Folate status in Italian blood donors: relation to gender and smoking

Arturo Cafolla,* Francesco Dragoni,* Gabriella Girelli,* Maria Elena Tosti,° Alessandro Costante,*
Donatella Pastorelli,* Giorgio Bedogni,# Steve Scott@

*Dipartimento di "Biotecnologie Cellulari ed Ematologia" University "La Sapienza", Rome, Italy;
°Laboratory of Epidemiology, Istituto Superiore di Sanità, Rome, Italy; #Istituto Scienza della Nutrizione,University of
Modena, Italy; @Abbott Diagnostics, Wiesbaden-Delkenheim, Germany

ABSTRACT

Background and Objectives. Folate deficiency in the general population is associated with a risk of cardiovascular disease and various cancers. The aim of this study was to evaluate folate status in Italian blood donors and its relationship with gender and smoking habit.

Design and Methods. A prospective study of 201 first visit donors (99 males and 102 females) was undertaken to evaluate folate status by measuring serum folate (SF) and red blood cell folate (RCF) levels and relating these with gender and smoking habit (100 smokers and 101 non-smokers).

Results. The rates of SF level less than 6.8 nmol/L and RCF less than 340 nmol/L were 9.9% and 25.3%, respectively in Italian blood donors. Mean RCF level was significantly lower ($p<0.05$) in females than in males and in smokers compared to in non-smokers ($p<0.001$). The risk of reduced RCF levels in smokers was related to the number of cigarettes smoked per day, more than nine cigarettes increased the relative risk (RR) of low RCF level to 2.93 (95% C.I.: 1.34-6.41).

Interpretation and Conclusions. This study suggests that folate deficiency, evaluated by RCF and SF levels, is widespread in Italian blood donors. Moreover, RCF values seem related to gender in non-smokers and modified by smoking habit, according to the number of cigarettes smoked per day.
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Key words: red blood cell folate, serum folate, Italian blood donors, gender, smoking habit

Renewed interest in the evaluation of folate status in the general population has been generated by the finding of an inverse association between folate status deficiency and the risk of cardiovascular disease[1] and various cancers.[2]

Folic acid in food exists in both monoglutamate and polyglutamate forms,[3] the latter being converted during digestion, to monoglutamate by pteroylpolyglutamate hydrolase, an enzyme which is most active at pH 5.5 in the presence of zinc.[4] The absorption, which occurs predominantly in the jejunum[5] is related to diet and daily vitamin intake, which can quickly modify the serum folate (SF) level. The intra-erythrocyte folate content is 30 fold higher than that of serum folate; the transport and incorporation of serum folate into erythroblasts is facilitated by folate receptors during erythropoiesis.[6] Progressive deficiency of SF will, therefore, first induce a decrease in the red blood cell folate (RCF) concentration.[7] On the other hand myelodysplastic patients with dyserythropoiesis due to BFU-E/CFU-E damage have been shown to have pathologically high levels of RCF and SF, both inversely correlated to hemoglobin level.[8] Additionally, a reduced RCF concentration can result from a low level of vitamin B_{12} even when the SF concentration itself is normal.[9]

A study reported that cyanide intake associated with cigarette smoking adversely affected folate and vitamin B_{12} status; reduced concentrations of serum B_{12} vitamin and RCF were more frequently found in smokers compared to in non-smokers.[10] It has recently been reported that a SF below 6.8 nmol/L is a mild deficiency whereas a RCF level below 340 nmol/L indicates a more advanced folate deficiency.[11] Folate deficiency may make a large contribution to the increased cardiovascular risk associated with hyperhomocysteinemia[1] and low SF concentration is a major cause of increased plasma homocysteine.[12] This latter epidemiological study showed that 23% of control subjects had plasma folate concentrations lower than 6.8 nmol/L. Micronutrient deficiencies of vitamins are actually considered one of the major causes of DNA damage.[13] Folic acid is essential for the synthesis and repair of DNA, the instability of which increased in human lymphocytes cultured under folate–deficient conditions.[14,15] Moreover, subjects with a low folate level have a higher risk of developing hematologic malignancies.[16]

The aim of this prospective study was to evaluate folate status in Italian blood donors. Furthermore we evaluated whether SF and RCF levels were related to gender and smoking habit.

Design and Methods

Subjects studied

A total of 201 blood donors who attended the Blood Bank (*Department of Biotechnology and Hematology, Rome, Italy*) and gave informed consent were analyzed in this study. According to law Italian donors are tested three times year for viral infections, hematologic parameters and ferritin. Subjects consecu-

Correspondence: Arturo Cafolla, M.D., via R. Fucini 204, 00137 Rome, Italy. E-mail: cafolla@bce.med.uniroma1.it

tively enrolled were grouped by sex and smoking habit; all these who were taking drugs, vitamin supplementation or oral contraceptive therapy were excluded. The sample group comprised 99 males and 102 females between 20 and 60 years of age (mean age: 37.8±10.4 years). Out of 102 females, eleven (six smokers and five non-smokers) were older than 50 years without menses. One hundred of the blood donors (49 males and 51 females) were current smokers, with the number of cigarettes smoked per day ranging from 5 to 40. None of the subjects in the smoking group had smoked for at least three hours prior to blood collection. The remaining 101 donors (50 males and 51 females) had no history of smoking either cigarettes or other tobacco products. Each subject filled in a questionnaire on medical history and lifestyle, specifically designed to obtain information about the daily intake of wine, fruit and vegetables. The amount of fruit and vegetables was categorized according to the Guidelines of the *Italian Society of Nutrition*[17] as follows: sub-optimal (score=1), optimal (score=2) and supra-optimal (score=3). Pregnancy status and smoking habits were recorded for each donor.

Hemoglobin electrophoresis and estimation of HbA2 were carried out on all subjects to exclude abnormalities of globin synthesis and thalassemia syndromes.

Specimen collection

Informed consent was obtained from each donor prior to the collection of samples. For the measurements of serum folate, vitamin B_{12} and ferritin, 10 mL of blood were collected into plain (non-anticoagulated) tubes. For the determination of red blood cell folate and the full blood count a further 5 mL of blood were collected into tubes with EDTA anticoagulant.

Analytical measurements

A full blood count (Abbott Diagnostics Cell-Dyn CD 3500 automated hematology system) was performed within three hours of collection. Within this same time, vitamin B_{12}, ferritin, serum and red blood cell folate were measured using a microparticle enzyme immunoassay (MEIA) method with IMx (Abbott Diagnostics)[18] according to standard practice. The RCF was determined on red blood cell lysate obtained by incubating, at room temperature for 90 minutes, 50 μL of whole blood with 100 μL of lysis reagent containing 1% ascorbic acid. The pH of the red blood cell lysate was 4.4.

Data analysis

All data were analyzed using BMDP statistical software.[19] Fisher's exact probability test and the chi-squared test were used to evaluate differences in the distribution of ordinal or categorized parameters between the investigated groups. The non-parametric Mann-Whitney U test was used to determine the differences in hematologic parameters in the male and female groups of blood donors and the smoking and the non-smoking groups. A multiple logistic regression model was used to evaluate the relative risk (RR) of low RCF levels and the 95% confidence intervals in the various groups divided according to gender and number of cigarettes smoked per day. The level of statistical significance was set at $p<0.05$.

Results

Subjects and smoking pattern

None of blood donors had been taking vitamin supplementation in the six months prior to this study. Alcohol intake was less than 250 mL of wine per day and none of the donors drank spirits. This habit was homogeneous for all groups independent of sex, age and smoking habit. None of the women was pregnant. All blood donors ate fresh fruit and vegetables at least once daily; out of 201 subjects 79 (39.3%) had a sub-optimal, 76 (37.8%) an optimal and 46 (22.9%) a supra-optimal intake of fresh fruit. In the smokers, the intake of fresh fruit was sub-optimal in 43 (43%), optimal in 37 (37%) and supra-optimal in 20 (20%). The intake of fresh fruit among non-smokers was sub-optimal in 36 (35.6%), optimal in 41 (40.6%) and supra-optimal in 24 (23.8%). Out of 201 subjects, 147 (73.1%) had a sub-optimal, 46 (22.9%) an optimal and 8 (4%) a supra-optimal intake of vegetables. According to smoking habit, among the smokers the intake of vegetables was sub-optimal in 67 (67%), optimal in 30 (30%) and supra-optimal in 3 (3%), whereas among the non-smokers the intake of vegetables was sub-optimal in 79 (78.2%), optimal in 19 (17.8%) and supra-optimal in 3 (3%). No significant difference was observed between smokers and non-smokers concerning the intake of fresh fruit or vegetables.

The age distribution of the subjects in the groups of smokers and non-smokers was similar (Table 2). The mean number of cigarettes smoked daily was 19±7 (range: 5-40) in male smokers and 14±7 (range: 5-30) in female smokers; the non-parametric Mann-Whitney test confirmed a significant ($p = 0.01$) difference between the number of cigarettes smoked by men and women.

No evidence of either hemoglobinopathy or thalassemia was found in any blood donor.

Table 1. Comparison of hematologic parameters according to sex.

	Males	Females	p
Number of subjects	99	102	
Hemoglobin (g/dL)	14.44±0.85	12.92±0.72	<0.001
Hematocrit (%)	43.42±2.78	38.65±2.5	<0.001
Serum ferritin (r. r.: 9-190 mg/L)	57±31	28±19	<0.001
Serum folate (r. r.: 6.8-26.5 nmol/L)	10.7±3.6	11.3±3.0	n. s.
RBC folate (r. r.: 397-1334 nmol/L)	518±190	458±136	<0.05
Vitamin B_{12} (r. r.: 210-940 pmol/L)	434±174	422 ±162	n. s.

Values are in SI units. r.r.: reference ranges; results are shown as mean values±SD. Data comparisons made by Mann-Whitney U test; n.s. = not significant (p > 0.05).

Table 2. Comparison of hematologic parameters according to smoking habit.

	Non-smokers	Smokers	p
Number of subjects	101	100	
Mean age (years)	34.5±10.8	37.1±9.6	n.s.
Hemoglobin (gr/dL.)	13.58±1.10	13.76±1..08	n.s.
Hematocrit (%)	40.5±3.6	41.5±3.5	n.s.
Serum ferritin (r. r.: 9-190 mg/L)	41±29	44±31	n.s.
Serum folate (r. r.: 6.8-26.5 nmol/L)	11.3±3.6	10.7±3.0	n.s.
RBC folate (r. r.: 397-1334 nmol/L)	531±187	445±132	<0.001
Vitamin B_{12} (r. r.: 210-940 pmol/L)	423±182	431±152	n.s.

Values are in SI units. r.r.: reference ranges; results are shown as mean values±SD. Data comparisons made by Mann-Whitney U test; n.s. = not significant (p> 0.05).

Analytical measurements

Out of the 201 blood donors, 51 (25.3%) had a RCF level lower than 340 nmol/L and 20 (9.9%) subjects had a SF level lower than 6.8 nmol/L. Hematologic parameters of the donors, divided according to sex, are reported in Table 1. All subjects had a vitamin B_{12} level over 210 pg/L. Serum ferritin concentration was significantly ($p<0.001$) lower in females than in males; however, the women's mean serum ferritin concentration was higher than the reference lower limit. Five female non-smokers older than 50 without menses had a mean serum ferritin concentration of 52±10 µg/L and a mean RCF of 499±53 nmol/L, not different to that observed among males. Serum ferritin and RCF of females older than 50 were both significantly ($p<0.05$) higher than those observed among females with menses (mean serum ferritin 23±14 µg/L; mean RCF 405±45 nmol/L). The level of RCF was also slightly lower in females but there were no sex-related differences for either SF or serum vitamin B_{12} concentrations.

The mean serum ferritin, SF and vitamin B_{12} concentrations analyzed according to smoking habits (Table 2) did not differ significantly between the group of 101 non-smokers and the group of 100 smokers (each group comprising a balanced number of males and females). Out of 20 subjects who had a SF concentration below 6.8 nmol/L, 14 were smokers and six non-smokers.

Out of 100 smokers, 30 (30%) had a RCF level below 340 nmol/L, whereas among the non-smokers 21 (20.8%) did ($p=$ n. s.). The mean RCF level of smokers was significantly lower (445 nmol/L) than that of non-smokers (531 nmol/L, $p<0.001$) (Table 2).

Dividing the distribution of RCF levels into percentiles, the 10th percentile was 316 nmol/L in female non-smokers and 312 nmol/L in female smokers. In the present study, 5/50 (10%) male non-smokers had an RCF level lower than 364 nmol/L, and this level was the 10th percentile for this group. However, among the 49 male smokers, 26 (53%) had a RCF lev-

Table 3. Comparison of evaluated parameters in smokers and non-smokers according to sex.

Sex Parameter	Non-smokers	Smokers	p
Male (n= 99)			
Hemoglobin	14.4±0.85	14.6±0.86	n. s.
Hematocrit	43.2±2.8	43.6±2.7	n. s.
Serum ferritin	58±30	57±32	n. s.
Serum folate	11.2±3.8	10.4±3.8	n. s.
RBC folate	592±198	443±132	< 0.001
Vitamin B_{12}	419±201	438±143	n. s.
Female (n = 102)			
Hemoglobin	12.9±0.70	13.0±0.73	n. s.
Hematocrit	38.1±2.3	39.4±2.6	n. s.
Serum ferritin	24±13	28±19	n. s.
Serum folate	11.4±3.2	11.3±2.6	n. s.
RBC folate	470±137	463±120	n. s.
Vitamin B_{12}	420±164	404±161	n. s.

Results shown as mean values±SD; n.s.: not significant.

el less than 364 nmol/L; the 10th percentile for male smokers was 294 nmol/L.

In order to investigate these observations further, the male and female groups were analyzed separately to determine whether the apparent effect of smoking on RCF was independent of the gender of the smokers. Male smokers had a significantly lower mean level of RCF (443 nmol/L) than non-smokers (592 nmol/L), but no such difference was seen between female smokers (463 nmol/L) and non-smokers (470 nmol/L) (Table 3). Among non-smokers the mean RCF value was significantly higher in males than in females (592±32 vs 470±20 nmol/L, $p<0.001$). No other parameter examined (hemoglobin, hematocrit, serum ferritin and vitamin B_{12}) was influenced by smoking in either sex (Table 3).

Multiple logistic regression confirmed the significant influence that smoking habit had on RCF levels in males (Table 4) and suggested that a relative risk of reduced RCF became significant if more than nine cigarettes were smoked per day.

Discussion

Many studies have shown an inverse association between folate status and the risk of cardiovascular disease, thrombosis and various cancers; others studies have led to a renewed interest in the evaluation of RCF as an indicator of folate deficiency risk in women[20] and RCF is considered a significant marker of a risk of folate deficiency.[21] Folate status is related to daily intake and polymorphisms of methylenetetrahydrofolate reductase (MTHFR).[22] Low plasma levels of folic acid were associated with an increased risk of carotid artery stenosis, independently of homocysteine plasma levels.[23,24] Folic acid is essential for the synthesis and repair of DNA; lymphocytes cultured under folate-deficient conditions were unable to repair oxidative DNA damage efficiently.[14] Increased chromosome fragility was found to be associated with smoking and blood folate level.[25] Previous studies separately reported the relationship

Table 4. Relative risk of reduced RCF according to sex and number of cigarettes in smokers.

Rate ratio and 95%c.i. for low red cell folate stratified for sex.

		R.R	95% C.I.
Smokers	Men	4.32	1.76-10.6
	Women	1.47	0.67-3.25

Rate ratio and 95% C.I. for low red cell folate in smokers according to number of cigarettes smoked per day.

Cigarettes N°	R.R	95% C.I.
0	1.00	
1-9	1.01	0.34-3.02
10-19	2.93	1.34-6.41
>20	2.86	1.37-5.98

Rate ratio and 95% C.I. for low red cell folate in smokers.

	R.R	95% C.I.
Sex	1.57	0.88-2.81
Smoke	2.41	1.35-4.30

between folate status and smoking among 123 non-pregnant women[26] and the influence of smoking on folate intake and blood folate concentration among 72 elderly men.[27]

The present study, performed among 201 subjects balanced for sex and smoking habit, shows that many Italian blood donors have folate deficiency, as evaluated by low levels of SF and RCF, and this is particularly true among smokers, as already reported.[28] Although the rate (9.9%) of SF is lower than that (23%) observed among USA controls subjects,[12] a large proportion of Italian blood donors do not have an adequate intake of fruit and vegetables, according to guidelines from the *Italian Society of Nutrition*. In this study out of 201 blood donors, 76 (39.3%) had a sub-optimal intake of fresh fruits and 147 (73%) a sub-optimal intake of vegetables. Although the intake of fresh fruit and vegetables was not statistically different between smokers and non-smokers, the mean RCF level of smokers was significantly lower than that of non-smokers. This study confirms that smokers had a reduced RCF level; moreover it suggests that RCF concentration is related to the number of cigarettes smoked per day and that this does not seem a transient effect of smoke.[10] In fact, the multivariate analysis showed that the relative risk of low RCF in smokers increased almost three-fold when the number of cigarettes smoked per day was more than nine. Moreover, the relative risk of low RCF level seems significantly related to smoking habit but not to gender in smokers. Instead, this study suggests that the normal values and reference range of RCF in non-smokers should be redefined according to sex. Thus, according to sex, RCF level could be defined as low

when it is less than 340 nmol/L in males and less than 316 nmol/L in females. Excluding a smoking related effect, in non-smokers the difference between RCF folate levels in males and females was statistically significant. Since both vitamin B_{12} and serum folate levels in female groups were similar to those in the male groups, the lower values of RCF and serum ferritin in females younger than 50 should be related to other factors. An epidemiological study reported that menstrual blood loss seems to be an important factor for the development of anemia in women of fertile age;[29] according to this hypothesis, the present study confirms this, showing that mean RCF and serum ferritin levels were significantly lower in women with menses compared to in women older than 50 without menses. Although there were only 5 female non-smokers older than 50 without menses, these 5 women had similar serum ferritin and RCF levels to those of male non-smokers over the age of 50 years.

The present study suggests that folate deficiency, better evaluated by RCF level than by SF level, is widespread in Italian blood donors. Moreover, RCF values seem to be related to gender in non-smokers and modified by the number of cigarettes smoked per day.

Potential implications for clinical practice

- Folate status, better evaluated by red blood cell folate (RCF) than serum folate alone, should be evaluated in all blood donors, especially in those who smoke.
- Folate status plays a key role in homocysteine metabolism and DNA repair mechanisms.
- In cases of proven folate deficiency a planned program of increased daily intake of folate could reduce the effects of folate deficiency on homocysteine metabolism (thrombotic risk) and DNA repair mechanisms.

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