Original Research
Nutrafoods (2016) 15:245-251
DOI 10.17470/NF-016-1010-4

Altered gender-related homocysteine metabolism and lipid panel in healthy heavy smokers: a possible role for nutraceutical supplementation

Luisella Vigna[1], Chiara Fenoglio[2], Fabrizia Bamonti[3], Cristina Novembrino[4], Rachele De Giuseppe[5], Cinzia Dellanoce[6], Laura Coassin[1], Giuliana Cighetti[7], Rita Maiavacca[4], Dario Gregori[8]

Correspondence to:
Luisella Vigna
luisella.vigna@policlinico.mi.it

Keywords:
Cigarette smoking,
Homocysteine,
Folate,
Cobalamin,
Gender,
Nutritional supplement

Introduction

In recent years, clinical and epidemiological studies have suggested that increased concentrations of total plasma homocysteine (tHcy) could be an additional independent risk factor for cardiovascular disease [1]. It has been established that a very high tHcy concentration due to inborn metabolic errors causing enzyme alterations in homocysteine metabolism can lead to serious thrombotic events at a young age [2]. Mild to moderate hyperhomocysteinemia is recognized as a potential risk factor for adult cardiovascular disease, and many observational studies, and case and placebo-controlled trials, are seeking to determine whether it is a cause or an effect of the disease [3]. Hyperhomocysteinemia can be caused by genetic and/or acquired factors due to lifestyle (i.e., nutritional habits), renal insufficiency and age [3]. It is known that low levels or deficiency in our diet of some group B vitamins, in particular cobalamin and/or folate (required cofactors for further homocysteine metabolism through the remethylation pathway), result in moderate elevations in tHcy levels.

Adequate cobalamin levels and early diagnosis of deficiency are crucial because of the latent nature of the resulting megaloblastic anaemia, which can be obscured by high folate intake. A delayed diagnosis of vitamin B12 (B12) deficiency can result in, sometimes irreversible, neuronal cell damage. However, serum cobalamin concentration does not reliably rule out functional B12 deficiency [4], so a new approach to diagnosing subtle cobalamin deficiency determines cobalamin status as holotranscobalamin (HoloTC, or active B12) concentration [4–6]. HoloTC, the transcobalamin cobalamin complex, representing the biologically active form of the vitamin (about 10–30% of total serum B12) is recognised by ubiquitous specific membrane receptors [4–6] and could have high diagnostic value as a storage marker [4–6].

[1] Dipartimento di Medicina Preventiva, Servizio di Promozione della Salute dei Lavoratori, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 35, 20122 Milan, Italy phone: +39 02.55032592
[2] Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 35, 20122 Milan, Italy
[3] Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano, Milan, Italy
[4] Laboratorio Centrale di Analisi Chimico Cliniche e Microbiologia, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, via F. Sforza 35, 20122 Milan, Italy
[5] Dipartimento di Sanità Pubblica, Medicina Sperimentale e Forense, Università degli Studi di Pavia, via Bassi 21, 27100 Pavia, Italy
[6] CNR Istituto di Fisiologia Clinica, Ospedale Niguarda, Piazza Ospedale Maggiore 3, 20162 Milan, Italy
[7] Dipartimento di Scienze Biomediche e Cliniche "Luigi Sacco", Università degli Studi, Milan, Italy
[8] Dipartimento Scienze Cardiologiche Toraciche Vascolari, Unit of Biostatistics, Epidemiology and Public Health, Università degli Studi di Padova, via Giustiniani 2, 35128 Padova, Italy

Received: February 05, 2016 / Accepted: June 10, 2016

Tobacco smoking is a very unhealthy habit and well-established lifestyle factor closely and independently associated with an increased risk of cardiovascular diseases [7]. It has been reported that cigarette smoking is associated with elevated tHcy concentrations [8] and is also negatively associated with serum folate (s-Fol) levels [9]. Elevated tHcy concentrations could be a direct effect of toxic substances in tobacco smoke, often accompanied by a poor diet and/or low intake of most vitamins [10]. Recently, assessment of glutathionyl-haemoglobin (Hb-SSG) levels showed the extent of biological damage caused by smoking tobacco [11]. There is growing evidence that the reactive oxygen species generated by cigarette smoking may contribute to smoking-related diseases (i.e., emphysema, atherogenesis and cancer). In particular, the study of Vassalle *et al*. showed that smoking was a gender-related cause of impaired oxidative status [12]. Moreover, the study by Frei *et al*. demonstrated that the gas phase oxidants of cigarette smoke can oxidize naturally occurring plasma antioxidants, protein thiol groups and lipids [13], while differences between men and women in whole-body lipid metabolism and in circulating blood lipid concentrations are reported by Williams [14].

The main objectives of the present study were to evaluate homocysteine metabolism and the lipid panel in Italian healthy heavy smokers focusing on the differences between male and female subjects and to verify the diagnostic value of HoloTC as a storage marker of vitamin B12 status. Moreover, the possible role played by nutraceutical supplements in counteracting metabolism alterations will be discussed.

Methods

Study population

Sixty-one healthy heavy smokers (26 women and 35 men, median age 51 years, IQR 43–60), with no indication of respiratory dysfunction or symptoms of other illnesses, were recruited for the study and presented to the Pneumology Department of the Niguarda Ca' Granda Hospital in Milan for an annual check-up. Exclusion criteria were pregnancy or lactation, hypertension, dyslipidemia, chronic disease, chronic medication including oral contraceptives, gastrointestinal disorders, unstable psychiatric health, alcohol abuse or use of dietary supplements. The subjects were defined as current heavy smokers on the basis of the number of cigarettes smoked per day (>20) for at least 10 years prior to enrolment.

This study was conducted in accordance with the guidelines of the Declaration of Helsinki for Research on Human Subjects and was approved by the Human Ethics Committee at the S. Giuseppe e Sacra Famiglia di Erba Hospital, Milan (protocol number 27/05/CE/smc).

Each participant signed an informed consent form and was then interviewed about his or her general health, usual dietary intake, lifestyle, alcohol and smoking habit using the Nutrition Status Assessment Score (NSAS) questionnaire [15]. This questionnaire includes 13 items on the number of cigarettes smoked, use of alcohol (safe levels: 20 g/day for women, 30 g/day for men), physical activity and the consumption of common Italian foods. Self-reported weight and height were used to estimate body mass index (BMI). Some (16%) of study smokers self-reported moderate alcohol consumption while the other 84% were non-drinkers or light drinkers. Items were scored on a Likert-type scale from 0 to 13. Two different scales were used: NSAS Total (all factor loadings) and NSAS Fruit and Vegetables (FV) [16].

Blood samples were drawn after an overnight fast. Two blood specimens from each subject were collected in light-protected tubes. Serum and plasma samples were frozen and stored at -20° C until analyzed. Plasma tHcy, serum B12, s-Fol and RBC-Fol concentrations were determined by immunoenzymatic assay using commercial kits on an automated AxSYM analyser (Abbott, Abbott Park, IL, USA), as previously reported [16]. Serum HoloTC levels were determined by a new immunoenzymatic assay [17] using the Active B12 kit on the same automated AxSYM analyser (Abbott). Intra- and inter-assay variability was less than 5% and 8%, respectively, for all assay procedures. The lipid panel (total cholesterol (tCh), high density lipoprotein cholesterol (HDL-Ch) and triglycerides (TG)) was measured on a Modular Analytics automated analyser (Roche, Switzerland) [16].

The reference interval (6.8–10.5 µM/l) for tHcy was calculated using the plasma concentrations of 105 healthy non-smokers (57% females, 43% males, mean age 45±12) as controls. All other reference intervals or cut-off values are those currently used in our laboratory [16, 17].

Statistical analysis

Data were presented as median and inter-quartile range (IQR) to account for skewness in data distribution. Unadjusted comparisons between males and females were performed using a Wilcoxon test. To account for the possible confounding effects of patients' baseline characteristics in comparing genders, *p* values were also computed adjusting for age, number of cigarettes smoked, nutritional score and BMI at baseline. For this purpose, a linear regression model was used, and adjusted variables were included in the model irrespective of their statistical significance, due to their usage as statistical confounders. Results with $p>0.05$ were reported as not significant (NS) in the tables. The relationship between serum B12 and HoloTC was calculated using a local regression smoother [18]. All analyses were performed using R software [19].

Results

The results of a standard complete blood count (CBC) panel showed all subjects had normal haematological status (data not shown).

Male subjects, approximately the same age as female subjects (males 53 years old, IQR 44–61.5 vs females 51 years old, IQR 42.25–59; p=NS), had a higher BMI (males 24.5, IQR 23.85–24.85 vs females 22.55, IQR 21.92–24.22; $p<0.001$) but the same NSAS total score (males 6, IQR 4–8 vs females 5, IQR 4–6; p=NS). The two NSAS scales showed good association with factors known to be related to nutritional habits, such as BMI and cigarette smoking [15, 16].

Table 1 shows that 87% of smokers had slight or moderate hyperhomocysteinemia (>10.5 µmol/l). RBC-Fol concentrations were below the reference interval in 52.5% of all smokers, whereas s-Fol was below the reference interval in only 15% of cases.

HoloTC concentrations were below the reference cut-off value (40 pmol/l) in about one third (30%) of smokers, while 16.4% had B12 concentrations below the reference cut-off value (180 pmol/l).

Moreover, smokers showed an altered lipid panel: hypercholesterolemia (tCh was elevated in 72.1%) and decreased HDL-Ch concentrations were seen in about 18% of subjects. As shown in Table 2, unadjusted *p* values for crude comparison of analytes between males and females and *p* values adjusted for BMI, number of cigarettes smoked, age and nutritional score were evaluated. B12, tHcy and HDL cholesterol levels showed significant gender-related differences, confirmed by both analyses. In

Analyte	Median (IQR)	Reference values
Vitamin B12 (pM)	272.2 (211.3–350.8) [16.4%]*	164–835 pM
Holotranscobalamin (pM)	58.0 (44.7–77.7) [29.5%]*	>40 pM
Serum folate (nM)	12.9 (10.0–17.6) [15%]*	7–28 nM
Erythrocyte folate (nM)	441.2 (355.6–597.7) [52.5%]*	421–1462 nM
Homocysteine (µM)	10.8 (8.0–13.2) [87.0%]*	6.8–10.5 µM
Total cholesterol (mg/dl)	229.2 (205.7–260.5) [72.1%]*	<200 mg/dl
HDL cholesterol (mg/dl)	51.7 (44.6–63.3) [18.0%]*	>40 mg/dl

*Subjects with values higher or lower than the reference interval or cut-off value
Data are presented as median and interquartile range (IQR)

Table 1 - Analytes evaluated in healthy heavy smokers

Analyte	Median (IQR)		Unadj. *p* value	Adj. *p* value
	M	F		
Vitamin B12 (pM)	252 (199–314)	310 (242–419)	0.037	0.010
Holotranscobalamin (pM)	52.5 (42.5–76.2)	59.8 (49.8–76.0)	NS	NS
Serum folate (nM)	12.9 (9.6–16.5)	12.9 (10.2–19.8)	NS	NS
Erythrocyte folate (nM)	404 (318–529)	519 (366–602)	NS	NS
Homocysteine (µM)	11.6 (8.8–16.0)	9.0 (6.4–10.9)	0.002	0.022
Total cholesterol (mg/dl)	226.5 (205.6–253.5)	233.5 (208.3–260.2)	NS	NS
HDL cholesterol (mg/dl)	47.4 (41.3–54.9)	62.7 (52.7–68.2)	<0.001	0.001

Data are presented as median and interquartile range (IQR) and stratified by gender. Unadjusted *p* values for the crude comparison between males and females are reported as well as *p* values for the same comparison adjusted for body mass index, age, number of cigarettes smoked and nutritional score. *F* female, *M* male

Table 2 - Analytes evaluated in healthy heavy smokers by gender

particular, B12 levels were significantly lower in men than in women (p<0.05) and low cobalamin status was more frequently found in men than in women (HoloTC <40 pmol/l: men 34%, women 23%; B12 <180 pmol/l: men 17%, women 11.5%). Hyperhomocysteinemia was more common in male (68%) than in female subjects (27%) and this seemed to be related to RBC-Fol concentrations, which were lower in men than in women, although not significantly so. The lipid panel was similarly altered in both groups, with low HDL-Ch levels more common in men than in women. Correlation analysis showed weak association between HoloTC and B12 (R^2=0.196; p<0.01); no correlation was observed between HoloTC and the other parameters considered.

Discussion

The present study evaluated parameters of homocysteine metabolism and the lipid panel in healthy middle-aged heavy smokers living in Northern Italy and also highlighted some gender-related differences. Moreover, inclusion of the new HoloTC assay, as part of a routine clinical chemistry panel of tHcy status, appeared to be clinically and diagnostically useful. The majority of these healthy heavy smokers showed slightly or moderately elevated tHcy concentrations, indicating impaired Hcy metabolism and confirming other authors' findings [8]. Our population demonstrated a modest increase in tHcy levels, and, interestingly, disturbances in vitamin status correlated with tHcy concentration. It is unclear whether this is a direct effect of smoking or of an inadequate diet in smokers.

All the subjects (none of whom were vegetarian or vegan) followed a normal Mediterranean diet and consumed B12 sources such as meat, eggs and dairy products, as confirmed by slightly elevated serum tCh concentrations (median 229 mg/dl, IQR 206–261 mg/dl). However, the findings of this study confirm a lack of vitamins in otherwise healthy smokers. Both the Total and FV NSAS scales correlated with major dietary proxies (BMI, alcohol use and smoking status). In fact, although the median estimated BMI was 24 (IQR 22.4–24.7) indicating an ideal body weight, the NSAS FV questionnaire showed that only 20% of these subjects ate at least five servings of fruit and non-starchy vegetables daily. Epidemiological evidence suggests that a deficiency in bioavailable antioxidants and other micronutrients can increase the risk of developing pathological conditions, particularly cardiovascular disease, stroke, metabolic disorders and cancer [16, 20–22]. The lifestyles of smokers differ from those of non-smokers in that smokers consume fewer sources of antioxidants (i.e., fruit and vegetables) and therefore may have a lower antioxidant capacity because of their diet [16, 20–22]. Cigarette smoking, a well-known risk factor for cardiovascular disease and cancer, has been correlated with a lower consumption of fruit and vegetables [21]. In accordance with other authors [12, 21, 22], we found a negative correlation between the number of cigarettes smoked and vitamin intake (data not shown). The nested case–control study of the European Concerted Action Project suggested that smokers with hyperhomocysteinemia and reduced levels of metabolically related B vitamins are at greatly increased risk of cardiovascular disease [17].

All smokers in our study showed haematological parameters within the reference intervals, indicating the absence of anaemia; moreover, the medium globular values (MCV) of all subjects were inside the reference interval, confirming the healthy levels of alcohol consumption of some smokers. However, alterations in vitamin status were found in these subjects. It is now recognized that cobalamin deficiency is more common than previously thought [3–6]. In order to prevent neurological and/or haematological complications, it is important to avoid B12 deficiency, which requires reliable methods of measurement. Other authors have reported that total serum B12 (tB12) concentrations may not correctly indicate cobalamin status [4, 17]. Several recent studies have shown that HoloTC, the ubiquitous bio-available serum cobalamin fraction, could be an early, sensitive and more reliable indicator of metabolically active B12 status than serum tB12 concentration [4, 5, 17]. Preliminary clinical data suggest that tB12 and HoloTC provide different types of information, as

B12 variability is mostly independent of HoloTC [23, 24]. It is important to note that in the present study only a weak correlation was found between HoloTC and B12: not all cases of low HoloTC levels showed low B12 levels nor did all cases of low B12 show low HoloTC levels (only seven cases (11.5%) had low HoloTC and B12 levels). This study indicates that HoloTC concentrations are mostly independent of serum cobalamin values, even though the latter remain the current clinical standard to evaluate B12 status. Measurement of both HoloTC and total serum cobalamin together provides better screening for B12 deficiency than either assay alone [17], especially in cases of suspected and/or subclinical B12 deficiency in otherwise healthy subjects at risk because of inadequate nutrition and/or unhealthy lifestyle.

Interestingly, in this study the significant differences between men and women's tHcy concentrations and B12 status were confirmed in both unadjusted and adjusted statistical analyses. In particular, moderate hyperhomocysteinemia together with cobalamin deficiency was more common in men than in women. Therefore, our results support the monitoring of Hcy metabolism in order to evaluate early vitamin deficiency.

The diagnostic accuracy of HoloTC and total serum cobalamin measurements for screening for B12 deficiency are probably equal. Analysing the metabolic markers of cobalamin status, Miller *et al.* proposed a graded predictive classification system for vitamin deficiency (probable, possible and unlikely). The most important category was 'possible deficiency' (either tB12 or HoloTC concentrations being low) in which the measurement of both parameters would help identify subjects at greater risk [17]. This classification may well be used by physicians to plan further diagnostic testing and/or treatment and, in some cases, to avoid over-treatment.

A reduction in plasma tHcy concentrations due to short-term folic acid supplementation has been observed in chronic smokers [20]. Folates, along with B12, are necessary for correct cellular metabolism. Insufficient folate status can result in several disorders in humans including anaemia, birth defects, psychiatric disorders and cancer [24, 25]. RBC-Fol is a reliable indicator of long-term folate status and general dietary intake, while s-Fol provides a good indication of recent dietary intake [25, 26]. Assessment of both parameters, as in this and our previous studies, gives a more accurate estimate of folate status than relying on s-Fol alone [20, 26]. Several mechanisms (decreased dietary intake, reduced absorption, diminished hepatic uptake and increased urinary excretion) may explain folate deficiency in smokers [20, 21]. However, in agreement with the European Concerted Action Project, a possible interaction between chemical components of cigarette smoke and folate coenzymes could lower folate status and promote hyperhomocysteinemia, increasing the risk of cardiovascular disease [20, 21]. In the present study, although almost all the smokers had adequate s-Fol levels, about 30% had suboptimal baseline RBC-Fol concentrations [20]. The important role of dietary folate or folic acid supplementation for our health has resulted in mandatory food fortification in several countries, including the USA and Canada. However, there is concern in other countries that folate fortification alone might not be sufficient to modify elevated tHcy, as adequate B12 status is also required for good health [24]. Our population showed median levels of both s-Fol and RBC-Fol within the relevant reference intervals, but interestingly more than 50% of subjects, independently of gender, had RBC-Fol concentrations below the reference interval, indicating a general lack of folate in the diets of smokers. When considered separately, low RBC-Fol concentrations were more common in male than in female subjects, possibly explaining the higher frequency of hyperhomocysteinemia in men than in women (Table 2). Finally, adequate folate and vitamin B12 levels are essential for cellular metabolism, with insufficiency associated with several disorders [20–25].

In the present study, moderate dyslipidemia was present in most subjects. Comparisons between men and women, both crude and adjusted for BMI, age, number of cigarettes smoked and nutritional score, showed that the frequency of increased tCh concentrations was very similar in the two groups. However, HDL-Ch concentrations were significantly lower in men than in women and low HDL-Ch levels were more common in male than in female subjects.

HDL-Ch concentrations are generally higher in premenopausal women than in men of the same age, while low HDL-Ch levels are reported to be a more significant predictor of risk in older women than in older men [27].

Numerous observational and clinical trials, reported by Mosca [27], have showed that concentrations of LDL-Ch, the major atherogenic lipoprotein in both men and women, can be used to predict the risk of cardiovascular disease, which in our population appears to be the same in both genders.

TG concentrations are also significantly associated with cardiovascular risk. The present study showed normal TG levels in most of the smokers, probably due to the beneficial effects of a Mediterranean diet [28].

These findings clearly support the evidence of gender-related differences in lipid and homocysteine metabolism [14, 29] and the suggestion that women have better diets.

Elevated tHcy concentrations can result from inadequate vitamin intake [10]. Long-term smoking has been associated with an increased risk of developing chronic disease and several authors recently reported that the diets of smokers are more unbalanced than those of non-smokers [10]. Generally, healthy lifestyle habits, such as a prudent diet, moderate drinking and not smoking, may help to prevent pathological conditions [10]. However, it has been reported that healthy smokers showed a marked increase in plasma antioxidant status after giving up smoking [10].

Current studies support the role of dietary antioxidants in disease prevention and health promotion. Increases in plasma concentrations of low molecular weight or soluble micro-antioxidants (vitamins C and E, phenolic compounds and carotenoids) and macro-nutrients (polymeric phenolic compounds, or polyphenols and carotenoids linked to plant food macromolecules) yield bioavailable metabolites through the action of microbiota. This has significant local and/or systemic effects and strengthens the immune system [30]. Moreover, a reduction in risk factors identified by histological and blood biochemical changes can indicate the clinical efficacy of suitable supplementation.

Several interesting studies have shown that improving dietary habits (including the consumption of a juice powder concentrate rich in exogenous macromolecular antioxidants) may have beneficial effects on parameters related to gastrointestinal health as well as systemic effects, especially in relation to cardiometabolic alterations [20–22, 30]. Moreover, moderate consumption of red wine is associated with a lower risk of cardiovascular disease (increased HDL levels, better sensitivity to insulin, improvements in factors influencing blood clotting), the well-known 'French paradox'.

However, standard dosages of vitamins and/or nutraceuticals supplementation have not been defined, so doses should be calculated according to each patient's observed deficit, co-morbidities and preventive/therapeutic targets. Moreover, coadministration should be carefully managed in light of possible antioxidant synergy so that the benefits far outweigh any potential side effects. In conclusion, the present pilot study confirms the diagnostic value of homocysteine and lipid panels when evaluating cardiovascular risk in asymptomatic and seemingly healthy heavy smokers with no evidence of total vitamin B12 deficiency but with clear signs of gender-related unbalanced Hcy metabolism and an impaired total lipid profile.

Finally, use of homocysteine and lipid panels can facilitate the early detection of subjects likely to develop disease who could benefit from early antioxidant treatment supplied by tailored nutraceutical supplements.

These interesting results may prompt us to carry out further studies on a larger population to confirm these preliminary findings.

Acknowledgements

The authors thank Mrs Mary Coduri for English correction and Miss Gloria Defilippi for help with preparing and assaying samples.

References

1. Wu X-Q, Ding J, Ge A-Y *et al.* (2013) Acute phase homocysteine related to severity and outcome of atherothrombotic stroke. Eur J Intern Med 24:362–367
2. Finkelstein JD (2006) Inborn errors of sulfur-containing amino acid metabolism. J Nutr 136:1750S–1754S
3. Selhub J (2006) The many facets of hyperhomocysteinemia: studies from the Framingham cohorts. J Nutr 136:1726S–1730S

4. Herrmann W, Obeid R, Schorr H, Geisel J (2003) Functional vitamin B12 deficiency and determination of holotranscobalamin in populations at risk. Clin Chem Lab Med 41:1478–1488
5. Herrmann W, Schorr H, Obeid R, Geisel J (2003) Vitamin B-12 status, particularly holotranscobalamin II and methylmalonic acid concentrations, and hyperhomocysteinemia in vegetarians. Am J Clin Nutr 78:131–136
6. Vigna L, Novembrino C, De Giuseppe R *et al.* (2011) Nutritional and oxidative status in occupational obese subjects. Med J Nutr Metab 4:69–74
7. White WB (2007) Smoking-related morbidity and mortality in the cardiovascular setting. Prev Cardiol 10:1–4
8. Sobczak A, Wardas W, Zielinska-Danch W, Pawlicki K (2004) The influence of smoking on plasma homocysteine and cysteine levels in passive and active smokers. Clin Chem Lab Med 42:408–418
9. Pounis GL, Di Castelnuovo AF, de Lorgeril M *et al.* (2014) Folate intake and folate serum levels in men and women from two European populations: the IMMIDIET project. Nutrition 30:822–830
10. Bamonti F, Novembrino C, Ippolito S *et al.* (2006) Increased free malondialdehyde concentrations in smokers normalise with a mixed fruit and vegetable juice concentrate: a pilot study. Clin Chem Lab Med 44:391–395
11. Rubino FM, Della Noce C, Vigna L *et al.* (2013) Measurement of glutathionylated haemoglobin by MALDI-ToF mass spectrometry as a biomarker of oxidative stress in heavy smokers and in occupational obese subjects. Int J Anal Mass Spectrom Chromatogr 1:22–30
12. Vassalle C, Novembrino C, Maffei S *et al.* (2011) Determinants of oxidative stress related to gender: relevance of age and smoking habit. Clin Chem Lab Med 49:1509–1513
13. Frei B, Forte TM, Ames BN *et al.* (1991) Gas phase oxidants of cigarette smoke induce lipid peroxidation changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. Biochem J 277:133–138
14. Williams CM (2004) Lipid metabolism in women. Proc Nutr Soc 63:153–160
15. Calvelli L, Accinni R, Gregori D *et al.* (2011) A quick and effective questionnaire to value dietary intakes and oxidative stress related to food in pneumological patients. Progr Nutr 13:55–64
16. Novembrino C, Cighetti G, De Giuseppe R *et al.* (2011) Effects of encapsulated fruit and vegetable juice powder concentrates on oxidative status in heavy smokers. J Am Coll Nutr 30:49–56
17. Miller JW, Garrod MG, Rockwood AL *et al.* (2006) Measurement of total vitamin B12 and holotranscobalamin, singly and in combination, in screening for metabolic vitamin B12 deficiency. Clin Chem 52:278–285
18. Cleveland WS, Grosse E, Shyu WM (1992) Local regression models. In: Chambers JM, Hastie TJ (eds) Statistical models. S. Wadsworth & Brooks/Cole, Pacific Grove, CA, chapter 8
19. R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org
20. Bamonti F, Pellegatta M, Novembrino C *et al.* (2013) An encapsulated juice powder concentrate improves markers of pulmonary function and cardiovascular risk factors in heavy smokers. J Am Coll Nutr 32:18–25
21. Kawashikma A, Madarame T, Koike H *et al.* (2007) Four week supplementation with mixed fruit and vegetable juice concentrates increased protective serum antioxidants and folate and decreased plasma homocysteine in Japanese subjects. Asia Pac J Clin Nutr 16:411–421
22. Tungtrongchitr R, Pongpaew P, Soonthornruengyot M *et al.* (2003) Relationship of tobacco smoking with serum vitamin B12, folic acid and haematological indices in healthy adults. Public Health Nutr 6:675–681
23. Clarke R, Sherliker P, Hin H *et al.* (2007) Detection of vitamin B12 deficiency in older people by measuring vitamin B12 or the active fraction of vitamin B12, holotranscobalamin. Clin Chem 53:963–970
24. Morris MS, Jacques PF, Rosemberg IH *et al.* (2007) Folate and vitamin B12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr 85:193–200
25. Pitkin RM (2007) Folate and neural tube defects. Am J Clin Nutr 85:285S–288S
26. Bamonti-Catena F, Buccianti G, Porcella A *et al.* (1999) Folate measurements in patients on regular hemodialysis treatment. Am J Kidney Dis 33:492–497
27. Mosca L (2005) Management of dyslipidemia in women in the post-hormone therapy era. J Gen Intern Med 20:297–305
28. Sofi F, Abbate R, Gensini GF *et al.* (2010) Accruing evidence on benefits of adherence to the Mediterranean diet on health: an updated systematic review and meta-analysis. Am J Clin Nutr 92:1189–1196
29. Gonzalez-Gross M, Sola R, Albers U *et al.* (2007) B-vitamins and homocysteine in Spanish institutionalized elderly. Int J Vitamin Nutr Res 77:22–33
30. Perez-Jimenez J, Diaz-Rubio ME, Saura-Calixto F (2015) Contribution of macromolecular antioxidants to dietary antioxidant capacity: a study in the Spanish Mediterranean diet. Plant Food Hum Nutr 70:365–370