European Journal of Clinical Nutrition (2011) 65, 1302–1308
 0954-3007/11
www.nature.com/ejcn

ORIGINAL ARTICLE

Increase in the prevalence of the *MTHFR 677* TT polymorphism in women born since 1959: potential implications for folate requirements

A Agodi[1], M Barchitta[1], G Valenti[1], R Marzagalli[1], V Frontini[2] and AE Marchese[2]

[1]*Department GF Ingrassia, University of Catania, Catania, Italy and* [2]*Laboratorio Analisi, POS. Bambino, Azienda Ospedaliero-Universitaria 'Policlinico-Vittorio Emanuele', Catania, Italy*

Background/Objectives: Folate has been recognized to ensure reproductive health and there is a growing body of epidemiological evidence suggesting that the methylenetetrahydrofolate reductase (*MTHFR*) 677T allele and reduced dietary folate may increase the risk of cervical cancer. The main focus of our survey was to investigate the distribution of the *MTHFR* C677T polymorphism in relation to women's year of birth and to assess their folate intake and folic acid supplementation.
Subjects/Methods: During a 6-months period, 307 healthy women of childbearing age in Catania, Italy, were enrolled in the cross-sectional study. Folate intake was estimated by a semiquantitative food frequency questionnaire and DNA extracted from blood samples for *MTHFR* C677T genotyping.
Results: A TT genotype frequency of 20.5% with an increase in the prevalence of the TT genotype in the cohort of women born since 1959 was shown. The prevalence of inadequate folate intake was 51.5%, significantly higher in non-pregnant women (83.4%) than in pregnant ones (12.3%) with a decrease during the three trimesters of pregnancy (from 25.7 to 5.0%; $P=0.013$). The use of folic acid supplements improved during the three trimester of pregnancy (from 71.4 to 95.0%; $P=0.001$).
Conclusions: Healthy young women may have higher folate needs due to increasing prevalence of the T allele and reduced folate intake compared with older groups. However, clinicians should be cautious when recommending supplements to women in late pregnancy due to the possible implications in the pregnancy outcome.
European Journal of Clinical Nutrition (2011) **65,** 1302–1308; doi:10.1038/ejcn.2011.125; published online 6 July 2011

Keywords: diet; food frequency questionnaire; gene polymorphism

Introduction

Adequate maternal nutrition during the periconceptional period as well as in pregnancy are key focus of attention in public health because of the increased needs and greater vulnerability of pregnant women to the effects of micronutrient deficiency or imbalance (Ortiz-Andrellucchi *et al*., 2009). The effect of folate status on pregnancy outcomes has long been recognized. Folate is now viewed not only to prevent megaloblastic anemia in pregnancy but also to ensure reproductive health, disease prevention and health maintenance (Tamura and Picciano, 2006). Although there is a growing body of epidemiological evidence suggesting that folate deficiency contributes to cancer risk at several sites, findings on cervical cancer risk have been inconsistent (Flatley *et al*., 2009).

Epidemiological and molecular studies have shown a causal relation between infection with high-risk human papillomaviruses (HPVs) and cervical cancer. Nutritional status and food consumption may be important HPV cofactors that increase risk of persistence and progression to cervical intraepithelial neoplasia (CIN; Garcia-Closas *et al*., 2005). Previous research has shown that high folate status significantly influences the natural history of infections with high-risk HPV (Piyathilake *et al*., 2004) and lower likelihood of developing high-risk HPV-associated CIN grades higher than 2 (CIN 2+; Piyathilake *et al*., 2007, 2009). The apparent role of folate in cervical carcinogenesis has lead to the interest in folate metabolism enzymes that may be the cofactors

Correspondence: Professor A Agodi, Department GF Ingrassia, University of Catania, Via S. Sofia n. 87, Catania 95123, Italy.
E-mail: agodia@unict.it
Received 22 November 2010; revised 25 May 2011; accepted 31 May 2011; published online 6 July 2011

linking folate deficiency and cervical carcinogenesis. Methylenetetrahydrofolate reductase (MTHFR) regulates the metabolism of folate and methionine, which are important factors in DNA methylation and synthesis. The *MTHFR* C677T polymorphism can lead to abnormal DNA methylation and DNA synthesis, possibly leading to an increased risk of cancer. However, the effect of *MTHFR* polymorphisms on cancer and precancer susceptibility remains controversial.

We have previously reported a decreased risk for CIN of individuals homozygous for the *MTHFR* T allele (Agodi *et al*., 2010). However, some studies have supported the existence of gene–folate status interactions in the etiology of cervical cancer, and specifically it has been reported that *MTHFR* T allele and reduced dietary folate may increase the risk for cervical squamous intraephitelial lesions (Goodman *et al*., 2001), while, on the contrary, a study conducted after folic acid fortification reported that *MTHFR* polymorphism is associated with reduced risk of CIN 2 or 3 (Henao *et al*., 2005).

The present cross-sectional study was conducted on women of childbearing age in Catania, Italy, where no folic acid fortification has been introduced but only supplementation in the periconceptional period is recommended. The main focus of our survey was to investigate the distribution of the *MTHFR* C677T polymorphism in relation to women's year of birth and to assess their folate intake and folic acid supplementation by the use of a food frequency questionnaire (FFQ).

Materials and methods

Study population

During a 6-months period, 307 healthy women of childbearing age, referred to the Laboratory of the S Bambino Hospital, Catania, Italy, an obstetric center for preconceptional, prenatal and/or postpartum care, were enrolled in the study. All women gave their informed consent to participate in the study. The study protocol was approved by the involved institutions. Fasting venous blood samples were collected, from each women, in EDTA-containing tubes and aliquots were stored at −80 °C until analysis.

Data were collected by trained interviewers using a structured questionnaire to obtain information on demographic and lifestyle data, including smoking habits and obstetrical history. The use of multimineral/multivitamin from supplements, including folic acid supplements, were derived from questions addressing the consumption of dietary supplements during the periconceptional period and pregnancy. Women were asked whether they used vitamin and mineral supplements and which supplement (type or brand) they used, and thus classified as taking or not taking supplements.

Body mass index was calculated as weight (kg) divided by height (m^2), based on criteria from the World Health Organization (1995). Pre-pregnancy body mass index was based on self-reported pre-pregnancy weight.

Estimation of folate intake

Folate intake, during the past month, was estimated by a semiquantitative 46-item FFQ that uses the previous month as a reference period. The FFQ comprises eight food group items: cereals, bread and snacks; fish and egg; pasta and soup; dairy products; vegetables; fruit; sweets; beverages. An indicative photo album showing standard portion sizes (small, medium and large) was used to estimate the amount of each food item. Data from the FFQ were stored in a database created using the software SPSS, version 14.0 (SPSS, Chicago, IL, USA). Dietary folate intake was derived from the FFQ by multiplying the frequency of intake for any food item by its respective portion size (g). Because of the lack of folate data in the Italian food composition database, the table of alimentary composition of the US Department of Agriculture was used to determine micronutrient level (Food and Nutrition Board, Institute of Medicine, 2001). Contribution of folic acid from supplements was not included in the estimates of total folate intake.

Prevalence of folate deficiency was estimated by comparing folate intake with the estimated average requirements for folate (Food and Nutrition Board, Institute of Medicine, 2001).

Furthermore, estimated folate intakes were ranked by tertiles using the dietary assessment approach and the rankings were compared across baseline characteristics of enrolled women.

The validity of folate intake estimates from the FFQ was assessed. In brief, a random sub-sample of 30 women from the study population (validation study subgroup) was asked to complete both the FFQ and, afterwards, a 4-days weighted dietary record (WDR) that covers 3 weekdays and 1 weekend day. A booklet with pictures of common food items and mixed dishes were used to facilitate the estimation of portion sizes. Folate intake from WDR was calculated using the methods describe for FFQ analysis. Associations between individual folate intake estimated from the FFQ and the WDR were assessed by Spearman's correlation coefficient and by ratios between intakes based on the test and reference method.

MTHFR *C677T and A1298C genotyping*

Genomic DNAs were extracted from whole blood using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare, Niskayuna, NY, USA) according to the manufacturer's protocol and stored at −20 °C.

Determination of *MTHFR* C677T and A1298C polymorphisms was performed using the TaqMan allelic discrimination Assay, using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Allelic discrimination was carried out by measuring fluorescence intensity at the endpoint. The results of the measurement and subsequent genotype were evaluated with SDS software version 2.3 (Applied Biosystems).

Statistical analyses

Statistical analyses were performed using the software SPSS, version 14.0 (SPSS). The χ^2-test was used for the statistical comparison of proportions. Continuous variables were tested using Student's *t*-test. The basic significance level was fixed at $P<0.05$.

To ascertain if population sample was in Hardy–Weinberg equilibrium for the *MTHFR* polymorphisms, a χ^2-test was performed.

Results

Population characteristics

During the study period, between July and October 2008, all women of childbearing age attending the laboratory to have a blood test were invited to participate in the study. A total of 307 women were enrolled. The main characteristics of the women enrolled in the study are shown in Table 1. The mean age was 28.9 years (median 28 years; range: 14–49 years). The majority (94%) of women were born in Italy and 45% were pregnant. Particularly, 43.8% of pregnant women were in the third trimester of pregnancy.

Folate intake

A total of 43.3% of enrolled women reported the use of folic acid supplements or of multimineral/multivitamin supplements containing folic acid. Particularly, 87.0% of pregnant women and 7.7% of non-pregnant women taking folic acid supplements ($P=0.000$). Reported use of folic acid supplements increased significantly from 71.4% of women in the first trimester of pregnancy to 88.1% in the second trimester and up to 95.0% in the third trimester (Table 2; $P=0.001$).

In the validation study subgroup, the mean folate intake, estimated by the FFQ, was 215.5 μg per day, and of 164.2 μg per day estimated by the WDR. Median intake estimated by the FFQ was 222.0 μg per day, and of 161.0 μg per day estimated by the WDR. Intake median ratio was of 1.38 (FFQ/WDR), thus, intake of folate was 38% higher, as estimated by FFQ versus WDR. Spearman's correlation coefficient was 0.59 ($P=0.002$).

For the overall population, the mean folate intake, assessed by the FFQ, was 208.5 μg per day (median 196.0 μg per day; range: 47.4–939.7 μg per day), 222.8 μg per day (median 204.4 μg per day; range: 67.2–939.7 μg per day) for pregnant women and of 196.9 μg per day (median 186.7 μg per day; range: 47.4–579.3 μg per day) for non-pregnant women ($P=0.026$). Mean folate intake increased, but not significantly, during the three trimester of pregnancy (Table 2).

Considering only diet and comparing the folate intake with estimated average requirement, the prevalence of inadequate folate intake was 90.2%, 89.9% and 90.5%, respectively, for the overall sample, for pregnant women and for non-pregnant women. The prevalence of inadequate folate intake was 94.3%, 88.1% and 88.3%, respectively, in the first, in the second and in the third trimester of pregnancy.

Table 1 Characteristics of study participants ($N=307$)

	n *(%)*	*Mean*	*s.d.*	*Range*
Nationality				
Italian	289 (94.2)			
European countries	9 (2.9)			
Non-European countries	9 (2.9)			
Age (years)		28.9	7.6	14–49
Education (years of schooling)				
<5	1 (0.3)			
5	24 (7.8)			
8	134 (43.6)			
13	122 (39.7)			
>13	26 (8.5)			
Employment status				
Employed	89 (29.0)			
Unemployed	10 (3.2)			
Student	34 (11.1)			
Housewife	174 (56.7)			
Smoking[a]				
Current smokers	94 (30.9)			
Non-smokers	171 (56.3)			
Former smokers	39 (12.8)			
Body mass index[b]		24.9	5.5	15.6–47.7
Underweight	22 (7.2)			
Normal weight	160 (52.1)			
Overweight	76 (24.8)			
Obese	49 (16.0)			
Pregnancy				
No	169 (55.0)			
Yes	138 (45.0)			
Trimester of pregnancy[c]				
First	35 (25.5)			
Second	42 (30.7)			
Third	60 (43.8)			

[a]Data missing for three women.
[b]Based on criteria from the World Health Organization (1995).
[c]Data missing for one woman.

Taking into account the use of supplements, and thus classifying each woman as taking or not taking supplements, the prevalence of inadequate folate intake decreased to 51.5% overall and during the three trimesters of pregnancy ($P=0.013$).

Following the percentile distribution, the population was divided into four groups according to birth date and prevalence of inadequate folate intake—considering only diet, without taking into account intake from supplements—was compared between groups. An increase of the prevalence of inadequate folate intake (from 88.3 to 91.7%) was observed in the group of women born in 1985–1994 when compared with the older group born in 1959–1973.

Inadequate folate intake was significantly larger among working women (61.7%) than housewives (43.7%; $P=0.002$)

Table 2 Folic acid supplements and folate intakes

Folic acid supplements use	n *(%)*	P-*value*
Overall	133 (43.3)	
Non-pregnant women	13 (7.7)	0.000[a]
Pregnant women	120 (87.0)	
First trimester	25 (71.4)	0.001[a]
Second trimester	37 (88.1)	
Third trimester	57 (95.0)	
Folate intake (μg per day)	*Mean*	P-*value*
Overall population	208.5	
Non-pregnant women	196.9	0.026[b]
Pregnant women	222.8	
First trimester	197.3	>0.05[b]
Second trimester	223.9	
Third trimester	235.6	
Prevalence of inadequate folate intake[c]	n *(%)*	P-*value*
Overall population	158 (51.5)	
Non-pregnant women	141 (83.4)	0.000[a]
Pregnant women	17 (12.3)	
First trimester	9 (25.7)	0.013[a]
Second trimester	5 (11.9)	
Third trimester	3 (5.0)	

Tertiles of folate intake	*First tertile*	*Second tertile*	*Third tertile*	P-*value*
Overall population (limit, μg per day)	155.7	234.1	939.7	
n (%)	102 (33.2)	103 (33.6)	102 (33.2)	
Folic acid supplements (yes)	40 (39.2)	42 (40.8)	51 (50.0)	0.244[a]
Low-medium educational level	49 (48.0)	59 (57.3)	48 (47.1)	0.271[a]
Medium-high educational level	53 (52.0)	44 (42.7)	54 (52.9)	
Working women	41 (40.2)	49 (47.6)	43 (42.2)	0.543[a]
Housewife women	61 (59.8)	54 (52.4)	59 (57.8)	
Normal weight women	49 (48.0)	50 (48.5)	61 (59.8)	0.164[a]
Overweight or obese women	53 (52.0)	53 (51.5)	41 (40.2)	

[a]χ^2-test.
[b]Student's *t*-test.
[c]Prevalence of inadequate folate intake was estimated by comparing folate intake with the estimated average requirement for folate (Food and Nutrition Board, Institute of Medicine, 2001).

who tended to take folic acid supplements (51.1%) more than women working outside (33.1%; $P=0.002$), also after controlling for educational level. Inadequacy was significantly larger among overweight or obese women (58.5%) than among women with normal weight (45.0%; $P=0.022$).

Furthermore, estimated folate intakes were ranked by tertiles and resulting rankings were compared across baseline characteristics of enrolled women. No statistically significant association was observed (Table 2).

Distribution of MTHFR *C677T and A1298C polymorphisms*

The distribution of *MTHFR* genotypes for the C677T and A1298C polymorphisms is shown in Table 3. The allelic distribution of both *MTHFR* polymorphisms follows the Hardy–Weinberg equilibrium expectations ($P=0.26$). The relative frequencies of the 677T- and 1298C-mutated alleles were 43.8% and 30.5%, respectively. Overall, 20.5% of the subjects were homozygous for the 677TT-mutated genotype and 7.8% for the 1298CC-mutated genotype. The heterozygous status for both mutations was similar, 46.6% for the C677T mutation and 45.3% for the A1298C mutation.

Six of the nine possible genotype combinations were found for the two *MTHFR* polymorphisms, whereas no subjects had the *MTHFR 677CT/1298CC*, *677TT/1298CC* or *677TT/1298AC* genotype combination.

Genotype frequencies and age

Following the age percentile distribution, genotype frequencies of *MTHFR* polymorphisms were compared between groups. For the C677T polymorphism an increase of the TT genotype (from 14.3 to 28.2%; $P=0.03$) was observed in the group of women born in 1980–1984 when compared with the group of women born in 1959–1973 (Table 3). Furthermore, an increase of the T-allelic frequency was shown from

Table 3 Distribution of *MTHFR* C677T and A1298C genotypes and allele frequencies[a]

	Genotype frequencies, n *(%)*			*Allele frequencies,* n *(%)*[b]	
C677T	CC 101 (32.9)	CT 143 (46.6)	TT 63 (20.5)	C 345 (56.2)	T 269 (43.8)
A1298C	AA 144 (46.9)	AC 139 (45.3)	CC 24 (7.8)	A 427 (69.5)	C 187 (30.5)

[a]The allelic distribution of both *MTHFR* polymorphisms follows the Hardy–Weinberg equilibrium expectations.
[b]Total number of alleles for each polymorphism: 614.

Table 4 Distribution of C677T and A1298C genotype and allelic frequencies in the four groups according to birth date

MTHFR	*Frequencies by date of birth,* n *(%)*			
	1959–1973	*1974–1979*	*1980–1984*	*1985–1994*
C677T				
CC	32 (41.6)	21 (30.9)	25 (32.1)	23 (27.4)
CT	34 (44.2)	36 (52.9)	31 (39.7)	42 (50.0)
TT	11 (14.3)[a]	11 (16.2)	22 (28.2)[a]	19 (22.6)
C allele[b]	98 (63.6)	78 (57.3)	81 (51.9)	88 (52.4)
T allele[b]	56 (36.4)[c,d]	58 (42.7)	75 (48.1)[c]	80 (47.6)[d]
A1298C				
AA	36 (46.8)	31 (45.6)	40 (51.3)	37 (44.0)
AC	33 (42.9)	33 (48.5)	32 (41.0)	41 (48.8)
CC	8 (10.4)	4 (5.9)	6 (7.7)	6 (7.1)
A allele[b]	105 (68.2)	95 (69.8)	112 (71.8)	115 (68.4)
C allele[b]	49 (31.8)	41 (30.2)	44 (28.2)	53 (31.6)

[a]χ^2-test: $P = 0.03$.
[b]Total number of alleles for each polymorphism: 614.
[c]χ^2-test: $P = 0.04$.
[d]χ^2-test: $P = 0.04$.

36.4% in the group of women born in 1959–1973 to 48.1% in the group born in 1980–1984 ($P = 0.04$) and to 47.6% in the group born in 1985–1994 ($P = 0.04$).

For the A1298C polymorphism no significant differences were observed in allelic or genotypic frequencies between the different groups (Table 4).

Discussion

The *MTHFR* C677T has been a focus of increasing interest worldwide since its genetic identification in 1995, and accurate information on its distribution can contribute to studies of gene–disease associations. Our study, conducted in a sample of the women population in Sicily (southern Italy), reports a TT genotype frequency of 20.5%, consistently with previously published works. Notably, an increase in the prevalence of the TT genotype in the cohort of women born since 1959 was shown, although this finding should be interpreted with caution given the sample size.

A variation in the prevalence of the TT homozygous genotype was previously reported, with the presence of a north to south gradient. Particularly in Europe, this prevalence has been shown to increase from the lowest values in the north (4–7%), to the highest frequencies in southern Italy (20.1 and 19.9% in Sicily; Wilcken *et al*., 2003; Guéant-Rodriguez *et al*., 2006). The mechanisms generating this gradient are not clearly known, although it has been hypothesized that they could involve, at least in part, gene–nutrient interactions and that in Europe dietary folate may have influenced the prevalence of the T allele (Guéant-Rodriguez *et al*., 2006). In fact, some results obtained in subgroups of the European populations confirmed a south to north decreasing gradient of dietary folate intake and of the T-allele frequency: a high frequency of the TT genotype in the presence of a high concentration of folate in plasma and a low frequency in the presence of low folate concentrations have been reported (Guéant-Rodriguez *et al*., 2006).

Our cross-sectional study was conducted on women of childbearing age in Catania, Italy, in which no folic acid fortification has been introduced but only supplementation in the periconceptional period is routinely recommended since the 1980s. The observed increase of the T-allelic frequency in the group of women in study, born in the last quarter of the last century, has been already associated with some selective advantage in a previous research conducted on a Spanish population (Mayor-Olea *et al*., 2008). Particularly, it has been reported that selection in favor of the T allele could be due to the increased fetal viability in early stages of embryonic development due to an increase in folic acid and vitamin supplements intake by women in the periconceptional period that began to be established in Spain in the last quarter of the 20th century as well as it started in other European countries including Italy (Mayor-Olea *et al*., 2008).

Previous studies have suggested that the *MTHFR* TT homozygosity may confer a survival advantage in populations with adequate dietary folate consumption (Munoz-Moran *et al*., 1998; Rosenberg *et al*., 2002; Guéant-Rodriguez *et al*., 2006). The T allele is associated with a greater risk of neural tube defects in those geographical areas or ethnic groups with a high frequency of this genotype, but that risk seems to be neutralized by a diet rich in folate, such as the Mediterranean diet (Zetterberg, 2004). In fact, in southern Italy, the TT genotype is common, but the rate of neural tube defects is not particularly high (International Clearinghouse for Birth Defects Monitoring Systems, 2001), probably because environmental and nutritional factors are likely to modulate the risk (Wilcken *et al*., 2003).

In the present study, using the validated FFQ, taking into account the folic acid supplements and comparing the

overall intake with estimated average requirement, the prevalence of inadequate folate intake was 51.5%, significantly higher in non-pregnant women (83.4%) than in pregnant ones (12.3%). Furthermore, the prevalence of inadequate folate intake significantly decreased during the three trimesters of pregnancy (from 25.7 to 5.0%; $P=0.013$). These findings may be the result of the higher use of folic acid supplements in pregnancy (87.0%) compared with the lower use in non-pregnant women (7.7%). Our results show that the use of folic acid supplements increased significantly during the three trimester of pregnancy (from 71.4 to 95.0%; $P=0.001$). Intake of multimineral/multivitamin from supplements during pregnancy is an attractive option to improve the nutritional status of pregnant women. However, although it has been recently reported that the daily use of multivitamin and mineral supplements (mainly folic acid) during any stage in pregnancy is not associated with lower birthweight and taking supplements in the third trimester has been associated with a threefold increased risk of preterm delivery (Alwan *et al*., 2010). Even if those findings need to be confirmed by other cohorts and/or trials, they suggest that clinicians should be cautious when recommending multivitamin–mineral supplements to women in late pregnancy due to the possible implications in the pregnancy outcome.

Nutritional status and food consumption may be important HPV cofactors that increase risk of persistence and progression to CIN. Using the validated FFQ, a high prevalence of folate intake inadequacy has been shown along with an increase in mutated *MTHFR* C677T polymorphism that may modulate the risk of cancer according to folate status (Henao *et al*., 2005; Piyathilake *et al*., 2007; Flatley *et al*., 2009). According to our study, healthy young women may have higher folate needs due to the increased prevalence of the T allele and reduced folate intake compared with older groups.

In interpreting the findings of this study, its limitations should be considered. First, we have not analyzed biomarkers of folate status, as our aim was to measure habitual folate intake over an extended period of time, although biomarkers of folate status could have helped us to better define folate inadequacy and to validate our FFQ. All dietary assessment instruments are associated with different, and sometimes considerable, random and systematic measurement errors (Kristal and Potter, 2006; Willett and Hu, 2007; Ortiz-Andrellucchi *et al*., 2009) and thus, when possible, biomarkers have to be incorporated into the validation of dietary assessment methods (Jenab *et al*., 2009), because folate biomarkers are very robust and sensitive markers of folate status. However, it has been reported that biomarkers are affected by different sources of error than FFQs, such as variation over time probably reflecting changes in intake, and, on the contrary, the advantages of using FFQs include measuring usual food intake over an extended period, allowing for large study samples (Johansson *et al*., 2010). Besides, this study was not designed to measure other important protective micronutrients, such as other B-vitamins for example, riboflavin) or antioxidants (for example, vitamins C, A, E and carotenes), and this could be an important limitation of the study, nevertheless the FFQ used in our study was designed and validated using WDR, in order to estimate only folate intake, with a correlation coefficient of 0.59, in accordance with other FFQ validation studies (Erkkola *et al*., 2001; French *et al*., 2001; Flood *et al*., 2004; Johansson *et al*., 2010).

Therefore, future studies, carefully designed to address the above limitations, are needed to further our knowledge about the critical role of maternal nutrition and, in particular, of micronutrients to reproductive health. Furthermore, potential implications for folate requirements are highlighted by our results. Healthy young women may have higher folate needs due to reduced folate intake compared with older groups and moreover they show an increasing prevalence of the T allele, which in turn may require supplement recommendation. Besides, appropriate timing of supplement use should be advised in order to prevent a poor pregnancy outcome, as it has been previously suggested to occur in the late pregnancy (Alwan *et al*., 2010). Well-designed-randomized controlled trials are needed to investigate the role of multivitamin supplement use in late pregnancy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported in part by grants from the University of Catania (Progetti di Ricerca di Ateneo to AA).

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