



## DNA Methylation

# Apolipoprotein E DNA methylation and late-life disease

Ida K Karlsson,<sup>1\*</sup> Alexander Ploner,<sup>1</sup> Yunzhang Wang,<sup>1</sup> Margaret Gatz,<sup>1,2</sup> Nancy L Pedersen<sup>1,2</sup> and Sara Hägg<sup>1</sup>

<sup>1</sup>Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden and

<sup>2</sup>Department of Psychology, University of Southern California, Los Angeles, CA, USA

\*Corresponding author. Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Box 281, Stockholm SE 171-77, Sweden. E-mail: Ida.Karlsson@ki.se

Editorial decision 22 January 2018; Accepted 7 February 2018

## Abstract

**Background:** This study aims to investigate if DNA methylation of the apolipoprotein E (*APOE*) locus affects the risks of dementia, Alzheimer's disease (AD) or cardiovascular disease (CVD).

**Methods:** DNA methylation across the *APOE* gene has previously been categorized into three distinct regions: a hypermethylated region in the promoter, a hypomethylated region in the first two introns and exons and a hypermethylated region in the 3'exon that also harbours the *APOE*  $\epsilon$ 2 and  $\epsilon$ 4 alleles. DNA methylation levels in leukocytes were measured using the Illumina 450K array in 447 Swedish twins (mean age 78.1 years). We used logistic regression to investigate whether methylation levels in those regions affect the odds of disease.

**Results:** We found that methylation levels in the promoter region were associated with dementia and AD after adjusting for sex, age at blood draw, education, smoking and relatedness among twins [odds ratio (OR) 1.32 per standard deviation increase in methylation levels, 95% confidence interval (CI) 1.08–1.62 for dementia; OR 1.38, 95% CI 1.07–1.78 for AD). We did not detect any difference in methylation levels between CVD cases and controls. Results were similar when comparing within discordant twin pairs, and did not differ as a function of *APOE* genotype.

**Conclusions:** We found that higher DNA methylation levels in the promoter region of *APOE* increase the odds of dementia and AD, but not CVD. The effect was independent of *APOE* genotype, indicating that allelic variation and methylation variation in *APOE* may act independently to increase the risk of dementia.

**Key words:** Epigenetics, methylation, apolipoprotein E, dementia, Alzheimer's disease, cardiovascular disease, ageing

### Key Messages

- Increased DNA methylation in the promoter region of apolipoprotein E is associated with dementia and Alzheimer's disease.
- DNA methylation in the apolipoprotein E locus is not associated with cardiovascular disease.
- Results were similar within twin pairs discordant for the disorder.
- There was no difference in the effect as a function of apolipoprotein E genotype.

## Introduction

Apolipoprotein E (*APOE*) is associated with several diseases, most strongly so with Alzheimer's disease (AD) and cardiovascular disease (CVD).<sup>1</sup> The *APOE* gene is polymorphic with three alleles,  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$ , that translate into different isoforms of the protein exhibiting different binding affinities. The  $\epsilon 4$  allele is the most important genetic risk factor for AD, whereas the  $\epsilon 2$  allele has a modest protective effect. Conversely, both the  $\epsilon 2$  and the  $\epsilon 4$  alleles increase the risk of CVD. The *APOE* protein is a key player in lipid metabolism, both peripherally and in the central nervous system (CNS).<sup>1</sup> Despite extensive research on the subject, the mechanisms through which *APOE* confers disease risk are not fully understood.

Epigenetics refers to regulation of gene expression through reversible mechanisms. Methylation is the most widely studied epigenetic mechanism, and refers to addition of a methyl group to cytosine nucleotides in the genome. CpG (cytosine/guanine) islands are regions of DNA with an unusually high CG content. They are most often found in promoter regions, whereby the level of methylation correlates negatively with gene expression.<sup>2</sup> Epigenetic changes are key elements in the ageing process, and longitudinal changes in methylation are explained mainly by accumulation of environmental factors throughout the life course.<sup>3</sup> Epigenetic mechanisms have been implicated in both dementia and CVD, and have been proposed as a mechanism through which gene-environment interactions may work.<sup>4,5</sup>

The promoter region of *APOE* contains several CpG sites, and the 3' exon contains a CpG island and also harbours the  $\epsilon 2$  and  $\epsilon 4$  single nucleotide polymorphisms (SNPs). Because the SNPs are C/T substitutions, they may have additional regulatory properties through methylation.<sup>6</sup> In light of this, this study aims to investigate if methylation variation in the *APOE* locus affects the risk of dementia and CVD.

## Methods

### Study population

Our study material includes participants from two sub-studies of the Swedish Twin Registry (STR):<sup>7</sup> the Swedish Adoption/Twin Study of Aging (SATSA)<sup>8</sup> and the Study of

Dementia in Swedish Twins (HARMONY),<sup>9</sup> both described in detail previously. Briefly, SATSA is a longitudinal study of 859 twins started in 1984, which includes physical and cognitive examinations at a 3-year rolling interval across 30 years of time. HARMONY is a cross-sectional study collected between 1998 and 2003, using telephone screening for cognitive dysfunction of all twins in the STR aged 65 or older. All individuals who screened positive for cognitive dysfunction, their co-twin and a cognitively healthy control sample of twin pairs, were invited to participate in a clinical phase with physical and cognitive examination ( $n = 1557$ ). In total, methylation data were available for 447 individuals. All participants provided informed consent, and the study was approved by the Regional Ethics Board at Karolinska Institutet, Stockholm.

### Assessment of dementia

Dementia ascertainment was performed similarly in SATSA and HARMONY.<sup>8,9</sup> All suspected cases and their co-twins were referred to a clinical work-up, including cognitive testing, physical and neurological examinations, informant interviews, reviews of medical records and laboratory tests. Final dementia diagnosis was set at multidisciplinary consensus conferences, according to DSM-III-R<sup>10</sup> or DSM-IV<sup>11</sup> criteria. Dementia was further differentially diagnosed as AD according to the NINCDS/ADRDA criteria.<sup>12</sup>

In addition, dementia information after end of follow-up was extracted from the National Patient Register (NPR), the Cause of Death Register (CDR) and the Prescribed Drug Register (AD only). All registries are nationwide and linked to the STR through the national personal identification number. Further information on registries and ICD codes used can be found in the Supplement, available at *IJE* online.

### Assessment of CVD

Information about CVD was extracted from the NPR and the CDR. Diagnoses included are ischaemic and haemorrhagic stroke, atherosclerosis, unstable angina, claudication, myocardial infarction, ischaemic heart disease and the surgical procedures coronary artery bypass grafting and percutaneous transluminal coronary angioplasty.

## APOE methylation

In the HARMONY study, blood samples were collected as part of the clinical work-ups between 1999 and 2003. In the SATSA study, blood samples collected as part of the third (collected 1992-94), fifth (1999-2002), sixth (2002-05), eighth (2008-10) and ninth (2010-12) examinations were used. Hence, up to five measurements per individual were available, yielding a total of 1094 samples (Table S2, available as [Supplementary data](#) at *IJE* online).

Methylation levels in leukocytes were analysed using the Infinium Human Methylation 450K BeadChip (Illumina Inc., San Diego, CA, USA). The raw data were pre-processed using a multi-step quality control pipeline, including adjustments for cell counts and batch effects, described in the Supplement, available at *IJE* online.

Methylation levels at each CpG site were obtained as M-values, i.e. the logit2-transformed ratios of methylated probe intensity to total (methylated plus unmethylated) probe intensity.<sup>13</sup> Thirteen CpG sites residing within the APOE locus were included on the BeadChip and selected for further analyses. We categorized these CpG sites into three previously suggested regions, based on location and methylation level:<sup>14</sup> region 1 (CpG 1-3: cg14123992, cg04406254, cg01032398) is a hypermethylated region located in the promoter; region 2 (CpG 4-9: cg26190885, cg12049787, cg08955609, cg18768621, cg19514613, cg06750524) is a hypomethylated region in the first two exons and introns; and region 3 (CpG 10-13: cg16471933, cg05501958, cg18799241, cg21879725) is a hypermethylated region in the fourth exon (Figure 1).

## Covariates

Covariates included in the primary analyses were age at blood draw, sex, education and smoking at time of blood draw. Education was dichotomized into less or more than 7 years, corresponding to basic or higher education in Sweden at the time. Individuals were categorized according to smoking status as current, former or non-smokers at the time of blood sample. In sub-analyses including APOE genotype, individuals were categorized into three groups:  $\epsilon 3/\epsilon 3$  ( $n = 236$ );  $\epsilon 2$  carriers ( $n = 52$ ) ( $\epsilon 2/\epsilon 2$  and  $\epsilon 2/\epsilon 3$ ); and  $\epsilon 4$  carriers ( $\epsilon 3/\epsilon 4$  and  $\epsilon 4/\epsilon 4$ ) ( $n = 126$ ). APOE genotype was missing for 15 individuals, and in addition those with  $\epsilon 2/\epsilon 4$  ( $n = 18$ ) were excluded from these analyses in order to distinguish the effect of each allele.

## Statistical analyses

For the main analyses, the latest available blood sample from each individual was used. A linear regression model was used to test whether methylation levels differed by age

category and APOE genotype. Unconditional logistic regression was used for the association between APOE methylation levels and dementia, AD and CVD. The mean M-values of each of the three regions were modelled as exposure. Additional analyses were performed where each CpG site was modelled separately as exposure. Models were adjusted for age at blood draw, sex, education and smoking. Robust sandwich estimators were used to account for relatedness between twins. Conditional logistic regression was used to compare differences in methylation levels within discordant twin pairs. This co-twin control model was adjusted for age at blood draw, education and smoking.

To identify potential differences between stroke and non-stroke CVD, sensitivity analyses were performed using these subcategories as outcome. All individuals having suffered from a stroke before blood draw were excluded from analyses of non-stroke CVD, and controls with a non-stroke CVD diagnosis before blood draw were excluded from analyses of stroke (stroke cases with a previous diagnosis of non-stroke CVD were not excluded).

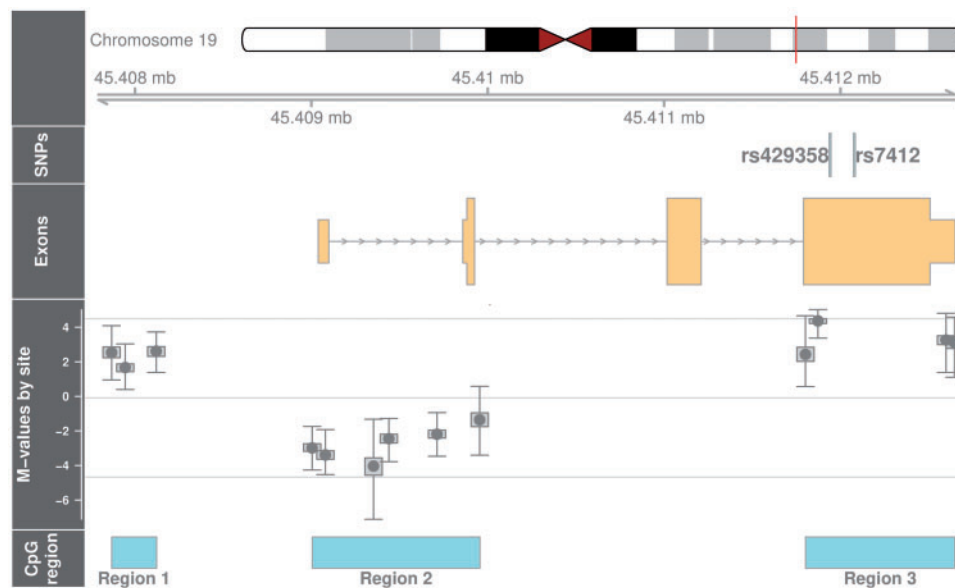
## APOE genotype

To examine whether the association between APOE methylation and the three outcomes differed by APOE genotype, we: (i) modelled APOE methylation and APOE genotype separately for each of the three outcomes; (ii) modelled APOE methylation and genotype jointly; (iii) added an interaction term between APOE genotype and the CpG regions; and (iv) used the likelihood ratio test (LRT) to test whether the interaction model improved the model fit over the joint model. The models were adjusted for age at blood draw, sex, education and smoking.

## Prevalent and incident disease

For comparison of disease with onset before and after blood draw: dementia, AD and CVD were divided into prevalent cases if they had already been diagnosed at the time of blood draw, and incident cases if they received their diagnosis after the blood sample was collected; and were modelled separately using unconditional logistic regression adjusted as above.

To further investigate methylation levels in relation to incident dementia and AD, we used all 1094 available blood samples and performed competing risk analysis with dementia and AD as outcome, and death as the competing risk. Individuals were followed from age at first blood sample, and for those with more than one blood sample, methylation levels were treated as time-varying exposure. The model was further stratified into blood samples taken before age 70, 70-80 and above 80. For each age category,



**Fig 1.** Structure of the Apolipoprotein E locus and categorization of methylation regions. Chromosome location of the Apolipoprotein E gene in the top panel, followed by location of the  $\epsilon 2$  and  $\epsilon 4$  SNPs and the four exons. The fourth panel shows mean M-values of CpG sites within the gene. The CpG sites were categorized into three regions based on location and methylation status: region 1 in the promoter (cg14123992, cg044406254, cg01032398), region 2 in the first two exons and introns (cg26190885, cg12049787, cg08955609, cg18768621, cg19514613, cg06750524), and region 3 in the fourth exon (cg16471933, cg05501958, cg18799241, cg21879725), which harbors both a CpG island and the  $\epsilon 2$  and  $\epsilon 4$  alleles.

individuals were followed until 31 December 2014 or death, whichever occurred first.

The number of cases and controls included in each analysis is shown in Figure S1, available as [Supplementary data](#) at *IJE* online. All analyses were performed using STATA 13.<sup>15</sup> Figure 1 of the *APOE* locus was created in R 3.3.2<sup>16</sup> using the Gviz package.<sup>17</sup>

## Results

### Study population

Out of the 447 individuals with methylation data, we identified 135 cases of dementia (AD: 82/61%). Among those, 84 individuals (62%) were already diagnosed at time of blood draw, and 51 (38%) were incident. The corresponding numbers for AD were 51/62% and 31/38%, respectively. Mean age at dementia diagnosis was 81.0 years. We identified 40 twin pairs discordant for dementia and 22 twin pairs discordant for AD among the 156 complete pairs.

A total of 205 individuals were diagnosed with CVD. Out of those, 112 (55%) cases were diagnosed before blood draw, and 93 (45%) were incident. Among the cases, 83 (40%) had a diagnosis of stroke and 163 (80%) had a diagnosis of non-stroke CVD (41 individuals had a diagnosis of both). Mean age at CVD diagnosis was 77.5 years. A total of 69 twin pairs discordant for CVD were identified.

Dementia, AD and CVD cases were older at blood draw compared with controls (Table S3, available as

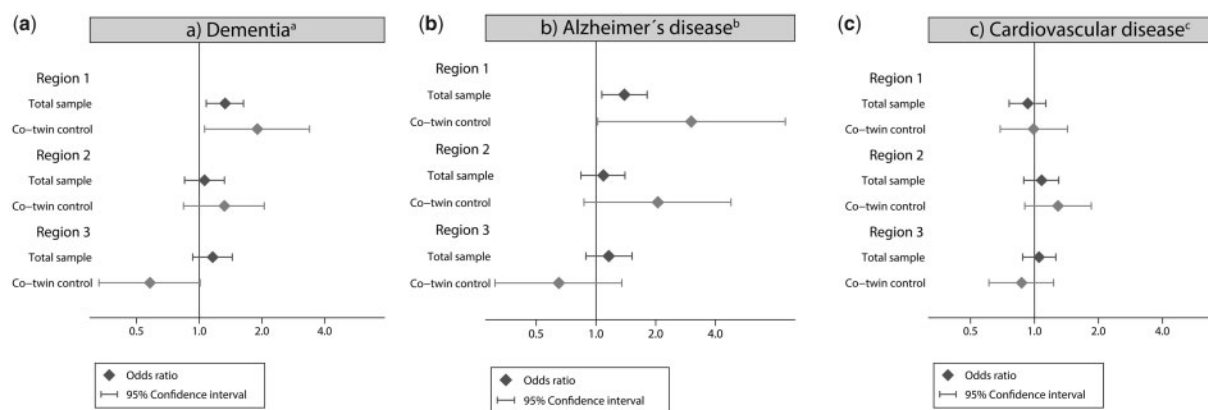
[Supplementary data](#) at *IJE* online). Dementia and AD cases were more likely than controls to have lower education and to be carriers of the *APOE*  $\epsilon 4$  allele. Dementia cases were also more likely to be female, and AD cases were more likely to be smokers.

### *APOE* methylation

The M-value distribution of each CpG site across the *APOE* gene is shown in Figure 1, and were in agreement with the results from Ma *et al.*<sup>14</sup> Mean M-values of the three CpG regions were 2.23 [standard error (SE) 0.01] for region 1, -2.72 (SE 0.01) for region 2 and 3.27 (SE 0.01) for region 3. Little variation was found in methylation levels in relation to *APOE* genotype or age (Table S4, available as [Supplementary data](#) at *IJE* online), with the exception of methylation in the promoter region of *APOE*, which decreased slightly with age ( $P = 0.05$ ). In agreement with these results, the mQTL database<sup>18</sup> did not find the  $\epsilon 2$  (rs7412) or  $\epsilon 4$  (rs429358) SNP to influence methylation levels in CpG sites within the *APOE* locus.

### *APOE* methylation in dementia

Methylation in the promoter region of *APOE* (CpG region 1) was associated with dementia and AD, with one standard deviation (SD) increase in M-value leading to 1.33 times higher odds of dementia [95% confidence interval



**Figure 2.** Odds ratios and 95% confidence intervals of a) dementia, b) Alzheimer's disease, and c) cardiovascular disease in relation to M-value of CpG regions in the Apolipoprotein E gene. Analyses of the total sample are adjusted for age, sex, education, smoking, and relatedness among twins. Co-twin control analyses are adjusted for age, education, and smoking. <sup>a</sup>Total sample: 135 cases, 312 controls; Co-twin control: 40 discordant pairs. <sup>b</sup>Total sample: n=82 cases, n=312 controls; Co-twin control: n=22 discordant pairs. <sup>c</sup>Total sample: n=205 cases, n=242 controls; Co-twin control: n=69 discordant pairs.

(CI) 1.08-1.63] and 1.39 times higher odds of AD [95% CI 1.07-1.81] (Figure 2). Co-twin control analyses revealed an even stronger association with *APOE* promoter methylation; a twin with one SD increase in methylation level had 1.90 (95% CI 1.06-3.38) times higher odds of dementia than their co-twin, and 3.02 (95% CI 1.02-9.00) times higher odds of AD (Figure 2). No association was found between methylation levels in the second or third region and dementia or AD, although cg26190885 in region 2 was associated with AD in the co-twin control model (Table S5, available as [Supplementary data](#) at *IJE* online). Estimates for the effect of each individual CpG site are presented in Table S5.

### *APOE* genotype

Including genotype in the models had little effect on the associations between methylation and dementia or AD. As expected, *APOE* genotype significantly predicted dementia and AD, and the association remained stable when methylation was included in the model (Table S6, available as [Supplementary data](#) at *IJE* online). Stratification on genotype did not indicate that the association between methylation and dementia and AD was genotype specific (Table 1; and Table S6), and the LRT showed that including an interaction between genotype and methylation did not improve the model fit compared with the joint model (dementia:  $P = 0.80$  for CpG region 1,  $P = 0.31$  for CpG region 2,  $P = 0.41$  for CpG region 3; AD:  $P = 0.40$  for CpG region 1,  $P = 0.28$  for CpG region 2,  $P = 0.20$  for CpG region 3).

### Prevalent and incident disease

Sub-analyses of the association with prevalent and incident disease separately revealed a stronger association with promoter methylation in incident compared with prevalent

dementia (Table 2). Competing risk regression stratified by age categories showed that only *APOE* methylation before age 70 increases the risk of dementia. Estimates were similar for AD but did not reach significance (Table S7, available as [Supplementary data](#) at *IJE* online).

### *APOE* methylation in CVD

None of the CpG regions were associated with CVD, nor was there an effect when CpG sites were analysed separately (Figure 2; Table S5). Results were similar in CVD-discordant twin pairs. Sensitivity analyses of stroke and non-stroke CVD did not find an effect of *APOE* methylation (Table S8, available as [Supplementary data](#) at *IJE* online).

### *APOE* genotype

*APOE* genotype did not predict CVD in this sample, and including genotype in the models did not affect the estimates of methylation on CVD. Stratification by *APOE* genotype did not reveal a genotype-specific association between DNA methylation and CVD (Table 1; and Table S6), and the LRT showed that including an interaction term between *APOE* genotype and methylation did not improve the model fit ( $P = 0.51$  for CpG region 1,  $P = 0.39$  for CpG region 2,  $P = 0.35$  for CpG region 3).

### Prevalent and incident disease

Analysis of incident and prevalent disease separately found no differences in the association of *APOE* methylation with CVD (Table S7).

## Discussion

We found that methylation in the promoter region of the *APOE* gene was associated with dementia and AD, but not



**Table 1.** Odds ratio of disease in relation to methylation of the Apolipoprotein E gene, stratified by Apolipoprotein E genotype

	CpG region 1		CpG region 2		CpG region 3	
<b>Dementia<sup>a</sup></b>						
Interaction p-value	0.47		0.62		0.99	
<i>APOE</i> $\epsilon$ 2	1.38	(0.66–2.88)	1.03	(0.54–1.93)	1.09	(0.55–2.16)
<i>APOE</i> $\epsilon$ 3	1.11	(0.83–1.49)	1.14	(0.86–1.52)	1.05	(0.77–1.41)
<i>APOE</i> $\epsilon$ 4	<b>1.49</b>	<b>(1.02–2.18)</b>	1.38	(0.96–1.97)	1.05	(0.75–1.48)
<b>Alzheimer's disease<sup>b</sup></b>						
Interaction p-value	0.34		0.65		0.72	
<i>APOE</i> $\epsilon$ 2	1.29	(0.57–2.92)	1.19	(0.66–2.14)	0.97	(0.46–2.03)
<i>APOE</i> $\epsilon$ 3	1.06	(0.70–1.60)	1.33	(0.92–1.93)	0.87	(0.55–1.35)
<i>APOE</i> $\epsilon$ 4	<b>1.71</b>	<b>(1.06–2.76)</b>	<b>1.61</b>	<b>(1.09–2.39)</b>	1.11	(0.77–1.58)
<b>Cardiovascular disease<sup>c</sup></b>						
Interaction p-value	0.40		0.97		0.54	
<i>APOE</i> $\epsilon$ 2	0.90	(0.51–1.57)	0.79	(0.45–1.36)	0.73	(0.41–1.32)
<i>APOE</i> $\epsilon$ 3	1.17	(0.89–1.55)	0.85	(0.66–1.10)	0.99	(0.75–1.30)
<i>APOE</i> $\epsilon$ 4	0.87	(0.59–1.27)	0.85	(0.58–1.26)	1.08	(0.77–1.50)

Abbreviations: *APOE*, Apolipoprotein E.

Odds ratios (95% confidence intervals) of dementia, Alzheimer's disease, and cardiovascular disease in relation to M-value of CpG regions in the *APOE* gene, stratified by *APOE* genotype. *APOE*  $\epsilon$ 2 include genotypes  $\epsilon$ 2/  $\epsilon$ 2 and  $\epsilon$ 2/  $\epsilon$ 3,  $\epsilon$ 3 genotype  $\epsilon$ 3/  $\epsilon$ 3, and  $\epsilon$ 4 genotypes  $\epsilon$ 3/  $\epsilon$ 4 and  $\epsilon$ 4/  $\epsilon$ 4. The models are adjusted for age at blood sample, sex, education, smoking, and relatedness among twins.

<sup>a</sup>n=123 cases, n=291 controls.

<sup>b</sup>n=74 cases, n=291 controls.

<sup>c</sup>n=188 cases, n=226 controls.

CVD. Similar results were seen both in the total sample and within discordant twin pairs, indicating that the associations are not driven by genotype. In addition, no difference in the methylation levels or in the association between methylation and any of the outcomes was detected as a function of *APOE* genotype. The effect was stronger for incident dementia than for prevalent, and when blood samples were taken before the age of 70.

Whereas many studies have investigated the effect of genetic variants of *APOE* on dementia and CVD, little work has been done on methylation variation in *APOE* in relation to disease. The null finding for *APOE* methylation and CVD is consistent with one previous study, where no difference in *APOE* promoter methylation was found between 15 coronary heart disease patients and 15 controls.<sup>19</sup> However, the sample size in this study is rather limited, and the possibility remains that lack of power hinders the detection of the signal. Furthermore, *APOE* genotype was not significantly associated with CVD in this sample, which may explain the negative finding.

No previous studies have investigated *APOE* methylation in relation to AD using both prospectively and retrospectively collected blood samples, but some have used posthumous brain tissue. Two epigenome-wide association studies of AD have been published, neither of which identified *APOE* as differentially methylated in AD.<sup>20,21</sup> Two studies focused on DNA methylation in AD-related genes,<sup>22,23</sup> one

of which found methylation of cg18799241 in the 3' exon of *APOE*, also included in this study, to be associated with AD pathology.<sup>23</sup> A study by Foraker *et al.*<sup>24</sup> focused on methylation across the 3' CpG island of the *APOE* gene, which they found was differentially methylated in the brains of 15 AD cases compared with 10 controls. The effect was cell type specific, and only present in neurons affected by AD. Conversely, we did not identify any difference in methylation across the 3' CpG island of *APOE* in blood samples from AD cases and controls, possibly due to cell-type specific effects. No previous studies have identified any difference in methylation in relation to AD in any of the CpG sites in the first or second region. In this study, we found an association between methylation and dementia or AD for both mean methylation of the promoter region and for the separate sites in the promoter. In addition, one of the sites in region 2 also differed within dementia-discordant twin pairs, and it may be that this effect is diluted in the main analysis due to heterogeneous effects of CpGs in the region. It should be mentioned that findings in the separate CpG sites should be interpreted with caution, as they were not corrected for the multiple testing of the 13 included sites.

Although overlapping confidence intervals limit the interpretation, the association between *APOE* promoter methylation and dementia was stronger in blood samples collected before disease onset, which highlights the importance of prospectively collected samples for a better

**Table 2.** Odds ratio and cause specific hazard rate of dementia in relation to methylation of the Apolipoprotein E gene, separated into prevalent and incident disease

	CpG region 1		CpG region 2		CpG region 3	
a) Logistic regression						
Incident dementia <sup>a</sup>	1.41	(1.02-1.96)	1.10	(0.82-1.48)	1.04	(0.76-1.44)
Prevalent dementia <sup>b</sup>	1.25	(0.97-1.62)	1.06	(0.83-1.37)	1.27	(0.97-1.66)
b) Competing risk regression						
<70 <sup>c</sup>	1.52	(1.14-2.03)	0.80	(0.49-1.33)	1.16	(0.76-1.77)
70–80 <sup>d</sup>	1.06	(0.79-1.41)	0.99	(0.77-1.29)	0.95	(0.74-1.20)
>80 <sup>e</sup>	1.24	(0.83-1.86)	1.18	(0.89-1.57)	0.93	(0.63-1.37)

a) Odds ratios (95% confidence intervals) of dementia in relation to M-value of CpG regions in the Apolipoprotein E gene, stratified into prevalent and incident dementia in relation to time of blood sample. The models are adjusted for age, sex, education, smoking, and relatedness among twins.

b) Cause specific hazard rate (95% confidence intervals) of incident dementia in relation to M-value of CpG regions in the Apolipoprotein E gene, stratified on age at blood sample. The model is adjusted for age, sex, education, smoking, and relatedness among twins.

<sup>a</sup>n=51 cases, n=312 controls.

<sup>b</sup>n=84 cases, n=312 controls.

<sup>c</sup>n=201 individuals, n=404 samples, n=3199 person years, n=25 dementia events.

<sup>d</sup>n=268 individuals, 383 samples, n=2421 person years, n=52 dementia events.

<sup>e</sup>n=137 individuals, 207 samples, n=745 person years, n=32 dementia events.

understanding of disease progression. Interestingly, the observed effect was most evident in blood samples collected before the age of 70, indicating that the differences in methylation can be detected long before diagnosis, the mean age of which was 81 in this sample. Alternatively, the greater effect for incident dementia may be driven by age at blood draw. Moreover, the preclinical dementia phase may begin years or even decades before diagnosis,<sup>25</sup> and it is hence not possible to say whether *APOE* methylation before onset is a cause or a consequence of the disease.

Promoter DNA methylation is known to suppress gene expression,<sup>2</sup> including *APOE*.<sup>14</sup> Our findings are in line with previous studies showing lower levels of circulating *APOE* to be related to incident dementia and AD in a genotype-independent manner, both in cross-sectional comparisons of AD cases and controls<sup>26</sup> and in incident disease.<sup>25,27</sup> *APOE* is not only involved in cholesterol metabolism both in the periphery and in the CNS, but also may be important in the clearance of amyloid- $\beta$ , the hallmark of AD.<sup>1</sup> Lower protein levels may thereby increase the risk of disease. Although *APOE* metabolism in the CNS is separated from that in the periphery by the blood-brain barrier, there is evidence that *APOE* levels in the two systems are correlated.<sup>25</sup> Circulating *APOE* levels, as well as DNA methylation in the *APOE* promoter, in dementia and AD hence warrant further investigation, as the latter may have potential both to elucidate disease mechanisms and to function as a biomarker to identify preclinical disease. Furthermore, identifying factors influencing the observed differences in methylation may help identify risk and protective factors as well as explain the gene-environment interactions often found between *APOE* and dementia risk factors.

One well-characterized monozygotic twin pair has been the basis for the only two previously published studies of DNA methylation in AD-discordant twins.<sup>28,29</sup> The studies used post-mortem brain samples from the two twins, and found a significant difference between the twins in global DNA methylation and hydroxymethylation in cortical neurons. Although our within-pair analyses suffered from low power, the effect of promoter methylation was even stronger when comparing twins discordant for dementia and AD than in the total sample. This not only supports the importance of the association, but also shows that the effect is not due to genetic and shared environmental factors, but is even stronger when these are controlled for.

The results presented here are based on a well-established cohort with longitudinal data. Although the number of discordant twin pairs was low, the use of twins further strengthens the study as it provides the possibility to account for factors that population-based studies cannot account for. Still, the sample is rather small, and low power may underlie some of the non-significant findings. In addition, an independent sample for validation was not available. It is challenging to find a suitable sample, but future studies are needed to replicate the findings. The use of nationwide disease registers enables us to identify cases after the end of study, but also carries some limitations. There is a risk that the more severe cases are more likely to receive a diagnosis in the registers, whereas milder forms are missed. Thus, misclassification may be introduced, leading to estimates biased towards the null. Another problem worth mentioning is investigating methylation levels in blood samples in relation to neurological disease. Although *APOE*

methylation has been shown to be rather consistent across cell types,<sup>14</sup> it is possible that the methylation patterns differ substantially between leukocytes and neuronal cells. Cell type composition was unfortunately not available, but we used the Houseman method to predict cell type composition.<sup>30</sup> However, it could still be that parts of the effect detected represent differences in leukocyte composition due to other factors. Nevertheless, studies on methylation patterns in blood are valuable, as it is possible to collect longitudinal samples from living individuals. These studies also have the potential to identify differences in the preclinical phase of disease, and hence to identify new biomarkers as well as to shed light on disease pathology.

In conclusion, we found DNA methylation in the promoter region of *APOE* to be increased in dementia and AD, but not in CVD. The observed effect was independent of genotype, indicating that methylation and genotype may act independently to increase disease risk. This is in line with the lower circulatory levels of *APOE* observed in both preclinical and clinical dementia, and may provide important insights into the causes and consequences of *APOE* expression in relation to dementia.

## Supplementary Data

Supplementary data are available at *IJE* online.

## Funding

This work was supported by: U.S. National Institutes of Health (grant numbers AG10175, AG08754, and AG028555); the Swedish Research Council (grant numbers 2013-086989 and 2015-03255); Karolinska Institutet Delfinansiering grant for doctoral student education; Loo & Hans Osterman Foundation, Foundation for Geriatric Diseases; the Swedish Council for Working Life and Social Research (2013-2292); and the Strategic Research Program in Epidemiology at Karolinska Institutet.

**Conflict of interest:** None of the authors has any financial or personal relationships or affiliations that might inappropriately influence their decisions, work or manuscript.

## References

- Giau VV, Bagyinszky E, An SS, Kim SY. Role of apolipoprotein E in neurodegenerative diseases. *Neuropsychiatr Dis Treat* 2015; 11:1723–37.
- Pal S, Tyler JK. Epigenetics and aging. *Sci Adv* 2016;2: e1600584.
- Tan Q, Heijmans BT, Hjelmborg JV, Soerensen M, Christensen K, Christiansen L. Epigenetic drift in the aging genome: a ten-year follow-up in an elderly twin cohort. *Int J Epidemiol* 2016;45:1146–58.
- Lunnon K, Mill J. Epigenetic studies in Alzheimer's disease: current findings, caveats, and considerations for future studies. *Am J Med Genet B Neuropsychiatr Genet* 2013;162B: 789–99.
- Muka T, Koromani F, Portilla E *et al.* The role of epigenetic modifications in cardiovascular disease: A systematic review. *Int J Cardiol* 2016;212:174–83.
- Yu CE, Cudaback E, Foraker J *et al.* Epigenetic signature and enhancer activity of the human *APOE* gene. *Hum Mol Genet* 2013; 22:5036–47.
- Magnusson PK, Almqvist C, Rahman I *et al.* The Swedish Twin Registry: Establishment of a biobank and other recent developments. *Twin Res Hum Genet* 2012;16:317–29.
- Finkel D, Pedersen N. Processing speed and longitudinal trajectories of change for cognitive abilities: The Swedish Adoption/Twin Study of Aging. *Neuropsychol Dev Cogn B Aging Neuropsychol Cogn* 2004;11:325–45.
- Gatz M, Fratiglioni L, Johansson B *et al.* Complete ascertainment of dementia in the Swedish Twin Registry: the HARMONY study. *Neurobiol Aging* 2005;26:439–47.
- American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders: DSM-III-R*. Washington, DC: American Psychiatric Association, 1987.
- American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders: DSM-IV*. Washington, DC: American Psychiatric Association, 1994.
- McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984;34:939–44.
- Du P, Zhang X, Huang CC *et al.* Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* 2010;11:587.
- Ma Y, Smith CE, Lai CQ *et al.* Genetic variants modify the effect of age on *APOE* methylation in the Genetics of Lipid Lowering Drugs and Diet Network study. *Aging Cell* 2015;14: 49–59.
- StataCorp. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP, 2013.
- R Core Team. *R: A language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing, 2015.
- Hahne F, Ivanek R. Visualizing genomic data using Gviz and Bioconductor. *Methods Mol Biol* 2016;1418:335–51.
- Gaunt TR, Shihab HA, Hemani G *et al.* Systematic identification of genetic influences on methylation across the human life course. *Genome Biol* 2016;17:61.
- Sharma P, Kumar J, Garg G *et al.* Detection of altered global DNA methylation in coronary artery disease patients. *DNA Cell Biol* 2008;27:357–65.
- De Jager PL, Srivastava G, Lunnon K *et al.* Alzheimer's disease: early alterations in brain DNA methylation at *ANK1*, *BIN1*, *RHBDF2* and other loci. *Nat Neurosci* 2014;17:1156–63.
- Lunnon K, Smith R, Hannon E *et al.* Methylomic profiling implicates cortical deregulation of *ANK1* in Alzheimer's disease. *Nat Neurosci* 2014;17:1164–70.
- Yu L, Chibnik LB, Srivastava GP *et al.* Association of brain DNA methylation in *SORL1*, *ABCA7*, *HLA-DRB5*, *SLC24A4*,



- and BIN1 with pathological diagnosis of Alzheimer Disease. *JAMA Neurol* 2015;**72**:15–24.
23. Chibnik LB, Yu L, Eaton ML *et al.* Alzheimer's loci: epigenetic associations and interaction with genetic factors. *Ann Clin Transl Neurol* 2015;**2**:636–47.
24. Foraker J, Millard SP, Leong L *et al.* The APOE gene is differentially methylated in Alzheimer's disease. *J Alzheimers Dis* 2015;**48**:745–55.
25. Wolters FJ, Koudstaal PJ, Hofman A, van Duijn CM, Ikram MA. Serum apolipoprotein E is associated with long-term risk of Alzheimer's disease: The Rotterdam Study. *Neurosci Lett* 2016;**617**:139–42.
26. Wang C, Yu JT, Wang HF *et al.* Meta-analysis of peripheral blood apolipoprotein E levels in Alzheimer's disease. *PLoS One* 2014;**9**:e89041.
27. Rasmussen KL, Tybjaerg-Hansen A, Nordestgaard BG, Frikke-Schmidt R. Plasma levels of apolipoprotein E and risk of dementia in the general population. *Ann Neurol* 2015;**77**:301–11.
28. Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD. Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. *PLoS One* 2009;**4**:e6617.
29. Chouliaras L, Mastroeni D, Delvaux E *et al.* Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer's disease patients. *Neurobiol Aging* 2013;**34**:2091–99.
30. Houseman EA, Accomando WP, Koestler DC *et al.* DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* 2012;**13**:86.