

## Gluten-Free Products Are Insufficient Source of Folate and Vitamin B<sub>12</sub> for Coeliac Patients

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### Summary

The gluten-free diet, the only treatment in coeliac disease, can be nutritionally unbalanced and deficient in several nutrients. Gluten-free products contain much lower levels of B vitamins, especially lower folate concentrations than their gluten-containing counterparts. Folate intake is considered as a major dietary determinant of plasma homocysteine concentration in healthy population. Elevated homocysteine is an independent risk factor for cardiovascular disease and has been associated with osteoporotic fractures, which are an increased risk factor in coeliac disease. The aim of this study is to determine dietary folate intake and plasma homocysteine concentration as metabolic markers of suboptimal intake of folate and B<sub>12</sub> in Croatian coeliac patients living on a gluten-free diet. Subjects were 52 coeliac patients (83 % female, age 35±13) adhering to a gluten-free diet. Blood samples were analyzed for plasma homocysteine, serum and red blood cell folate and serum B<sub>12</sub>. Quantitative food frequency questionnaire was used to measure dietary folate intake. Mean dietary folate intake was 206 µg of dietary folate equivalents (DFE), which was far below the national recommendation of 400 µg of DFE (or 200 µg of folic acid). Mean homocysteine was (9±2) µmol/L (range from 5.42 to 13.90 µmol/L), while elevated homocysteine concentrations (>10 µmol/L) were found in 34 % of subjects. In conclusion, coeliac patients adhering to gluten-free diet included in this study showed low folate intake and suboptimal folate and vitamin B<sub>12</sub> status, possibly due to low folate content in gluten-free products. Therefore, folate fortification or enrichment of gluten-free products could be beneficial for coeliac patients and it would be of great interest for the food industry.

*Key words:* gluten-free diet, homocysteine, vitamin B<sub>12</sub>, folate intake, coeliac disease

### Introduction

Coeliac disease (CD) is a chronic inflammatory multi-systemic disorder that occurs as a result of an immune response to ingested gluten in genetically predisposed individuals. Gluten is a protein found in wheat, rye and barley. Therefore, CD is treated by excluding all dietary

sources of gluten. Adherence to the gluten-free diet is necessary to prevent malabsorption and it results in symptomatic, serologic and histological remission in the majority of patients (1). The gluten-free diet is based on gluten-free products, which are made from gluten-free flour. Several types of gluten-free flour are nowadays present on Croatian market; however, they are composed of dif-

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ferent types of starch. Some of them consist of wheat starch where gluten has been removed, or of naturally gluten-free starch such as corn or potato starch. Therefore, the nutritive quality of gluten-free products is important. Previous studies suggest that gluten-free diet could be nutritionally unbalanced and deficient in several nutrients (2–4). Gluten-free products contain much lower folate concentrations than their gluten-containing counterparts (5,6), therefore such diet can cause vitamin deficiency. B vitamins, especially folate and cobalamin (B<sub>12</sub>), are major determinants of plasma homocysteine (Hcy) concentration (7). Epidemiological evidence suggests a relationship between elevated total plasma Hcy concentration and a risk of cardiovascular diseases (8–11), increased risk of neurodegenerative conditions such as stroke, Alzheimer's and Parkinson's disease (12,13) as well as osteoporotic fractures (14,15). Some of these conditions, such as osteoporotic fractures, are more prevalent in CD patients (16).

The aim of this study is to determine dietary folate intake and plasma homocysteine concentration as a metabolic marker of B vitamins in Croatian coeliac patients living on a gluten-free diet.

## Subjects and Methods

### Subjects

The subjects were 52 coeliac patients (83 % female, age range from 18 to 69). Inclusion criteria were biopsy-proven CD, adherence to gluten-free diet and age ( $\geq 18$  years old), while exclusion criterion was the use of drugs known to interfere with folate metabolism. Patients were recruited through the Croatian Society for Coeliac Disease. All participants signed an informed consent form. The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia.

### Methods

Fasting venous blood samples were drawn from subjects to determine Hcy, serum cobalamin, serum folate and red blood cell (RBC) folate concentration. Blood samples were collected in tubes containing 7.5 % tripotassium EDTA (BD Vacutainer Systems, Plymouth, UK) and centrifuged at 2000 rpm for 5 min. Two aliquots of serum per subject were stored at  $-20$  °C until analysis. Plasma Hcy concentration, which included both the bound and unbound fractions of Hcy, was determined with the fluorescence polarization immunoassay. Mildly elevated Hcy was considered as  $>10$   $\mu\text{mol/L}$ . Serum B<sub>12</sub> concentration was measured by radioimmunoassay; normal value range was between 208.0 and 963.5 pg/mL. Folate concentration in RBC and serum was measured using an ion capture assay; normal value range being between 572–1843 and 16–35 nmol/L, respectively. All parameters were measured on the Abbott AxSYM System according to the manufacturer's instructions (Abbott AxSYM System, Abbott Park, IL, USA) at the Children's Hospital Zagreb. Intra-assay coefficient of variation for Hcy, vitamin B<sub>12</sub> and folate was 3.1, 3.3 and 3.5 %, respectively.

Dietary intake of folate was assessed by using validated 39-item food frequency questionnaire (FFQ) as de-

scribed elsewhere (17). FFQ was slightly modified by replacing gluten products such as bread, pasta and breakfast cereals with gluten-free products. The questionnaire was mailed to the subjects and once completed was returned. Subsequently, blood samples were taken. Folate intake was calculated as dietary folate equivalents (DFE) by using food composition tables (18), while for gluten-free products, not included in the food composition tables, folate content was taken from the nutrition information on the product labels or from manufacturers. Folate supplement use was recorded separately in a questionnaire that included brand name, composition, and amount taken for each supplement. Average daily supplement intake of folate was computed from the questionnaire and was added to the folate intake from food to provide total daily folate intake.

### Statistical analysis

Data having normal distribution are presented as mean values  $\pm$  S.D. Differences among mean values were assessed using Student's *t*-test. The distribution of total daily folate intake was positively skewed; therefore, log-transformed and presented as median and 95 % confidence interval. Pearson's correlation was used to examine the correlation between folate intake and biochemical parameters;  $p < 0.05$  was considered significant. Multiple regression analysis was used to examine the influence of individual factors on Hcy concentration. All statistical analyses were performed with STATISTICA software v. 8 (19).

## Results and Discussion

Characteristics of coeliac patients are presented in Table 1. Coeliac patients were divided into two groups according to folate supplement usage. Significant difference was observed between supplement users and non-users only for RBC folate and total daily folate intake ( $p < 0.05$ ). Therefore, for other observed parameters, coeliac patients were treated as one group. Subjects included in this research were adhering to gluten-free diet on average for 9 years (range from 0.25 to 30 years). The mean total plasma Hcy concentration was  $(9 \pm 2)$   $\mu\text{mol/L}$ . All observed biochemical parameters were in the recommended range for supplement users, while for non-users elevated Hcy ( $>10$   $\mu\text{mol/L}$ ) was found in 34 % of the subjects, low plasma folate in 25 % patients, low red blood folate in 25 % and low serum cobalamin in 14 % of the subjects. These results are in accordance with the previous studies among this population. Hallert *et al.* (20) reported that despite a 10-year gluten-free diet, 37 % of coeliac patients still have lower folate levels and higher Hcy levels, indicating a poor vitamin status.

FFQ was used to determine folate intake from food. No statistical difference was observed between supplement users and non-users regarding folate intake from food ( $p > 0.05$ ) (Table 1). Mean folate intake from food was 206  $\mu\text{g}$  DFE, which was far below the national recommendation of 400  $\mu\text{g}$  DFE (or 200  $\mu\text{g}$  of folic acid), but higher than in coeliac patients reported by Hallert *et al.* (20) and Grehn *et al.* (2). Green leafy and leguminous vegetables supplied 56 % of dietary folate intake, while grain products contributed less than 10 % of dietary in-

Table 1. Selected characteristics of coeliac patients

Variables	Coeliac patients not using folate supplements (N=44) <sup>a</sup>	Coeliac patients using folate supplements (N=8) <sup>b</sup>	All patients (N=52) <sup>c</sup>	p
Age/year	36±3	38±12	35±13	0.370
GF diet/year	9±8	5±4	9±1	0.129
c(Hcy)/(μmol/L)	9±2	9±2	9±2	0.534
c(F(S))/(nmol/L)	22±8	28±10	23±9	0.076
c(F(RBC))/(nmol/L)	761±280	1256±292	839±333	<0.001*
γ(B <sub>12</sub> )/(pg/mL)	339±143	349±137	341±141	0.863
Dietary folate/(μg DFE/day)	203±73	224±163	206±91	0.565
Total folate intake <sup>d</sup> /(μg DFE/day)	197.61 (18.97–225.45)	731.83 (455.66–5785.90)	220.96 (122.68–1041.25)	<0.0001*

all values are expressed as mean±S.D.

GF=gluten-free, Hcy=homocysteine, F(S)=serum folate, F(RBC)=red blood cell folate

<sup>a</sup>includes 37 females and 7 males, <sup>b</sup>includes 6 females and 2 males, <sup>c</sup>includes 43 females and 9 males,

<sup>d</sup>includes folate intake from food and supplements (positively skewed, therefore presented as median±95 % CI)

\*significant (p<0.05)

take. According to Grehn *et al.* (2) relative contribution of folate was lower in gluten-free bread *vs.* gluten-containing bread, despite the fact that the intake of bread was similar among coeliac patients and healthy controls in Sweden. It should be noted that in Sweden neither gluten-free nor gluten products are folate-enriched.

Folate supplements which were used by 15 % of subjects (N=8) were added to folate intake from food to provide total daily folate intake. As expected, differences between total daily folate intake between supplement users and non-users were observed (p<0.05). Patients used folate supplement in dosages of 100 to 680 μg DFE, while two patients used extra high dosage of 8500 μg DFE.

Pearson's correlation was done to examine the relationship between total daily folate intake and biochemical parameters that reflect Hcy concentration, namely serum folate and RBC folate. For all patients positive correlation was found between total daily folate intake and folate status (serum and RBC folate), while correlation with Hcy was negative but it did not reach statistical significance (Table 2). Positive correlation was also found between total folate intake and serum folate among supplement users, which can be explained by the fact that the most recent intake reflects on serum folate levels, which was considerably higher in supplement users (Table 1). Hallert *et al.* (21) showed that vitamin B supplements normalized Hcy and improved subjective health status of coeliac patients.

Furthermore, correlation was made to examine the association between Hcy, biochemical parameters and the duration of gluten-free diet (Fig. 1). Statistically significant correlations were found between the concentration of Hcy and both folate fractions (in serum and in RBC) and with B<sub>12</sub> (p<0.05) for all patients. These correlations were also observed for non-users of supplements (not shown). We assumed that the duration of gluten-free diet could also influence Hcy concentration, since it was found that the exclusion of gluten from the diet of CD patients can increase folate status and thus normalize Hcy levels (22). However, no correlations were found between Hcy concentrations and the number of years the participants adhered to gluten-free diet (p=0.788) (Fig. 1).

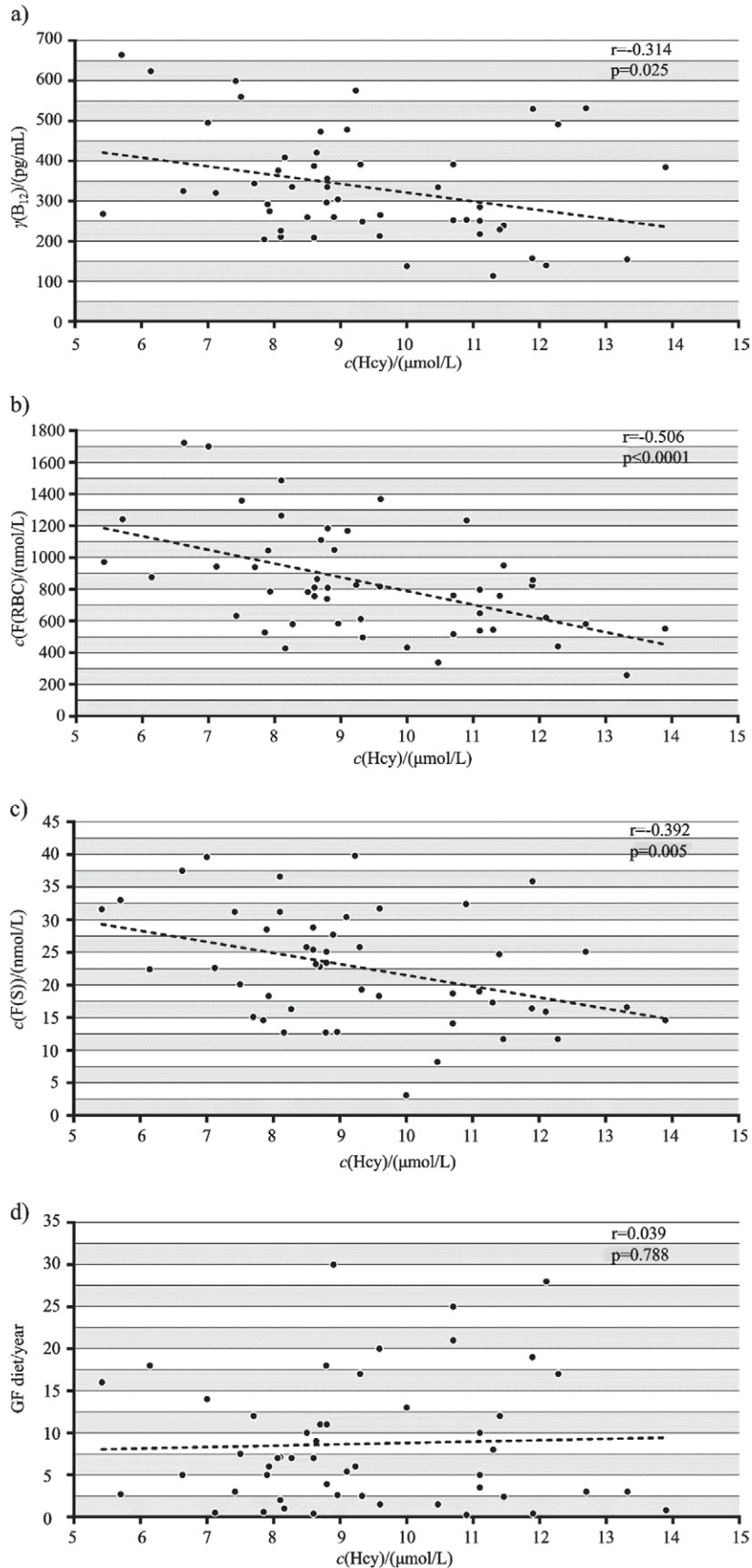
To further define the role of all observed biochemical and dietary parameters on Hcy concentration, a multiple regression analysis was performed where Hcy was dependent variable and age, duration of gluten-free diet (years), serum folate, RBC folate, serum B<sub>12</sub> and total dietary folate intake were the independent variables. The results show that 31.3 % of the variation in the total plasma Hcy concentration could be explained by RBC folate and age (p<0.0001). While serum folate reflects the most recent intake, RBC folate reflects the long-term intake because folate accumulates during erythropoiesis and appears to be retained through the life span of the cell (23). It is well established that Hcy concentration increases with age (24,25).

Table 2. Correlation between folate intake and serum values

Variables	Coeliac patients not using folate supplements (N=44)		Coeliac patients using folate supplements (N=8)		All patients (N=52)	
	r	p	r	p	r	p
Total folate intake (log[DFE (μg/day)]) <i>vs.</i> Hcy	-0.245	0.112	-0.636	0.090	-0.267	0.059
Total folate intake (log[DFE (μg/day)]) <i>vs.</i> serum folate	0.206	0.185	0.742	0.035*	0.431	0.002*
Total folate intake (log[DFE (μg/day)]) <i>vs.</i> RBC folate	0.136	0.385	0.669	0.069	0.563	<0.0001*

Hcy=homocysteine, RBC=red blood cell, total folate intake includes folate intake from food and supplements (positively skewed, therefore log-transformed), DFE=dietary folate equivalents

\*significant (p<0.05)



**Fig. 1.** Correlation between plasma homocysteine and red blood cell folate, serum folate, serum B<sub>12</sub> and years of adherence to gluten-free diet

Hcy=homocysteine, F(RBC)=red blood cell folate, F(S)=serum folate, GF=gluten-free

Previous research had shown that in healthy subjects high dietary intake of folate reduces Hcy concentration in plasma (26). Folate is absorbed in the jejunum, the proximal segments of which can be inflamed and damaged in active CD. Therefore, elevated Hcy concentration is frequent in newly diagnosed patients and vitamin deficiencies caused by malabsorption are the most important determinants of this condition (27,28). However, as reported before (2,20) and confirmed by this study, coeliac patients adhering to gluten-free diet for several years can also develop folate deficiency, possibly due to low folate content in gluten-free products.

To increase folate intake in all population, especially among women to prevent the development of neural tube defects, food fortification is mandatory in some countries such as the USA, Canada, Australia and Ireland. Folic acid is usually added to staple food such as flour for bread making purposes. However, because coeliac patients can only consume gluten-free flour, it does not apply to them. Nowadays, CD is well recognized and prevalence of CD is about 1 % in the general population (29,30). Therefore, folate fortification or enrichment of gluten-free products/flour could be beneficial for coeliac patients and of great interest for food industry. Our study has several limitations, primarily the small sample size and single centre. Unfortunately, these limitations were the result of specific inclusion criteria such as adherence to gluten-free diet, age and medical condition of coeliac patients. Another limitation was the lack of control group. However, previous research conducted among young healthy Croatian population showed no clinical deficit in vitamin B status (31,32). All of these limitations were taken into account. This research could contribute to better understanding of the impact of gluten-free diet on the nutritional status of coeliac patients in Croatia.

## Conclusion

Results of this study show that coeliac patients adhering to gluten-free diet included in this study have inadequate folate intake and low folate and vitamin B<sub>12</sub> status, which was in correlation with mildly elevated Hcy concentrations. These results may support the need for dietary counselling for coeliac patients to increase the intake of folate-rich foods and enriched gluten-free products.

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