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Authors

Paniz, Clóvis
Lucena, Maylla Rodrigues
Bertinato, Juliano Felix
et al.

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Daily supplementation with 5 mg of folic acid in Brazilian patients with hereditary spherocytosis

Clovis Paniz[1], **Maylla Rodrigues Lucena**[2], **Juliano Felix Bertinato**[1], **Felipe Rebello Lourenço**[3], **Bruna Cipriano A. Barros**[2], **Guilherme Wataru Gomes**[1], **Maria Stella Figueiredo**[2], **Rodolfo Cançado**[4], **Vera Lúcia Blaia D'Avila**[5], **Christine M Pfeiffer**[6], **Zia Fazili**[6], **Ralph Green**[7], **Valdemir Melechco Carvalho**[8], **Elvira Maria Guerra-Shinohara**[1,2]

[1]Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Análises Clínicas e Toxicológicas, São Paulo, Brasil

[2]Universidade Federal de São Paulo, Disciplina de Hematologia e Hemoterapia, São Paulo, Brasil

[3]Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Farmácia, São Paulo, Brasil

[4]Faculdade de Medicina da Santa Casa de São Paulo, Seção de Hematologia e Oncologia, São Paulo, Brasil

[5]Pontifícia Universidade Católica de São Paulo, Faculdade de Medicina e Ciências da Saúde, Divisão de Hematologia, Sorocaba, Brasil

[6]National Center for Environment Health, CDC, Atlanta, GA, USA

[7]Department of Pathology and Laboratory Medicine, UC Davis School of Medicine, Sacramento, CA, USA

[8]Grupo Fleury, São Paulo, Brasil

Abstract

Hereditary spherocytosis (HS) patients have increased rates of erythropoiesis and higher folate requirements. In a case-control study in HS patients, we evaluated the associations between the use of 5 mg FA daily and serum concentrations of folate, unmetabolized folic acid (UMFA), interleukin (IL)-6, IL-8, IL-10, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α); and mRNA expression of *dihydrofolate reductase* (*DHFR*), *methylene tetrahydrofolate reductase* (*MTHFR*), *IL8*, *IFNG* and *TNFA* genes. Total serum folate and folate forms were measured in 27 HS patients (21 users [HS-U] and 6 non-users [HS-NU] of supplemental FA) and 54 healthy

Corresponding author: Clóvis Paniz, Department of Clinical and Toxicological Analyses, School of Pharmaceutical Sciences, University of Sao Paulo, Brazil, Av. Professor Lineu Prestes, 580, Bloco 17, CEP: 05508-000, São Paulo, SP, Brasil, Phone: +55 11 3091 3785 Fax: 11 3813 2197, clovis.paniz@yahoo.com.br.

Contributors CP designed the research study, collected the samples and data, performed the laboratorial determinations, analyzed the data and wrote the paper. MRL and JFB collected the samples and data, performed the laboratorial determinations and analyzed the data. FRL performed statistical analysis and analyzed the data. BCAG and VMC performed MMA determinations and analyzed the data. GWG performed the laboratorial determinations and reviewed the paper. MSF, RC, VLBD included the patients and performed collect of clinical data. CMP and ZF performed UMFA and folate forms by LC/MS and reviewer the paper. RG designed the research study and did an expert review of the paper. EMG designed the research study, analyzed the data, performed statistical analysis and wrote the paper.

Competing interests The authors have no conflicts of interest to declare.

controls not consuming 5 mg/d supplemental FA. Each patient was matched to two controls based on age, sex and BMI. The mononuclear leukocyte mRNA expression of relevant genes and their products were determined. Serum folate, UMFA, 5-methyl-tetrahydrofolate (5-methyl-THF), and tetrahydrofolate (THF) concentrations were significantly higher in HS-U compared with matched healthy controls (P<0.001, n=42). HS-NU had lower serum folate concentrations than matched healthy controls (P=0.044, n=12). HS-U and HS-NU presented similar hematological and biochemical markers profiles. No differences were found between HS-U and HS-NU for cytokine serum concentrations and mRNA expression genes. DHFR mRNA expression was higher in HS-U than in HS-NU. The use of high daily doses of FA for treatment of HS patients may be excessive and is associated with elevated serum UMFA and elevated DHFR mRNA expression. It is not known whether long-term high dose folic acid use by HS patients might have adverse health effects.

Keywords

folic acid; unmetabolized folic acid; hereditary spherocytosis; *DHFR*; cytokines

INTRODUCTION

Hereditary spherocytosis (HS) is an inherited hemolytic anemia most prevalent in Caucasians, affecting around 1 in 2,000 individuals. The HS phenotype may arise from one of several mutations that lead to defects or deficiency in one or more proteins present in red blood cell (RBC) membranes, such as band 3, protein 4.2, ankyrin and β-spectrin.[1] As a result, RBCs lose small vesicles of cytoplasmic membrane containing lipids and proteins, reducing their membrane surface area, and leading to a spherical-shaped (spherocytes).[1] The abnormal shape leads to reduced deformability of the cells and consequent premature trapping and destruction by the spleen. Shortened red cell lifespan results in increased compensatory rates of red cell production.[2, 3]

To ensure adequate nutrient requirements for increased erythropoietic demands, folic acid (FA) is usually prescribed for patients with chronic hemolysis. However, guidelines are lacking regarding the optimal dose of FA.[1, 4]

FA is a synthetic form of folate that must be reduced by the enzyme dihydrofolate reductase (DHFR) to be incorporated into the active cellular pool of folate.[5] The activity of DHFR could be a limiting factor for FA reduction in people consuming greater than the Tolerable Upper Intake Level (UL) of 1 mg/day of the vitamin in the form of folic acid,[6] leading to the appearance of unmetabolized FA (UMFA) in the circulation. Although no definitive adverse outcomes have been linked to high FA intakes, a recent study carried out by our group showed that intake of 5 mg of FA daily for 3 months by healthy individuals reduced the number and the cytotoxic activity of natural killer (NK) cells and was associated with elevated mRNA expression of *interleukin-8* (*IL8*) and *tumor necrosis factor-α* (*TNFA*) genes.[7]

In Brazil, patients with HS and other chronic hemolytic states are being prescribed 5 mg of FA daily, which is 5 times higher than the UL for healthy individuals.[8] It is not known what the effects of this high FA dose are in chronically supplemented individuals.

Thus, the aims of this study were to assess whether there is an association between elevated UMFA and proinflammatory cytokinelevels when HS patients were treated long-term with 5 mg of FA daily.

METHODS

Twenty-seven HS patients seen at hematology centers in the cities of Sao Paulo and Sorocaba (Brazil) were recruited for this study and 54 healthy people were recruited as a control group from the same community. Each patient was matched to two controls by age, sex and body mass index (BMI), the main covariates that may confound the analysis. Although patients were prescribed 5 mg of FA daily, six of them self-reported not taking FA during the interview conducted by our team. We took advantage of the fact that some patients were not using FA daily because of non-compliance with the treatment, which enabled us to compare HS patients who were users (HS-U) and non-users (HS-NU).

The Institutional Ethics Committee approved this study (CNS 466/12, CAEE 04389512.2.000.0067), and a written informed consent was obtained from all subjects prior to their inclusion in the study. The analysis of blinded specimens by the CDC Nutritional Biomarkers Laboratory (serum folate forms) does not constitute engagement in human subject research. Other blood measurements were performed in laboratories at the University of Sao Paulo, Brazil.

The exclusion criteria were: age less than 18 years, chronic alcoholism, acute or chronic diseases, pregnancy, use of immunosuppressive drugs, and people who donated or received blood transfusion within 6 months of the study. Furthermore, control subjects who used FA, either in the form of multivitamins or vitamin-minerals mixtures in the last 6 months were also excluded from the study.

Venous blood samples were obtained by venipuncture from each participant after an overnight fast (8-10 hours). Serum folate was determined by a microbiologic assay with the use of *Lactobacillus casei* (chloramphenicol-resistant strain NCIB 10463) and FA (F7876 Sigma Aldrich folic acid 98%) as calibrator.[9] Concentrations of serum folate forms [UMFA, tetrahydrofolate (THF), 5-methyl-THF, 5-formyl-THF, 5,10-methenyl-THF, and MeFox (an oxidation product of 5-methyl-THF)] were analyzed by high performance liquid chromatography-tandem MS (HPLC-MS/MS).[10] Serum total folate was calculated as the sum of these folate forms excluding MeFox. Plasma methylmalonic acid (MMA) was assessed by HPLC-MS/MS. Other biochemical analyses, as well as hematological, mRNA expression analysis and NK cell count, were performed as described previously.[7]

BMI was calculated by dividing weight (kg) by height^2 (m) and the measures were performed as previously described.[11] Subjects were classified as underweight (BMI <18.5 kg/m^2), normal weight (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2) and obese (BMI ≥30.0 kg/m^2).[12]

Dietary intake assessment

Dietary intake was assessed by two 24-hour dietary recalls (24-HR) in each period of the study, the first one on the day of blood collection and the second one a few days after blood collection, as previously described.[7, 11]

Statistical analyses

Statistical analyses were carried out using SPSS version 22.0 (IBM, USA) and GraphPad Prism™ version 5.04 (GraphPad Software, Inc., USA) software.

Serum folate concentration <7.0 nmol/L was defined as folate deficiency.[13] The value of 45 nmol/L (20 ng/mL) for serum folate was considered as a cutoff due to being the upper limit of the assay calibration curve in many serum folate assays. Elevated serum UMFA was defined as a serum concentration >1.98 nmol/L, according to the 95th percentile in the distribution of values obtained for fasted persons in the NHANES 2007-2008 survey.[14]

Elevated plasma total homocysteine (tHcy) was defined as a plasma concentration >13.9 μmol/L.[15] Vitamin B12 deficiency was defined as a serum B12 concentration <256 pmol/L, while functional vitamin B12 deficiency was defined as a B12 concentration <256 pmol/L and MMA >271 nmol/L.[16] Anemia was defined as a hemoglobin concentration <120 g/L for women and <130 g/L for men.[17]

HS disease severity was classified adapting the criteria previously described,[18] by using values of hemoglobin (Hb), reticulocyte count (Ret) and serum total bilirubin (TBi). Thus, HS patients were stratified as Trait: Hb normal, Ret <3% and TBi <17 μmol/L; Mild: Hb 110-150 g/L, Ret 3-6 % and TBi 17-34 μmol/L; Moderate: Hb 80-120 g/L, Ret 6-10% and TBi 34-51 μmol/L; Severe: Hb 60-80 g/L, Ret >10% and TBi >51 μmol/L. To be accepted in a category the patient needed at least two parameters compatible with the classification. When the three parameters (Hb, Ret and TBi) were not enough to establish the disease staging, Hb was used as the arbiter criterion for classification.

The chi-square test, or the likelihood ratio and Fisher's exact tests were used to analyze the frequencies of categorical variables. The Mann-Whitney test was used to compare numeric variables between groups.

Spearman correlations between serum UMFA and serum cytokine concentrations and among serum cytokine concentrations were performed in each group studied.

In order to define a cutoff for serum folate that represents a higher likelihood of having elevated UMFA concentrations, the area under the ROC curve was obtained for UMFA >1.98 nmol/L as a dependent variable and serum folate concentration measured by HPLC-tandem mass spectrometry as an independent variable. The selected cutoff maximized sensitivity and specificity.

The level of significance was set at $\alpha = 5\%$, $P \leq 0.05$.

RESULTS

Among the 27 HS patients enrolled in this study, 21 were users of 5 mg FA daily (HS-U), while 6 were nonusers (HS-NU). Regarding BMI, only 1 woman in HS-U, 1 woman in HS-NU and their respective controls were classified as obese. The characteristics of the HS patients and control subjects are shown in Table 1.

Natural food folate intake, folic acid intake and total food folate intake, expressed as dietary folate equivalents (DFEs), did not differ significantly among HS-U, HS-NU and their controls (Table 2).

Serum folate, UMFA, 5-methyl-THF, THF, and MeFox concentrations were significantly higher in HS-U compared with their controls ($P < 0.001$) (Table 2). On the other hand, HS-NU patients had lower serum folate concentrations ($P = 0.044$) and did not show differences regarding UMFA, 5-methyl-THF, THF and MeFox concentrations compared with their controls (Table 2). In addition, HS-U had higher serum folate, UMFA, and 5-methyl-THF than HS-NU patients (Figure 1).

In HS-U patients, serum UMFA represented on average 11.4% of total folate (HPLC-MS/MS), while it represented on average 2.44% in their controls, 3.97% in HS-NU patients, and 2.23% in HS-NU controls. The contribution of 5-methyl-THF to serum total folate (HPLC-MS/MS) ranged from 63.3% in HS-U to more than 90% in HS-NU and both control groups.

No difference was found between HS-U or HS-NU patients and their controls concerning serum vitamin B12 and tHcy concentration ($P > 0.050$). However, MMA concentration was higher in HS-U patients than in their healthy controls ($P=0.003$). Vitamin B12 deficiency was observed in 23.8% of HS-U patients, but the deficiency frequency was similar to their healthy controls. Even when functional deficiency of vitamin B12 was observed, there was no difference between HS-U and their healthy controls (Table 2).

We found no significant difference between HS-U and HS-NU patients for any of the hematological or biochemical parameters, including the inflammation markers (Table 3). HS patients (users or non-users of 5 mg of FA daily) presented lower RBC counts and hemoglobin concentration, and higher values of reticulocytes and serum lactate dehydrogenase activity when compared with their controls (Table 3). Most HS-U and HS-NU patients were anemic (76.2% and 83.3%, respectively) compared with only 3 (5.9%) healthy control subjects. Only HS-U patients had higher WBC and NK cell counts than their healthy controls (Table 3). When HS-U patients were classified according to the severity of clinical presentation of the disease (trait, mild, moderate or severe), no significant differences in NK cell counts were observed.

Higher concentrations of IL-8 and TNF-α were found in HS-U patients compared with their healthy controls ($P < 0.001$), while HS-NU patients presented higher TNF-α concentrations ($P = 0.028$) but similar IL-8 concentrations compared with their healthy controls (Table 3). No differences were observed between HS patients and their controls regarding serum concentrations of IL-6, IL-10, interferon-γ (IFN- γ) and high sensitivity C-reactive protein (Hs-CRP) ($P > 0.05$) (Table 3).

We found no correlation between the serum cytokine concentrations with serum UMFA in 54 controls (Table 4). However, there were inverse and moderate correlations (close to significance) between UMFA and IL-10 (rho= −0.400, P=0.072), and UMFA and IFN-γ (rho= −0.396, P=0.076) (Table 4).

Higher *DHFR* mRNA expression was found in HS-U compared with HS-NU patients. No differences were found between the two groups for MTHFR, IFNG, TNFA and IL8 mRNA expression genes (Figure 2).

The inflammatory biomarkers and mRNA expression evaluated in this study did not show significant differences when HS patients were classified according to the severity of their clinical presentation.

The cutoff for serum folate obtained by ROC analysis was 54 nmol/L (sensitivity=100% and specificity=91.7%) and the area under the curve (AUC) was 0.982 (CI 95%: 0.957-1.000), P<0.001.

DISCUSSION

To our knowledge, this is the first study that evaluated serum folate forms and inflammatory markers in HS patients receiving high doses of FA, exceeding the UL (1 mg/d). Our findings show that daily use of 5 mg of FA among HS patients resulted in unusually high serum folate concentrations and its non-metabolized form UMFA compared with healthy controls not consuming supplemental FA. Furthermore, a high proportion of elevated UMFA concentrations was found in HS-U patients when compared with their controls. The data that we obtained on patients not using folate was fortuitous and a result of patient noncompliance and the inclusion of six HS patients not using FA supplementation made it possible for us to assess the same biochemical markers when only one variable changed compared with the control group (i.e., disease status), albeit in a small group. We observed lower serum folate concentrations in HS-NU when compared with their healthy controls, confirming that HS patients require an additional supply of FA. However, 5 mg of FA daily may be an excessive dose and it may be more prudent to substantially reduce the daily dose. Titration of the ideal safe dose would require further studies to ensure adequacy of folate intake through monitoring of homocysteine or others metabolite status.

The appropriate FA dose is a subject of discussion for hemolytic anemias, including HS, especially according to disease severity. A few studies have described different doses, ranging from 1 mg/day, 2.5 mg/day, 5 mg once a week, and even 100 μg/kg/day.[4, 19, 20] The FA dose recommended by the Brazilian government for women in the periconceptional period is 5 mg daily,[21] and this dose has also been used for patients requiring higher amounts of FA due to HS or other hemolytic anemia over their entire lives.

In a previous study, we demonstrated that the daily use of 5 mg of FA by healthy individuals over 90 days, in a non-controlled intervention, led to increased UMFA concentrations, increased mRNA expression of the *DHFR* gene and of the cytokine *IL8* and *TNFA* genes, and reduced number and cytotoxicity of NK cells.[7] The present study confirmed the increased mRNA expression of the *DHFR* gene. We hypothesize that the enzyme may be

upregulated due to saturation in the presence of excess FA. However, the present study did not find higher *IL8* or *TNFA* mRNA gene expression in HS-U compared to HS-NU patients, possibly due to limited sample size. The much higher serum IL-8 and TNF-α concentrations in the two HS groups suggest that this may be a result of the disease (HS) and not from the use of FA. To our knowledge, this is the first study that showed the inflammatory cytokine profile, including IL-6, IL-8, IL-10, IFN-γ and TNF-α, in HS patients chronically subjected to high concentrations of FA. In the present study, NK cell counts in HS-U patients were higher than in healthy controls. This contrasts with our previous finding in which the NK counts decreased in healthy volunteers after they consumed 5mg FA daily for 90 days.[7] Unfortunately, we were not able to study sequential changes in WBC and NK number and activity in HS patients before and following initiation of FA supplementation, nor were we able to determine NK cell cytotoxicity in HS patients in the present study.

While we found no differences in serum vitamin B12 concentrations and functional deficiency of this vitamin between HS-U patients or HS-NU patients and their respective controls, HS-U patients had higher MMA concentrations than their controls. On the other hand, concentrations of tHcy were similar between HS groups and their controls. This observation raises the possibility that high concentrations of folate caused by intake of supplemental FA may negatively affect B12 status, as has been noted in other reports [22-24].

The serum folate concentration that is associated with elevated UMFA concentrations (>1.98 nmol/L) is not yet known. We found that a serum folate cutoff of >54 nmol/L showed a high sensitivity and specificity for predicting elevated UMFA concentrations >1.98 nmol/L. This cutoff is close to 45 nmol/L, suggested previously as a high folate cutoff [13], and could be used to distinguish between individuals with normal compared with elevated UMFA, since UMFA determination is not easily available due to high cost.

The major limitation of our study is the relatively small number of HS patients, which is due to the low prevalence of this disease in our region. However, we highlight as strengths of this study the assessment of hematological and inflammatory cytokine profiles of patients with hemolytic anemia with low-grade inflammation using chronically high amounts of FA.

From this case-control study, we conclude that daily doses of 5 mg of FA, for treatment of HS patients was associated with elevated serum folate forms and elevated DHFR mRNA expression. We were not able to show an effect of UMFA excess on modulation of immune response in HS patients. More studies with a larger number of patients are needed to verify possible health effects associated with the long-term use of high FA doses, which exceed the UL in patients with chronic hemolytic anemias. This may be particularly relevant in individuals with functional asplenia or post-surgical splenectomy such as might occur in sickle cell disease or HS, who are already more susceptible to infections, particularly those caused by unicellular parasitic infections.

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The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Summary box

What is already known about this subject:

- Hereditary spherocytosis (HS) patients present with chronic anemia and require greater amounts of folate for maintaining adequate erythropoiesis.
- Most of these patients receive 5 mg/day of folic acid, a synthetic molecule that needs to be reduced to tetrahydrofolate by the enzyme DHFR before it can be be incorporated into the active cellular pool of folate.
- This high dose of folic acid (5-fold the Tolerable Upper Intake Level of folic acid for healthy subjects), and part of this folic acid circulates in blood as unmetabolized form.
- There are concerns about the risk of such high concentration, especially in relation to unmetabolized folic acid (UMFA).

What are the new findings:

- In our study we demonstrate, high concentrations of unmetabolized folic acid (UMFA) and total serum folate in HS patients.
- To our knowledge, this is the first time UMFA was determined in HS patients. In a previous study by us, we demonstrated high folic acid could affect NK cell activity.
- We demonstrate higher *DHFR* mRNA expression in HS patients using 5 mg/day of folic acid compared with HS patient that are not using folic acid.
- We performed a hematologic and inflammatory profile in HS patients.

How might it impact on clinical practice in the foreseeable future?

- Differently than previously thought, excess folic acid is not completely eliminated in the urine and persistently high circulating concentrations can be observed in these patients.
- We believe that the doses of folic acid used routinely in HS patients need to be reduced to minimize potential health risks.
- Since determination of UMFA is not available in routine laboratories, determination of total serum folate may be useful for monitoring therapy in these patients. Thus, we propose an upper limit showing high sensitivity, and specificity cutoff for serum folate concentration that is associated with elevated UMFA concentrations.

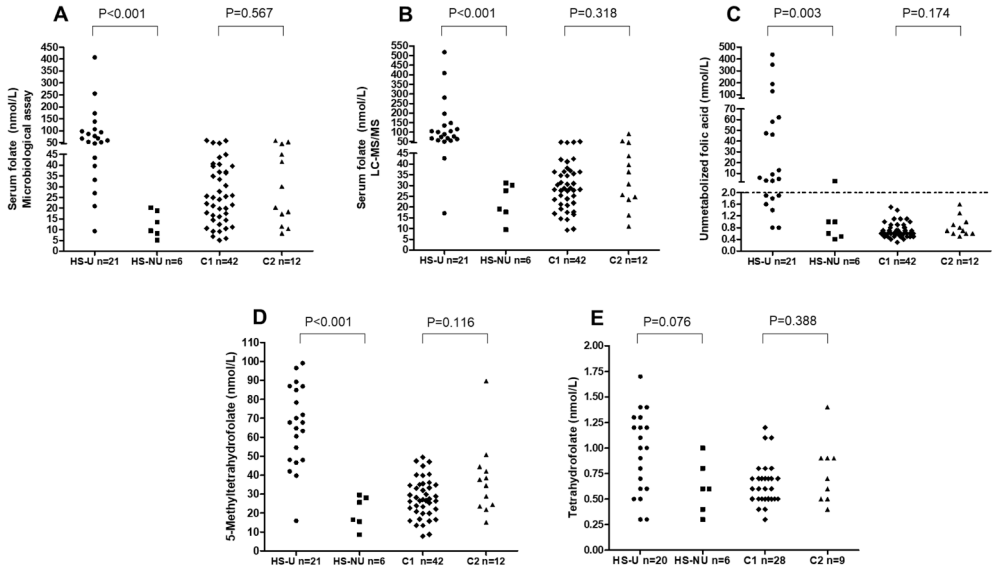

Figure 1.

Concentrations of folate forms in patients with hereditary spherocytosis who are users (HS-U) and non-users (HS-NU) of 5 mg of folic acid daily and healthy controls of the HS-U (C1) and HS-NU (C2) groups. (A) serum folate determined by microbiological assay; (B, C, D, E) serum folate, unmetabolized folic acid, 5-methyltetrahydrofolate and tetrahydrofolate, respectively, were determined by HPLC-tandem mass spectrometry. Mann-Whitney test was performed to compare the two groups. Patient above dashed horizontal line in C panel presented increased unmetabolized folic acid (cutoff point of 1.98 nmol/L).[14]

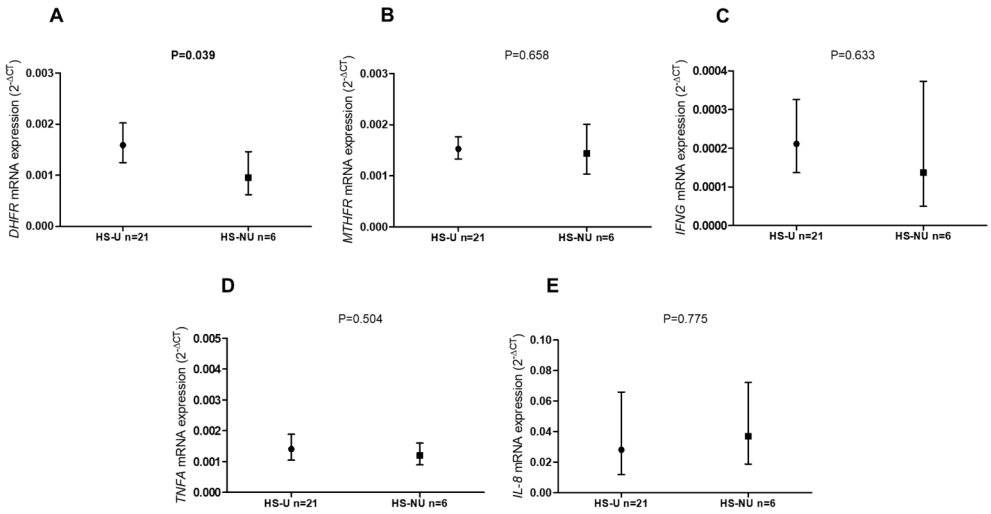

Figure 2.

mRNA expression of (A) Dihydrofolate reductase (*DHFR*), (B) Methylenetetrahydrofolate reductase (*MTHFR*), (C) Interferon-gamma (*IFNG*), (D) Tumor necrosis factor-α (*TNFA*), and (E) Interleukin-8 (*IL-8*) genes in hereditary spherocytosis patients who are users (HS-U) and non-users (HS-NU) of 5 mg of folic acid daily. Geometric mean values and 95% confidence intervals were shown.

Table 1.

Characteristics of hereditary spherocytosis (HS) patient groups and their controls

	HS-U (n = 21)	Controls (n = 42)	P value	HS-NU (n = 6)	Controls (n = 12)	P value
Age (years)	39.2 (33.3–46.1)	38.3 (34.3–42.6)	0.721	39.3 (23.2–66.5)	39.5 (29.0–53.8)	0.777
BMI (kg/m^2)	23.6 (22.3–24.9)	24.0 (23.1–25.1)	0.726	25.8 (18.9–35.3)	25.3 (22.1–29.0)	0.815
Sex (female)	17 (81.0)	34 (81.0)	1.000 ***	4 (66.7)	8 (66.7)	1.000 ***
Self-reported skin color						
White	16 (76.2)	31 (73.8)	0.862 **	5 (83.3)	9 (75.0)	1.000 ***
Black	2 (9.5)	3 (7.1)		0	0	
Mulatto	3 (14.3)	8 (19.0)		1 (16.7)	3 (25.0)	
Smokers	1 (4.8)	6 (14.3)	0.408 ***	1 (16.7)	1 (8.3)	1.000 ***
Daily use of 5mg folic acid	21 (100)	0	**<0.001** *	0	0	
Splenectomized	5 (23.8)	0	**<0.001** *	0	0	
Classification of spherocytosis *						
Trait	4 (14.8)			0		
Mild	7 (25.9)			3 (50.0)		
Moderate	9 (33.4)			3 (50.0)		
Severe	7 (25.9)			0		

HS-U: hereditary spherocytosis patients who were using 5mg of folic acid daily; HS-NU: hereditary spherocytosis patients who were not using folic acid. Age and body mass index (BMI) are presented as geometric mean values and 95% confidence intervals. The groups were compared by Mann-Whitney test. Categorical variables are shown as number of subjects and percentage (in parentheses).

* Chi-square test

** likelihood ratio test and

*** Fisher's exact test. No differences were found when comparing the data from the HS-U and HS-NU groups: age (P=0.977); BMI (P=0.838); sex (P=0.588), self-reported skin color (P=0.591); smokers (P=0.402); splenectomized (p=0.545); classifications of spherocytosis (P=0.656).

Table 2.

Folate and energy intake and concentrations of vitamins and metabolites (MMA and tHcy) in hereditary spherocytosis (HS) patient groups and their controls

	HS-U (n = 21)	Control (n = 42)	P value	HS-NU (n = 6)	Control (n = 12)	P value
Folate and energy intake						
Energy (Kcal)	1,781 (1,554-2,041)	1,906 (1,770-2,054)	0.503	2,055 (1,532-2,757)	1,851 (1,598-2,145)	0.482
Total folate (μg DFE/d) [5]	421 (379–468)	405 (374–439)	0.431	424 (364–495)	398 (267–591)	0.743
Natural food folate intake (μg/d)	195 (175–217)	193 (180–207)	0.976	191 (151–243)	205 (179–235)	0.888
Folic acid (μg/d)	136 (122–152)	118 (102–136)	0.164	113 (57–227)	129 (104–161)	0.851
Serum biomarkers						
Serum folate (nmol/L) [1]	66.1 (45.0–97.2)	22.2 (18.2–27.0)	< **0.001**	11.3 (6.5–19.6)	24.6 (16.0–38.0)	**0.044**
Serum total folate [2]	97.3 (68.3–138)	27.4 (24.0–31.1)	< **0.001**	20.9 (13.0–33.7)	35.4 (25.7–48.7)	0.079
UMFA (nmol/L) [2]	11.1 (4.3–28.4)	0.7 (0.6–0.7)	< **0.001**	0.8 (0.4–1.7)	0.8 (0.6–1.0)	0.879
5-methyl-THF (nmol/L) [2]	61.6 (51.0–74.4)	25.8 (22.5–29.5)	< **0.001**	19.0 (11.5–31.2)	33.6 (24.2–46.6)	0.079
THF (nmol/L) [2]	0.8 (0.7–1.1)	0.6 (0.5–0.7)	**0.008**	0.6 (0.4–0.9)	0.7 (0.5–0.9)	0.475
MeFox (nmol/L) [2]	7.7 (5.4–10.9)	1.2 (1.0–1.5)	< **0.001**	2.6 (1.3–5.2)	1.4 (0.9–2.4)	0.209
High Serum folate [1,3] (>45.0 nmol/L)	15 (71.4%)	4 (9.5%)	< **0.001** *	0	3 (25.0%)	0.515 **
High Serum folate [2,3] (>45.0 nmol/L)	19 (90.5%)	4 (9.5%)	< **0.001** *	0	3 (25.0%)	0.515 **
High UMFA [2] (>1.98 nmol/L) (14)	14 (66.6%)	0	< **0.001** *	1 (16.7)	0	1.000 **
Deficiency of serum folate (<7.0 nmol/L) [1]	0	3 (7.1%)	0.545 **	1 (16.7%)	0	0.128 **
Deficiency of serum folate (<7.0 nmol/L) [2]	0	0		0	0	
Total homocysteine (μmol/L) [4]	8.4 (7.4–9.5)	9.8 (8.8–10.9)	0.112	10.3 (7.2–14.8)	10.3 (8.7–12.2)	1.000
Vitamin B12 (pmol/L) [1]	357 (293–436)	365 (327–408)	0.988	353 (222–562)	382 (304–481)	0.815
Vitamin B12 <256 pmol/L	5 (23.8%)	6 (14.3%)	0.483 **	2 (33.3%)	1 (8.3%)	0.245 **
MMA (nmol/L)	308 (198–479)	144 (119–175)	**0.003**	303 (111–826)	134 (88–202)	0.191
MMA >271 nmol/L	8 (38.1%)	6 (14.3%)	0.052 **	3 (50.0%)	1 (8.3%)	0.083

	HS-U (n = 21)	Control (n = 42)	P value	HS-NU (n = 6)	Control (n = 12)	P value
Functional vitamin B12 deficiency (vitamin B12 <256 pmol/L and MMA >271 nmol/L)	1 (4.8%)	2 (4.8%)	1.000 **	1 (16.7%)	0	0.333 **

HS-U: hereditary spherocytosis patients who were using daily 5mg of folic acid; HS-NU: hereditary spherocytosis patients who were not using folic acid; UMFA: unmetabolized folic acid; THF: tetrahydrofolate; MeFox: oxidation product of 5-methy-THF; MMA: methylmalonic acid; tHcy: plasma total homocysteine. The 5,10-methenyl-THF and 5-formyl-THF were below the limit of detection (0.2 nmol/L).

1 Microbiological assay

2 LC/MS-MS

3 Maximum value detectable in calibration curve for serum folate. All forms of folate, vitamin B12 and total homocysteine were presented as geometric mean values and 95% confidence intervals

4 Chemiluminescence

5 Presented as dietary folate equivalents (DFEs), 1μg of DFE= 1 μg of natural folate sources = 0.6 μg of folic acid from fortified foods. Groups were compared using the Mann-Whitney test. Categorical variables were shown as number of subjects and percentage (in parentheses).

*** Chi-square test

**** likelihood ratio. No differences were found when comparing folate and energy intake data from the HS-U and HS-NU groups. However serum folate forms concentrations were higher in HS-U when compared with HS-NU: serum folate (both assays P<0.001); UMFA (P=0.003); 5-methyl-THF (P<0.001); MeFox (P=0.009), and no differences were found between two groups THF (P=0.076); total homocysteine (P=0.102); vitamin B12 (P=0.930) and MMA (P=0.779).

Table 3.

Hematological and biochemical parameters in subgroups of patients with hereditary spherocytosis and their controls

	HS-U (n=21)	Control (n=42)	P value	HS-NU (n=6)	Control (n=12)	P value
RBC (x10^{12}/L)	3.53 (3.22–3.86)	4.48 (4.35–4.61)	**<0.001**	3.64 (3.28–4.03)	4.59 (4.31–4.90)	**<0.001**
Hemoglobin (g/L)	109 (101–118)	135 (131–139)	**<0.001**	112 (94–133)	137 (128–147)	**0.007**
Reticulocytes (%)	5.0 (3.3–7.6)	0.9 (0.8–1.1)	**<0.001**	3.7 (1.3–10)	0.9 (0.8–1.2)	**0.021**
Reticulocytes (x10^{11}/L)	1.76 (1.20–2.59)	0.42 (0.36–0.50)	**<0.001**	1.34 (0.46–3.92)	0.43 (0.34–0.54)	**0.028**
Anemia	16 (76.2)	3 (7.1)	**<0.001** *	5 (83.3)	0	**0.001** **
WBC (x10^{9}/L)	9.1 (7.3–11.3)	6.4 (5.9–6.8)	**<0.001**	6.6 (4.0–10.8)	6.6 (5.8–7.4)	0.708
Lymphocytes (x10^{9}/L)	3.2 (2.5–4.2)	2.5 (2.3–2.7)	0.076	2.2 (1.1–4.5)	2.3 (1.9–2.7)	0.543
NK cells x10^{9}/L	0.91 (0.68–1.24)	0.52 (0.44–0.63)	**<0.001**	0.74 (0.28–1.98)	0.47 (0.32–0.70)	0.119
LDH (U/L)	635 (568–710)	405 (377–436)	**<0.001**	628 (517–763)	449 (396–508)	**0.004**
Hs-CRP (mg/dL)	0.16 (0.10–0.24)	0.20 (0.13–0.31)	0.640	0.14 (0.04–0.51)	0.20 (0.08–0.46)	0.480
IL-6 (pg/mL)	1.28 (0.83–1.98)	0.94 (0.72–1.22)	0.076	1.22 (0.30–4.88)	1.37 (1.04–1.81)	0.482
IL-8 (pg/mL)	26.00 (14.40–47.10)	8.66 (7.31–10.30)	**<0.001**	38.3 (9.58–153)	15.4 (8.60–27.60)	0.122
IL-10 (pg/mL)	2.51 (1.50–4.21)	1.63 (1.02–2.60)	0.113	3.71 (0.94–14.70)	2.21 (1.11–4.41)	0.303
IFN-γ (pg/mL)	4.80 (3.48–6.63)	4.39 (3.27–5.88)	0.965	5.66 (3.31–9.67)	4.44 (2.65–7.45)	1.000
TNF-α (pg/mL)	9.95 (7.51–13.2)	5.39 (4.65–6.25)	**<0.001**	12.1 (7.44–19.7)	5.42 (3.50–8.38)	**0.028**

HS-U: hereditary spherocytosis patients, who were using daily 5mg of folic acid, HS-NU: hereditary spherocytosis patients, who were not using folic acid. RBC: red blood cells; WBC: white blood cells; LDH: lactate dehydrogenase; Hs-CRP: high-sensitivity serum C-reactive protein; IL-6: serum interleukin-6; IL-8: serum interleukin-8; IL-10: serum interleukin-10; IFN-γ; serum interferon-γ; TNF-α; serum tumor necrosis factor-α. Variables were presented as geometric mean values and 95% confidence interval. Anemia was presented as number of subjects (percentage). Anemia was characterized by hemoglobin <130 g/L and <120 g/L, respectively for man and woman. Groups were compared using Mann-Whitney test.

*Chi-square test

**Likelihood ratio. No differences were found when comparing the data from the HS-U and HS-NU groups for RBC (P=0.793); hemoglobin (P=0.502); reticulocytes % (P=0.255) and number (P=0.448); frequency of anemia (P=1.000); WBC (P=0.153); lymphocytes (P=0.221); NK cells (P=0.976); LDH (P=0.861); Hs-CRP (P=0.977); IL-6 (P=0.521); IL-8 (P=0.502); IL-10 (P=0.220); IFN-γ (P=0.641) and TNF-α (P=0.540).

Table 4.

Spearman correlations between serum concentations of UMFA and cytokines in HS-U and control groups

	IL-6	**IL-8**	**IL-10**	**IFN-γ**	**TNF-α**
HS-U patients (n=21)					
UMFA	rho=−0.003, P=0.989	rho=0.219, P=0.339	rho= −0.400, P=0.072	rho= −0.396, P=0.076	rho= 0.213, P=0.354
IL-6		rho=0.359, P=0.110	rho= 0.390, P=0.081	rho= 0.374, P=0.095	rho= 0.258, P=0.259
IL-8			rho= 0.051, P=0.827	rho= −0.166, P=0.471	rho= 0.177, P=0.444
IL10				rho= **0.455, P=0.038**	rho= 0.175, P=0.447
IFN-γ					rho= −0.335, P=0.137
Controls (n=54)					
UMFA	rho= 0.193, P= 0.170	rho= 0.175, P=0.213	rho= −0.081, P=0.566	rho=−0.021, P=0.885	rho=−0.052, P=0.715
IL-6		rho= **0.423, P=0.001**	rho= **0.629, P<0.001**	rho= **0.460, P<0.001**	rho= 0.217, P=0,115
IL-8			rho= 0.160, P=0.249	rho= 0.132, P=0.342	rho= 0.197, P=0.153
IL10				rho= **0.456, P=0.001**	rho= 0.059, P=0.670
IFN-γ					rho= 0.170, P=0.219

rho= Spearman correlation index. Spearman correlations were not done in HS-NU due small number of patients (n=6).