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B-vitamins intake, DNA-methylation of One Carbon Metabolism and homocysteine pathway genes and myocardial infarction risk: The EPICOR study

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Abstract

Background and aims

Several epidemiological studies highlighted the association between folate and B-vitamins low intake and cardiovascular diseases (CVD) risk. Contrasting results were reported on the relationship between folate intake and DNA-methylation. Folate and B-vitamins may modulate DNA-methylation of specific enzymes which are included in the One-Carbon Metabolism (OCM) and in the homocysteine (Hcy) pathways. The aim of the study was to evaluate whether DNA-methylation profiles of OCM and Hcy genes could modulate the myocardial infarction (MI) risk conferred by a low B-vitamins intake.

Methods and results

Study sample (206 MI cases and 206 matched controls) is a case-control study nested in the prospective EPIC cohort. Methylation levels of 33 candidate genes where extracted by the whole epigenome analysis (Illumina-HumanMethylation450K-BeadChip). We identified three differentially methylated regions in males (TCN2 promoter, CBS 5′ UTR, AMT gene-body) and two in females (PON1 gene-body, CBS 5′ UTR), each of them characterized by an increased methylation in cases. Functional in silico analysis suggested a decreased expression in cases. A Recursively Partitioned Mixture Model cluster algorithm identified distinct methylation profiles associated to different MI risk: high-risk vs. low-risk methylation profile groups, OR = 3.49, p = 1.87 × 10−4 and OR = 3.94, p = 0.0317 in males and females respectively (multivariate

logistic regression adjusted for classical CVD risk factors). Moreover, a general inverse relationship between B-vitamins intake and DNA-methylation of the candidate genes was observed.

Conclusions

Our findings support the hypothesis that DNA-methylation patterns in specific regions of OCM and Hcy pathways genes may modulate the CVD risk conferred by folate and B-vitamins low intake.

Keywords

DNA-methylation;

B-vitamins;

Myocardial infarction;

One Carbon Metabolism;

Homocysteine

Abbreviations

CVD, cardiovascular disease;

Hcy, homocysteine;

OCM, One Carbon Metabolism;

DMR, differentially methylated region;

RPMM, recursively partitioned mixture model;

BMI, body mass index (BMI);

WHR, waist-hip ratio;

CHD, coronary heart disease

Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality, morbidity and hospitalization in both genders in Europe and North America [1]. Diabetes mellitus, hypercholesterolemia, smoking, hypertension, obesity and physical inactivity are the primary risk factors for these diseases [2] and [3]. Other risk factors concur to the etiology such as low socioeconomic status, unhealthy dietary habits, alcohol or drugs abuse, lipoproteins, left ventricular hypertrophy. Non-modifiable risk factors include age, male gender, ethnicity and family history [4].

Several studies focused on the inverse relationship between B-vitamins intake and CVD risk [5]. As an example, it is well established that folates and some B-vitamins (B2, B6 and B12, folic acid) introduced with diet can reduce serum homocysteine (Hcy) levels promoting its re-methylation to methionine [6], and that an elevated plasma Hcy level is an independent risk factor for CVD [7].

In humans and in animal models, global decreased DNA-methylation was observed in atherosclerotic lesions, a condition linked to low intake of folates, methionine-rich diet, and elevated plasma Hcy levels [8], although human supplementation studies reported contrasting results [9] and [10]. DNA-methylation of CpG sites in the gene promoter region is an important determinant of gene expression, having an inverse relationship [11]. The mechanism by which folate and B-vitamins intake may modulate DNA-methylation depends on the activity of specific enzymes, many of which are included in the One Carbon Metabolism (OCM) [12]. The OCM is a complex network of biochemical reactions, involving the transfer of one-carbon groups needed for DNA-methylation and nucleotide synthesis [13], with the production of several metabolic intermediates in the Hcy and folate metabolic pathways. Specifically, in the Hcy pathway the de-methylation of the methionine-derived s-adenosyl methionine provides methyl groups for DNA-methylation, and generates S-adenosyl-homocysteine and ultimately Hcy. Folates and B-vitamins play a pivotal role as enzymatic co-factors.

The aim of this study was to investigate the possible role of DNA-methylation of genes in folate-dependent-OCM and Hcy pathways as a mediator of the CVD risk conferred by a low intake of folates and B-vitamins.

Methods

Study sample

The study sample includes 206 myocardial infarction (MI) cases and 206 matched controls from prospective case-control study nested in the EPIC cohort [14]. Details on matching parameters, outcome definition, laboratory analysis, methylation measurements, dietary and lifestyle information, and candidate genes selection are provided in Supplementary Methods (Text S1).

Study design

We first investigated the relationship between candidate genes DNA-methylation levels and folic acid (B9-vitamin), riboflavin (B2-vitamin), niacin (B3-vitamin), and pyridoxine (B6-vitamin) intake. Then, the association between DNA-methylation levels and MI risk was evaluated. Methylation values of CpG located in genes resulted significant from previous analyses were used in a cluster algorithm to group subjects with similar methylation profiles. Finally, we evaluated the association between the different clusters and MI risk using multivariate logistic regression analysis, adjusted for the classical CVD factors. The correlation between candidate genes DNA-methylation and gene-expression levels in several tissues was also investigated through data mining in publicly available databases. Details on statistical methods are provided in Supplementary Methods.

This study complies with the Declaration of Helsinki principles, and conforms to ethical requirements. All volunteers signed an informed consent form at enrollment. The EPIC study protocol was approved by Ethics Committees at the International Agency for Research on Cancer (Lyon, France) and at the Human Genetics Foundation (Turin, Italy).

Results

In the broader context of a genome-wide DNA-methylation analysis performed on 206 MI cases and 206 matched controls, we selected 33 genes involved in Hcy metabolism and OCM pathways (Table 1), for a total of 575 CpG sites. Sample characteristics are reported, according to stratification by gender (Table 2). The average follow-up was 12.80 (±2.15) years and 12.23 (±2.53) years for cases and controls respectively. The average time between recruitment and MI for cases was 5.98 (±3.58) years.

Table 1.

List of candidate genes.

Gene ID	Gene name	Gene selection criteria
AHCY	adenosylhomocysteinase	ahsa00270:Cysteine and methionine metabolism
ALDH1L1	aldehyde dehydrogenase 1 family, member L1	ahsa00670:One carbon pool by folate
AMT	aminomethyltransferase	ahsa00670:One carbon pool by folate
APOE	apolipoliterature and/or gene function/interaction E	gene function and/or protein‑protein interaction
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	ahsa00670:One carbon pool by folate
BHMT	betaine-homocysteine methyltransferase	ahsa00270:Cysteine and methionine metabolism
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	gene function and/or protein‑protein interaction
CBS	cystathionine-beta-synthase	ahsa00270:Cysteine and methionine metabolism
CTH	cystathionase (cystathionine gamma-lyase)	ahsa00270:Cysteine and methionine metabolism
DHFR	dihydrofolate reductase	ahsa00670:One carbon pool by folate
DNMT1	DNA (cytosine-5-)-methyltransferase 1	ahsa00270:Cysteine and methionine metabolism
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	ahsa00670:One carbon pool by folate
FOLR1	folate receptor 1	gene function and/or protein‑protein interaction
FOLR2	folate receptor 2	gene function and/or protein‑protein interaction
FTCD	formiminotransferase cyclodeaminase	gene function and/or protein‑protein interaction
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase,	ahsa00670:One carbon pool by folate

Gene ID	Gene name	Gene selection criteria
	phosphoribosylaminoimidazole synthetase	
MAT1A	methionine adenosyltransferase I, alpha	ahsa00270:Cysteine and methionine metabolism
MAT2B	methionine adenosyltransferase II, beta	ahsa00270:Cysteine and methionine metabolism
MTHFD1	methylenetetrahydrofolate dehydrogenase (NADP + dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	ahsa00670:One carbon pool by folate
MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP + dependent) 1-like	ahsa00670:One carbon pool by folate
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase	ahsa00670:One carbon pool by folate
MTHFD2L	methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2-like	gene function and/or protein‐protein interaction
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)	ahsa00670:One carbon pool by folate
MTHFS	5,10-methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)	ahsa00670:One carbon pool by folate
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase	ahsa00270:Cysteine and methionine metabolism,ahsa00670:One carbon pool by folate
MTRR	5-methyltetrahydrofolate-homocysteine methyltransferase reductase	gene function and/or protein‐protein interaction
NNMT	nicotinamide N-methyltransferase	gene function and/or protein‐protein interaction
PON1	paraoxonase 1	gene function and/or protein‐protein interaction
RFC1	replication factor C (activator 1) 1, 145 kDa	gene function and/or protein‐protein interaction
SHMT1	serine hydroxymethyltransferase 1 (soluble)	ahsa00670:One carbon pool by folate
SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)	ahsa00670:One carbon pool by folate

Gene ID	Gene name	Gene selection criteria
TCN2	transcobalamin II; macrocytic anemia	gene function and/or protein‐protein interaction
TYMS	thymidylate synthetase	ahsa00670:One carbon pool by folate

a

KEGG, Kyoto Encyclopedia of Genes and Genomes, Nucleic Acids Res. 2000; 28:27‐30.

Table 2.

Sample characteristics.

	Controls		Cases			Controls		Cases	
Males	N = 139		N = 139		Females	N = 67		N = 67	
Centre	N		N		Centre	N		N	
Turin	87		87		Turin	11		11	
Varese	38		38		Varese	42		42	
Ragusa	14		14		Ragusa	3		3	
					Naples	11		11	
Lifestyle variables	N	%	N	%	Lifestyle variables	N	%	N	%
Smokeb					Smokeb				
Never	36	61%	23	39%	Never	49	61%	31	39%
Former	62	56%	48	44%	Former	7	54%	6	46%
Current	41	38%	68	62%	Current	11	27%	30	73%
Coffee (cup/day)					Coffee (cup/day)b				
Non drinkers	4	67%	2	33%	Non drinkers	4	57%	3	43%
(0‐2)	33	57%	25	43%	(0‐2)	21	72%	8	28%
(2‐4)	65	55%	54	45%	(2‐4)	32	54%	27	46%

Males	Controls		Cases		Females	Controls		Cases	
	N = 139		N = 139			N = 67		N = 67	
(4-5)	15	43%	20	57%	(4-5)	8	42%	11	58%
More than 5	22	37%	38	63%	More than 5	2	10%	18	90%
Alcohol (gr./day)b					Alcohol (gr./day)				
(0-12)	50	45%	61	55%	(0-6)	44	48%	48	52%
(12-24)	39	67%	19	33%	(6-12)	5	45%	6	55%
More than 24	50	46%	59	54%	More than 12	18	58%	13	42%
Anthropometrics	Mean	±SD	Mean	±SD	Anthropometrics	Mean	±SD	Mean	±SD
Age (years)	49.88	7.10	49.84	7.17	Age (years)	53.75	8.06	54.06	7.89
BMI (kg/m2)a	26.36	3.13	27.26	2.95	BMI (kg/m2)	26.51	5.32	27.25	5.09
WHR (waist-hip ratio)	0.93	0.06	0.94	0.06	WHR (waist-hip ratio)a	0.80	0.06	0.83	0.06
Blood Pressure (mm Hg)					Blood Pressure (mm Hg)				
Diastolic	84.96	10.28	85.03	9.05	Diastolic	85.30	8.37	86.75	10.14
Systolic	134.96	18.77	135.66	15.23	Systolica	135.90	17.30	145.55	23.50
Lipids (mg/dL)					Lipids (mg/dL)				
LDL Cholesterola	137.69	39.15	151.40	39.04	LDL Cholesterol	150.37	36.54	160.20	47.68
HDL Cholesterola	56.55	13.52	50.54	11.78	HDL Cholesterol	65.73	16.01	61.32	15.53
Total Cholesterola	224.23	47.18	235.52	43.50	Total Cholesterol	240.82	41.47	249.72	51.27
Triglycerides	149.91	93.21	167.93	90.88	Triglycerides	123.61	51.66	141.01	107.44
B vitamins intake									
Folic Acid (μ g/die)a	317.11	96.10	288.01	102.10	Folic Acid (μ g/die)	284.09	103.82	253.35	94.24
Pyridoxine (mg/die)a	2.31	0.64	2.10	0.69	Pyridoxine (mg/die)	1.83	0.63	1.72	0.60

	Controls		Cases			Controls		Cases	
Males	N = 139		N = 139		Females	N = 67		N = 67	
Riboflavin (mg/die)	1.65	0.47	1.61	0.51	Riboflavin (mg/die)	1.60	0.64	1.50	0.54
Niacin (mg/die)	22.62	6.3	21.29	7.15	Niacin (mg/die)	17.25	5.77	17.00	4.87

a

T test p-value < 0.05.

b

Chi-squared test p-value < 0.05.

Significant differences between cases and controls were observed for smoking status, alcohol consumption, BMI, LDL cholesterol, HDL cholesterol, total cholesterol, folic acid and pyridoxine intake in males; smoking status, WHR, systolic pressure and coffee consumption in females. We also ascertained a lower B-vitamins intake in cases, both in males and in females (Table 2).

Interestingly, the methylation status of more than 97% of the examined probes negatively correlated with pyridoxine, riboflavin, niacin and folic acid intake both in males and females (data not shown), although some correlations were not statistically significant.

In males, six DMRs were identified in the training set, three of which (TCN2 gene promoter, CBS gene 5′ UTR, AMT gene body; Table 3) were confirmed in the test set (see Supplementary Methods for analysis details). In females, four DMRs were identified in the training set, two of which were confirmed in the test set (PON1 gene body/1st exon, CBS gene 5′ UTR; Table 3). For all these chromosomal regions a negative correlation between CpG methylation and B-vitamins intake was observed (Table S1), as well as a higher DNA-methylation in cases vs. controls (Table S2).

Table 3.

Differentially methylated regions in males and females respectively (training-set). Regions which were confirmed in the-test set are in bold.

CHR	BP STARTa	BP ENDa	SIZE	n Probes	Gene	RIb	SEb	RSb
Males								
20	32891026	32891428	403	10	AHCY	0.49	0.09	5.47
1	70876492	70877088	597	11	CTH	0.27	0.06	4.36

CHR	BP STARTa	BP ENDa	SIZE	n Probes	Gene	RIb	SEb	RSb
3	49459855	49460177	323	8	AMT	0.32	0.10	3.31
21	44480624	44480711	88	3	CBS	0.14	0.05	3.13
22	31002892	31003283	392	8	TCN2	0.18	0.06	2.92
21	44494906	44495288	383	5	CBS	0.30	0.10	2.88
6	151346268	151346409	142	3	MTHFD1L	0.37	0.16	2.37
Females								
5	78407418	78408347	930	11	BHMT	0.60	0.07	8.17
7	94953653	94954202	550	9	PON1	0.54	0.08	6.48
3	49459855	49460177	323	8	AMT	0.46	0.12	3.99
21	44494997	44495288	292	4	CBS	0.22	0.11	2.00

a

NCBI 37/hg19.

b

RI = regulation index of the region indicate the percentage of regulated loci; SE = standard error of estimated RI; RS = regulation score defined as RI/SE.

The correlation between gene-expression levels and DNA-methylation profiles of CpGs within the DMRs was examined by mining the MENT database (see methods): despite only two of the 30 identified CpGs (Table S2) were found in the database, it should be noted that the methylation levels of all the CpGs within each of the four identified DMR positively correlate (as tested by pairwise comparisons), and it might be expected they likely have the same relationship with gene expression levels. Cg20191453 (AMT gene body) showed a strong negative correlation with gene-expression in brain, colon, ovary, and rectum (Table S3); cg07404485 (PON1 gene body) showed a significant inverse relationship with gene-expression in ovary only, while no significant result was found for other tissues, although the same trend was clearly observed in colon, prostate, and rectum.

Lastly, the RPMM method identified four clusters in males and five in females based on the DNA-methylation profiles of the DMRs confirmed in the test set. The estimation of the risk conferred by the different methylation profiles was comparable at the three levels of adjustment (without adjustment, adjusting for matching variables, adjusting for matching variables and cardiovascular risk factors; Table 4). Specifically, focusing on the last model, the OR was 3.49 (95% CI 1.81‑6.73; p = 1.87 × 10−4) when comparing the two extreme groups in males (Table 4), and OR = 3.94 (95% CI 1.13‑13.75; p = 0.0317) when comparing the two extreme groups in females (Table 4).

Table 4.

Multivariate logistic regression analysis in males and females taking the CLUSTER 1 with lower MI risk as reference: clusters were identified through RPMM method.

Males	Or (95% CI)a	p	Or (95% CI)b	p	Or (95% CI)c	p
CLUSTER 2	1.16 (0.54-2.49)	0.7101	1.17 (0.54-2.54)	0.6934	1.26 (0.53-2.99)	0.6005
CLUSTER 3	2.29 (1.10-4.75)	0.0266	2.36 (1.12-4.96)	0.0233	2.85 (1.25-6.52)	0.0130
CLUSTER 4	2.90 (1.60-5.26)	4.49 × 10−4	2.97 (1.63-5.42)	3.85 × 10−04	3.49 (1.81-6.73)	1.87 × 10−04

Females	OR (95% CI)a	p	OR (95% CI)b	p	OR (95% CI)c	P
CLUSTER 2	1.75 (0.68-4.49)	0.2455	1.75 (0.68-4.54)	0.2488	1.85 (0.58-5.90)	0.3001
CLUSTER 3	1.86 (0.54-6.37)	0.3250	1.98 (0.55-7.16)	0.2952	3.00 (0.81-11.05)	0.0995
CLUSTER 4	2.48 (0.84-7.30)	0.1003	2.53 (0.85-7.52)	0.0944	3.04 (0.69-13.41)	0.1427
CLUSTER 5	4.18 (1.45-12.02)	0.0080	4.49 (1.52-13.28)	0.0067	3.94 (1.13-13.75)	0.0317

a

Without adjustment.

b

Adjusted for matching variables.

c

Adjusted for matching variables and CVD risk factors.

Discussion

In this study, we examined the association between DNA-methylation and B-vitamins intake in relation to MI risk, focusing on the risk-effect of DNA-methylation profiles of 33 genes involved in Hcy and OCM pathways.

We ascertained a lower intake of folic acid, pyridoxine, riboflavin and niacin both in male and female MI cases, and a general inverse relationship between B-vitamins intake and DNA-methylation of genes in OCM and Hcy pathways, in agreement with previous studies [10]. The analyses of anthropometric and lifestyle data showed statistically significant differences between cases and controls for known CVD risk factors, such as smoking status, alcohol and coffee consumption, obesity indices, blood pressure and lipids levels.

We identified five significant DMRs (cases vs. controls), always with higher DNA-methylation in cases: TCN2 and AMT methylation profiles were significant in males only, PON1 in females only, while, interestingly enough, CBS gene was found differentially methylated both in males and females.

A significant inverse correlation between DNA-methylation of the identified DMRs and gene-expression levels was assessed in several tissues through data mining. A similar scenario can be speculated for blood-derived DNA, suggesting a putative decrease of gene expression in MI cases, that who consistently display a higher DNA-methylation. The gene-functions also support the hypothesis of a CVD risk conferred by a lower gene expression in cases compared to controls.

CBS activity in the trans-sulfuration pathway is known to mediate the balance between the hyperomocysteinemia-driven vascular damage and the production of the beneficial hydrogen sulphide (H2S), which proved effective in free radical scavenging, inflammation suppression and endothelial protection [15]. The minor allele of a polymorphism within the promoter of CBS gene was recently associated with promoter hypo-methylation and a marked increase in CBS transcription, putatively conferring protection due to low levels of Hcy maintained by CBS activity [16].

Although genetic variation was extensively investigated in the PON1 gene, involved in the prevention of LDL lipid peroxidation, no clear conclusion was drawn on the effect of genetic polymorphisms on the onset of Coronary Heart Disease (CHD), while it was suggested that PON1 activity and concentration may instead play a major role determining CHD [17]. On the same line, a recent extensive literature survey and pooled analysis highlighted that decreased PON1 activity was significantly associated with CHD risk [18].

Deleterious genetic variants in TCN2 gene, involved in vitamin B12 metabolism, were suggested to further aggravate the effects of low vitamin B12 levels and consequently high Hcy plasma concentrations, even if with contrasting results [19] and [20], while haplotypes in TCN2 were associated with premature ischemic stroke [21].

Concerning AMT gene, genetic variants were associated with glycine encephalopathy [22] and neural tube defects [23], but no evidence of its involvement in the onset of CVD was described up to now.

Applying a RPMM cluster algorithm, we showed that different methylation profiles are associated to different MI risks, both in males and in females, and the effect is independent from the classical CVD risk factors. This model has been successfully used previously in other studies [24] and proved to be an efficient strategy for the analysis of methylation data derived by Illumina technology [25]. Notably, the methylation profiles of the above mentioned genes are still statistically significant risk factor also after adjusting for B-vitamins intake, matching variables and other CVD risk factors.

Conclusions and future directions

Our findings further support the hypothesis that DNA-methylation of specific regions of genes involved in OCM and Hcy metabolic pathways can be a mediator of the CVD risk conferred by low B-vitamins intake.

To understand if supplementation of B-vitamins in high risk subjects may be effective in preventing CVD, further investigations are needed to evaluate the effects of the interaction between B-vitamins intake and other exposures, such as smoking status or alcohol consumption, on DNA-methylation.

Limitations of the study

Notwithstanding our study had the power to detect only large differences at a single CpG level between cases and controls, the DMRs identified were statistically robust and consistent because discovered in a training set and validated in a test set. Despite we investigated the relationship between DNA-methylation and gene-expression in several tissues using publicly available datasets, no data are currently available on blood. Further investigations are needed to confirm that the inverse relationship observed in different tissues can be extended to blood.

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Appendix A. Supplementary data

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