primers included a 5' biotin label to facilitate subsequent analysis by pyrosequencing.

Quantitative DNA methylation analysis using pyrosequencing

Following PCR amplification, sequencing was performed using a PSQ 96MA pyrosequencer (Qiagen, Hilden, Germany), as per manufacturer's protocol. For all loci, assays were performed in duplicate and values averaged between the duplicates. Only samples that were passed by the pyrosequencer were included and to further ensure a high degree of accuracy, only runs in which single peak heights were in excess of 200 were included. Samples in which the duplicates differed by >2% methylation were repeated one additional time and the average taken across the three replicates.

Primer design was performed using the manufacturer's provided PyroMark software. For initial assessment of all assays, pyrosequencing was carried out on samples of known methylation status (5, 10, 15, 20, 50, 70 and 100% methylated) which were produced by diluting peripheral blood derived DNA (from a young adult volunteer) into *in-vitro* methylated DNA (Millipore, Watford, UK).

DNA methylation was assessed at five promoter associated CpG islands (*EPHA10*, *HAND2*, *HOXD4*, *TUSC3* and *TWIST2*) using quantitative pyrosequencing analysis. The five genes were selected from a larger panel of 15 genes previously assessed for age-related methylation in peripheral blood samples from Newcastle 85+ Study participants (Gautrey et al, unpublished). These five loci were selected as they showed the most extensive and variable methylation in 85 year olds, and exhibited a steady and near linear age-related increase in methylation levels in samples taken from multiple age groups (range birth to 85 years old) (Gautrey et al,

unpublished), suggesting that these loci would represent good indicator loci for age-related methylation. For each locus between three and six consecutive CpG sites were measured and the methylation value for each locus was taken as the mean of the values for all CpG sites measured at that locus. Methylation values at consecutive CpG sites were strongly correlated, with correlation coefficients between consecutive CpG sites ranging from 0.93-0.98 (*TWIST2*), 0.87-0.93 (*TUSC3*), 0.63-0.87 (*HAND2*), 0.80-0.86 (*HOXD4*), and 0.42-0.64 (*EPHA10*). Methylation levels across the five loci also correlated (correlation coefficients between 0.20 and 0.60). Methylation levels across the five loci were combined (see below) to produce an overall estimate of the level of age-related CpG island methylation for each participant.

Of the 759 participants from whom DNA samples were collected, 480 were analysed for CpG island methylation. The samples analysed were chosen primarily on the basis of previous cancer diagnosis (n=113), incident cancer diagnosis (n=72) or as a control group without cancer diagnosis (n=212). Also included were current smokers (n=24) and participants with a diagnosis of type 2 diabetes (n=58) or dementia (n=36). 35 participants were included in two of these categories and hence the total number of samples analysed for CpG island methylation was 480. This set of samples was analysed primarily to allow an investigation of the link between altered DNA methylation and cancer in the elderly (Gautrey et al, unpublished). This was then expanded to include some non-cancer endpoints (smoking, type 2 diabetes, dementia) although only cancer diagnosis was found to correlate with DNA methylation status.

Derivation of overall CpG island methylation level

1 The overall level of CpG island methylation for each participant was calculated
2 by averaging the values for each of the five age-related loci examined. To ensure an
3 equal contribution for each locus, the values for individual loci were first scaled such
4 that each had an average value across the population equal to the average value of
5 all five loci. The scaled values for each of the five loci were then averaged to give an
6 overall value for each participant.

7 8 Other data reported

9 Pre-existing diagnoses of diabetes mellitus and cancer (excluding non-
10 melanoma skin cancer) were extracted from the general practice records. A count of
11 chronic diseases was calculated for each participant (Collerton et al. 2009); the full
12 list of 18 diseases included is shown in the footnote to Table 1. Cognitive status was
13 assessed using the standardised mini-mental state examination (Molloy and
14 Standish 1997). Body mass index was calculated from measured weight and height
15 (derived from demi-span). Ethnicity, place of residence, years of education, smoking
16 status (current smoker, ex-smoker, never), and difficulties with activities of daily living
17 were obtained by self-report. A disability score (maximum 17) was calculated from
18 the total number of activities of daily living performed with difficulty, or requiring an
19 aid or appliance or personal help (Collerton et al. 2009).

20 The levels of lymphocyte subpopulations in peripheral blood samples taken
21 from Newcastle 85+ participants were determined using flow-cytometry (Becton
22 Dickinson FACScan Flow Cytometer), as described before (Martin-Ruiz et al. 2011).

23 24 Data Analysis

25 The analysed sample (i.e. participants with both Fried status and CpG island
26 methylation data available) was compared to the sample excluded due to missing
27 methylation data. The Chi-square or Fisher exact tests, as indicated, were used for

nominal variables and the Mann-Whitney U test for ordinal and non-parametric continuous variables. Potential correlation between LINE-1 and CpG island methylation was assessed using the Pearson correlation coefficient. To explore the relationship between frailty and methylation, ordinal logistic regression models were fitted with Fried frailty status as the dependent variable and quartiled methylation level as the independent variable. The middle two methylation quartiles were combined and used as the reference category to facilitate identification of potential relationships at the top and bottom of the distribution. The odds ratios obtained represent the odds of being in a frailer Fried category i.e. pre-frail rather than robust or frail rather than pre-frail. The test of parallel lines was used to check that the proportional odds assumption was satisfied. Two models are reported: Model 1 was adjusted for sex, % lymphocytes and previous diagnosis of cancer (excluding non-melanoma skin cancer); and Model 2 was adjusted for sex, % lymphocytes, previous diagnosis of cancer (excluding non-melanoma skin cancer), educational status, smoking status and number of chronic diseases. Choice of potential confounders was made on the basis that DNA methylation in this cohort correlates with % lymphocytes ($R^2=0.37$, $p\text{-value}<1\times10^{-40}$; median % lymphocyte count 28.8% of total white blood cells, interquartile range 22.5-34.4%) and cancer diagnosis (Gautrey et al, unpublished), as well as evidence that age-related methylation is influenced by gender (Tapp et al. 2013). Data from six participants were excluded from the final analysis as the methylation data and % lymphocyte data came from different blood samples and the participants thus couldn't be included in either model 1 or model 2. P-values less than 0.05 were taken as statistically significant. Data analysis was conducted using SPSS Version 19 (IBM Inc., Chicago, USA).

Results

Of the 552 participants with frailty status assigned, CpG island methylation data was available for 321; these 321 participants formed the sample for the principal analyses. The characteristics of the 321 participants are shown in Table 1, together with a comparison to the sample with frailty status assigned but who were not analysed for CpG island methylation (n=231). Frailty was found in 21.8% (70/321) of participants, with 58.6% (188/321) defined as pre-frail and the remainder as robust.

Frailty status in 85 year olds is associated with gene promoter associated CpG island methylation.

There was substantial inter-individual variation in CpG island methylation levels, with values ranging from 4.8% to 29.1%, and a mean level of 10.7% (standard deviation 3.0%) (Fig. 1A). Compared to participants in the combined middle (referent) quartiles, those in the lowest quartile of gene promoter associated CpG island methylation level had lower odds of frailty. This was observed using the model adjusted for factors known to influence DNA methylation (i.e. sex, % lymphocytes and cancer diagnosis) (Model 1: odds ratio (95% confidence interval) 0.47 (0.26-0.85), p-value=0.013) and in the model additionally adjusted for educational status, smoking status and number of chronic diseases (Model 2: odds ratio (95% confidence interval) 0.46 (0.25-0.86), p-value=0.016) (Table 2).

As frailty status is derived using five separate criteria, it is possible that the association found above may be largely driven by an association with a single criterion, as opposed to the frailty syndrome as a whole. We therefore further investigated whether CpG island methylation was associated with the individual criteria used to define the frailty syndrome (shrinking/weight loss, poor

endurance/exhaustion, low physical activity, weakness, and slow walking speed). The relationship found between CpG island methylation and the frailty syndrome appeared to be mirrored in four out of the five frailty criteria (shrinking excepted), although associations only reached statistical significance for weakness and low physical activity (slow walking speed was close to significance) (Supplementary Table 3).

Frailty status in 85 year olds is not associated with LINE-1 methylation levels

The relationship between frailty and DNA methylation at gene promoter associated CpG islands could suggest a role for DNA methylation induced changes in gene expression in the development of frailty. Alternatively, it may represent a more general association between frailty and genome-wide levels of DNA methylation. To explore this possibility the analysis was extended to include methylation levels at LINE-1 elements. These are the most common repeat elements present in the human genome and have previously been shown to represent a good surrogate for genome-wide methylation levels (Weisenberger et al. 2005).

Sufficient DNA was available to assess LINE-1 methylation in the majority (84.1%, 270/321) of samples with both Fried frailty status and CpG island methylation data. Comparison of the participants with/without LINE-1 methylation data showed them to be similar, other than a lower level of disability in the group with LINE-1 data (Supplementary Table 2). LINE-1 methylation levels varied from 74.7% to 85.2%, with a mean level of 80.9% (standard deviation 1.8%) (Fig. 1B). The LINE-1 methylation levels in the Newcastle 85+ study cohort were similar to those reported for previously analysed cohorts, including elderly individuals (Zhu et al. 2012). No

1 significant correlation was found between the level of CpG island methylation and
2 LINE-1 methylation ($R=-0.096$, $p=0.12$, Fig. 1C).

3 In contrast with the results for gene promoter associated CpG island
4 methylation, there was no evidence for an association between LINE-1 methylation
5 and frailty status (Table 2). This implies that frailty is specifically associated with
6 methylation changes focussed on gene promoter regions, where it is likely to
7 influence gene expression patterns, and not with DNA methylation levels as a whole.

Discussion

Frailty is becoming a major health and societal issue, as the world's population ages. However, frailty does not appear to be an inevitable consequence of ageing. Whilst a quarter to a third of people aged 85 and older are frail, even at this age the majority of the population are not frail (Collerton et al. 2012; Fried et al. 2001; Song et al. 2010). The underlying causes of frailty, and the reasons for inter-individual differences in the experience of frailty within people of the same age, are poorly understood.

Epigenetic mechanisms are an attractive candidate as one of the underlying causes of frailty. Unlike genetic factors, epigenetic mechanisms are sensitive to environmental factors, including nutrition and lifestyle, and the epigenome in elderly individuals will have been re-modelled in response to their lifetime exposures to different environmental factors (Mathers et al. 2010). In addition, epigenetic factors, and in particular DNA methylation, vary significantly both cross-sectionally within populations of the same age and across the life-course (Rakyan et al. 2010).

In this study we have examined the potential associations between frailty and both promoter specific CpG island methylation and genome-wide methylation (using LINE-1 elements as a surrogate). We found that lower levels of promoter specific CpG island methylation were associated with reduced frailty. Promoter methylation is associated with gene silencing (Bird and Wolffe 1999) and hence the differences in promoter methylation between frailty categories observed here may reflect differences in expression of the corresponding genes. If such differences in methylation patterns occur in other genes and in multiple tissue types, the consequential differences in gene expression could play a role in the breakdown of

1 many physiological processes, which is thought to be a key mechanism underlying
2 frailty (Bortz 2002; Fried et al. 2001; Lipsitz 2002; Walston et al. 2006).

3 A second possibility is that alterations in methylation act as a biological
4 marker for frailty but without playing a direct causative role, for example if there was
5 a common driving force behind the altered methylation and the development of
6 frailty. In either case it would be important to determine the extent to which altered
7 DNA methylation can function as a predictive marker, allowing the detection of
8 individuals at risk of developing frailty. This will be assessed within the longitudinal
9 data from the Newcastle 85+ study, by examining the extent to which DNA
10 methylation levels at baseline predict frailty status at subsequent phases of the
11 study. Furthermore, long-term studies, initiated in younger populations, will be
12 required to robustly assess the potential utility of altered DNA methylation as a
13 predictive marker for frailty as intervention strategies to reduce or prevent frailty may
14 be more effective if implemented earlier in the life-course.

15 The Fried frailty syndrome is based on five criteria and it is possible that the
16 association between CpG island methylation levels and frailty could be primarily
17 driven by an association with one or a subset of these criteria. Analysis using the
18 individual criteria found that the relationship between CpG island methylation and the
19 frailty syndrome appeared to be mirrored in four out of the five individual criteria
20 (shrinking excepted), although associations only reached statistical significance for
21 weakness and low physical activity (slow walking speed was close to significance). It
22 is not possible to formally assess whether the association with CpG island
23 methylation is stronger for any of the specific criteria than for the syndrome of frailty,
24 particularly as the low number of participants in the frail category for the individual
25 criteria reduces the power of this analysis. Whilst there is heterogeneity in the initial

1 manifestation of frailty, weakness is the most common first manifestation, with
2 weakness, low physical activity and slowness tending to precede shrinking and
3 exhaustion (Xue et al. 2008). This underlines the central role of sarcopaenia in the
4 frailty phenotype (Xue 2011). Different patterns of accumulation of individual frailty
5 criteria may represent different causative pathways for frailty, with certain criteria
6 being more sensitive than others to particular physiological, molecular or cellular
7 dysregulations resulting from disease and/or ageing (Xue 2011).

8 We also found that the association between frailty and methylation was
9 specific for promoter associated regions, as methylation levels at LINE-1 assays
10 (used as a surrogate for genome-wide methylation) were not associated with frailty
11 status. This observation is in apparent conflict with the recent findings of Bellizzi et al
12 (Bellizzi et al. 2012) who reported an inverse association between genome-wide
13 DNA methylation and frailty. The apparent difference in findings between the two
14 studies may reflect the different methodologies used to assess genome-wide DNA
15 methylation levels. As their surrogate for global DNA methylation, Bellizzi et al
16 (Bellizzi et al. 2012) used the so-called CpGlobal assay which is based on differential
17 cutting of DNA by two restriction endonucleases viz. HpaII (which is sensitive to
18 cytosine methylation) and MspI (which cuts regardless of methylation status).
19 Previous studies have demonstrated the utility of LINE-1 methylation as a marker of
20 genome-wide methylation (Weisenberger et al. 2005) and the results obtained in this
21 study are very similar to results previously reported for LINE-1 methylation in similar
22 age groups (Zhu et al. 2012). In contrast, the extensive variation in genome-wide
23 methylation levels reported in the Bellizzi et al study is not consistent with previously
24 published data, either using the LINE-1 approach or directly measuring total 5-
25 methylcytosine levels by HPLC (Fuke et al. 2004; Weisenberger et al. 2005).

Furthermore, the large within-individual changes, over seven years of the lifespan, which were reported by Bellizzi et al (Bellizzi et al. 2012) are not consistent with the far more subtle temporal changes in global DNA methylation previously reported by Bjornsson et al, who interrogated within-individual changes in global DNA methylation over 11-16 years of adulthood. These authors used the LUMA assay, which, like the CpGlobal assay used by Bellizzi et al., is also based on differential cutting of DNA by the restriction endonucleases HpaII and MspI (Bjornsson et al. 2008). In addition, the HpaII sites targeted by these assays are highly enriched within CpG islands (Bjornsson et al. 2008) and thus may be more reflective of CpG island methylation levels than genome-wide methylation status.

This study suggests that the level of accumulation of CpG island methylation may influence the development of frailty. However, the factors which drive the population variability in CpG island methylation, and the relative importance of genetic and environmental factors, remains unknown. Multiple studies have examined the possibility that DNA methylation levels may be influenced by polymorphisms in enzymes regulating one carbon metabolism, which is crucial for the production of the methyl donors required for the DNA methylation reaction (Struck et al. 2012), or in the DNA methyltransferase genes themselves. Most of these studies have focussed on cancer associated DNA methylation changes, and no clear impact of SNPs on DNA methylation levels has so far been identified (Huidobro et al. 2013). Recent studies have also examined such SNPs in healthy individuals. This has identified some potential associations with key metabolites in the DNA methylation pathway, but what, if any, effect this has on DNA methylation patterns is still unclear (DeVos et al. 2008; Ho et al. 2011). In addition, several studies, using genome wide approaches, have reported associations between SNPs

1 and methylation at CpG sites proximally located to the SNP (Boks et al. 2009; Zhang
2 et al. 2010). However, such SNPs have not been found to correlate with methylation
3 at multiple loci throughout the genome (Boks et al. 2009) and so could not underlie
4 the correlations we found in methylation levels between different loci and would likely
5 have little impact on the average methylation value used in this study.

6 A number of other factors could influence DNA methylation levels in the
7 elderly, such as changes in the relative proportions of the different cell types found in
8 peripheral blood. Indeed it has been well documented that lymphocyte levels fall in
9 elderly individuals (Huppert et al. 1998) and furthermore a number of diseases
10 common in the elderly population, such as cardiovascular disease (Nunez et al.
11 2011), as well as level of activity (DiPenta et al. 2004) can potentially result in
12 alterations in the relative proportions of the different cell types. In this study we found
13 that the percentage of lymphocytes (as a fraction of total white blood cells) was
14 significantly associated with DNA methylation levels. This correlation was specifically
15 for total lymphocytes, as the relative proportions of T or B lymphocytes within this
16 population was not associated with DNA methylation levels (Gautrey et al,
17 unpublished). To address this, the analysis presented in this paper was adjusted for
18 the percentage of lymphocytes and thus inter-individual variation in relative cell
19 counts should not have impacted on the results presented here.

20 Strengths of this study include its population-based sample, which included
21 the institutionalised, and the domiciliary nature of the assessment which avoids the
22 selection bias inherent in clinic-based assessment of this age group. As all
23 participants were the same age, all variation detected will represent inter-individual
24 variation and will not be due to differences in chronological age. Furthermore, by
25 studying a population of the very old, we are likely to have maximised the extent of

1 age-associated methylation changes. In addition, our analyses were adjusted for a
2 range of potential confounders, including disease burden.

3 Some limitations deserve comment. The analysed sample differs from the
4 sample excluded due to missing data. Higher levels of care home residence,
5 cognitive impairment and disability in the excluded sample relate to the previously
6 reported problems operationalizing the Fried model in this age group (Collerton et al.
7 2012), with lower levels of smoking and disease burden resulting from the
8 methylation sampling strategy. However, our findings were robust to adjustment for
9 the key confounders. As in many other investigations (Avila-Funes et al. 2008;
10 Cawthon et al. 2007; Cigolle et al. 2009; Ensrud et al. 2007; Santos-Eggimann et al.
11 2009; Wilhelm-Leen et al. 2010), our Fried operationalization was a modification of
12 the Cardiovascular Health Study methodology as the exact variables were not
13 collected. However our frailty prevalence was identical to that reported in the
14 Cardiovascular Health Study for this age group (Fried et al. 2001).

15 Overall, our results suggest that promoter specific CpG island methylation
16 levels, but not genome-wide DNA methylation levels, are associated with frailty in the
17 very old and should be further investigated as a potential underlying cause and/or
18 predictive marker for the development of frailty.

Acknowledgements

Thanks are especially due to the 85 year olds of Newcastle and North Tyneside, and their families and carers, for the generous donation of their time and personal information to make the study possible. In addition we thank the: research nurses (Brenda Balderson, Sally Barker, Julie Burrows, June Edwards, Julie Ferguson, Gill Hedley, Joan Hughes, Judith Hunt, Julie Kimber and Victoria Raynor); biomarker technicians (Sam Jameson, Claire Kolenda, Craig Parker and Anna Tang); data manager (Pauline Potts); project secretary (Lucy Farfort); and the North of England Commissioning Support Unit (formerly NHS North of Tyne, working on behalf of Newcastle and North Tyneside Primary Care Trusts and Northumberland Care Trust) and local general practices.

The Newcastle 85+ Study was funded by a combined grant from the UK Medical Research Council and the Biotechnology and Biological Sciences Research Council (G0500997), the Dunhill Medical Trust (R124/0509), and a grant from the Newcastle Healthcare Charity. The research was also supported by the National Institute for Health Research Newcastle Biomedical Research Centre, based at Newcastle upon Tyne Hospitals NHS Foundation Trust and Newcastle University. HG was funded by a PhD studentship from the Biotechnology and Biological Sciences Research Council and SvO was funded by a PhD studentship from the National Institute for Health Research Newcastle Biomedical Research Centre and a project grant from the Newcastle Healthcare Charity and Newcastle upon Tyne hospitals NHS Charity.

References

- Avila-Funes JA, Helmer, C, Amieva, H, Barberger-Gateau, P, Le Goff, M, Ritchie, K, Portet, F, Carriere, I, Tavernier, B, Gutierrez-Robledo, LM, Dartigues, JF (2008) Frailty among community-dwelling elderly people in France: the three-city study *The journals of gerontology. Series A, Biological sciences and medical sciences* 63:1089-1096.
- Bandeem-Roche K, Xue, QL, Ferrucci, L, Walston, J, Guralnik, JM, Chaves, P, Zeger, SL, Fried, LP (2006) Phenotype of frailty: characterization in the women's health and aging studies *The journals of gerontology. Series A, Biological sciences and medical sciences* 61:262-266.
- Bellizzi D, D'Aquila, P, Montesanto, A, Corsonello, A, Mari, V, Mazzei, B, Lattanzio, F, Passarino, G (2012) Global DNA methylation in old subjects is correlated with frailty *Age* 34:169-179.
- Bird AP (1996) The relationship of DNA methylation to cancer *Cancer surveys* 28:87-101.
- Bird AP, Wolffe, AP (1999) Methylation-induced repression--belts, braces, and chromatin *Cell* 99:451-454.
- Bjornsson HT, Sigurdsson, MI, Fallin, MD, Irizarry, RA, Aspelund, T, Cui, H, Yu, W, Rongione, MA, Ekstrom, TJ, Harris, TB, Launer, LJ, Eiriksdottir, G, Leppert, MF, Sapienza, C, Gudnason, V, Feinberg, AP (2008) Intra-individual change over time in DNA methylation with familial clustering *JAMA : the journal of the American Medical Association* 299:2877-2883.
- Boks MP, Derks, EM, Weisenberger, DJ, Strengman, E, Janson, E, Sommer, IE, Kahn, RS, Ophoff, RA (2009) The relationship of DNA methylation with age, gender and genotype in twins and healthy controls *PloS one* 4:e6767.
- Bollati V, Schwartz, J, Wright, R, Litonjua, A, Tarantini, L, Suh, H, Sparrow, D, Vokonas, P, Baccarelli, A (2009) Decline in genomic DNA methylation through aging in a cohort of elderly subjects *Mechanisms of ageing and development* 130:234-239.
- Bortz WM, 2nd (2002) A conceptual framework of frailty: a review *The journals of gerontology. Series A, Biological sciences and medical sciences* 57:M283-288.
- Cawthon PM, Marshall, LM, Michael, Y, Dam, TT, Ensrud, KE, Barrett-Connor, E, Orwoll, ES, Osteoporotic Fractures in Men Research, G (2007) Frailty in older men: prevalence, progression, and relationship with mortality *Journal of the American Geriatrics Society* 55:1216-1223.
- Cigolle CT, Ofstedal, MB, Tian, Z, Blaum, CS (2009) Comparing models of frailty: the Health and Retirement Study *Journal of the American Geriatrics Society* 57:830-839.
- Collerton J, Barrass, K, Bond, J, Eccles, M, Jagger, C, James, O, Martin-Ruiz, C, Robinson, L, von Zglinicki, T, Kirkwood, T (2007) The Newcastle 85+ study: biological, clinical and psychosocial factors associated with healthy ageing: study protocol *BMC geriatrics* 7:14.

- Collerton J, Davies, K, Jagger, C, Kingston, A, Bond, J, Eccles, MP, Robinson, LA, Martin-Ruiz, C, von Zglinicki, T, James, OF, Kirkwood, TB (2009) Health and disease in 85 year olds: baseline findings from the Newcastle 85+ cohort study *BMJ (Clinical research ed)* 339:b4904.
- Collerton J, Martin-Ruiz, C, Davies, K, Hilkins, CM, Isaacs, J, Kolenda, C, Parker, C, Dunn, M, Catt, M, Jagger, C, von Zglinicki, T, Kirkwood, TB (2012) Frailty and the role of inflammation, immunosenescence and cellular ageing in the very old: cross-sectional findings from the Newcastle 85+ Study *Mechanisms of ageing and development* 133:456-466.
- Costello JF, Plass, C (2001) Methylation matters *Journal of medical genetics* 38:285-303.
- Davies K, Collerton, JC, Jagger, C, Bond, J, Barker, SA, Edwards, J, Hughes, J, Hunt, JM, Robinson, L (2010) Engaging the oldest old in research: lessons from the Newcastle 85+ study *BMC geriatrics* 10:64.
- DeVos L, Chanson, A, Liu, Z, Ciappio, ED, Parnell, LD, Mason, JB, Tucker, KL, Crott, JW (2008) Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations *The American journal of clinical nutrition* 88:1149-1158.
- DiPenta JM, Johnson, JG, Murphy, RJ (2004) Natural killer cells and exercise training in the elderly: a review *Canadian journal of applied physiology = Revue canadienne de physiologie appliquee* 29:419-443.
- Ensrud KE, Ewing, SK, Taylor, BC, Fink, HA, Stone, KL, Cauley, JA, Tracy, JK, Hochberg, MC, Rodondi, N, Cawthon, PM, Study of Osteoporotic Fractures Research, G (2007) Frailty and risk of falls, fracture, and mortality in older women: the study of osteoporotic fractures *The journals of gerontology. Series A, Biological sciences and medical sciences* 62:744-751.
- Fraga MF, Esteller, M (2007) Epigenetics and aging: the targets and the marks *Trends Genet* 23:413-418.
- Fried LP, Tangen, CM, Walston, J, Newman, AB, Hirsch, C, Gottdiener, J, Seeman, T, Tracy, R, Kop, WJ, Burke, G, McBurnie, MA, Cardiovascular Health Study Collaborative Research, G (2001) Frailty in older adults: evidence for a phenotype *The journals of gerontology. Series A, Biological sciences and medical sciences* 56:M146-156.
- Fuke C, Shimabukuro, M, Petronis, A, Sugimoto, J, Oda, T, Miura, K, Miyazaki, T, Ogura, C, Okazaki, Y, Jinno, Y (2004) Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study *Annals of human genetics* 68:196-204.
- Gopalakrishnan S, Van Emburgh, BO, Robertson, KD (2008) DNA methylation in development and human disease *Mutation research* 647:30-38.
- Ho V, Massey, TE, King, WD (2011) Thymidylate synthase gene polymorphisms and markers of DNA methylation capacity *Molecular genetics and metabolism* 102:481-487.
- Huidobro C, Fernandez, AF, Fraga, MF (2013) The role of genetics in the establishment and maintenance of the epigenome *Cellular and molecular life sciences : CMLS* 70:1543-1573.

- Huppert FA, Solomou, W, O'Connor, S, Morgan, K, Sussams, P, Brayne, C (1998) Aging and lymphocyte subpopulations: whole-blood analysis of immune markers in a large population sample of healthy elderly individuals *Experimental gerontology* 33:593-600.
- Kulminski A, Yashin, A, Arbeev, K, Akushevich, I, Ukraintseva, S, Land, K, Manton, K (2007) Cumulative index of health disorders as an indicator of aging-associated processes in the elderly: results from analyses of the National Long Term Care Survey *Mechanisms of ageing and development* 128:250-258.
- Lipsitz LA (2002) Dynamics of stability: the physiologic basis of functional health and frailty *The journals of gerontology. Series A, Biological sciences and medical sciences* 57:B115-125.
- Martin-Ruiz C, Jagger, C, Kingston, A, Collerton, J, Catt, M, Davies, K, Dunn, M, Hilkens, C, Keavney, B, Pearce, SH, den Elzen, WP, Talbot, D, Wiley, L, Bond, J, Mathers, JC, Eccles, MP, Robinson, L, James, O, Kirkwood, TB, von Zglinicki, T (2011) Assessment of a large panel of candidate biomarkers of ageing in the Newcastle 85+ study *Mechanisms of ageing and development* 132:496-502.
- Mathers JC, Strathdee, G, Relton, CL (2010) Induction of epigenetic alterations by dietary and other environmental factors *Advances in genetics* 71:3-39.
- Mitnitski A, Song, X, Skoog, I, Broe, GA, Cox, JL, Grunfeld, E, Rockwood, K (2005) Relative fitness and frailty of elderly men and women in developed countries and their relationship with mortality *Journal of the American Geriatrics Society* 53:2184-2189.
- Molloy DW, Standish, TI (1997) A guide to the standardized Mini-Mental State Examination *International psychogeriatrics / IPA 9 Suppl* 1:87-94; discussion 143-150.
- Nunez J, Minana, G, Bodi, V, Nunez, E, Sanchis, J, Husser, O, Llacer, A (2011) Low lymphocyte count and cardiovascular diseases *Current medicinal chemistry* 18:3226-3233.
- Rakyan VK, Down, TA, Maslau, S, Andrew, T, Yang, TP, Beyan, H, Whittaker, P, McCann, OT, Finer, S, Valdes, AM, Leslie, RD, Deloukas, P, Spector, TD (2010) Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains *Genome research* 20:434-439.
- Rockwood K, Song, X, Mitnitski, A (2011) Changes in relative fitness and frailty across the adult lifespan: evidence from the Canadian National Population Health Survey *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne* 183:E487-494.
- Romero-Ortuno R, O'Shea, D, Kenny, RA (2011) The SHARE frailty instrument for primary care predicts incident disability in a European population-based sample *Quality in primary care* 19:301-309.
- Santos-Eggimann B, Cuenoud, P, Spagnoli, J, Junod, J (2009) Prevalence of frailty in middle-aged and older community-dwelling Europeans living in 10 countries *The journals of gerontology. Series A, Biological sciences and medical sciences* 64:675-681.

- Song X, Mitnitski, A, Rockwood, K (2010) Prevalence and 10-year outcomes of frailty in older adults in relation to deficit accumulation *Journal of the American Geriatrics Society* 58:681-687.
- Struck AW, Thompson, ML, Wong, LS, Micklefield, J (2012) S-adenosyl-methionine-dependent methyltransferases: highly versatile enzymes in biocatalysis, biosynthesis and other biotechnological applications *Chembiochem : a European journal of chemical biology* 13:2642-2655.
- Tapp HS, Commane, DM, Bradburn, DM, Arasaradnam, R, Mathers, JC, Johnson, IT, Belshaw, NJ (2013) Nutritional factors and gender influence age-related DNA methylation in the human rectal mucosa *Aging cell* 12:148-155.
- Walston J, Hadley, EC, Ferrucci, L, Guralnik, JM, Newman, AB, Studenski, SA, Ershler, WB, Harris, T, Fried, LP (2006) Research agenda for frailty in older adults: toward a better understanding of physiology and etiology: summary from the American Geriatrics Society/National Institute on Aging Research Conference on Frailty in Older Adults *Journal of the American Geriatrics Society* 54:991-1001.
- Weber M, Hellmann, I, Stadler, MB, Ramos, L, Paabo, S, Rebhan, M, Schubeler, D (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome *Nature genetics* 39:457-466.
- Weisenberger DJ, Campan, M, Long, TI, Kim, M, Woods, C, Fiala, E, Ehrlich, M, Laird, PW (2005) Analysis of repetitive element DNA methylation by *MethylLight* *Nucleic acids research* 33:6823-6836.
- Wilhelm-Leen ER, Hall, YN, Deboer, IH, Chertow, GM (2010) Vitamin D deficiency and frailty in older Americans *Journal of internal medicine* 268:171-180.
- Xue QL (2011) The frailty syndrome: definition and natural history *Clinics in geriatric medicine* 27:1-15.
- Xue QL, Bandeen-Roche, K, Varadhan, R, Zhou, J, Fried, LP (2008) Initial manifestations of frailty criteria and the development of frailty phenotype in the Women's Health and Aging Study II *The journals of gerontology. Series A, Biological sciences and medical sciences* 63:984-990.
- Zhang D, Cheng, L, Badner, JA, Chen, C, Chen, Q, Luo, W, Craig, DW, Redman, M, Gershon, ES, Liu, C (2010) Genetic control of individual differences in gene-specific methylation in human brain *American journal of human genetics* 86:411-419.
- Zhu ZZ, Hou, L, Bollati, V, Tarantini, L, Marinelli, B, Cantone, L, Yang, AS, Vokonas, P, Lissowska, J, Fustinoni, S, Pesatori, AC, Bonzini, M, Apostoli, P, Costa, G, Bertazzi, PA, Chow, WH, Schwartz, J, Baccarelli, A (2012) Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis *International journal of epidemiology* 41:126-139.

Figure legend

Fig. 1. Methylation levels in Newcastle 85+ Study participants. A) Distribution of CpG island methylation levels (averaged across 5 genes) in the Newcastle 85+ Study participants (n=321). B) Distribution of LINE-1 methylation levels in the Newcastle 85+ Study participants (n=270). C) Correlation between LINE-1 methylation and CpG island methylation (n=270).

Figure 1
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Table 1. Comparison of sample populations with or without CPG island methylation data

	Sample with Fried status and CpG island methylation data (%) (n=321)	Sample with Fried status but without CPG island methylation data (%) (n=231)	p value
Female	57.3 (184)	64.1 (148)	0.110 ^a
White ethnicity	99.4 (319)	100.0 (231)	0.512 ^b
Place of residence			
Standard housing	86.3 (277)	82.7 (191)	0.179 ^a
Sheltered housing	12.5 (40)	13.9 (32)	
Resident in care home	1.2 (4)	3.5 (8)	
Years of education			0.237 ^c
12+	12.9 (41)	12.1 (28)	
10-11	24.1 (77)	19.5 (45)	
0-9	63.0 (201)	68.4 (158)	
Smoking status			
Never smoker	30.5 (98)	40.3 (93)	0.002 ^a
Ex-smoker	61.1 (196)	57.6 (133)	
Current	8.4 (27)	2.2 (5)	
Body mass index (kg/m ²)			0.012 ^c
<18.50	5.4 (17)	9.8 (21)	
18.50-24.99	48.4 (152)	53.3 (114)	
25.00-29.99	33.4 (105)	28.0 (60)	
≥30.00	12.7 (40)	8.9 (19)	
Cognitive function (mini-mental state examination score)			0.337 ^c
Normal (26-30)	81.1 (258)	84.1 (191)	
Mildly impaired (22-25)	15.1 (48)	13.7 (31)	
Moderately impaired (18-21)	3.8 (12)	2.2 (5)	
Severely impaired (0-17)	0.0 (0)	0.0 (0)	
Diabetes Mellitus	21.5 (69)	4.3 (10)	<0.001 ^a
Cancer (any previous diagnosis; excluding non-melanoma skin cancer)	25.2 (81)	3.0 (7)	<0.001 ^a
Chronic disease count, median (IQR) ^d	5 (3-6)	4 (3-5)	0.001 ^b
Disability score, median (IQR) ^e	2 (0-5)	2 (0-6)	0.713 ^c
Fried frailty status, % (n)			0.591 ^c
Robust	19.6 (63)	16.0 (37)	
Pre-frail	58.6 (188)	62.8 (145)	
Frail	21.8 (70)	21.2 (49)	

Notes : Data are % (n) except where specified; denominators vary due to missing values

^aChi-square test for no significant difference between samples with and without methylation data

^bFisher exact test for no significant difference between samples with and without methylation data

^cMann-Whitney U test for no significant difference between samples with and without methylation data

^d18 diseases: hypertension, ischaemic heart disease, cerebrovascular disease, peripheral vascular disease, heart failure, atrial flutter or fibrillation, arthritis, osteoporosis, chronic obstructive pulmonary disease or asthma, other respiratory disease, diabetes mellitus, hypothyroidism or hyperthyroidism, cancer diagnosed within past 5 years (excluding non-melanoma skin cancer), eye disease, dementia, Parkinson's Disease, renal impairment and anaemia.

^eNumber of activities of daily living performed with difficulty or requiring an aid, appliance or personal help, maximum score 17.

Table 2. Association between frailty and CpG island methylation levels

		Model 1 odds ratio (95% confidence interval)	Model 1 p value	Model 2 odds ratio (95% confidence interval)	Model 2 p value
CpG island methylation	≤ 8.50	0.47 (0.26 to 0.85)	0.013	0.46 (0.25 to 0.86)	0.016
	8.51-11.95	Reference		Reference	
	≥ 11.96	1.63 (0.88 to 3.04)	0.124	1.54 (0.76 to 3.10)	0.232
LINE-1 methylation	≤ 79.88	0.87 (0.51 to 1.57)	0.629	0.71 (0.38 to 1.34)	0.291
	79.89-82.22	Reference		Reference	
	≥ 82.23	0.89 (0.51 to 1.57)	0.693	0.79 (0.44 to 1.41)	0.420

Ordinal logistic regression models were fitted with Fried status as the dependent variable and quartiled methylation as the independent variable. The middle two methylation quartiles were combined and used as the reference category.

Model 1 is adjusted for sex, % lymphocytes and any cancer except non-melanoma skin cancer.

Model 2 is adjusted for sex, % lymphocytes, any cancer except non-melanoma skin cancer, education, smoking and total disease count.

Supplementary Material

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