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The effects of long-term daily folic acid and vitamin B₁₂ supplementation on genome-wide DNA methylation in elderly subjects

Dieuwertje E. G. Kok^{1*}, Rosalie A. M. Dhonukshe-Rutten¹, Carolien Lute¹, Sandra G. Heil², André G. Uitterlinden^{3,4}, Nathalie van der Velde^{3,5}, Joyce B. J. van Meurs³, Natasja M. van Schoor⁶, Guido J. E. J. Hooiveld¹, Lisette C. P. G. M. de Groot¹, Ellen Kampman¹ and Wilma T. Steegenga¹

Abstract

Background: Folate and its synthetic form folic acid function as donor of one-carbon units and have been, together with other B-vitamins, implicated in programming of epigenetic processes such as DNA methylation during early development. To what extent regulation of DNA methylation can be altered via B-vitamins later in life, and how this relates to health and disease, is not exactly known. The aim of this study was to identify effects of long-term supplementation with folic acid and vitamin B₁₂ on genome-wide DNA methylation in elderly subjects. This project was part of a randomized, placebo-controlled trial on effects of supplemental intake of folic acid and vitamin B₁₂ on bone fracture incidence (B-vitamins for the PRevention Of Osteoporotic Fractures (B-PROOF) study). Participants with mildly elevated homocysteine levels, aged 65–75 years, were randomly assigned to take 400 µg folic acid and 500 µg vitamin B₁₂ per day or a placebo during an intervention period of 2 years. DNA was isolated from buffy coats, collected before and after intervention, and genome-wide DNA methylation was determined in 87 participants ($n = 44$ folic acid/vitamin B₁₂, $n = 43$ placebo) using the Infinium HumanMethylation450 BeadChip.

Results: After intervention with folic acid and vitamin B₁₂, 162 (versus 14 in the placebo group) of the 431,312 positions were differentially methylated as compared to baseline. Comparisons of the DNA methylation changes in the participants receiving folic acid and vitamin B₁₂ versus placebo revealed one single differentially methylated position (cg19380919) with a borderline statistical significance. However, based on the analyses of differentially methylated regions (DMRs) consisting of multiple positions, we identified 6 regions that differed statistically significantly between the intervention and placebo group. Pronounced changes were found for regions in the *DIRAS3*, *ARMC8*, and *NODAL* genes, implicated in carcinogenesis and early embryonic development. Furthermore, serum levels of folate and vitamin B₁₂ or plasma homocysteine were related to DNA methylation of 173, 425, and 11 regions, respectively. Interestingly, for several members of the developmental *HOX* genes, DNA methylation was related to serum levels of folate.

Conclusions: Long-term supplementation with folic acid and vitamin B₁₂ in elderly subjects resulted in effects on DNA methylation of several genes, among which genes implicated in developmental processes.

Keywords: DNA methylation, Folic acid, Vitamin B₁₂, B-vitamins, One-carbon metabolism, Intervention trial, Infinium 450k BeadChip, Elderly, Cancer, Development, Epigenetics

* Correspondence: dieuwertje.kok@wur.nl

¹Division of Human Nutrition, Wageningen University, PO Box 8129, 6700 EV Wageningen, The Netherlands

Full list of author information is available at the end of the article

Background

During early development, DNA methylation is one of the epigenetic phenomena responsible for programming of gene expression profiles [1]. It has been shown that the plasticity of epigenetic regulation can be determined by environmental factors, such as parental diet and lifestyle, during the critical windows in early development [1–3]. B-vitamins play an essential role in one-carbon metabolism and have, as such, also been implicated in the regulation of DNA methylation and DNA synthesis [1, 4–6].

Recently, neonatal folate-sensitive regions of differential methylation were identified based on the maternal folate status during the last trimester of pregnancy [7]. Maternal use of folic acid before and during pregnancy has been previously associated with specific DNA methylation patterns in the infants [8–10]. Also, maternal vitamin B₁₂ levels have been shown to influence global DNA methylation in cord blood, whereas infants' levels of vitamin B₁₂ were associated with distinct gene-specific DNA methylation patterns [11]. These findings illustrate and confirm the potential role of B-vitamins on DNA methylation during early development [12, 13].

The “developmental origins of health and disease” hypothesis states that adult diseases often originate from aberrant epigenetic programming during early development [14, 15]. To what extent environmental exposures later in life can induce changes in DNA methylation and how this relates to development of adult diseases is an emerging field of research. Although some studies demonstrate plasticity of DNA methylation in adults [16, 17], it is still largely unknown to what extent B-vitamins involved in one-carbon metabolism can affect DNA methylation throughout the life cycle. Although not consistently, suboptimal levels of B-vitamins and homocysteine, a major key player in one-carbon metabolism, in adult life have been implicated in several clinical phenotypes [18], such as an increased risk of osteoporosis [19, 20], vascular diseases [21, 22], cognitive impairment [23], and cancer [24–26]. There is a growing body of evidence that the association between B-vitamins and disease risk implies a narrow range of an optimal B-vitamin status, and depends, in case of cancer, on the presence of preclinical neoplastic lesions [27–30]. Detailed insight into the effects of B-vitamins on DNA methylation is therefore urgently required in order to elucidate the mechanisms underlying the suggested effects of these B-vitamins on health and disease in adult life.

The aim of the current study was to determine the effects of a long-term daily supplementation with folic acid and vitamin B₁₂ on genome-wide DNA methylation in leukocytes of elderly subjects.

Results

Biochemical analyses

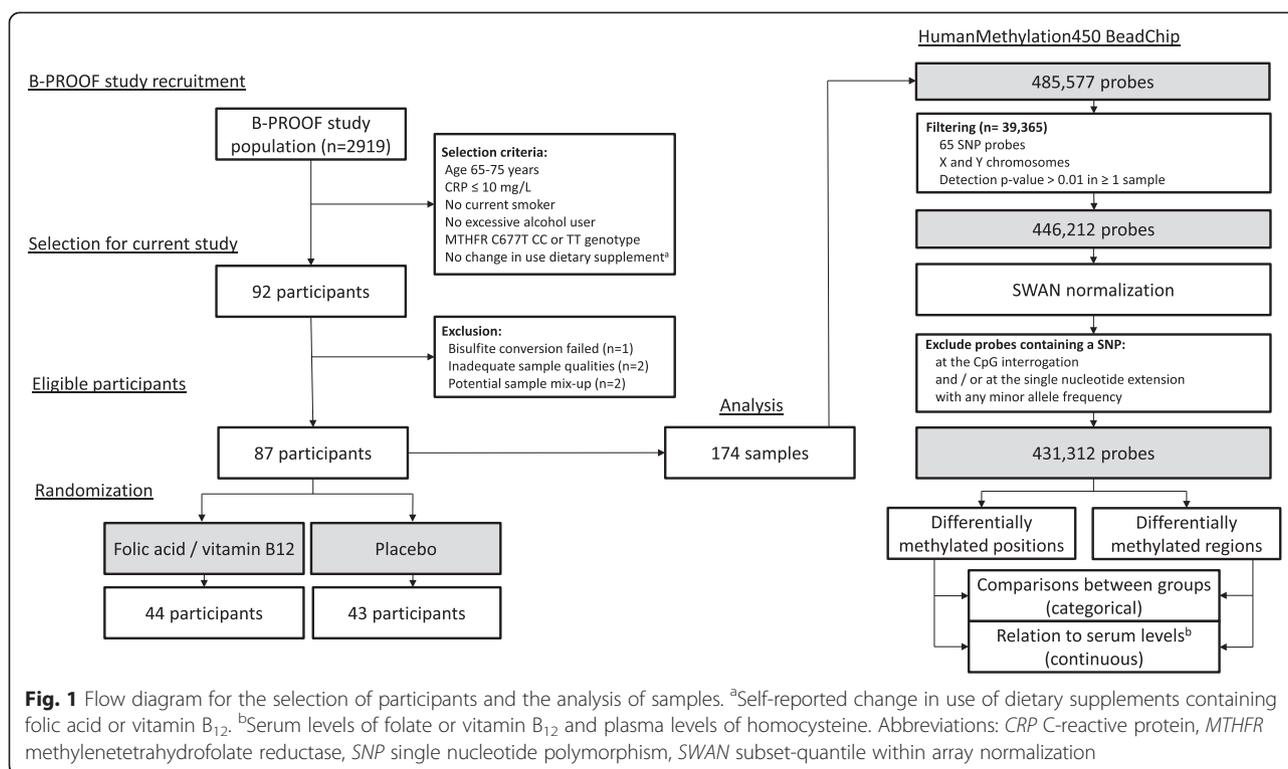
As shown in Fig. 1, 44 participants were randomized to the intervention with folic acid and vitamin B₁₂, while 43 participants were assigned to the placebo group. Baseline characteristics of the participants are presented in Table 1. Inherent to the selection procedure for our study, participants with the methylenetetrahydrofolate reductase (*MTHFR*) (C677T) CC and TT genotypes were equally distributed among the groups. Age, body mass index, and serum levels of folate, vitamin B₁₂, and plasma homocysteine at baseline did not differ between the two groups. The 2-year intervention with folic acid and vitamin B₁₂ resulted in increased serum levels of both B-vitamins (folate median change 34.0 nmol/L; vitamin B₁₂ median change 319 pmol/L) as well as decreased levels of homocysteine (median change -5.3 μmol/L). The changes in these levels were statistically significantly different from the ones observed in the placebo group (median change for folate 5.4 nmol/L, vitamin B₁₂ 30.4 pmol/L, and homocysteine -1.5 μmol/L, for all parameters $p < 0.0001$). Individual changes in levels of serum folate, vitamin B₁₂, and plasma homocysteine are presented in Fig. 2.

Leukocyte proportions

In order to assess whether the mean leukocyte composition changed as a result of the intervention or duration of the study, leukocyte proportions were estimated based on DNA methylation data. The composition of the leukocyte fraction was similar for participants receiving folic acid and vitamin B₁₂ or placebo and for both time points (Additional file 1: Figure S1). The Pearson correlation coefficient for estimated versus measured cell proportions in all follow-up samples ($n = 85$, 2 missing samples) was 0.66 for monocytes, 0.45 for lymphocytes (CD4+ and CD8+ T-lymphocytes, natural killer cells, and B-lymphocytes), and 0.46 for granulocytes (eosinophils, basophils, and neutrophils). No significant differences in measured or estimated cell proportions between the placebo and intervention group were measured at follow-up.

Differentially methylated positions

At baseline, there were no statistically significant differences in methylation between the placebo and the intervention group. After intervention with folic acid and vitamin B₁₂, 162 positions (versus 14 in the placebo group) of the 431,312 positions were differentially methylated as compared to baseline (Benjamini-Hochberg (BH)-adjusted p value < 0.05). Volcano plots showing the DNA methylation changes versus the statistical significance are presented in Fig. 3a, b. An overview of the top-100 of differentially methylated positions with corresponding p values and methylation changes is presented



in Additional file 2: Table S1. Mean changes in DNA methylation, as a result of the intervention, for these differentially methylated positions ranged from -4 to 5 % (Fig. 3e). Overall, positions within the CpG islands and around the transcription start sites were overrepresented among the positions that were differentially methylated after the intervention with folic acid and vitamin B₁₂ (Fig. 3c, d).

Differentially methylated positions that differ between groups

Comparisons between the DNA methylation changes in the participants receiving folic acid and vitamin B₁₂ versus participants receiving the placebo showed one differentially methylated position with a BH-adjusted p value <0.05 . This position (cg19380919) is located within a non-CpG dense region (open sea) on chromosome 2. Further exploration of the top-20 of differentially methylated positions showed that most pronounced differences (>5 %) were found for two probes on chromosome 4 and 1. For one probe (cg01129246) within the body of *GRXCRI*, mean DNA methylation increased with 2.6 % after the intervention with folic acid and vitamin B₁₂, whereas it decreased with 4.1 % in the placebo group. Furthermore, DNA methylation changes differed for cg06191076 within the promoter region of the *DIRAS3* gene (also known as *ARHI*), a suggested tumor suppressor gene and member of the ras superfamily (Table 2). For this position, mean DNA methylation increased after intervention with folic acid and

vitamin B₁₂ with 2.7 %, whereas it decreased with 2.5 % in the placebo group (Fig. 3f). Stratification for *MTHFR* genotype did only reveal 2 and 3 (TT and CC, respectively) positions with differential methylation after the intervention with folic acid and vitamin B₁₂, indicating that the power for stratified analyses may be insufficient as a consequence of the limited sample sizes in the subgroups.

Differentially methylated regions that differ between groups

Identification and characterization of differentially methylated regions (DMRs), consisting of multiple consecutive positions, showed that 6 regions differed for the participants receiving folic acid and vitamin B₁₂ versus placebo (BH-adjusted p value <0.05) (Table 3). Again, for a DMR of 11 positions within *DIRAS3* the most pronounced difference (maximal 5.2 %) in DNA methylation changes was found (Table 3 and Fig. 4). For this DMR, the mean percentage of DNA methylation increased after intervention with folic acid and vitamin B₁₂ (1.5 %), but decreased in the placebo group (-0.9 %). A similar pattern was found for *NODAL* (nodal growth differentiation factor) on chromosome 10, for which 2 consecutive positions located within the 3' untranslated region (3'UTR) were identified. After intervention with folic acid and vitamin B₁₂, the mean methylation for these positions increased with 1.2 %, whereas in the placebo group a modest decrease of -0.4 % was found.

Table 1 Characteristics of the participants at baseline and after 2 years of intervention with either folic acid and vitamin B₁₂ or placebo

	Folic acid and vitamin B ₁₂ (n = 44)	Placebo (n = 43)
Baseline characteristics		
Age at baseline (years)	70.8 ± 2.9	71.1 ± 3.0
Women (%)	25 (57 %)	22 (51 %)
Body mass index (kg/m ²)	27.2 (24.4–29.7)	28.7 (25.7–31.2)
Smoking (%) ^a		
Former	25 (57 %)	31 (72 %)
Never	19 (43 %)	12 (28 %)
MTHFR C677T genotype (%) ^b		
CC	22 (50 %)	20 (47 %)
TT	22 (50 %)	23 (54 %)
Biochemical analyses		
Serum folate levels (nmol/L)		
Baseline	16.2 (13.1–24.4)	17.3 (14.1–21.5)
After intervention	52.3 (45.1–67.6)	23.1 (18.3–26.9)
Change	34.0 (26.5–47.6)	5.4 (2.0–9.6)
Serum vitamin B ₁₂ levels (pmol/L)		
Baseline	279 (232–358)	300 (204–365)
After intervention	595 (467–814)	325 (230–449)
Change	319 (197–462)	30.4 (–12.6–89.5)
Plasma homocysteine levels (μmol/L)		
Baseline	14.7 (13.1–16.3)	14.9 (13.1–16.3)
After intervention	9.6 (8.5–11.1)	13.7 (11.8–16.6)
Change	–5.3 (–6.7 to –3.4)	–1.5 (–2.4–0.6)

Data are presented as median (interquartile ranges) or numbers (%). ^aCurrent smokers were not selected for the study population. ^bSingle nucleotide polymorphism: rs1801133, participants with the CT genotype were not selected for the current study

Relation with serum or plasma levels of B-vitamins and homocysteine

Changes in serum levels of folate or vitamin B₁₂ and plasma homocysteine after the 2-year intervention period were fairly subjected to interindividual variation resulting in an “overlap” between the intervention and placebo group (Fig. 2). In addition to the comparisons for the DNA methylation changes between these two groups, we therefore explored the relation between DNA methylation and serum folate, vitamin B₁₂ or plasma homocysteine levels in a continuous manner. Applying the conservative threshold for significance of BH-adjusted *p* values <0.05 revealed no statistically significant positions related to folate levels. Exploration, however, of the top-35 positions for which DNA methylation tends to be related to serum folate levels showed a probe (cg24973150, *p* = 5.15E-05) located within a CpG island in the promoter region (TSS200) of the *HOXB7* gene, which has been recently associated with the risk of neural tube defects [31]. Most (32 of the top-35 positions) of the observed positions were positively related to serum levels of folate, indicating that DNA methylation increased with higher levels of serum folate. In addition to the identification of individual positions, we subsequently identified 173 DMRs that were related to folate levels. One of the most prominent regions, consisting of 22 probes and a mean BH-adjusted *p* value of 0.001, belongs to the promoter region of *HOXA4*, another member of the homeobox family. An overview of DNA methylation changes in *HOXB7*, *HOXA4*, and the other *HOX* genes in one of the four *HOX* gene clusters is presented in Fig. 5, showing that mean DNA methylation for the majority of these *HOX* genes tends to be increased after intervention with folic acid and vitamin B₁₂, whereas it mostly remained stable or decreased in the placebo group.

Likewise, for plasma levels of homocysteine, 11 DMRs were identified. As expected, an inverse association between DNA methylation and plasma levels of homocysteine was found for most (32 of the top-35 positions) of the positions related to homocysteine levels. In comparison to folate and homocysteine, most pronounced

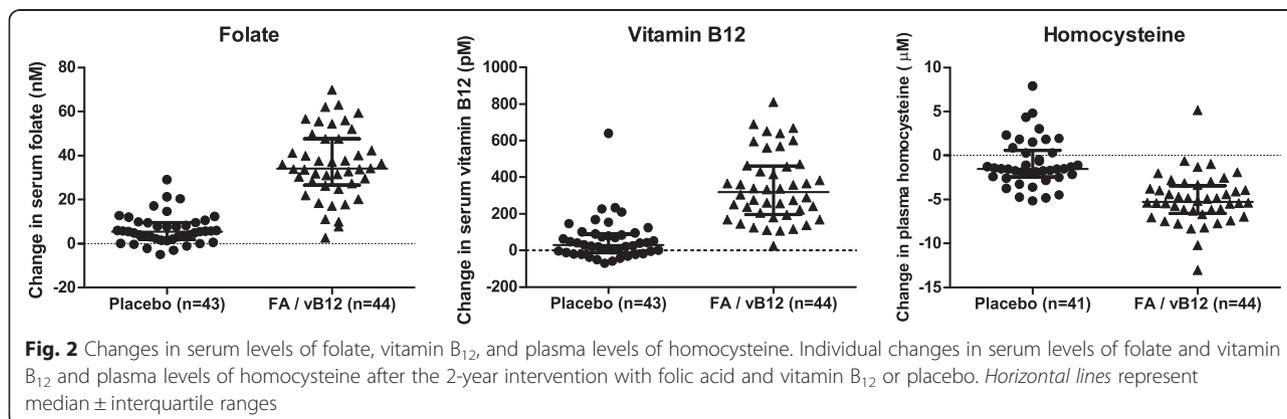


Fig. 2 Changes in serum levels of folate, vitamin B₁₂, and plasma levels of homocysteine. Individual changes in serum levels of folate and vitamin B₁₂ and plasma levels of homocysteine after the 2-year intervention with folic acid and vitamin B₁₂ or placebo. Horizontal lines represent median ± interquartile ranges

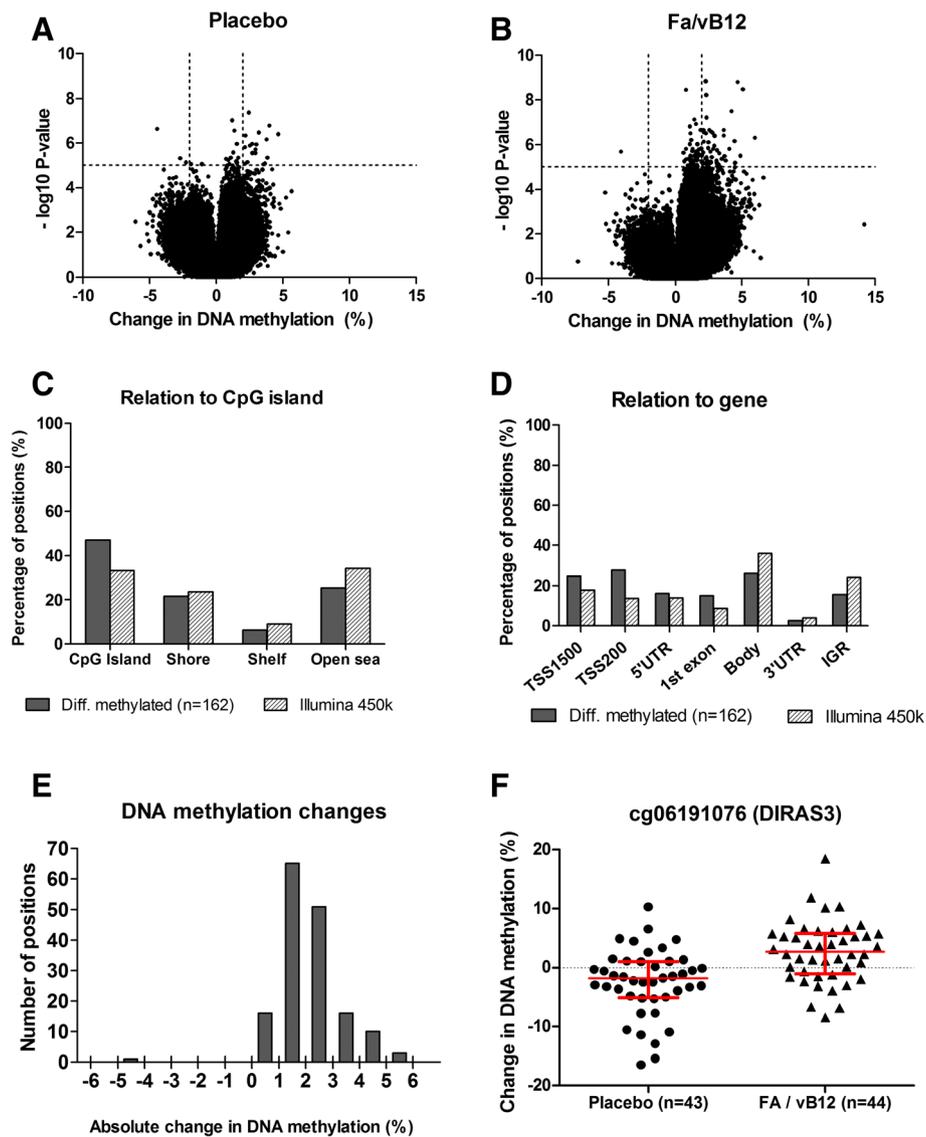


Fig. 3 Differentially methylated positions after the intervention with folic acid and vitamin B₁₂. Volcano plots show the statistical significance versus the changes in DNA methylation after the intervention with (a) the placebo or (b) folic acid and vitamin B₁₂. Dashed lines represent 2 % methylation changes and a *p* value of 1.0E-05. Features of the positions (*n* = 162) that were differentially methylated after the intervention with folic acid and vitamin B₁₂ are presented in (c) percentages of positions expressed per relationship to CpG islands for the differentially methylated positions (*n* = 162) as well as for all considered positions on the Infinium HumanMethylation450 Beadchip (*n* = 431,312), and (d) percentage of positions expressed per relationship to the nearest gene(s). e Number of positions according to the presented categories for absolute changes in DNA methylation for the 162 positions that were differentially methylated after the intervention with folic acid and vitamin B₁₂. f Individual changes in DNA methylation for probe cg06191076 located within *DIRAS3*. Horizontal lines represent median ± interquartile ranges of DNA methylation, which is expressed as a beta value (0–100 %). Abbreviations: *TSS200* 200 base pairs around the transcription start site, *TSS1500* 1500 base pairs around the transcription start site, *3'UTR* 3' untranslated region, *5'UTR* 5' untranslated region, *IGR* intergenic region

relations with DNA methylation were, however, found for serum levels of vitamin B₁₂. In total, 425 regions were identified for which DNA methylation was related to serum vitamin B₁₂ levels (BH-adjusted *p* value <0.05). An overview of the top-3 most statistically significant positions related to folate, vitamin B₁₂, or homocysteine levels is presented in Fig. 6. A detailed list with differentially methylated positions and regions related to either

folate, vitamin B₁₂, or homocysteine levels is included in Additional file 3: Table S2.

Discussion

The current study shows that long-term supplementation with folic acid and vitamin B₁₂ resulted in DNA methylation changes in leukocytes of older persons. We identified several differentially methylated positions as

Table 2 The top-20 of differentially methylated positions representing changes in DNA methylation that differ between participants receiving folic acid and vitamin B₁₂ or placebo

CpG	Chr	Gene	Gene feature	<i>p</i> value	Adjusted <i>p</i> value	Mean change in methylation (%)	
						Folic acid and vitamin B ₁₂	Placebo
cg19380919	2	–	–	9.9E-08	0.0426	1.0	–1.4
cg05308617	3	ARMC8	TSS200	8.4E-07	0.1810	0.6	–0.6
cg06016645	10	NODAL	3'UTR	1.6E-06	0.2323	1.5	–0.8
cg11567849	6	REV3L	Body	2.7E-06	0.2495	0.8	–1.0
cg10367137	10	–	–	3.8E-06	0.2495	0.7	–1.1
cg18514573	6	–	–	4.0E-06	0.2495	0.6	–0.6
cg03330920	16	IFT140	Body	4.6E-06	0.2495	1.9	–2.1
cg01129246	4	GRXCR1	Body	4.9E-06	0.2495	2.6	–4.1
cg16895973	11	TOLLIP	Body	6.0E-06	0.2495	1.3	–2.6
cg09708616	6	–	–	6.3E-06	0.2495	1.4	–1.3
cg04530976	6	ZBTB9	Body	6.4E-06	0.2495	0.8	–0.9
cg27610561	20	SLC2A10	TSS1500	8.2E-06	0.2665	1.0	–1.6
cg05119686	10	–	–	8.7E-06	0.2665	1.5	–1.1
cg03761210	20	NECAB3;C20orf144	Body	8.7E-06	0.2665	0.3	–0.3
cg07292606	2	TEX261	Body	9.4E-06	0.2665	2.0	–0.9
cg27194173	10	ADK	Body	9.9E-06	0.2665	1.9	–0.9
cg06191076	1	DIRAS3	TSS200	1.3E-05	0.3192	2.7	–2.5
cg00066153	11	ACRV1	1stExon; 5'UTR	1.3E-05	0.3192	1.1	–0.9
cg20455570	7	OSBPL3	5'UTR	1.7E-05	0.3888	1.2	–1.0
cg24106824	17	MRPL12	3'UTR	2.0E-05	0.4105	1.1	–0.6

Mean changes in methylation are presented as beta-values ranging from 0–100 %. The adjusted *p* values for the comparisons between the two groups control the false discovery rate using the Benjamini-Hochberg procedure. Abbreviations: *Chr* chromosome, *TSS200* 200 base pairs around the transcription start site, *TSS1500* 1500 base pairs around the transcription start site, *3'UTR* 3' untranslated region, *5'UTR* 5' untranslated region

well as regions for which the change in DNA methylation differed between the participants receiving folic acid and vitamin B₁₂ versus placebo. Furthermore, the DNA methylation levels of several genomic loci were found to correlate to serum levels of either folate, vitamin B₁₂, or plasma homocysteine. Most prominent DNA methylation patterns associated with supplemental intake or status of

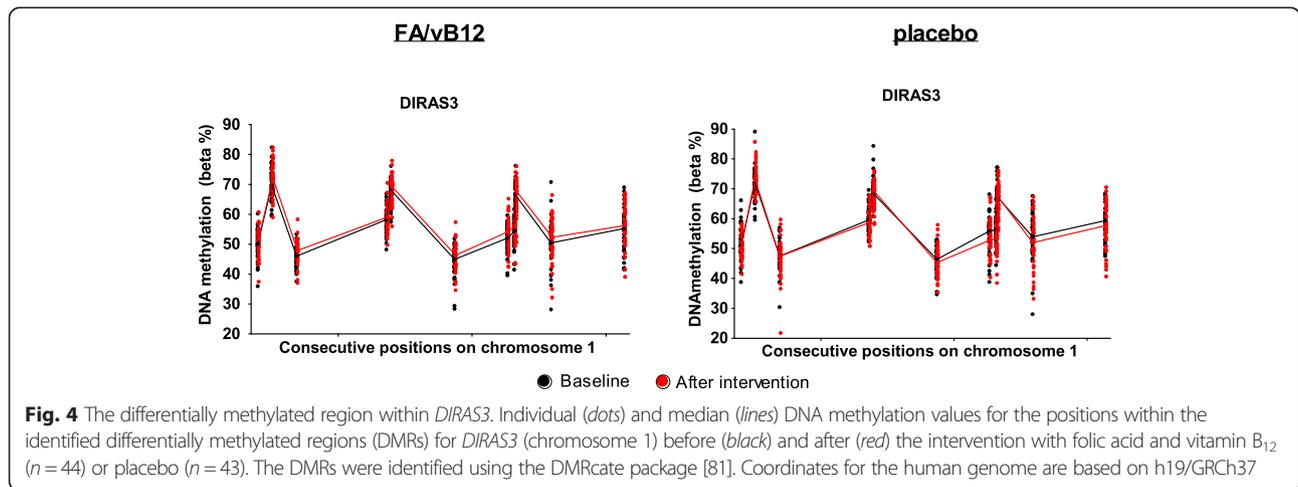
these B-vitamins seemed to be related to developmental processes as well as carcinogenesis.

Based on comparisons between the two groups (folic acid and vitamin B₁₂ versus placebo), *DIRAS3* was identified as a gene of interest with a DMR consisting of 11 consecutive positions. *DIRAS3*, also known as *ARHI*, is a maternally imprinted member of the ras superfamily

Table 3 Differentially methylated regions with DNA methylation changes that differ between participants receiving folic acid and vitamin B₁₂ or placebo

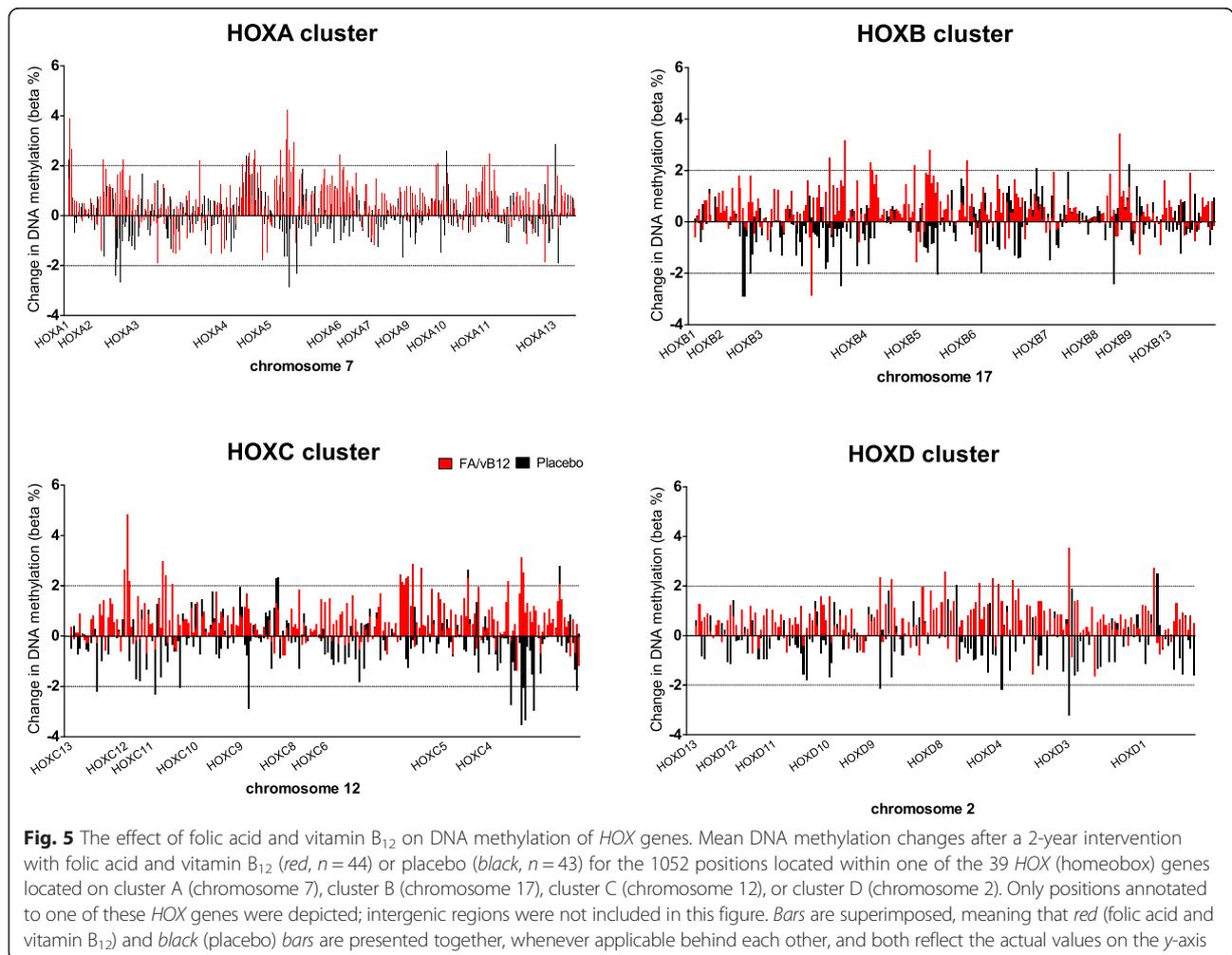
Gene	Gene feature	Chr	hg19 coordinates	Probes	Minimal <i>p</i> value	Mean <i>p</i> value	Maximal difference in methylation change (%) ^a
DIRAS3	5'UTR,1stExon,TSS200	1	68516080-68516627	11	0.0095	0.0175	5.2
–	–	6	85478576-85478807	2	0.0155	0.0210	3.6
NODAL	3'UTR	10	72191686-72191804	2	0.0198	0.0231	2.3
ARMC8	TSS1500,TSS200	3	137905836-137906122	7	0.0361	0.0400	1.3
PRDM16	Body	1	3130485-3130550	3	0.0361	0.0361	2.8
GNA12	Body	7	2801424-2801732	3	0.0387	0.0437	–2.4

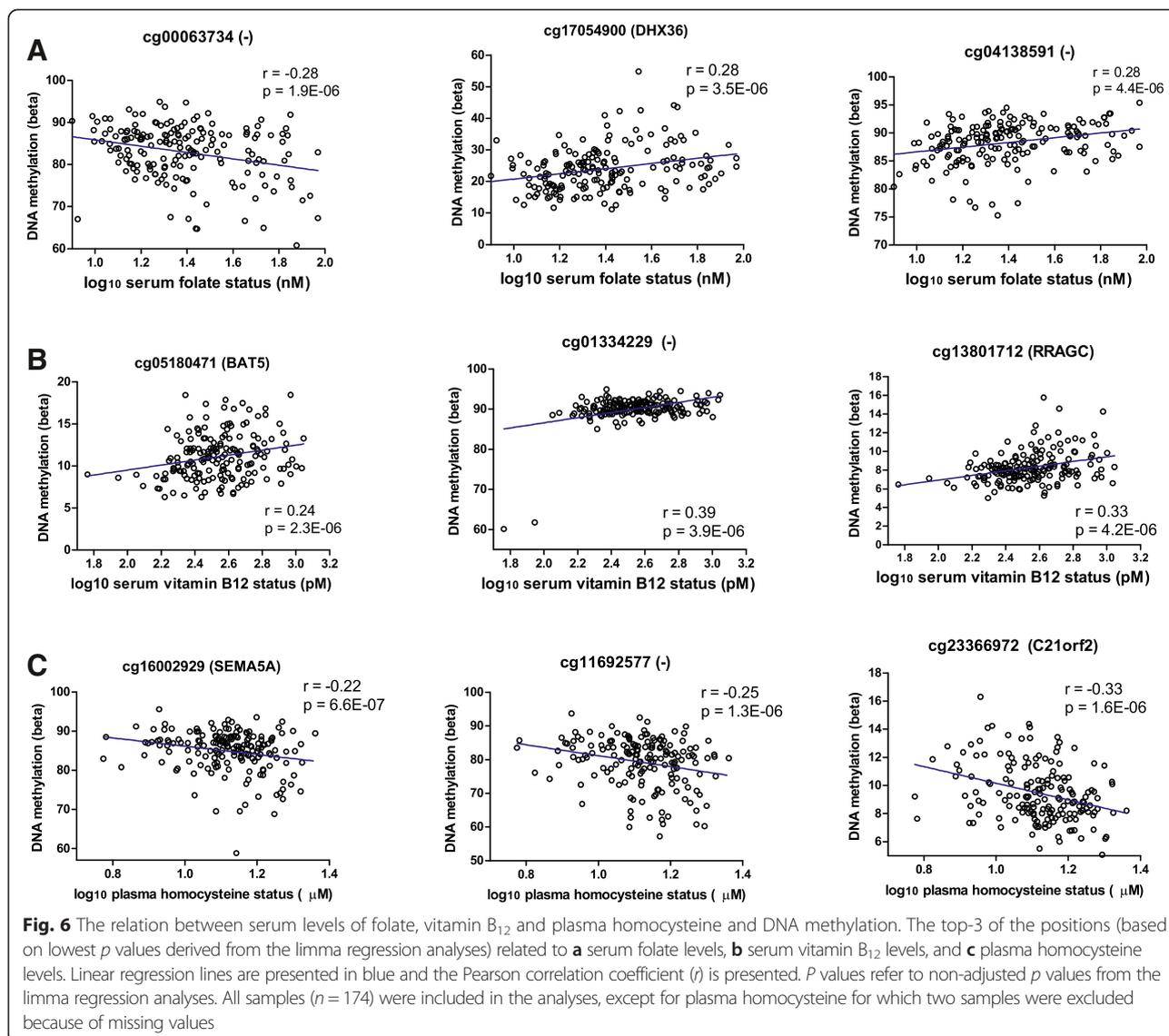
The DMRs were identified using the DMRcate package available through Bioconductor. The *p* values considered the false discovery rate and were adjusted using the Benjamini-Hochberg procedure. The minimal and mean *p* value as well as the maximal difference in DNA methylation change were calculated based on the indicated number of probes for each region. ^aThe maximal difference was calculated for the DNA methylation change after intervention with folic acid and vitamin B₁₂ versus placebo. Abbreviations: *Chr* chromosome, *DMR* differentially methylated region, *hg19* coordinates for the human genome based on the Genome Reference Consortium Human Build 37 (hg19/GRCh37), *TSS200* 200 base pairs around the transcription start site, *TSS1500* 1500 base pairs around the transcription start site, *3'UTR* 3' untranslated region, *5'UTR* 5' untranslated region



and is considered to be a tumor suppressor gene [32]. Downregulation of *DIRAS3* expression has been described for several forms of cancer and has been specifically associated with progression and invasive behavior of neoplastic cells [33–35]. To what extent transcriptional

downregulation of *DIRAS3* occurs via epigenetic mechanisms is not exactly known, although previous work indicated that regulation by miRNAs, chromatin remodeling as well as promoter hypermethylation may be responsible for this effect [35–37].





Another gene identified based on its DMR responsive to the intervention with folic acid and vitamin B₁₂ is the nodal growth differentiation factor (*NODAL*), a member of the transforming growth factor- β (*TGF-beta*) superfamily. Like *DIRAS3*, *NODAL* has been recognized for its role in cancer progression, with abundant expression of *NODAL* associated with cellular migration, invasion, and metastatic behavior [38–41].

Noteworthy, *NODAL* is not only known for its role in cancer progression as it has been originally identified as a morphogen and regulator of mesoderm formation and organization of axial structures during early-stage embryogenesis [42, 43]. Upregulation of *NODAL* expression as a consequence of tobacco or nicotine exposure in differentiating human embryonic stem cells (hESCs) suggests that the *NODAL* signaling routes are vulnerable to environmental exposures or stimuli during embryonic

development [44]. Interestingly, it has been recently demonstrated that expression of *NODAL* is regulated via an epigenetic regulatory element for which dynamic changes in DNA methylation were described throughout embryonic development [45]. Although this epigenetic regulatory element was found upstream of the *NODAL* gene, whereas our small DMR was located in the 3' UTR, these results support our findings that *NODAL* may be sensitive to endogenous or environmental exposures.

Besides *NODAL*, also positions or regions within other developmental genes have been identified as potentially responsive to the intervention with folic acid and vitamin B₁₂ or were related to serum folate levels in our study. The homeobox B7 (*HOXB7*) gene was identified for its relation with serum folate levels of the participants in the current study. *HOXB7* is a member of the homeobox family [46] and occurs in a cluster with other

homeobox B genes. For *HOXA4*, a DMR was related to serum folate levels. The highly conserved *HOX* genes are considered important transcriptional regulators during embryonic development, where they are mainly responsible for vertebral axial patterning [47, 48].

Epigenetic control of *HOX* genes in developmental processes related to health and disease has been described previously [47, 49]. Hypomethylation of *HOXB7* has been recently recognized as a risk factor for neural tube defects in a case-control study [31]. It is firmly established that periconceptual folic acid supplementation decreases the risk of neural tube defects in the offspring [50, 51]. To what extent *HOX* or other developmental genes are key targets for the prevention of neural tube defects through folic acid supplementation has not been described extensively so far [52]. The clinical relevance of our findings with respect to mechanisms underlying prevention of neural tube defects, however, is questionable since we found modest increases (~2–5 %) in DNA methylation of the *NODAL* or *HOX* genes after supplementation in our elderly population. Case-control studies have previously shown that DNA methylation of several *HOX* genes differed up to 29 % between children with myelomeningocele (a form of spina bifida) and healthy controls [31]. To what extent epigenetic control of developmental genes during fetal development [1] can be translated into an aging population, and vice versa, is not clear. Thus, our careful and explorative hypotheses about the effects of folic acid and vitamin B₁₂ on epigenetic processes in relation to programming of (early) developmental genes warrant further investigation and confirmation in future research with a specific focus on the suggested interaction between genetic, biological, and nutritional factors [53].

As stated before, aberrant DNA methylation or expression of several *HOX* genes, *NODAL* and *DIRAS3* have been implicated in the etiology of cancer [54, 55]. Members of the homeobox family as well as *NODAL* are specifically associated with cancer progression and metastatic behavior [56–59]. Reactivation of embryonic pathways in cancer cells may contribute to uncontrolled differentiation, proliferation, invasion, and metastatic behavior. To what extent the suggested increases in DNA methylation of tumor-related genes such as *DIRAS3*, *NODAL*, *HOXB7*, *HOXA4*, and other *HOX* genes may contribute to the pathogenesis of cancer cannot be concluded from the current study. Theoretically, our findings, however, support the hypothesis that folic acid and possibly other B-vitamins specifically increase cancer risk if preclinical neoplastic lesions already exist, pointing toward an effect on progression rather than development of cancer [27, 29]. Our findings may also explain the explorative results of the entire B-vitamins for the Prevention Of Osteoporotic Fractures (B-PROOF) study, where a slightly increased cancer risk was reported after

intervention with folic acid and vitamin B₁₂ as compared to the placebo group (hazard ratio 1.56; 95 % confidence interval 1.04–2.30) [60]. Taken altogether, our data suggest that supplementation with folic acid and vitamin B₁₂, and consequential changes in serum levels of the corresponding B-vitamins or plasma homocysteine, in older subjects is associated with changes in DNA methylation of several genes implicated in normal developmental processes, which may be reactivated or deregulated during carcinogenesis. It should be noted that the observed effects on DNA methylation were marginal and that biological and functional consequences with respect to gene expression cannot be validated with the current study. On the other hand, previous studies from our groups and others have already shown that nutritional or environmental exposures often result in small, but biologically meaningful, effects on DNA methylation in comparison to the tremendously deviating methylation patterns observed in complex diseases such as cancer [61–63].

Potential limitations of our study include the relatively heterogeneous study population with respect to characteristics that may have occurred or changed during the intervention period, such as changes in dietary habits, use of drugs, and presentation of diseases. It should be noted, however, that these characteristics represent and reflect the general elderly population, which is therefore an advantage with respect to the generalizability of our findings. For disease-related findings, it is questionable whether our results observed in leukocytes also reflect the situation in the tissue of interest, since DNA methylation patterns are highly tissue specific [64–66]. Finally, all participants in our study received 15 µg of vitamin D₃ per day in order to ensure a normal vitamin D status. Therefore, we cannot exclude the possibility that some DNA methylation changes may be predominantly attributable to vitamin D rather than folic acid and vitamin B₁₂ [67]. However, since all participants, both from the intervention group and the placebo group, received vitamin D in their study tablets (also containing the placebo or folic acid/vitamin B₁₂), this did not hinder our main analyses dedicated to comparisons between these two groups. Although, the results from our study were fairly consistent with other studies and current insights, strict replication of our findings was not feasible so far given the unique design of our long-term intervention study with folic acid as well as vitamin B₁₂ among elderly subjects.

Strengths of our study include the analyses of genome-wide DNA methylation both before and after long-term supplementation, which allow comparisons within individuals over time. Furthermore, our population consisted of older subjects with mildly elevated homocysteine levels. Elevated homocysteine levels, which are common among elderly subjects in The Netherlands [68],

may point toward inadequate levels or deficiencies of folate and vitamin B₁₂ [69]. Therefore, especially, this population may have benefited from the prolonged supplementation with these B-vitamins and thus enabled detection of changes in DNA methylation patterns.

Conclusions

Long-term supplementation with folic acid and vitamin B₁₂ in elderly subjects with mildly elevated homocysteine levels resulted in changes in DNA methylation of several genes implicated in normal developmental processes and carcinogenesis. These findings may provide unique leads for further research unraveling the mechanisms underlying the effects of B-vitamins on health and disease during the life cycle.

Methods

Design and recruitment

The current study was part of a double-blind, randomized, and placebo-controlled trial on the effects of supplemental intake of folic acid and vitamin B₁₂ on fracture incidence (B-PROOF) in The Netherlands [60, 70]. Design of the B-PROOF study and recruitment of the participants have been described in detail previously [70]. Briefly, men and women, aged 65 years and older, with an elevated homocysteine level (12–50 µmol/L) were eligible to participate in this study. Between September 2008 and March 2011, over 69,000 subjects were screened and 2919 participants were finally enrolled in the B-PROOF study (Fig. 1). All participants provided written informed consent and the study protocol was approved by the institutional Medical Ethics Committee. Participants were randomly assigned to take 400 µg folic acid and 500 µg vitamin B₁₂ (Orthica, Almere, The Netherlands) per day or a placebo (Orthica, Almere, The Netherlands) during an intervention period of 2 years. Moreover, all participants received a daily dose of 15 µg vitamin D₃, in the same tablet as the placebo or B-vitamins, to ensure a normal vitamin D status.

For the current study, a subgroup of participants was selected from the B-PROOF study cohort. From the 331 participants, who were among the first who completed the intervention period of 2 years, 92 participants were selected for DNA methylation analyses. In order to obtain a relatively homogenous study population, our predefined selection criteria included: age between 65 and 75 years and no reported changes in use of dietary supplements containing folic acid or vitamin B₁₂ during the study. Levels of the C-reactive protein (CRP) at baseline should be ≤10 mg/L, because elevated levels of CRP as a consequence of inflammation may indicate aberrant leukocyte counts. Furthermore, participants who were current smokers, or excessive alcohol users (at least once a week ≥6 glasses of alcohol or 5–7 days/week ≥4 glasses of alcohol) [71] at baseline were not selected for the

current study. All participants had elevated homocysteine levels, which is in line with inclusion criteria of the B-PROOF study. Given the essential role of MTHFR in folate-mediated one-carbon metabolism, the functional C677T single nucleotide polymorphism (SNP) of the *MTHFR* gene was considered as well for the selection procedure. Participants with the *MTHFR* (C677T) CC ($n = 46$) and TT ($n = 46$) genotypes were equally and exclusively represented in the current study. The *MTHFR* SNP was determined using the Illumina Omni-express array (Illumina Inc., San Diego, CA, USA).

Sample collection and biochemical analyses

At baseline and after 2 years of intervention, blood samples were collected from all 92 participants. Serum levels of folate and vitamin B₁₂ were determined using electrochemiluminescence immunoassays (Elecsys 2010, Roche, Almere, The Netherlands). Depending on the study center, plasma levels of homocysteine were measured using the Architect i2000 RS analyzer (VU University Medical Center, Amsterdam), HPLC (Wageningen University, Wageningen), or LC-MS/MS method (Erasmus MC, Rotterdam). According to a cross-calibration, outcomes of the three centers did not differ significantly [60].

Genome-wide DNA methylation analysis

DNA was isolated from buffy coats and 500 ng genomic DNA was bisulfite converted using the EZ DNA Methylation Kit according to the manufacturer instructions (Zymo Research, Freiburg, Germany). Bisulfite converted DNA was used for the genome-wide DNA methylation analyses. Genome-wide DNA methylation was determined in a total of 184 samples from 92 participants using the Infinium HumanMethylation450 BeadChip (Illumina). All samples were processed and analyzed at the Genetic laboratory of Internal Medicine of the Erasmus MC, Rotterdam, The Netherlands. Initial quality control of the samples revealed that one participant had to be excluded because bisulfite conversion for the baseline sample failed. Furthermore, two participants were excluded since their baseline samples showed (1) an average intensity signal in the methylated or unmethylated channel below our predefined threshold of 1966 and (2) a percentage of probes with a call rate <97.5 %. Clustering based on the 65 known SNP probes on the BeadChip as well as multidimensional scaling stratified for gender, revealed two potential sample mix-ups. The corresponding participants of these samples were excluded resulting in samples from 87 participants available for final analysis (Fig. 1).

Raw idat.files were subsequently used for analysis in the R statistical environment (R 3.1.2) through the minfi package (version 1.12.0) available from Bioconductor [72]. Preprocessing of the data included filtering of 65 control probes containing known SNPs and probes annotated to

the X and Y chromosome. Probes with a detection p value of >0.01 in at least one sample were filtered, as these probes did not surpass the background signal. Finally, probes containing an SNP (based on the dbSNP version 137) [73] at the CpG interrogation and/or the single nucleotide extension were excluded, resulting in 431,312 probes included in the analyses (Fig. 1).

Filtered data were normalized using the SWAN normalization procedure [74] available through the minfi package [72]. The datasets were annotated based on the ilmn12.hg19 annotation [75]. The M-values, which are the log₂ ratios of the methylated versus the unmethylated probe intensities, were used for statistical testing as recommended by Du et al. [76]. For visualization purposes, methylation status of the individual probes was expressed as a β -value. These β -values range from 0 or 0 % (fully unmethylated) to 1 or 100 % (fully methylated). Phenotypic data and idat-files from the genome-wide DNA methylation analysis are available through the NCBI's Gene Expression Omnibus (GEO) repository [77] with accession number GSE74548.

Differentially methylated positions

Initially, differentially methylated positions, representing changes in DNA methylation over time, were identified in both the placebo as well as the intervention group using a multi-level linear regression analysis within the limma package [78, 79]. For these analyses, subjects were treated as random effects using the “duplicateCorrelation” function in order to conduct the comparisons in a pairwise manner [78, 80].

Differences in changes in DNA methylation between the placebo and intervention group were identified using this multi-level linear regression analysis as well [78, 80]. The contrast ((Placebo_followup-Placebo_baseline)-(Intervention_followup-Intervention_baseline)) allowed the comparison between DNA methylation changes in the two groups.

Besides the comparisons between the two groups, differentially methylated positions were identified as a function of serum folate, vitamin B₁₂, or plasma homocysteine levels using the dmpFinder function for continuous phenotypes (available from the minfi package [72]). For these analyses, log₁₀-transformed serum levels of folate, vitamin B₁₂ levels, or plasma homocysteine at baseline and after intervention were considered as continuous variables in the linear regression analyses ($n = 174$).

Differentially methylated regions

Identification of differentially methylated regions (DMRs) was conducted using the DMRcate package available through Bioconductor [81]. DMRs were defined as regions with a maximal distance of 1000 nucleotides between

consecutive probes [81]. Identified DMRs consisting of a single probe were excluded for further analyses. Unless otherwise stated, we have taken into account the issue of multiple testing for all analyses by considering p values which are adjusted for the false discovery rate (FDR) by using the Benjamini-Hochberg (BH) procedure [82].

Statistical considerations

Samples with a heterogeneous cell population are a major issue for epigenomic analyses, since DNA methylation shows a pronounced cell-type specific pattern [65]. In order to determine whether the leukocyte fractions differed between the groups or baseline and follow-up samples, we predicted the leukocyte composition using the Houseman method [83, 84] implemented in minfi [72]. For a subgroup of participants ($n = 85$, follow-up samples only), automated cell counts (lymphocytes, monocytes and basophil, eosinophil and neutrophil granulocytes) were available. Pearson correlation coefficients were calculated for the predicted and measured counts of these specific leukocyte fractions. The statistical analyses were adjusted for the different cell fractions by inclusion of predicted percentages of CD4+ and CD8+ T-lymphocytes, B-lymphocytes, natural killer cells, monocytes, and granulocytes as covariates in the linear regression models. Also, the plates used for the bisulfite conversion (four in total) were included as covariates in the models.

Since numerous demographic and biochemical variables were not normally distributed, these data were summarized as median and interquartile ranges (IQR) or numbers and percentages. The Mann-Whitney U test was used to compare characteristics and changes in serum levels of folate, vitamin B₁₂, or plasma homocysteine between the intervention group and the placebo group. The Statistical package for Social Sciences (SPSS version 22) was, unless otherwise stated, used for all the statistical analyses regarding the descriptive and biochemical parameters.

Additional files

Additional file 1: Figure S1. The mean proportion of the leukocyte fractions in the buffy coat samples collected before and after intervention with folic acid and vitamin B12 ($n=44$) or placebo ($n=43$). Estimates of the leukocyte fractions were based on the Houseman method.

Additional file 2: Table S1. Top-100 positions that are differentially methylated after intervention with folic acid and vitamin B12. Overview of the top-100 positions that are differentially methylated (Benjamini-Hochberg adjusted p -value <0.05) after intervention with folic acid and vitamin B12 (FA/vB12). Changes for DNA methylation in the intervention group as well as the placebo group are presented as % beta-values and can range from 0-100 %.

Additional file 3: Table S2a and S2b. Differentially methylated positions and regions related to serum levels of folate, vitamin B12 of plasma homocysteine. Overview of the top-10 of a) differentially methylated positions as well as b) regions related to serum levels of folate, vitamin B12 of plasma homocysteine.

Abbreviations

3'UTR: 3' untranslated region; 5'UTR: 5' untranslated region; BH: Benjamini-Hochberg; B-PROOF: B-vitamins for the prevention of osteoporotic fractures; CpG: cytosine-phosphate-guanine; CRP: C-reactive protein; DMR: differentially methylated region; FA/vB12: folic acid and vitamin B₁₂; FDR: false discovery rate; hESC: human embryonic stem cell; HPLC: high-performance liquid chromatography; IQR: interquartile range; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MTHFR: methylenetetrahydrofolate reductase; SNP: single nucleotide polymorphism; SWAN: subset-quantile within array normalization; TSS: transcription start site.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DK performed the analyses and drafted the manuscript. RDR participated in the design and coordination of the study. CL coordinated the sample collection and processing. SH, JM, and GH contributed to the analysis and interpretation of the data and helped to draft the manuscript. RDR, AU, LG, NS, and NV conceived or coordinated the B-PROOF study and critically revised the manuscript. EK and WS initiated and coordinated the current project, revised the data analyses, and helped to draft the manuscript. All authors read and approved the final manuscript.

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Author details

¹Division of Human Nutrition, Wageningen University, PO Box 8129, 6700 EV Wageningen, The Netherlands. ²Department of Clinical Chemistry, Erasmus University Medical Center, Rotterdam, The Netherlands. ³Genetic Laboratory Internal Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands. ⁴Department of Epidemiology, Erasmus University Medical Center, Rotterdam, The Netherlands. ⁵Department of Internal Medicine, Section of Geriatrics, Academic Medical Center, Amsterdam, The Netherlands. ⁶Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VU University Medical Center, Amsterdam, The Netherlands.

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References

- Xu J, Sinclair KD. One-carbon metabolism and epigenetic regulation of embryo development. *Reproduction, Fertility and Development*. 2015. doi:10.1071/RD14377.
- Hanson MA, Gluckman PD. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiol Rev*. 2014;94(4):1027–76. doi:10.1152/physrev.00029.2013.
- Steegers-Theunissen RPM, Twigt J, Pestinger V, Sinclair KD. The periconceptual period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. *Hum Reprod Update*. 2013;19(6):640–55. doi:10.1093/humupd/dmt041.
- Jung A, Kampman E. Nutrition, epigenetics, and cancer: an epidemiological perspective. *Nutrition in Epigenetics*. Oxford, UK: Wiley-Blackwell; 2011. p. 329–43.
- Liu JJ, Ward RL. Folate and one-carbon metabolism and its impact on aberrant DNA methylation in cancer. *Adv Genet*. 2010;71:79–121. doi:10.1016/B978-0-12-380864-6.00004-3.
- Mandaviya PR, Stolk L, Heil SG. Homocysteine and DNA methylation: a review of animal and human literature. *Mol Genet Metab*. 2014;113(4):243–52. doi:10.1016/j.ymgme.2014.10.006.
- Amarasekera M, Martino D, Ashley S, Harb H, Kesper D, Strickland D et al. Genome-wide DNA methylation profiling identifies a folate-sensitive region of differential methylation upstream of ZFP57-imprinting regulator in humans. *FASEB journal:official publication of the Federation of American Societies for Experimental Biology*. 2014. doi:10.1096/fj.13-249029.
- Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, et al. Periconceptual maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. *PLoS One*. 2009;4(11):e7845. doi:10.1371/journal.pone.0007845.
- Hoyo C, Murtha AP, Schildkraut JM, Jirtle RL, Demark-Wahnefried W, Forman MR, et al. Methylation variation at IGF2 differentially methylated regions and maternal folic acid use before and during pregnancy. *Epigenetics*. 2011;6(7):928–36.
- Haggarty P, Hoad G, Campbell DM, Horgan GW, Piyathilake C, McNeill G. Folate in pregnancy and imprinted gene and repeat element methylation in the offspring. *Am J Clin Nutr*. 2013;97(1):94–9. doi:10.3945/ajcn.112.042572.
- McKay JA, Groom A, Potter C, Coneyworth LJ, Ford D, Mathers JC, et al. Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: role for folate gene variants and vitamin B12. *PLoS One*. 2012;7(3):e33290. doi:10.1371/journal.pone.0033290.
- McKay JA, Mathers JC. Diet induced epigenetic changes and their implications for health. *Acta Physiologica*. 2011;202(2):103–18. doi:10.1111/j.1748-1716.2011.02278.x.
- Vickers MH. Early life nutrition, epigenetics and programming of later life disease. *Nutrients*. 2014;6(6):2165–78. doi:10.3390/nu6062165.
- Barker DJP. Fetal and infant origins of adult disease. London: British Medical Journal; 1992.
- Barker DJP. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition*. 1997;13(9):807–13. doi:10.1016/S0899-9007(97)00193-7.
- Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr*. 1998;128(7):1204–12.
- Rampersaud GC, Kauwell GP, Hutson AD, Cerda JJ, Bailey LB. Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. *Am J Clin Nutr*. 2000;72(4):998–1003.
- Lucock M, Yates Z. Folic acid fortification: a double-edged sword. *Curr Opin Clin Nutr Metab Care*. 2009;12(6):555–64. doi:10.1097/MCO.0b013e32833192bc.
- van Wijngaarden JP, Doets EL, Szczecińska A, Souverein OW, Duffy ME, Dullemeijer C, et al. Vitamin B(12), folate, homocysteine, and bone health in adults and elderly people: a systematic review with meta-analyses. *J Nutri Metabol*. 2013;2013:486186. doi:10.1155/2013/486186.
- McLean RR, Hannan MT. B vitamins, homocysteine, and bone disease: epidemiology and pathophysiology. *Curr Osteoporos Rep*. 2007;5(3):112–9.
- Huo Y, Li J, Qin X, Huang Y, Wang X, Gottesman RF, et al. Efficacy of folic acid therapy in primary prevention of stroke among adults with hypertension in China: the CSPPT randomized clinical trial. *JAMA*. 2015;313(13):1325–35. doi:10.1001/jama.2015.2274.
- Wang X, Qin X, Demirtas H, Li J, Mao G, Huo Y, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *Lancet*. 2007;369(9576):1876–82. doi:10.1016/S0140-6736(07)60854-X.
- Durga J, van Boxtel MPJ, Schouten EG, Kok FJ, Jolles J, Katan MB, et al. Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial. *Lancet*. 2007;369(9557):208–16. doi:10.1016/S0140-6736(07)60109-3.
- Basset JK, Severi G, Hodge AM, Baglietto L, Hopper JL, English DR, et al. Dietary intake of B vitamins and methionine and colorectal cancer risk. *Nutr Cancer*. 2013;65(5):659–67. doi:10.1080/01635581.2013.789114.
- Kim D-H, Smith-Warner S, Spiegelman D, Yaun S-S, Colditz G, Freudenheim J, et al. Pooled analyses of 13 prospective cohort studies on folate intake and colon cancer. *Cancer Causes Control*. 2010;21(11):1919–30. doi:10.1007/s10552-010-9620-8.
- Gibson TM, Weinstein SJ, Pfeiffer RM, Hollenbeck AR, Subar AF, Schatzkin A, et al. Pre- and postfortification intake of folate and risk of colorectal cancer in a large prospective cohort study in the United States. *Am J Clin Nutr*. 2011;94(4):1053–62. doi:10.3945/ajcn.110.002659.

27. Ulrich CM, Potter JD. Folate supplementation: too much of a good thing? *Cancer Epidemiol Biomarkers Prev.* 2006;15(2):189–93. doi:10.1158/1055-9965.epi-06-0054.
28. Kim YI. Folic acid fortification and supplementation—good for some but not so good for others. *Nutr Rev.* 2007;65(11):504–11.
29. van den Donk M, Pellis L, Crott JW, van Engeland M, Friederich P, Nagengast FM, et al. Folic acid and vitamin B-12 supplementation does not favorably influence uracil incorporation and promoter methylation in rectal mucosa DNA of subjects with previous colorectal adenomas. *J Nutr.* 2007;137(9):2114–20.
30. Cole BF, Baron JA, Sandler RS, Haile RW, Ahnen DJ, Bresalier RS, et al. Folic acid for the prevention of colorectal adenomas: a randomized clinical trial. *JAMA.* 2007;297(21):2351–9. doi:10.1001/jama.297.21.2351.
31. Rochtus A, Izzl B, Vangeel E, Louwette S, Wittevrongel C, Lambrechts D et al. DNA methylation analysis of homeobox genes implicates HOXB7 hypomethylation as risk factor for neural tube defects. *Epigenetics : official journal of the DNA Methylation Society.* 2015;1–10. doi:10.1080/15592294.2014.998531.
32. Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, et al. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci.* 1999;96(1):214–9. doi:10.1073/pnas.96.1.214.
33. Wang L, Hoque A, Luo RZ, Yuan J, Lu Z, Nishimoto A, et al. Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer. *Clin Cancer Res.* 2003;9(10 Pt 1):3660–6.
34. Chen J, Shi S, Yang W, Chen C. Over-expression of ARHI decreases tumor growth, migration, and invasion in human glioma. 2014;31(3):1–10. doi:10.1007/s12032-014-0846-2.
35. Li Y, Liu M, Zhang Y, Han C, You J, Yang J et al. Effects of ARHI on breast cancer cell biological behavior regulated by microRNA-221. 2013;34(6):3545–54. doi:10.1007/s13277-013-0933-6.
36. Yuan J, Luo RZ, Fujii S, Wang L, Hu W, Andreoff M, et al. Aberrant methylation and silencing of ARHI, an imprinted tumor suppressor gene in which the function is lost in breast cancers. *Cancer Res.* 2003;63(14):4174–80.
37. Yu Y, Fujii S, Yuan J, Luo RZ, Wang L, Bao J, et al. Epigenetic regulation of ARHI in breast and ovarian cancer cells. *Ann N Y Acad Sci.* 2003;983(1):268–77. doi:10.1111/j.1749-6632.2003.tb05981.x.
38. Quail DF, Zhang G, Walsh LA, Siegers GM, Dieters-Castator DZ, Findlay SD, et al. Embryonic morphogen nodal promotes breast cancer growth and progression. *PLoS One.* 2012;7(11):e48237. doi:10.1371/journal.pone.0048237.
39. Strizzi L, Hardy KM, Margaryan NV, Hillman DW, Sefter EA, Chen B, et al. Potential for the embryonic morphogen nodal as a prognostic and predictive biomarker in breast cancer. *Breast Cancer Res.* 2012;14(3):R75. doi:10.1186/bcr3185.
40. Chen J, Liu WB, Jia WD, Xu GL, Ma JL, Ren Y, et al. Embryonic morphogen nodal is associated with progression and poor prognosis of hepatocellular carcinoma. *PLoS One.* 2014;9(1):e85840. doi:10.1371/journal.pone.0085840.
41. Lee CC, Jan HJ, Lai JH, Ma HI, Hueng DY, Lee YC, et al. Nodal promotes growth and invasion in human gliomas. *Oncogene.* 2010;29(21):3110–23. doi:10.1038/ncr.2010.55.
42. Schier AF, Shen MM. Nodal signalling in vertebrate development. *Nature.* 2000;403(6768):385–9.
43. Schier AF. Nodal morphogens. *Cold Spring Harb Perspect Biol.* 2009;1(5):a003459. doi:10.1101/cshperspect.a003459.
44. Liszewski W, Ritner C, Aurigui J, Wong SSY, Hussain N, Krueger W, et al. Developmental effects of tobacco smoke exposure during human embryonic stem cell differentiation are mediated through the transforming growth factor- β superfamily member, nodal. *Differentiation.* 2012;83(4):169–78. doi:10.1016/j.diff.2011.12.005.
45. Arai D, Hayakawa K, Ohgane J, Hirokawa M, Nakao Y, Tanaka S, et al. An epigenetic regulatory element of the nodal gene in the mouse and human genomes. *Mech Dev.* 2015;136(0):143–54. doi:10.1016/j.mod.2014.12.003.
46. Holland PWH, Booth HAF, Bruford EA. Classification and nomenclature of all human homeobox genes. *BMC Biol.* 2007;5:47. doi:10.1186/1741-7007-5-47.
47. Barber BA, Rastegar M. Epigenetic control of Hox genes during neurogenesis, development, and disease. *Ann Anat.* 2010;192(5):261–74. doi:10.1016/j.aanat.2010.07.009.
48. Wellik DM. Hox genes and vertebrate axial pattern. *Curr Top Dev Biol.* 2009;88:257–78. doi: 10.1016/S0070-2153(09)88009-5.
49. Soshnikova N, Duboule D. Epigenetic temporal control of mouse Hox genes in vivo. *Science.* 2009;324(5932):1320–3. doi:10.1126/science.1171468.
50. Czeizel AE, Dudas I, Metneki J. Pregnancy outcomes in a randomised controlled trial of periconceptional multivitamin supplementation. *Final report. Arch Gynecol Obstet.* 1994;255(3):131–9.
51. Wolff T, Witkop CT, Miller T, Syed SB. Folic acid supplementation for the prevention of neural tube defects: an update of the evidence for the U.S. Preventive Services Task Force. *Ann Intern Med.* 2009;150(9):632–9.
52. Kappen C, Mello MA, Finnell RH, Salbaum JM. Folate modulates Hox gene controlled skeletal phenotypes. *Genesis.* 2004;39(3):155–66. doi:10.1002/gene.20036.
53. Imbard A, Benoit J-F, Blom HJ. Neural tube defects, folic acid and methylation. *Int J Environ Res Public Health.* 2013;10(9):4352–89. doi:10.3390/ijerph10094352.
54. Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. *Nat Rev Cancer.* 2010;10(5):361–71. doi:10.1038/nrc2826.
55. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence?. *Nat Rev Cancer.* 2002;2(10):777–85.
56. Liu S, Jin K, Hui Y, Fu J, Jie C, Feng S et al. HOXB7 Promotes malignant progression by activating the TGF β signaling pathway. *Cancer research.* 2014. doi:10.1158/0008-5472.can-14-3100.
57. Wu X, Chen H, Parker B, Rubin E, Zhu T, Lee JS, et al. HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition. *Cancer Res.* 2006;66(19):9527–34. doi:10.1158/0008-5472.can-05-4470.
58. Kovochich AN, Arensman M, Lay AR, Rao NP, Donahue T, Li X et al. HOXB7 promotes invasion and predicts survival in pancreatic adenocarcinoma. *Cancer.* 2013;119(3):10.1002/cncr.27725. doi:10.1002/cncr.27725.
59. Yuan H, Kajiyama H, Ito S, Yoshikawa N, Hyodo T, Asano E, et al. ALX1 induces snail expression to promote epithelial-to-mesenchymal transition and invasion of ovarian cancer cells. *Cancer Res.* 2013;73(5):1581–90. doi:10.1158/0008-5472.can-12-2377.
60. van Wijngaarden JP, Swart KM, Enneman AW, Dhonukshe-Rutten RA, van Dijk SC, Ham AC, et al. Effect of daily vitamin B-12 and folic acid supplementation on fracture incidence in elderly individuals with an elevated plasma homocysteine concentration: B-PROOF, a randomized controlled trial. *Am J Clin Nutr.* 2014;100(6):1578–86. doi:10.3945/ajcn.114.090043.
61. Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, et al. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. *PLoS Genet.* 2013;9(6):e1003572. doi:10.1371/journal.pgen.1003572.
62. Jacobsen SC, Brøns C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. *Diabetologia.* 2012;55(12):3341–9. doi:10.1007/s00125-012-2717-8.
63. Kupers LK, Xu X, Jankipersadsing SA, Vaez A, la Bastide-van Gemert S, Scholtens S et al. DNA methylation mediates the effect of maternal smoking during pregnancy on birthweight of the offspring. *International journal of epidemiology.* 2015. doi:10.1093/ije/dyv048.
64. Slieker RC, Bos SD, Goeman JJ, Bovée JWMG, Talens RP, van der Breggen R, et al. Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array. *Epigenetics Chromatin.* 2013;6:26. doi:10.1186/1756-8935-6-26.
65. Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol.* 2014;15(2):R31-R. doi:10.1186/gb-2014-15-2-r31.
66. Montano C, Irizarry R, Kaufmann W, Talbot K, Gur R, Feinberg A, et al. Measuring cell-type specific differential methylation in human brain tissue. *Genome Biol.* 2013;14(8):R94.
67. Fetahu IS, Höbaus J, Kállay E. Vitamin D and the epigenome. *Front Physiol.* 2014;5:164. doi:10.3389/fphys.2014.00164.
68. de Bree A, van der Put NMJ, Mennen LI, Verschuren WMM, Blom HJ, Galan P et al. Prevalences of hyperhomocysteinemia, unfavorable cholesterol profile and hypertension in European populations. *Eur J Clin Nutr.* 2005;59(4):480–8.
69. Refsum H, Smith AD, Ueland PM, Nexø E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. *Clin Chem.* 2004;50(1):3–32. doi:10.1373/clinchem.2003.021634.
70. van Wijngaarden JP, Dhonukshe-Rutten RA, van Schoor NM, van der Velde N, Swart KM, Enneman AW, et al. Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12 and folic acid on fracture incidence. *BMC Geriatr.* 2011;11:80. doi:10.1186/1471-2318-11-80.
71. Garretsen HFL. Probleemdrinken: Prevalentiebeëpalning, beïnvloedende factoren en preventiemogelijkheden: Theoretische overwegingen en onderzoek in Rotterdam (thesis in Dutch, with summary in English). 1983.

72. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, et al. Minfi: a flexible and comprehensive bioconductor package for the analysis of infinium DNA methylation microarrays. *Bioinformatics*. 2014;30(10):1363–9. doi:10.1093/bioinformatics/btu049.
73. Sherry ST, Ward M-H, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res*. 2001;29(1):308–11. doi:10.1093/nar/29.1.308.
74. Maksimovic J, Gordon L, Oshlack A. SWAN: subset-quantile within array normalization for illumina infinium HumanMethylation450 BeadChips. *Genome Biol*. 2012;13(6):R44. doi:10.1186/gb-2012-13-6-r44.
75. Hansen KD. IlluminaHumanMethylation450kanno.ilmn12.hg19: annotation for illumina's 450k methylation arrays. R package version 0.2.1.
76. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, et al. Comparison of beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinform*. 2010;11:587. doi:10.1186/1471-2105-11-587.
77. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207–10. doi:10.1093/nar/30.1.207.
78. Smyth GK. Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. *Bioinformatics and computational biology solutions using R and bioconductor*. New York: Springer; 2005. p. 397–420.
79. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. 2004. *Statistical Applications in Genetics and Molecular Biology*, Vol. 3, No. 1, Article 3.
80. Smyth GK, Michaud J, Scott HS. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics*. 2005;21(9):2067–75. doi:10.1093/bioinformatics/bti270.
81. Peters T, Buckley M, Statham A, Pidsley R, Samaras K, Lord R, et al. De novo identification of differentially methylated regions in the human genome. *Epigenetics Chromatin*. 2015;8(1):6.
82. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc Series B*. 1995;57(1):289–300. doi:10.2307/2346101.
83. Houseman E, Accomando W, Koestler D, Christensen B, Marsit C, Nelson H, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinform*. 2012;13(1):86.
84. Koestler DC, Christensen BC, Karagas MR, Marsit CJ, Langevin SM, Kelsey KT, et al. Blood-based profiles of DNA methylation predict the underlying distribution of cell types. *Epigenetics*. 2013;8(8):816–26. doi:10.4161/epi.25430.

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