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DNA methylation and cognitive functioning in healthy older adults

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Running title

DNA methylation and cognitive functioning

Key words

DNA methylation; epigenetics; cognitive performance; population-based study

1 **ABSTRACT**

2 Long-term supplementation with folic acid may improve cognitive performance in older
3 individuals. The relationship between folate status and cognitive performance might be mediated by
4 changes in methylation capacity, as methylation reactions are important for normal brain
5 functioning. Although aberrant DNA methylation has been implicated in neurodevelopmental
6 disorders, the relationship between DNA methylation status and non-pathological cognitive
7 functioning in humans has not yet been investigated. The present study investigated the associations
8 between global DNA methylation and key domains of cognitive functioning in healthy older adults.
9 Global DNA methylation, defined as the percentage of methylated to total cytosine, was measured
10 in leukocytes by LC-MS/MS, in 215 men and women, aged 50-70 years, who participated in the
11 FACIT study (clinical trial registration number NCT00110604). Cognitive performance was
12 assessed by means of the Visual Verbal Word Learning Task, the Stroop Colour-Word Interference
13 Test, the Concept Shifting Test, the Letter-Digit Substitution Test, and the Verbal Fluency Test.
14 Using hierarchical linear regression analyses adjusted for age, sex, level of education, alcohol
15 consumption, smoking status, physical activity, erythrocyte folate concentration, and *MTHFR*
16 677C→T genotype, global DNA methylation was not related to cognitive performance on any of
17 the domains measured. Our results do not support the hypothesis that global DNA methylation, as
18 measured in leukocytes, might be associated with cognitive functioning in healthy older individuals.
19

1 **Introduction**

2 Most cognitive functioning declines with advancing age, and identifying the risk factors for age-
3 related cognitive decline has become a topic of increasing interest. Previous research has indicated
4 that a low folate status might increase the risk of cognitive impairment¹. However, the potential
5 biological mechanisms underlying this relationship remain to be elucidated.

6 One possible mechanism that might explain the involvement of folate status in cognitive
7 performance is DNA methylation, which refers to the epigenetic modification of gene expression by
8 the addition of methyl groups to cytosine residues in DNA². Recent animal studies have suggested
9 that DNA methylation may be involved in regulating synaptic plasticity in hippocampal neurons,
10 thereby influencing learning and memory processes^{3,4}. In humans, both hypomethylation and
11 hypermethylation of DNA have been implicated in psychiatric disorders, including schizophrenia⁵,
12 neurodegenerative disorders, such as Alzheimer's disease⁶, and syndromes associated with mental
13 retardation, e.g. Fragile X syndrome⁷.

14 Methyl groups for DNA methylation are provided by the universal methyl donor *S*-
15 adenosylmethionine, which is synthesized from methionine⁸. Folic acid may increase the
16 availability of *S*-adenosylmethionine by promoting the conversion of homocysteine into
17 methionine, thereby influencing DNA methylation status⁹. Indeed, an intervention study in older
18 women has shown that low dietary folate intake was associated with global DNA hypomethylation,
19 which could be reversed by folate repletion¹⁰. In addition, the common *MTHFR* 677C→T
20 polymorphism, which mimics folate deficiency by impairing the conversion of homocysteine into
21 methionine, has also been related to DNA hypomethylation¹¹.

22 Given the role of folate metabolism in generating methyl donors for methylation processes,
23 and the involvement of DNA methylation in brain functioning, it seems reasonable to hypothesize
24 that folate status might influence cognitive functioning by exerting effects on DNA methylation.
25 However, the association between DNA methylation status and cognitive performance in the
26 general population has not yet been investigated. Therefore, the present study examined whether

- 1 leukocyte global DNA methylation was associated with cognitive performance in healthy older
- 2 adults.
- 3

1 **Methods**

2 ***Study population***

3 The present study was performed using data from the FACIT study, a randomized, double-blind,
4 placebo-controlled trial, originally designed to investigate the effects of 3-year folic acid
5 supplementation on the risk of cardiovascular disease¹². The study population consisted of 818
6 healthy men and women, aged 50-70 years at baseline. A detailed description of the study design
7 and the selection of participants can be found elsewhere¹².

8 Venous blood samples were collected at baseline. Leukocyte global DNA methylation was
9 determined in a subsample of 216 participants. First, the study population was stratified by *MTHFR*
10 677C→T genotype, to ensure equal distribution of *MTHFR* 677C→T genotypes in the final sample.
11 Thereafter, participants in the folate treatment group were randomly selected from the three strata
12 and individually matched with participants in the placebo group on the variables age, sex, smoking
13 status, and *MTHFR* 677C→T genotype, as these variables may influence DNA methylation^{11,13,14}.
14 Some samples were not measured due to human error in sample retrieval. Valid DNA methylation
15 data were available for 111 participants in the treatment group and 105 participants in the placebo
16 group. As valid data on cognitive functioning were lacking for one participant in the folate
17 treatment group, the final study sample consisted of 215 individuals.

18 This study was conducted according to the guidelines laid down in the Declaration of
19 Helsinki and all procedures involving human participants were approved by the Medical Ethics
20 Committee of Wageningen University. Written informed consent was obtained from all
21 participants.

22

23 ***Cognitive functioning***

24 Cognitive functioning on the domains of memory, sensorimotor speed, complex speed,
25 information processing speed, and word fluency was assessed by means of a comprehensive
26 neuropsychological test battery, consisting of the Visual Verbal Word Learning Task, the Stroop

1 Colour-Word Interference Test, the Concept Shifting Test, the Letter-Digit Substitution Test, and
2 the Verbal Fluency Test, as described before¹².

4 ***DNA methylation status and genotyping***

5 Genomic DNA was isolated from peripheral blood leukocytes at baseline. Global DNA methylation
6 was determined by LC-MS/MS, as described previously¹⁵. Genomic DNA methylation status was
7 calculated as the percentage of methylated to total cytosine (mCyt/tCyt) using the following
8 formula: $(\text{nmol mCyt}/[\text{nmol mCyt} + \text{nmol Cyt}]) \times 100\%$ ¹⁵.

9 *MTHFR* 677C→T genotype was determined by PCR with restriction fragment length
10 polymorphism analysis with *Hinfl*¹⁶, and was defined as common variant (CC or CT genotype) or
11 rare variant (TT genotype).

13 ***Blood measurements***

14 Fasting venous blood samples were collected at baseline, directly processed, and stored at -80°C.
15 Serum folate was measured using a chemiluminescent immunoassay (Diagnostic Products
16 Corporation, Los Angeles, CA, USA). Erythrocyte folate was determined in duplicate and the
17 average was taken to reduce measurement error. Erythrocyte folate concentrations were calculated
18 using the following formula: $(\text{unadjusted erythrocyte folate}/\text{hematocrit}) - ([1 -$
19 $\text{hematocrit}]/\text{hematocrit}) \times \text{serum folate}$. Plasma total homocysteine was determined by HPLC and
20 fluorimetric detection, as described previously¹⁷.

22 ***Demographic and lifestyle variables***

23 Level of education (low/middle/high) was measured by classifying formal schooling according to
24 the Dutch educational system¹⁸. Alcohol consumption (g/d) and current smoking (yes/no) were
25 ascertained by means of self-report questionnaires. BMI (kg/m^2) was calculated from height and
26 weight, and physical activity was estimated using the Physical Activity Scale for the Elderly¹⁹.

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Statistical analysis

Normality of data distributions was ascertained by normal P-P plots. Baseline data were used to assess the cross-sectional associations between total DNA methylation status and cognitive functioning. Independent samples *t* tests and univariate ANOVA were used to examine whether DNA methylation status varied according to sex, level of education, smoking status, or *MTHFR* 677C→T genotype.

Hierarchical linear regression analyses were performed for DNA methylation status in relation to each of the five cognitive performance indices. The analyses were corrected for sociodemographic and lifestyle variables that were considered potential confounders, i.e. age, sex, level of education, alcohol consumption, smoking status, physical activity, erythrocyte folate concentration, and *MTHFR* 677C→T genotype^{11,13,14,20}.

To investigate the possibility of a non-linear relationship between global DNA methylation and cognitive performance, the analyses were repeated with the quadratic term for DNA methylation status as the independent variable, adjusted for covariates and the linear term for DNA methylation status. The quadratic term for DNA methylation status was expressed as the residuals of regressing (DNA methylation)² on DNA methylation, i.e. the quadratic component that is orthogonal to the linear component of DNA methylation.

Statistical power for detecting associations between DNA methylation status and each of the dependent variables, assuming a small effect size of $f^2 = 0.03$, was 0.80. Statistical differences were considered significant at *P*-values <0.05. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

1 **Results**

2 Table 1 summarizes the characteristics of the study population. The percentage of methylated to
3 total cytosine residues in leukocyte DNA ranged from 4.0 to 5.6%, which was comparable to the
4 range reported by other population-based studies^{11,15}. The extent of global DNA methylation did not
5 vary according to sex ($t = -1.285$, $P = 0.200$), level of education ($F = 0.611$, $P = 0.544$), smoking
6 status ($t = 1.611$, $P = 0.109$), or *MTHFR* 677C→T genotype ($t = -0.907$, $P = 0.365$).

7 Hierarchical linear regression analyses corrected for age, sex, level of education, alcohol
8 consumption, smoking status, physical activity, erythrocyte folate concentration, and *MTHFR*
9 677C→T genotype did not reveal any significant associations between leukocyte global DNA
10 methylation and cognitive performance on any of the domains measured (Table 2). In addition,
11 repeating the analyses with the quadratic term for DNA methylation status as the independent
12 variable did not yield any significant results (data not shown), implying that global DNA
13 methylation did not show a non-linear relationship with cognitive performance.

1 Discussion

2 The present study did not offer support for the hypothesis that individual variation in cognitive
3 functioning in older adults might be related to the extent of leukocyte global DNA methylation.

4 Although there are no previous studies investigating the relationship between global DNA
5 methylation and cognitive functioning in healthy humans, aberrant DNA methylation has been
6 implicated in neurodevelopmental disorders⁷, psychiatric diseases⁵, and neurodegenerative
7 disorders⁶. In addition, animal research has suggested that DNA methylation status may be involved
8 in learning and memory processes, e.g. by regulating synaptic plasticity in hippocampal neurons^{3,4}.

9 The observed lack of a relationship between global DNA methylation and cognitive
10 performance in healthy adults might imply that there is no functional relationship between the
11 extent of cytosine methylation within DNA and individual differences in cognitive performance in
12 the general population. In line with earlier reports¹⁵, we observed that global DNA methylation has
13 a relatively narrow distribution in healthy individuals. These findings suggest that under non-
14 pathological conditions, there appears to be little interindividual variation in DNA methylation-
15 based regulation of gene expression, which decreases the likelihood that individual differences in
16 cognitive performances may be mediated by this epigenetic mechanism.

17 Although global DNA methylation might not be involved in cognitive functioning, the
18 present results do not rule out the possibility that DNA methylation at specific loci may be related
19 to cognitive performance. In humans, gene-specific alterations in DNA methylation patterns have
20 been associated with a number of pathological conditions characterized by cognitive deficits.

21 Animal studies have suggested that diet-induced folate deficiency may result in overexpression of
22 the Presenilin 1 gene by causing hypomethylation of its promoter region²¹. Increased expression of
23 this gene, which leads to elevated production of β -amyloid peptide, has been implicated in the
24 etiology of Alzheimer's disease²². In addition, schizophrenia has been associated with reduced
25 expression of the gene encoding the protein Reelin, which is involved in neurodevelopment and
26 synaptic plasticity, due to hypermethylation of the gene's promoter region⁵. However, although it

1 may be speculated that gene-specific changes in DNA methylation might underlie part of the
2 individual differences in non-pathological cognitive functioning, little is known about the genetic
3 correlates of cognitive performance in healthy humans.

4 An alternative explanation for the present null findings is that cognitive performance might
5 be related to short-term changes, i.e. within the range of hours, in DNA methylation patterns rather
6 than individual variation on the level of global DNA methylation. Indeed, animal studies have
7 reported that dynamic and reversible changes in DNA methylation, such as the transient
8 methylation and demethylation of DNA, are crucial for synaptic plasticity, learning, and memory
9 processes^{3,4}. It might be complicated, however, to measure such short-term changes in DNA
10 methylation in volunteers, which makes it rather difficult to test this possibility.

11 From a methodological perspective, **our study was limited by its cross-sectional nature**. In
12 addition, the fact that we determined global DNA methylation in leukocytes rather than brain tissue
13 should also be considered a limitation, as the extent of DNA methylation might differ between cells
14 derived from the periphery and the brain²³. However, no direct measures of DNA methylation status
15 in the central nervous system were available, given the inability to measure cerebrospinal fluid or
16 brain DNA methylation status in volunteers.

17 It might also be argued that due to the relatively small sample size, the present study might
18 have been underpowered to detect very modest associations. However, it should be noted that our
19 study had 80% power to detect a 3% change in the proportion of explained variance, which may be
20 considered a small effect size²⁴.

21 **The present study did not support the notion that folate metabolism might influence**
22 **cognitive performance through the mechanism of global DNA methylation, as measured in**
23 **leukocytes**. In line with the present findings, we found that long-term supplementation with folic
24 acid, which significantly improved cognitive performance in the FACIT population¹², did not have
25 any effect on **leukocyte** global DNA methylation status (A. Jung, Y. Smulders, P. Verhoef, F.J.
26 Kok, H. Blom, R. Kok, E. Schouten, E. Kampman, J. Durga, 2010; unpublished results). **This might**

1 be explained by the fact that methylation capacity is not exclusively dependent on folate status, as
2 methyl groups may also be provided by dietary intake of methionine, or by betaine-mediated
3 remethylation of homocysteine⁹.

4 To our knowledge, this is the first study to investigate the relationship between leukocyte
5 global DNA methylation and non-pathological cognitive functioning in healthy older adults. Future
6 studies focusing on gene-specific DNA methylation patterns or short-term changes in DNA
7 methylation status might contribute further to identifying the epigenetic mechanisms involved in
8 cognitive functioning.

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Table 1. Characteristics of the study population.

Characteristic	Total sample (<i>n</i> = 215)	95% CI
Age (years)	60.9	60.2; 61.6
Female sex (%)	34.9	
Level of education (% low / middle / high)	26.0 / 39.1 / 34.9	
Alcohol consumption (g/d) *	12.6	4.5; 23.5
Current smoker (%)	14.9	
BMI (kg/m ²)	26.7	26.2; 27.2
Physical activity (PASE score)	149.2	140.5; 158.0
Erythrocyte folate (nmol/l)	716.0	681.2; 750.8
Plasma total homocysteine (μmol/l)	13.4	12.9; 13.8
<i>MTHFR</i> 677C→T genotype (% CC / CT / TT)	34.9 / 32.6 / 32.6	
Leukocyte global DNA methylation status (%) [†]	4.6	4.6; 4.7

Values are means or %. PASE, Physical Activity Scale for the Elderly; *MTHFR*, 5,10-methylenetetrahydrofolate reductase.

* Median (interquartile range) is given because of skewed data distribution.

[†] Defined as the percentage of methylated to total cytosine (mCyt/tCyt).