

Montserrat Creus, Ramon Deulofeu, Joana Peñarrubia, Francisco Carmona and Juan Balasch\*

# Plasma homocysteine and vitamin B12 serum levels, red blood cell folate concentrations, C677T methylenetetrahydrofolate reductase gene mutation and risk of recurrent miscarriage: a case-control study in Spain

## Abstract

**Background:** Hyperhomocysteinemia and methylenetetrahydrofolate reductase (*MTHFR*) gene mutation have been postulated as a possible cause of recurrent miscarriage (RM). There is a wide variation in the prevalence of *MTHFR* polymorphisms and homocysteine (Hcy) plasma levels among populations around the world. The present study was undertaken to investigate the possible association between hyperhomocysteinemia and its causative genetic or acquired factors and RM in Catalonia, a Mediterranean region in Spain.

**Methods:** Sixty consecutive patients with  $\geq 3$  unexplained RM and 30 healthy control women having at least one child but no previous miscarriage were included. Plasma Hcy levels, *MTHFR* gene mutation, red blood cell (RBC) folate and vitamin B12 serum levels were measured in all subjects.

**Results:** No significant differences were observed neither in plasma Hcy levels, RBC folate and vitamin B12 serum levels nor in the prevalence of homozygous and heterozygous *MTHFR* gene mutation between the two groups studied.

**Conclusions:** In the present study RM is not associated with hyperhomocysteinemia, and/or the *MTHFR* gene mutation.

**Keywords:** folate; homocysteine; *MTHFR* polymorphism; recurrent miscarriage; vitamin B12.

\*Corresponding author: Juan Balasch, MD, Clinical Institute of Gynecology, Obstetrics and Neonatology, Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Faculty of Medicine-University of Barcelona, C/Casanova 143, 08036 Barcelona, Spain, Phone: +34 93 2275436, Fax: +34 93 2275454, E-mail: jbalasch@ub.edu

Montserrat Creus, Joana Peñarrubia and Francisco Carmona: Clinical Institute of Gynecology, Obstetrics and Neonatology, Hospital Clínic-Institut d'Investigacions Biomèdiques

August Pi i Sunyer (IDIBAPS), Faculty of Medicine-University of Barcelona, Barcelona, Spain

Ramon Deulofeu: Biochemistry Laboratory, Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Faculty of Medicine-University of Barcelona, Barcelona, Spain

## Introduction

Homocysteine (Hcy) is an intermediate product of methionine metabolism, an essential amino acid provided by dietary proteins. Part of Hcy is combined irreversibly with serine generating cystathionine, an enzyme-mediated reaction by the cystathionine beta-synthase (CBS) and the cofactor vitamin B6. However, most Hcy is re-methylated again forming methionine in a process requiring a proper function of several enzymes. Methionine synthase (MS) adds a methyl group to Hcy in the presence of vitamin B12 as a cofactor using the 5-methyltetrahydrofolate (5-methyl-THF) as co-substrate. 5-methyl-THF production needs an adequate supply of reduced folate and an appropriate methylenetetrahydrofolate reductase (*MTHFR*) function. Therefore, impaired enzyme function or cofactor deficiencies can cause elevated levels of Hcy. Hence hyperhomocysteinemia can be acquired by a deficiency of folates or vitamin B12 and B6 supply [1–4] or may be genetic due to a *MTHFR* gene mutation [5, 6].

Several published reports regarding the prevalence of hyperhomocysteinemia and *MTHFR* gene mutation in women with recurrent miscarriage (RM), have shown dissenting and inconclusive results as evidenced in a systematic review and meta-analysis of the literature [7]. The most common and studied *MTHFR* gene mutation is produced by the substitution of cytosine (C) (normal) by thymine (T) (mutated) at nucleotide 677 (677C>T), which converts an alanine residue to a valine. A homozygosity mutation of the gene encoding *MTHFR* (T/T) produces a thermolabile variant that results in an enzyme with

reduced stability and specific activity [8]. Homozygosity is associated with hyperhomocysteinemia [5, 6], and also heterozygosity among patients with low folate intake [9]. Furthermore, the presence of the *MTHFR* gene mutation itself has also been related to RM [10].

Hyperhomocysteinemia promotes vascular disease by a variety of mechanisms [11–15]. Hcy also has procoagulant properties [16–18]. In pregnancy, elevated maternal Hcy concentrations are associated with defective chorionic villous vascularization that interferes with embryonic development [19]. Although it is not clear whether hyperhomocysteinemia is a cause or only an associated risk factor for RM, some studies have suggested that reducing circulating Hcy levels in these patients by the addition of group B vitamin supplements may have a beneficial effect in the subsequent pregnancy [20–22]. Remarkably, to add further to the above discussed controversy, different studies have reported a wide variation in the prevalence of the *MTHFR* gene polymorphisms and Hcy plasma levels among populations around the world [23–28].

Therefore, this study was undertaken to investigate the overall factors associated with hyperhomocysteinemia, mainly the *MTHFR* C677T mutation, red blood cell (RBC) folate concentrations, and serum levels of vitamin B12 in patients with RM, a matter which, to the best of our knowledge, has not been previously reported in Spain.

## Materials and methods

### Subjects and study design

We included 60 patients aged 26–41 years (mean, 34 years) with a history of  $\geq 3$  (mean 3.44, range 3–5) consecutive spontaneous miscarriages of unknown etiology  $\leq 10$  weeks' gestation (RM group). All patients underwent a complete diagnostic work-up for RM including screening for systemic diseases, diabetes mellitus, thyroid dysfunction, polycystic ovary disease, a chromosome assessment of the woman and her partner, uterine abnormalities, endometrial and hormonal luteal phase defects, endometrial and cervical infection, and thrombophilia (antiphospholipid antibodies, plasma levels of protein S and C, antithrombin, factor V Leiden and prothrombin G20210A mutations, acquired protein C resistance). All were negative for the above reported investigations.

The control population consisted of 30 healthy women aged  $< 41$  years and having at least one healthy child (mean 1.37, range 1–3) but no previous miscarriage (control group). They were attending our center for routine gynecological examination during the study period.

Informed consent was obtained from all patients to be included in the current study which was approved by the Ethics Committee of the Hospital Clinic, Barcelona. All were Caucasian and from the same geographical area, the Autonomous Community of Catalonia, a Mediterranean region in Spain. All had normal renal function and

none taken vitamin supplementation or hormonal treatment at least 6 months before inclusion in the study.

The sample size and study design (2 cases/1 control) were decided arbitrarily but in keeping with previous studies on the subject leading to contradictory results [29–31].

In all women plasma Hcy, RBC folate, vitamin B12 serum levels and *MTHFR* gene polymorphisms were determined. In addition, other factors which may affect Hcy levels such as age of the patient, body mass index (BMI), renal function [32], and consumption of  $> 10$  cigarettes/day [33] were also taken into account.

### Sample and data collection

Laboratory determinations were performed at least 6 months after the last pregnancy. All blood samples were drawn between 08.30 and 10.00 h after overnight bed rest and fasting and without smoking (only water was allowed). To measure Hcy, blood was obtained in an EDTA-K tube and plasma was separated and stored at  $-80^{\circ}\text{C}$  until analysis. Vitamin B12 was measured in a light protected serum. RBC folate concentration was measured in whole blood obtained in a light protected EDTA-K tube that was hemolyzed in a solution of ascorbic acid and the intraerythrocytic content was calculated according to patient's hematocrit values.

After processing the samples the three parameters were measured by an automated electrochemoluminescence immunoassay system Advia-Centaur (Siemens, Barcelona, Spain) as previously reported [34–36]. The intra-assay coefficient variations (CV) for Hcy, vitamin B12 and RBC folate were  $< 6.8\%$ ,  $< 7.9\%$  and  $< 7.3\%$ , respectively. The inter-assay CV for Hcy, vitamin B12 and RBC folate were  $< 7\%$ ,  $< 9.8\%$  and  $< 10.3\%$ , respectively. Reference ranges in our laboratory are: 5–15  $\mu\text{mol/L}$  for homocysteine, 250–1050  $\text{pg/mL}$  for vitamin B12 and 250–1050  $\text{ng/dL}$  for RBC folate.

### DNA extraction and genotyping

*MTHFR* polymorphisms were analyzed in DNA extracted from heparinized or EDTA-K blood and measured by Real Time PCR using primers and probes contained in the lightMix-Kit *MTHFR* C677T, Cat No 40-0095-16 (Roche Diagnostics, Barcelona, Spain).

The primers used were: Pr Sense: AGG CCA GCC TCT GAC TG Pr Reverse: AGG ACG GTG CGG TGA GAG TG and fluorescent probes: Flu: TGA CCT GAA GCA CTT GAA GGA GAA GGT GTC – FL y LC-Red640-CGG GAG CCG ATT TCA TCA – PH. Using this system the wild type showed a melting peak at  $63^{\circ}\text{C}$  and the mutant a melting peak at  $54.5^{\circ}\text{C}$ .

### Statistics

Data were analyzed with the SPSS statistical software version 14.0. A comparative analysis of the data was performed, obtaining frequencies and percentages for qualitative variables and median and standard deviation (SD) for quantitative variables. The analysis of the differences between the two groups was performed using the Mann-Whitney test for quantitative variables and the  $\chi^2$ -test for qualitative variables. Then, a multivariate analysis using the enter mode was

performed to corroborate results. The analysis of the differences between RM patients with low and normal RBC folate levels and the control group was performed with the Kruskal-Wallis test for quantitative variables and the  $\chi^2$ -test for qualitative variables. Statistical significance was defined as a  $p < 0.05$ .

## Results

The baseline patient characteristics, including creatinine serum levels, were similar in the two groups studied (Table 1). However, as expected, the mean number of previous miscarriages and term pregnancies were significantly higher in RM and control groups, respectively, Hcy plasma levels were similar in both groups (Table 2). In the RM group only one patient (1.6%) had plasma Hcy values  $>15$   $\mu\text{mol/L}$ . This patient was 33 years old, had three previous spontaneous abortions between 8 and 10 weeks' gestation and was homozygous (TT) for the *MTHFR* gene mutation with normal vitamin B12 and RBC folate levels. In the control group, also only one patient (3.3%) aged 29 years and having two children had plasma Hcy values  $>15$   $\mu\text{mol/L}$ . She had a homozygous (TT) *MTHFR* mutation and normal vitamin B12 and RBC folate levels.

There were no significant differences in RBC folate levels between the two groups. Nevertheless, the number of patients with RBC folate levels lower than normal was significantly higher ( $p < 0.02$ ) in RM group as compared with controls (Table 2). Both groups were similar with respect to vitamin B12 serum levels and *MTHFR* gene polymorphisms (Table 2). Results presented in Table 2 were corroborated in a multivariate analysis model (Table 3).

Table 4 summarizes RBC folate levels, plasma levels of Hcy, serum concentrations of vitamin B12, and *MTHFR* gene polymorphisms in RM patients having RBC folate within the normal range, those RM subjects with low RBC, and controls. As expected, mean RBC folate levels were

Variable	RM (n=60)	Controls (n=30)	p-Value
Age, years	35 $\pm$ 4	35.8 $\pm$ 3.1	NS
Body mass index, kg/m <sup>2</sup>	23.9 $\pm$ 3.4	24.7 $\pm$ 3.9	NS
Tobacco $>10$ cigarettes/day	8 (13.3%)	4 (13.3%)	NS
Term pregnancies	0.4 $\pm$ 0.5	1.3 $\pm$ 0.6	$<0.001$
Previous miscarriages	3.4 $\pm$ 0.7	0	$<0.0001$
Serum creatinine, $\mu\text{mol/L}$	88.40	88.40	NS

**Table 1** Patients baseline characteristics.

Results are expressed as median $\pm$ SD or n (%). NS, not significant; RM, recurrent miscarriage.

Variable	RM (n=60)	Controls (n=30)	p-Value
Homocysteine, $\mu\text{mol/L}$	7.7 $\pm$ 2.3	8.1 $\pm$ 3.3	NS
RBC folate, ng/dL	374.6 $\pm$ 189.2	401.3 $\pm$ 135.7	NS
Patients with low RBC folate, n	16 (26.6%)	1 (3.3%)	$<0.02$
Vitamin B12, pg/mL	518.2 $\pm$ 180.7	477.3 $\pm$ 146.9	NS
<i>MTHFR</i>			
Homo+/+ (TT)	11 (18.3%)	4 (13.3%)	NS
Homo -/- (CC)	23 (38.3%)	13 (43.3%)	NS
Hetero +/- (CT)	26 (43.3%)	13 (43.3%)	NS

**Table 2** Homocysteine, red blood cell folate, vitamin B12 and *MTHFR* polymorphisms in the two groups studied.

Results are expressed as median $\pm$ SD or n (%). *MTHFR*, methylenetetrahydrofolate reductase; NS, not significant; RBC, red blood cell; RM, recurrent miscarriage.

significantly lower in RM patients having low values for this parameter as compared with RM group with normal RBC folate and control women. However, Hcy plasma levels, vitamin B12 serum levels, and *MTHFR* gene polymorphisms were similar in the three groups considered (Table 4).

## Discussion

RM is frustrating for both patients and clinicians because a causative etiology cannot be identified in more than 50% of the cases [37, 38]. At present, one of the possible causes increasingly investigated in the literature is thrombophilic status which may alter the placental circulation.

In normal pregnancy the plasma concentrations of the different proteins involved in blood coagulation undergo changes that result in a physiological procoagulant state in order to decrease hemorrhagic risk at delivery. However, this may increase the development of thromboembolic complications especially in cases with associated genetic or acquired risk factors for thrombosis [39–41].

Hyperhomocysteinemia and the *MTHFR* gene mutation are part of these thrombotic risk factors and a number

Parameter	Odds ratio	95% CI	p-Value
Homocysteine	1.061	0.981	1.141
RBC folate	1.002	0.999	1.005
Vitamin B12	0.999	0.996	1.002

**Table 3** Multivariate analysis model. RBC, red blood cell.

Variable	RM with low RBC folate (n=16)	RM with normal RBC folate (n=44)	Controls (n=30)	p-Value
RBC folate, ng/dL	172.1±40	451.1±167.5	401.3±135.7	<0.001
Homocysteine, µmol/L	8.12±2	7.6±2.4	8.1±3.3	NS
Vitamin B12, pg/mL	520.3±248.9	512±153.3	477.3±146.9	NS
MTHFR polymorphisms				NS
Homo+/+ (TT)	1 (6.2%)	10 (22.7%)	4 (13.3%)	
Homo -/- (CC)	8 (50%)	15 (34%)	13 (43.3%)	
Hetero +/- (CT)	7 (43.7%)	19 (43.1%)	13 (43.3%)	

**Table 4** Red blood cell folate, homocysteine, vitamin B12 and MTHFR polymorphisms in RM patients with low and normal red blood cell folate plasma levels and controls.

Results are expressed as median±SD or n (%). MTHFR, methylenetetrahydrofolate reductase; NS, not significant; RBC, red blood cell; RM, recurrent miscarriage.

of studies have investigated their potential association with RM [31, 42–52].

The frequency of the homozygous *MTHFR* genotype in Europe varies according to the geographical area studied, being of 6%–10% in the Nordic countries and of 13%–18% in the Mediterranean area. The Mediterranean diet, rich in folates, may also influence in the genotype difference between the north and south of Europe [53]. Low levels of vitamin B12 and/or folates in association with a homo or heterozygous *MTHFR* gene mutation can induce hyperhomocysteinemia; namely, this would occur as a result of an interaction between a genetic defect and the nutritional status [6, 11]. Given the above evidence, in the present study we evaluated not only plasma Hcy, but also vitamin B12 and folate levels and *MTHFR* gene polymorphisms.

The mean levels of plasma Hcy in the women included in our study were similar to those reported in other published series in the Spanish and French general population [54, 55]. In addition, the prevalence of hyperhomocysteinemia detected in our study (1.6% and 3.3% in the RM and control groups, respectively) is in agreement with that reported by other authors (2.5%) in a healthy Spanish general population using the same reference range values for plasma Hcy [54]. In both studies the cases of hyperhomocysteinemia detected were moderate (>15 and <30 µL/L). Finally, the current investigation provides further support to previous studies reporting a similar incidence of hyperhomocysteinemia in RM patients and healthy controls [44, 56].

It could be argued that, as previously suggested [57, 58], serum measurement of bioactive vitamin B12 fraction holotranscobalamin (holoTC) and methylmalonic acid (MMA) would be better diagnostic tools defining vitamin B12 deficiency. While the value of these tests are acknowledged mainly in the research setting, in the routine clinical setting definite biomarker(s) of vitamin B12

status remain(s) to be established [32, 59]. Thus, an expert opinion report indicates that, as a screening test, total Hcy measurements are less expensive, widely available, and reflect both cobalamin and folate status [32]. Also, a roundtable to discuss the measurement of vitamin B12 status biomarkers in NHANES, stressed that problems with sensitivity and specificity of individual biomarkers underscore the need for including at least one biomarker of circulating vitamin B12 (serum vitamin B12 or holoTC) and one functional biomarker (MMA or total Hcy) [59]. In our study, vitamin B12 and total Hcy were used according to our facilities. Vitamin B6 was not measured in our study given that the main determinants of total Hcy are intakes and plasma concentrations of vitamin B12 and folate, whereas the results regarding vitamin B6 are inconsistent [60, 61].

However, while serum folate, RBC folate, and plasma total Hcy are the three most commonly used biochemical indicators to assess folate status, serum folate is indicative of recent folate intake and short-term status [62, 63]. RBC folate better indicates folates stores and long-term status [62, 63] and was thus used in the current investigation. Finally, some components of Hcy metabolism such as 5-methyl-tetrahydrofolate (5MTHF) and S-adenosylmethionine (SAM) or S-adenosylhomocysteine (SAH) which may be involved in the pathogenesis of microvascular disease [64] were not investigated in our patients. However, in humans, little is known about the relationship between Hcy and its metabolites and microangiopathy and thus, it has been stressed that estimates of regulatory changes based on determinations of the plasma concentrations of these metabolites are still of limited value and must be used with caution [64, 65].

The role of the 677 C→T mutation in RM is controversial. We found a similar incidence of the various (CC, CT y TT) *MTHFR* polymorphisms as well as homozygous

and heterozygous mutations in the two groups studied. This is in keeping with findings previously reported by other authors [20, 66–68] and suggests that the *MTHFR* gene mutation does not represent an increased risk of RM in our geographical environment. Vitamin B12 does not seem to play a role in this sense in either the present or in previous studies [69, 44], however, there are others that do not confirm these results [31, 70].

It is interesting to note that even though the mean level of RBC folate in the study group was normal and similar to that found in the control group, the percentage of patients with RBC folate concentrations below the normal range was significantly higher in the RM than in the control group. However, no patient with low levels of RBC folate presented associated hyperhomocysteinemia. The mechanism by which folate deficiency can cause miscarriage is not clear but it is known that folate plays a critical role in DNA synthesis and the regulation of DNA, by providing carbon groups for synthesis of both purine and pyrimidine and also methyl groups required for DNA regulation by methylation. It has been postulated that the rapid cellular development of the embryo could be harmed by the lack of proper folate levels [71].

Some studies have reported an association between folate deficiency and increased risk of both spontaneous miscarriage [69, 72, 73] and RM [44]. In this respect, a previous study by George et al. [74] is of note showing that compared with a control women group, patients

with low folate levels were at increased risk of spontaneous miscarriage of fetuses with abnormal karyotype, whereas no significant association was observed with the risk of a normal fetal karyotype. These authors proposed that folate deficiency can produce an alteration in DNA synthesis and in chromosome structure from cells that are in a rapid division stage. However, in other published series low RBC folate levels did not seem to be involved in an increased risk of recurrent pregnancy loss [56]. Further studies are clearly needed to clarify the role of RBC folate as a possible independent risk factor in these women.

In summary, the present study based on our small series of Spanish women living in the Mediterranean region of Catalonia, failed to show an association between RM and hyperhomocysteinemia, the *MTHFR* gene mutation or vitamin B12 deficiency.

## Conflict of interest statement

**Authors' conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article.

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