

Relationship of tobacco smoking with serum vitamin B₁₂, folic acid and haematological indices in healthy adults

Rungsunn Tungtrongchitr¹, Praneet Pongpaew^{1,*}, Malida Soonthornruengyot¹, Duangkamol Viroonudomphol¹, Niyomsri Vudhivai¹, Anchalee Tungtrongchitr², Benjaluck Phonrat³, Somchai Pooudong¹ and Frank Peter Schelp⁴

¹Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Rajthevee, Bangkok 10400, Thailand: ²Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand: ³Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand: ⁴Center for Humanity and Health Sciences, Free University, Berlin and Humbolt University, Berlin, Germany

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Abstract

Objectives: To investigate the effects of tobacco smoking on serum vitamin B₁₂, folic acid and haematological parameters in healthy Thai smokers and non-smokers.

Design: Cross-sectional study of smokers and non-smokers in a military unit in Bangkok, Thailand.

Setting: A military unit in Thailand.

Subjects: One hundred and twenty-three male smokers from a military unit in Bangkok, who participated voluntarily in the study, were investigated. Sixty-six male non-smokers from the same unit were selected as controls. Fasting blood samples were collected for investigation of vitamin B₁₂, folic acid and haematological variables.

Results: The serum folic acid concentration of smokers was lower than that of non-smokers, but was not statistically significantly different. Haemoglobin was lower in smokers than in non-smokers; 16.3% of smokers were anaemic compared with only 3.0% of non-smokers. Anaemia was not related to folate deficiency. The white blood cell count was found to be higher in smokers than in non-smokers.

Conclusion: The results of this study suggest that there were low serum folic acid concentrations in smokers compared with non-smokers, which might contribute to the development of vascular and cardiovascular diseases. The higher white blood cell count might be indicative alterations in the immune functions of smokers.

Keywords

Vitamin B₁₂
Folic acid
Haematological parameters
Smokers

The number of smokers in the population of the Third World will increase from 4.5 billion to 7.1 billion by 2025^{1,2}. Smokers aged 45–64 years have a three times higher mortality rate compared with non-smokers, and those aged 65–84 years have a doubling of their mortality rate³. Smoking is a major health problem and risk factor for chronic disease. Tobacco use is by far the most important risk factor for most respiratory symptoms and chronic bronchitis^{4–8}. There are now tens of thousands of studies linking cigarette smoking to increased morbidity and mortality from cardiovascular diseases, various forms of cancer and chronic obstructive pulmonary disease⁹.

Abnormal serum folic acid and vitamin B₁₂ concentrations might be the cause of homocysteine elevation, which has been recognised as an independent risk factor for vascular disease in smokers¹⁰. The diseases can be normalised with vitamin supplementation. Polymorphonuclear transit time was found to be delayed in the lungs immediately after smoking¹¹. Via chemical inactivation,

exposure to cigarette smoke may result in folic acid deficiency that principally affects the bronchial epithelium, rendering it more susceptible to neoplastic transformation by the carcinogenic hydrocarbons of tobacco smoke¹². Several of the hundreds of chemical components of cigarette smoke, primarily organic nitrites, nitrous oxide, cyanates and isocyanates, have been shown to interact with folic acid and vitamin B₁₂ coenzymes, transforming them into biologically inactive compounds¹³. Therefore, the aim of the present study was to determine vitamin B₁₂, folic acid and haematological parameters in healthy Thai smokers compared with non-smokers.

Subjects and methods

Subjects

The subjects used for this study consisted of participants from a military unit in Bangkok, Thailand. The objectives of this study were explained to the volunteers.

One hundred and twenty-three male smokers, who participated voluntarily in the study, were investigated. Sixty-six male non-smokers from the same unit were selected as controls. The ages of the subjects were in the range of 19–60 years. Information on age, socio-economic status, lifestyle patterns such as consumption of alcohol, smoking and taking of medicines, including past and present illnesses, were obtained by questionnaires. The number of cigarettes smoked per day and the duration of cigarette smoking were multiplied together and expressed as 'cigarette-years'.

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, and informed consent was obtained from each participant.

In the morning, about 10 ml of venous blood was taken from each subject after an overnight fast. Heparinised blood was used to determine haematological variables. Haemoglobin concentration, haematocrit values and mean corpuscular haemoglobin concentration (MCHC) were determined. A serum aliquot was stored frozen at -20°C for vitamin B₁₂ and folic acid determinations.

Laboratory techniques

Haemoglobin concentrations in whole blood were determined by using the modified cyanmethaemoglobin method¹⁴. The haematocrit values were measured by a micro-method using calibrated heparinised capillary tubes. After filling the capillaries with blood, they were centrifuged for 5 min at 14 000 *g* in a micro-haematocrit centrifuge (IEC MB centrifuge model 3412, Massachusetts, USA). Then the haematocrit values were read using a micro-haematocrit reader (Hawksleye Son Ltd, Marlborough, UK). MCHC was also calculated by the formula:

$$\text{MCHC (g dl}^{-1}\text{)} = \frac{\text{haemoglobin}}{\text{haematocrit}} (\text{g dl}^{-1}) \times 100.$$

Platelets in peripheral blood smears were counted using the method of Nosanchuk *et al.*¹⁵. Reticulocytes were counted under an oil-immersion lens¹⁶.

The morphology of both red and white blood cells was determined using the Wedge method, which involved making blood films, staining with Wright's stain and examining under a microscope using an oil-immersion lens (1000 \times)¹⁶. A differential leucocyte count was also performed under a microscope using an oil-immersion lens (1000 \times)¹⁶.

Vitamin B₁₂ and folic acid were determined from 200 μl serum samples by radioimmunoassay using commercial kits (Dualcount solid phase no boil assay for vitamin B₁₂/folic acid, Diagnostic Products Corporation, Los Angeles, CA, USA). To further minimise analytical variation, the same technician performed all assays and single lots of reagents were used. The between-run coefficients of variation for each of the parameters

were less than 5% ($n = 30$ runs), corresponding to a between-run variance of 0.002. The concentrations of serum folate are reflected in dietary intake, but dietary assessment for folate intake was not recorded in this study and only the exclusion criterion of vitamin tablet intake was examined.

Statistical methods

All continuous data were examined for their distribution, skewness and kurtosis. As these were dispersed from a normal distribution, the results are expressed as median and range and non-parametric statistical analysis was used. For data processing, the Minitab computer program was utilised⁷. The Mann–Whitney *U*-test and the Wilcoxon rank sum *W*-test were used to compare the differences between smokers and non-smokers for continuous variables. The chi-square test was used to compare proportions.

To determine whether smoking was directly related to B₁₂ and folic acid levels, a covariance analysis was performed taking folic acid, vitamin B₁₂, alcohol drinking and age as independent variables and smoking as the dependent variable. The statistical software program SPSS 9.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for these computations.

Results

Tables 1 and 2 show the characteristics of the participants (smokers and non-smokers) and the distribution of smokers according to the quantity of cigarettes smoked (units in numbers of cigarette-years). Age and haematological variables of smokers and non-smokers are given in Table 3. Haemoglobin, haematocrit (packed red cell volume) and MCHC in smokers were slightly and significantly lower than in non-smokers. Serum folic acid concentrations of smokers were lower than those of non-smokers, but not statistically significantly different (Table 3). Mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) of smokers were not statistically significantly different from those of non-smokers. Serum vitamin B₁₂ levels were statistically significantly higher in the smokers than the non-smokers. The smokers had a significantly higher white blood cell count than the non-smokers, mainly in neutrophil and eosinophil levels (Table 3). Of smokers, 16.3% were anaemic compared with only 3% of non-smokers using a haemoglobin concentration below 13.0 g dl^{-1} as the cut-off point. Of smokers, 17.9% and 4.5% of non-smokers had a haematocrit below the cut-off point of 40%. According to their MCHC, 6.5% of smokers and 16.7% of non-smokers had MCHC below the cut-off point of 33 g dl^{-1} (Table 4). Regarding serum folic acid, 76 out of 122 (61.8%) of the smokers and 30 out of 66 (45.5%) of the non-smokers had serum folic acid levels below the cut-off point of 6.79 nmol l^{-1} (odds ratio = 1.98, $P = 0.026$) (Table 4).

Table 1 Descriptive data for smokers and non-smokers

	Smokers, n (%)	Non-smokers, n (%)
Age distribution (years)		
18–30	28/123 (22.8)	21/66 (31.8)
31–40	34/123 (27.6)	17/66 (25.8)
41–50	40/123 (32.5)	18/66 (27.3)
51–60	20/123 (16.3)	10/66 (15.2)
> 60	1/123 (0.8)	–
Education		
Primary	33/123 (26.8)	3/66 (5.5)
High	51/123 (41.5)	26/66 (39.4)
Vocational	16/123 (13.0)	14/66 (21.2)
Undergraduate/postgraduate	23/123 (18.7)	23/66 (34.9)
Status		
Low	22/122 (18.0)	13/66 (19.7)
Middle	49/122 (40.2)	20/66 (30.3)
High	51/122 (41.8)	33/66 (50.0)
Marital status		
Single	27/123 (22.0)	23/66 (34.8)
Married	92/123 (74.8)	41/66 (62.1)
Widow	2/123 (1.6)	1/66 (1.5)
Divorced	2/123 (1.6)	1/66 (1.5)
Alcohol drinking		
Not drink	11/123 (8.9)	30/66 (45.5)
Drink	106/123 (86.2)	32/66 (48.5)
Gave up	6/123 (4.9)	4/66 (6.1)
Tonic drink		
Not drink	50/119 (42.0)	49/66 (74.2)
Drink	69/119 (58.0)	17/66 (25.8)

Table 5 shows the correlation coefficients between haematological values, serum vitamin B₁₂ and serum folic acid for male smokers. Significant correlations were found between vitamin B₁₂, MCH, MCV and serum folic acid. There were significantly negative correlations between MCV and serum folic acid.

To determine whether smoking is directly related to increased vitamin B₁₂ concentration, or whether it is a confounder, a covariance analysis was carried out where folic acid, alcohol consumption and age were taken as independent variables and vitamin B₁₂ as the dependent variable (Table 6). Age and folic acid were not found to be significantly related, under these conditions, to vitamin B₁₂. When smoking was added to the model, a significant relationship to serum vitamin B₁₂ independent of alcohol consumption was found.

Table 2 Distribution of smokers according to the quantity of cigarettes smoked for the whole period of smoking (units in number of cigarette-years)*

Quantity of cigarettes smoked (cigarette-years)	n	%
1–5	36	29.3
6–10	34	27.6
11–15	30	24.4
16–20	18	14.6
>21	5	4.1

*Number of cigarettes per day multiplied by duration of smoking (years).

Discussion

Serum vitamin B₁₂ was significantly higher in smokers than in non-smokers. The median serum vitamin B₁₂ level was 464.9 pmol l⁻¹ in the smokers and 313.7 pmol l⁻¹ in the non-smokers (Table 3). It is known that hyperhomocystinaemia is linked to inadequate intake of vitamins, particularly B-group vitamins, and therefore may be amenable to nutritional intervention¹⁸. The study by Bostom and Lathrop¹⁹ is the only one in which concentrations of all three vitamins known to influence hyperhomocystinaemia were determined. It has been recognised that smoking affects the nutritional status of folic acid, vitamin B₁₂ and vitamin B₆^{20–23}, each of which regulates homocysteine metabolism²⁴. Alternatively, it may be because cigarette smokers have poorer diets than non-smokers²⁵; smokers are more likely to choose white bread, sugar, meat, butter, whole milk and eggs, and less likely to consume whole-wheat bread, high-fibre breakfast cereals, fruits and vegetables, than non-smokers^{26,27}. The usual dietary sources of vitamin B₁₂ are meat and meat products (including shellfish, fish, poultry and eggs) and to a lesser extent milk and milk products. The source of vitamin B₁₂ in animal products is via the animal's ingestion of micro-organisms containing vitamin B₁₂ or the vitamin B₁₂-producing activity of micro-organisms in the animal's alimentary tract being sufficiently high to result in absorption and storage in the animal's tissues²⁸. The results obtained in this study confirm that the highest value of serum vitamin B₁₂ is found in smokers. However, this result is not in accordance with the results of a study by Pagan *et al.*²⁹, who found serum vitamin B₁₂ concentrations to be significantly lower in smokers than in non-smokers. Univariate analysis of local and systemic vitamin B₁₂ concentrations showed significantly lower buccal mucosa vitamin B₁₂ concentrations in current smokers²². The 11.8% prevalence of subnormal cobalamin concentrations (<140 pmol l⁻¹) confirms that low vitamin B₁₂ concentrations are common in the elderly³⁰, although the prevalence may be even higher in Europe³¹, where the population is largely white.

Although there is wide documentation of the adverse effects of cigarette smoking on a variety of diseases and disturbances, the direct effects of smoking on nutrient concentrations are less well studied³². Detrimental effects of cigarette smoke on systemic concentrations of folic acid and vitamin B₁₂ have been known for decades. Many of these published studies, however, have not considered other factors, including dietary intake, which might explain the differences in folic acid or vitamin B₁₂ status among smokers and non-smokers^{20,33–36}. In this study, higher serum B₁₂ levels in smokers could not explain the relationships between the increased levels of white blood cells found in smokers. However, Bunting *et al.*³⁷ reported that high apoptotic leucocyte levels were found in subjects who had low serum cobalamin. Therefore, a high level of

Table 3 Median, range and 95% confidence interval (CI) of age, anthropometric variables, haematological measurements, vitamin B₁₂ and folic acid in smokers and non-smokers

Variable	Smokers (n = 123)		Non-smokers (n = 66)		P-value
	Median (range)	95% CI	Median (range)	95% CI	
Age (years)	40.0 (19.0–68.0)	38.0–42.0	37.0 (19–59)	32.0–42.0	0.326
Haemoglobin (g dl ⁻¹)	13.9 (11.4–16.6)	13.6–14.3	14.6 (12.3–15.8)	14.2–14.8	0.009*
Haematocrit	0.430 (0.350–0.525)	0.423–0.438	0.445 (0.380–0.484)	0.432–0.450	0.027*
MCHC (g dl ⁻¹)	32.6 (29.5–37.2)	32.2–32.8	33.0 (30–35.4)	32.8–32.2	0.046
MCH (pg)	28.4 (19.3–37)	27.5–28.8	28.3 (3.9–34.4)	27.6	0.855
MCV (fl)	86.3 (62.5–100.9)	81.6–87.4	85.5 (66.7–97.3)	84.0	0.828
Basophils (cells/100 WBC)	0 (0–12)	0–0	0 (0–1)	0–0	0.087
Eosinophils (cells/100 WBC)	3.0 (0–30)	2.0–3.0	2.0 (0–14)	1.0–3.0	0.070
Lymphocytes (cells/100 WBC)	33.0 (10–55)	30.00–35.00	35.50 (21–50)	32.60–38.0	0.215
Monocytes (cells/100 WBC)	5.0 (1–10)	4.0–5.0	4.0 (1–18)	4.0–5.0	0.455
Neutrophils (cells/100 WBC)	58.0 (23–85)	56.15–60.0	56.5 (38–74)	54.0–60.0	0.933
WBC count (× 10 ⁹ l ⁻¹)	7000 (3600–13 500)	6500–7385	6300 (3800–10 100)	5800–6840	0.013*
Platelet count (× 10 ⁹ l ⁻¹)	255 000 (63 000–585 000)	243 000–268 392	250 500 (138 000–365 000)	238 601–264 789	0.525
Serum B ₁₂ (pmol l ⁻¹)	464.9 (36.9–2435.4)	373.1–546.6	313.7 (31.0–1439.1)	279.2–333.4	0.001*
Serum folic acid (nmol l ⁻¹)	8.84 (0.99–32.86)	7.48–10.06	9.74 (0.48–23.79)	8.12–11.35	0.457

MCHC – mean corpuscular haemoglobin concentration; MCH – mean corpuscular haemoglobin; MCV – mean corpuscular volume; WBC – white blood cell.
*Significant difference using the Mann–Whitney U-test.

Table 4 Haematological parameters indicating anaemia* in smokers and non-smokers

Variable	Smokers		Non-smokers		Odds ratio	P-value
	n	%	n	%		
Haemoglobin	20/122	16.3	2/66	3.0	6.27	0.006
Haematocrit	22/122	17.9	3/66	4.5	4.53	0.010
MCHC	8/122	6.5	11/66	16.7	0.35	0.028
Serum B ₁₂	35/122	28.5	18/66	27.3	1.07	0.837
Serum folic acid	76/122	61.8	30/66	45.5	1.98	0.026

MCHC – mean corpuscular haemoglobin concentration.
*Values indicating haematological deficiencies are, for males: haemoglobin <13 g dl⁻¹; haematocrit <0.400; MCHC <33 g dl⁻¹; serum B₁₂ <147.6 pmol l⁻¹; serum folic acid <6.79 nmol l⁻¹.

serum cobalamin might be a factor in extending the lifetime of leucocytes and related to higher numbers of white blood cells. When computing covariance, where folic acid, alcohol consumption and age were taken as

independent variables and vitamin B₁₂ as a dependent variable, the model with the two independent variables did not significantly determine the variation of the dependent variable, vitamin B₁₂. However, when smoking was added to the model, this variable significantly determined the variation in serum vitamin B₁₂ (Table 6). Therefore, an increase in vitamin B₁₂ can be assumed to be a useful protective reaction for smokers. Another reason for the increased leucocytes found in smokers may be the accumulation of polymorphonuclear white blood cells in upper and lower respiratory tract infections, which most frequently occurs in smokers. This hypothesis may require further study.

Serum folic acid concentrations of the smokers were lower than those of the non-smokers, but not significantly different. The median of serum folic acid levels in smokers was 8.84 nmol l⁻¹, and 9.74 nmol l⁻¹ in non-smokers (Table 3). It is important to mention that plasma folic

Table 5 Correlation coefficients of haematological parameters, anthropometric parameters, serum B₁₂ and serum folic acid in smokers

	B ₁₂	Folic acid	Haemoglobin	Haematocrit	MCH	MCHC	Platelet	MCV
B ₁₂	1.000	0.274**	-0.096	-0.061	-0.045	-0.079	-0.051	-0.071
Folic acid	0.274**	1.000	0.010	0.034	-0.160*	-0.113	0.051	-0.174**
Haemoglobin	-0.096	0.010	1.000	0.794**	0.192**	0.160*	-0.058	0.217**
Haematocrit	-0.061	0.034	0.794**	1.000	0.027	-0.045	-0.036	0.090
MCH	-0.045	-0.160*	0.192**	0.027	1.000	0.598**	-0.157*	0.797**
MCHC	-0.079	-0.113	0.160*	-0.045	0.598**	1.000	-0.133*	0.435**
Platelet	-0.051	0.051	-0.058	-0.036	-0.157*	-0.133*	1.000	-0.119
MCV	-0.071	-0.174**	0.217**	0.090	0.797**	0.435**	-0.119	1.000

MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; MCV – mean corpuscular volume.
Significance level: *, P < 0.05; **, P < 0.01.

Table 6 Covariance analysis of folic acid, alcohol drinking and age as independent variables, and vitamin B₁₂ as dependent variable, and smoking as an additional independent variable to the model

Model	Coefficient			
	β	Standard error	T	Significance level
(Constant)	1.162	0.150	7.755	0.000
Folic acid	-1.214×10^{-2}	0.014	-0.862	0.390
Alcohol drinking	2.590×10^{-4}	0.039	3.255	0.001
Age	4.912×10^{-3}	0.003	1.523	0.130
(Constant)	1.284	0.093	13.766	0.000
Alcohol drinking	2.476×10^{-4}	0.000	3.525	0.001
Smoking	23.007	6.546	3.515	0.001

acid is related to recent consumption, while red blood cell folic acid is an indicator of folic acid stores³⁸. People who smoke cigarettes are known to differ from persons who have never smoked with respect to several lifestyle behaviours, including eating less healthful diets and drinking more alcohol³⁹⁻⁴³. This study found that 86.2% of the smokers drank alcohol compared with 48.5% of the non-smokers. Alcohol is a known antagonist of folic acid metabolism⁴⁴ and an interaction between alcohol and folic acid intake was reported in prospective studies of coronary heart disease, colon cancer and breast cancer. For each of these diseases, the benefit of folic acid intake in decreasing risk is stronger in alcohol users than in non-users⁴⁵⁻⁴⁸. With respect to folic acid metabolism, Sullivan and Herbert⁴⁹ were among the first investigators to recognise that ethanol has an effect on folic acid status that cannot be explained simply by a diminished intake of folic acid. The causes for this deficiency are presently unclear although a number of mechanisms have been proposed, including diminished dietary intake, poor absorption of polyglutamyl folic acids, decreased hepatic uptake and retention, increased urinary excretion of folic acid, impaired formation or hydrolysis of polyglutamates, and increased folic acid catabolism⁵⁰⁻⁵⁶. Several of the hundreds of chemical components of tobacco smoke have been shown to interact with folic acid coenzymes, transforming them into biologically inactive compounds¹³. These chemical interactions may have physiological significance, which is supported by reports of lowering circulating folic acid levels in smokers³⁴. Reactive oxygen species (ROS) can be produced by cigarette smoke-induced phagocytic cells and cause oxidative damage to DNA, proteins and lipids, which may be closely related to cancer, ageing and cardiovascular disease. ROS may act as an initiator or promoter in multi-stage chemical carcinogenesis⁵⁷. Our study also reported results in accordance with other studies in which white blood cell count in smokers was significantly higher than in non-smokers (30.0% higher leucocyte count). The higher white blood cell count might also cause the alteration of immune function.

In this study, 16.3% of smokers were anaemic compared with only 3% of non-smokers. Anaemia was not related to folic acid deficiency because the MCV of the smokers was

not statistically different from that of the non-smokers (Table 3). Folic acid malabsorption also causes folic acid deficiency, although anaemia and macrocytosis are not seen consistently. This malabsorption is caused by diseases affecting the jejunum, such as celiac diseases, tropical sprue, jejunal resection or infiltrative disease such as lymphoma of the upper intestine⁵⁸. Folic acid deficiency can also be due to an increased demand of any kind. The demand may be physiological such as haemolytic anaemia, malignant disease, inflammatory disease or psoriasis. Liver disease and alcoholism are usually associated with folic acid deficiency but the mechanisms are often multi-factorial. In addition to dietary inadequacy, varying degrees of intestinal malabsorption are induced by excessive alcohol consumption. Altered hepatic function is also a potential cause of folic acid deficiency because the liver is the major site of folic acid storage and metabolism. A recent report documented the elevation of plasma homocysteine in alcoholism⁵⁹.

This result implies that nutritional intervention plans that are specifically designed for the enhancement of folic acid status might be necessary for cigarette smokers in public health programmes. In the future, such programmes should face the challenge of providing appropriate care to maintain a high quality of life for this population group.

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References

- 1 United Nations. *World Population Prospects 1990*. New York: United Nations, 1991; 226-31.

- 2 United Nations. *Sex and Age Distributions of Populations. The 1990 Revision*. New York: United Nations, 1991; 4.
- 3 Doll R, Peto R, Wheatley K, Gray R, Sutherland I. Mortality in relation to smoking: 40 years' observations on male British doctors. *British Medical Journal* 1994; **309**: 901–5.
- 4 Huhti E. Prevalence of respiratory symptoms, chronic bronchitis and pulmonary emphysema in a Finnish rural population. Field survey of age group 40–64 in the Harjavalta area. *Acta Tuberculosea et Pneumologica Scandinavica* 1965; **61**(Suppl.): 1–111.
- 5 Flecher C, Peto R, Tinker C, Speizer FE. *The Natural History of Chronic Bronchitis and Emphysema*. London: Oxford University Press, 1976.
- 6 Lundback B, Nystrom L, Rosenhall L, Stjernberg N. Obstructive lung disease in northern Sweden: respiratory symptoms assessed in a postal survey. *European Respiratory Journal* 1991; **4**: 257–66.
- 7 American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine* 1995; **152**(Suppl.): S78–9.
- 8 Troisi RJ, Speizer FE, Willett WC, Trichopoulos D, Rosner B. Menopause, postmenopausal estrogen preparations, and the risk of adult-onset asthma. A prospective cohort study. *American Journal of Respiratory and Critical Care Medicine* 1995; **152**: 1183–8.
- 9 US Public Health Service, US Department of Health and Human Services, The Office of Disease Prevention and Health Promotion. *Disease Prevention and Health Promotion. The Facts*. Palo Alto, CA: Bull Publishing Co., 1988.
- 10 Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, *et al.* Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *Journal of the American Medical Association* 1997; **277**: 1775–81.
- 11 Francis KT, Thompson RW, Krumdieck CL. Reaction of tetrahydrofolic acid with cyanate from urea solutions: formation of an inactive folate derivative. *American Journal of Clinical Nutrition* 1977; **30**: 2028–32.
- 12 Khaled MA, Krumdieck CL. Association of folate molecules as determined by proton NMR: implications on enzymes binding. *Biochemical and Biophysical Research Communications* 1985; **130**: 1273–80.
- 13 Khaled MA, Watkins CL, Krumdieck CL. Inactivation of B₁₂ and folate coenzymes by butyl nitrite as observed by NMR: implications on one-carbon transfer mechanism. *Biochemical and Biophysical Research Communications* 1986; **135**: 201–7.
- 14 International Committee for Standardization in Hematology. Recommendations for reference method for hemoglobinometry in human blood (ICSH Standard EP 6/2: 1977) and specifications for international hemoglobin cyanide reference preparation (ICSH Standard EP 6/3: 1977). *Journal of Clinical Pathology* 1978; **31**: 139–43.
- 15 Nosanchuk JS, Chang J, Bennett JM. The analytic basis for the use of platelet estimates from peripheral blood smears. *American Journal of Clinical Pathology* 1978; **69**: 383–7.
- 16 Nelson DA, Morris MW. Basic methodology: Part IV. Hematology and coagulation. In: Henry JB, ed. *Clinical Diagnosis and Management by Laboratory Methods*, 17th ed. Philadelphia, PA: WB Saunders Company, 1984; 578–625.
- 17 Ryan TA, Brian LB, Ryan BF. *Minitab Student Handbook*, 2nd ed. Boston: PWS-Kent Publishing Company, 1985.
- 18 Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *Journal of the American Medical Association* 1993; **270**: 2693–8.
- 19 Bostom AG, Lathrop L. Hyperhomocysteinemia in end-stage renal disease: prevalence, etiology, and potential relationship to arteriosclerotic outcomes. *Kidney International* 1997; **52**: 10–20.
- 20 Dastur DK, Quadros EV, Wadia NH, Desai MM, Bharucha EP. Effect of vegetarianism and smoking on vitamin B₁₂, thiocyanate, and folate levels in the blood of normal subjects. *British Medical Journal* 1972; **3**: 260–3.
- 21 Ortega RM, Lopez-Sobaler AM, Gonzalez-Gross MM, Redondo RM, Marzana I, Zamora MJ, *et al.* Influence of smoking on folate intake and blood folate concentrations in a group of elderly Spanish men. *Journal of the American College of Nutrition* 1994; **13**: 68–72.
- 22 Piyathilake CJ, Macaluso M, Hine RJ, Richards EW, Krumdieck CL. Local and systemic effects of cigarette smoking on folate and vitamin B₁₂. *American Journal of Clinical Nutrition* 1994; **60**: 559–66.
- 23 Giraud DW, Martin HD, Driskell JA. Erythrocyte and plasma B-6 vitamers concentrations of long-term tobacco smokers, chewers, and nonusers. *American Journal of Clinical Nutrition* 1995; **62**: 104–9.
- 24 Refsum H, Ueland PM, Nygard O, Vollset SE. Homocysteine and cardiovascular disease. *Annual Review of Medicine* 1998; **49**: 31–62.
- 25 Dallongeville J, Marceaux N, Fruchart JC, Amouyel P. Cigarette smoking is associated with unhealthy patterns of nutrient intake: a meta-analysis. *Journal of Nutrition* 1998; **128**: 1450–7.
- 26 Larkin FA, Basiotis PP, Riddick HA, Sykes KE, Pao EM. Dietary patterns of women smokers and non-smokers. *Journal of the American Dietetic Association* 1990; **90**: 230–7.
- 27 Margetts BM, Jackson AA. Interactions between people's diet and their smoking habits: the Dietary and Nutritional Survey of British Adults. *British Medical Journal* 1993; **307**: 1381–4.
- 28 Herbert V. Vitamin B₁₂. *American Journal of Clinical Nutrition* 1981; **34**: 971–2.
- 29 Pagan K, Hou J, Goldenberg RL, Cliver SP, Tamura T. Effect of smoking on serum concentrations of total homocysteine and B vitamins in mid-pregnancy. *Clinica Chimica Acta* 2001; **306**: 103–9.
- 30 Carmel R. Cobalamin, the stomach, and aging. *American Journal of Clinical Nutrition* 1997; **66**: 750–9.
- 31 van Asselt DZ, de Groot LC, van Staveren WA, Blom HJ, Wevers RA, Biemond I, *et al.* Role of cobalamin intake and atrophic gastritis in mild cobalamin deficiency in older Dutch subjects. *American Journal of Clinical Nutrition* 1998; **68**: 328–34.
- 32 Preston AM. Cigarette smoking – nutritional implications. *Progress in Food & Nutrition Science* 1991; **15**: 183–217.
- 33 Linnell JC, Smith AD, Smith CL, Wilson J, Mathews DM. Effects of smoking on metabolism and excretion of vitamin B₁₂. *British Medical Journal* 1968; **2**: 215–6.
- 34 Witter FR, Blake DA, Baumgardner R, Mellittis ED, Niebyl JR. Folate, carotene, and smoking. *American Journal of Obstetrics and Gynecology* 1982; **144**: 857.
- 35 Nakazawa Y, Chiba K, Imatoh N, Kotorii T, Sakamoto T, Ishizaki T. Serum folic acid levels and antipyrine clearance rates in smokers and non-smokers. *Drug and Alcohol Dependence* 1983; **11**: 201–7.
- 36 Chen AT, Reidy JA, Annet JL, Welty TK, Zhou HG. Increased chromosome fragility as a consequence of blood folate levels, smoking status, and coffee consumption. *Environmental and Molecular Mutagenesis* 1989; **13**: 319–24.
- 37 Bunting RW, Selig MK, Dickersin GR. Apoptotic cells in peripheral blood from patients with low serum cobalamin. *Journal of Submicroscopic Cytology and Pathology* 1997; **29**: 223–7.

- 38 McNulty H. Folate requirements for health in women. *Proceedings of the Nutrition Society* 1997; **56**: 291–303.
- 39 Hays R, Stacy AW, DiMatteo MR. Covariation among health-related behaviors. *Addictive Behaviors* 1984; **9**: 315–8.
- 40 Castro FG, Newcomb MD, McCreary C, Baezconde-Garbanati L. Cigarette smokers do more than just smoke cigarettes. *Health Psychology* 1989; **8**: 107–29.
- 41 Kato I, Tominaga S, Suzuki T. Characteristics of past smokers. *International Journal of Epidemiology* 1989; **18**: 345–54.
- 42 Schoenborn CA, Boyd GM. Smoking and other tobacco use. *Vital and Health Statistics* 1989; **10**: 1–79.
- 43 Osler M. Social class and health behavior in Danish adults: a longitudinal study. *Public Health* 1993; **107**: 251–60.
- 44 Hillman RS, Steinberg SE. The effects of alcohol on folate metabolism. *Annual Review of Medicine* 1982; **33**: 345–54.
- 45 Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, *et al.* Folate, methionine, and alcohol intake and risk of colorectal adenoma. *Journal of the National Cancer Institute* 1993; **85**: 875–84.
- 46 Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, Willett WC. Alcohol, low-methionine–low-folate diets, and risk of colon cancer in men. *Journal of the National Cancer Institute* 1995; **87**: 265–73.
- 47 Rimm EB, Willett WC, Hu FB, Sampson L, Colditz GA, Manson JE, *et al.* Folate and vitamin B₆ from diet and supplements in relation to risk of coronary heart disease among women. *Journal of the American Medical Association* 1998; **279**: 359–64.
- 48 Zhang S, Hunter DJ, Hankinson SE, Giovannucci EL, Rosner BA, Colditz GA, *et al.* A prospective study of folate intake and the risk of breast cancer. *Journal of the American Medical Association* 1999; **281**: 1632–7.
- 49 Sullivan IW, Herbert V. Suppression of hematopoiesis by ethanol. *Journal of Clinical Investigation* 1964; **43**: 2048.
- 50 Halsted CH, Robles EA, Mezey E. Decreased jejunal uptake of labeled folic acid (³H-PGA) in alcoholic patients: roles of alcohol and nutrition. *New England Journal of Medicine* 1971; **285**: 701–6.
- 51 Halsted CH, Robles EA, Mezey E. Intestinal malabsorption in folate-deficient alcoholics. *Gastroenterology* 1973; **64**: 526–32.
- 52 Goldsmith RH, Iber FL, Miller PA. Nutritional status of alcoholics of different socioeconomic class. *Journal of the American College of Nutrition* 1983; **2**: 215–20.
- 53 Tamura T, Halsted CH. Folate turnover in chronically alcoholic monkeys. *Journal of Laboratory and Clinical Medicine* 1983; **101**: 623–8.
- 54 McMartin KE, Collins TD, Eisenga BH, Fortney T, Bates WR, Bairnsfather L. Effects of chronic ethanol and diet treatment on urinary folate excretion and development of folate deficiency in the rat. *Journal of Nutrition* 1989; **119**: 1490–7.
- 55 Naughton CA, Chandler CJ, Duplantier RB, Halsted CH. Folate absorption in alcoholic pigs: *in vitro* hydrolysis and transport at the intestinal brush border membrane. *American Journal of Clinical Nutrition* 1989; **50**: 436–41.
- 56 Shaw S, Jayatilleke E, Herbert V, Colman N. Cleavage of folates during ethanol metabolism. Role of acetaldehyde/xanthine oxidase-generated superoxide. *Biochemical Journal* 1989; **257**: 277–80.
- 57 Ames BN. Endogenous oxidative DNA damage, aging, and cancer. *Free Radical Research Communications* 1989; **7**: 121–8.
- 58 Chanarin I, Bennett MC. Absorption of folic acid and D-xylose as tests of small-intestinal function. *British Medical Journal*; 1962; **i**: 985–9.
- 59 Cravo ML, Gloria LM, Selhub J, Nadeau MR, Camilo ME, Resende MP, *et al.* Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12 and vitamin B-6 status. *American Journal of Clinical Nutrition* 1996; **63**: 220–4.