ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 3/2010
339–345
on-line at: www.actabp.pl

Regular paper

Correlation between folate and vitamin B_{12} and markers of DNA stability in healthy men: preliminary results

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The aim of this study was to find correlations between folate and vitamin B_{12} on baseline damage in white blood cells and their association with smoking, alcohol consumption and ageing. Thirty-six healthy vitamin non-deficient male subjects were selected in a randomized study. Comet assay (SCGE) and micronucleus (MN) assay were used as biomarkers of DNA damage. The amount of DNA damage was correlated with vitamin B_{12} and folic acid concentration. Positive, but non-significant correlation (canonical R = 0.61; $\chi^2=28.97$; $P=0.253$) was found between micronucleus (MN) frequency or comet assay parameters (SCGE) and five covariates (age, smoking, alcohol consumption, vitamin B_{12} and folate blood serum concentration). The highest MN frequency was observed in the group with the lowest vitamin B_{12} concentration ($F=3.59$; $P=0.024$). The SCGE assay failed to show significant correlation with vitamin B_{12} or folic acid concentration. Concentration of vitamin B_{12} was significantly correlated with incidence of micronuclei. Our results present background data that could be valuable for future genotoxicological monitoring.

Keywords: folic acid, vitamin B_{12}, micronucleus, comet assay, healthy men, DNA damage

Received: 29 Januray, 2010; revised: 21 April, 2010; accepted: 08 June, 2010; available on-line: 11 June, 2010

INTRODUCTION

Dietary habits play an important role in maintaining genome stability. An association between micronutrient intake (such as folate and vitamin B_{12}) and cancer incidence has been observed (Fenech, 2003a; Ames, 2001). Reduced thymidylate (TMP) synthesis has been associated with folate-sensitive fragile sites (Sutherland, 1988; Zingg & Jones, 1997), development of malignancies (Popescu, 2003) and with increased uracil misincorporation (Eto & Krumdieck, 1986; Fenech, 2001). Uracil removal can generate point mutations, lead to single and double DNA strand breaks, chromosome breakage, especially chromosome 21 aneuploidy (Beetstra *et al.*, 2005), micronucleus formation (Blount & Ames, 1995; Duthie, 1999) and malignant transformation (Basten *et al.*, 2006). Inadequate folate intake is associated with increased risk of neural tube defects, Down syndrome, cardiovascular disease, Alzheimer's disease and various cancers (Kažimirova *et al.*, 2006). An association between folate status and micronucleus frequency was also found in folate non-deficient individuals (Fenech *et al.*, 1998).

Vitamin B_{12} is required for the synthesis of *S*-adenosylmethionine (SAM), a common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (Zingg & Jones, 1997). Low concentrations of SAM lead to DNA hypomethylation, associated with abnormal gene expression (Fenech, 2003) and chromosomal segregation (Fenech, 2003) in specific regions such as the centromere, chromosomes 1, 9 and 16 (Tuck-Muller *et al.*, 2000), and fragile sites (Sutherland, 1988; Zingg & Jones, 1997). Very low intake of vitamin B_{12} leads to anaemia and nervous system damage (Kažimirova *et al.*, 2006). Folate and/or vitamin B_{12} deficiency can cause chromosome damage and micronucleus formation in humans *in vivo* (Kažimirova *et al.*, 2006) and in human cells *in vitro* (Fenech, 2001).

Genome stability is under the influence of exogenic factors (alcohol intake, smoking). Alcoholism is associated with significantly reduced levels of folate, vitamin B_{12} and B_6 in humans (Glória *et al.*, 1997). Excessive urinary folate excretion associated with acute or chronic ethanol ingestion predisposes to the development of folate deficiency (Hamid & Kaur, 2006). Smoking and ageing can also increase micronucleus frequency (Zijno *et al.*, 2003; Gabriel *et al.*, 2006).

The most widely used method for DNA damage assessment in human cells is the micronucleus (MN) assay based on counting MN in once-divided binucleated cultured cells after the cytokinesis block with cytochalasin B. MN originate mainly from chromosome breaks or whole chromosomes that have failed to engage with the mitotic spindle when the cell divides (Fenech *et al.*, 2003).

Another technique, alkaline comet assay (single-cell gel electrophoresis, SCGE) is an easy-to-use, quick and very sensitive method for detecting primary DNA strand breaks, that is, direct DNA damage within single cells (Tice *et al.*, 2000; Collins, 2004) and can be applied to proliferating and non-proliferating cells (Kassie *et al.*, 2000) to determine DNA damage as a result of endogenous factors, lifestyle (Morillas *et al.*, 2002; Hoffman & Speit, 2005), and occupational (Palus *et al.*, 1999; Zhu *et al.*,1999) and environmental exposure (Šrám *et al.*, 1998; Valverde *et al.*, 1999).

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Abbreviations: BRT, total number of nucleoplasmic bridges; BT, total number of nuclear buds; CBMN, cytochalasin B blocked micronucleus test; FA, folic acid; MN, micronucleus; NDI, nuclear division index; SAM, *S*-adenosylmethionine; SCGE, single-cell gel electrophoresis; TI, tail intensity; TL, tail length; TM, tail moment

The accuracy of any risk assessment, especially low-dose exposures, depends on both the resolution of the method, and the baseline data obtained in well-selected controls. The use of several independent endpoints improves the resolution and provides a more accurate risk assessment (Major *et al.*, 1998).

This study provides data on two biomarkers in peripheral blood samples collected from healthy male subjects randomly selected from the general population of the Republic of Croatia. The aim of the study was to characterise the variability in baseline damage in white blood cells, to investigate association with external and internal factors, and to establish background data useful as control values in future biomonitoring studies.

MATERIALS AND METHODS

Population characteristics. Thirty-six male blood donors (mean age 37.67 ± 9.26, age range: 22–56 years, see Table 1) of mixed social class and occupation (students, teachers, retired, clerks, drivers, waiters, policemen, and unemployed) were informed of the study scope and experimental details, have filled a standardised questionnaire designed to obtain relevant information on the current health status, medical history, and lifestyle, and gave their written consent, submitted and approved by the local Ethics Committee. The questionnaire included data on exposure to possible confounding factors: smoking, alcohol consumption, use of medicines, contraceptives, severe infections, or viral diseases over the past six months, vitamin intake, recent vaccinations, presence of known inherited genetic disorders and chronic diseases, family history of cancer, exposure to diagnostic X-rays, and previous radio- or chemotherapy. Two donors were excluded from the study (one for a recent virosis, and the other who had three diagnostic X-ray examinations during past year).

The enrolled subjects did not differ much in dietary habits and none had a history of occupational exposure to known genotoxic chemicals. Concerning the answers about regular daily alcohol consumption in the questionnaire, and since no one in the group exceeded the normal daily limit (1 dl of wine, 2 dl of beer, 0.3 dl of strong alcoholic drink), we have divided them into two groups, consumers and non-consumers.

Blood sampling. Peripheral blood (10 ml) was collected under sterile conditions by venipuncture, then 5 ml of heparinised blood was used for SCGE and MN assay. After collection, all blood samples were randomly coded and processed as quickly as possible (usually within 2 h following the blood sampling). Five milliliters of peripheral blood without an anticoagulant (BD vacutainer, Becton Dickinson, NJ, USA) was used for haematochemical analysis of plasma folate and vitamin B_{12} concentrations.

Table 1. Relevant demographic data of the study group

Parameter (units)	Value
Number of subject	36
Mean age (years)	37.67 ± 9.26
Age range (years)	22–56
Smokers (number and %)	13 (36.1)
Non-smokers (number and %)	23 (63.9)
Alcohol consumers (number and %)	22 (61.1)
Alcohol non-consumers (number and %)	14 (38.9)

Alkaline comet assay. SCGE was carried out under alkaline conditions, as described by Singh *et al.* (1988). Two fully-frosted microscopic slides per subject were prepared. Slides were stained with ethidium bromide (20 μg/ml, Sigma). All steps of the preparation of slides for the comet analysis were conducted under yellow light or in the dark. Slides were examined at 250 × magnifications with a fluorescence microscope (Zeiss, Germany, excitation filter of 515–560 nm and a barrier filter of 590 nm). A total of 200 comets per subject were scored (100 from each of two replica slides). Comets were randomly captured at a constant depth of the gel. The microscope was connected to a camera with a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd., UK). As a measure of DNA damage we used tail length (TL), tail moment (TM), and tail intensity (TI). TL was calculated from the centre of the head and presented in micrometers. TM was calculated using a computerised system, considering both the tail length and the fraction of DNA in the comet tail (tail intensity).

Micronucleus test. Cultures for the CBMN (cytochalasin B-blocked micronucleus) assay were set up in triplicate according to method of Fenech and Morley (1985). After 72 h of incubation the cell cultures received hypotonic treatment and were fixed according to Kapka *et al.* (2007). One thousand binucleated lymphocytes with well-preserved cytoplasm per subject were analyzed. The criteria used for the selection of binucleated cells, MN, BRT (total number of nucleoplasmic bridges), BT (total number of nuclear buds), and for the calculation of NDI (nuclear division index) have already been described by Fenech *et al.* (2003).

FA and vitamin B_{12} measurement. Serum levels of vitamin B_{12} and folic acid (FA) were measured with an electrochemiluminescence immunoassay Elecsys System 1010/2010 (Roche Diagnostics, Mannheim, Germany), based on competitive chemiluminescence immunoanalysis. The reference ranges in the general population in Croatia were 211–911 pg/l for vitamin B_{12}, and 1.1–20.0 ng/l for FA.

Statistical analyses. Statistical analyses were carried out using Statistica 5.0 package (StatSoft, Tulsa, USA). Each subject was characterized for the extent of DNA damage by considering the mean (± standard deviation) for the SCGE and MN parameters measured. Mann-Whitney U-test was used to test the differences between the groups. Analyses of variance, multiple regression and canonical correlation analyses were used for multiple comparisons between the groups to test the influence of age, smoking, alcohol, vitamin B_{12} and FA concentration (covariates) on the MN frequency (MNT), nucleoplasmic bridges (BRT) and buds (BT), mitotic index (NDI), tail length (TL), tail moment (TM), and tail intensity (TI) (dependent variables). For statistical analysis, subjects were divided into subgroups considering the age, and vitamin B_{12} and FA concentration. The level of statistical significance was set at $p<0.05$.

RESULTS

Relevant demographic data of the study on male group are shown in Table 1. Table 2 shows group data

Table 2. Means and standard deviations for the total number of micronuclei (MNT), nucleoplasmic bridges (BRT) and buds (BT) and mitotic index (NDI) per 1000 of binucleated cells

TL, tail length; TM, tail moment; TI, tail intensity for 200 comets; Y-alcohol consumers, N-alcohol non-consumers, NS-non smokers, S-smokers

Variable	Group (No. of people)	Vitamin B_{12} (ng/l)	Folic acid (μg/l)	MNT	BT	BRT	NDI	TL	TM	TI
Age	20–30 (9)	461.90±73.49	7.33±1.80	5.80±6.07	1.60±2.67	0.70±1.57	2.12±0.31	15.24±2.00	1.33±0.78	0.19±0.13
	31–40 (10)	439.10±133.18	6.82±2.05	7.50±5.72	1.60±2.50	1.30±2.36	2.16±0.20	14.38±0.63	1.87±0.83	0.24±0.10
	>40 (17)	451.19±153.68	7.08±3.74	10.13±7.39	2.25±2.24	0.94±1.73	1.97±0.36	14.48±0.81	2.18±3.73	0.17±0.06
Alcohol	N (22)	436.79±135.13	7.76±3.85	8.93±5.90	1.43±2.24	1.07±2.06	2.14±0.24	14.45±0.78	1.45±0.73	0.19±0.09
	Y (14)	459.73±124.06	6.65±1.88	7.73±7.25	2.18±2.48	0.91±1.74	2.02±0.35	14.80±1.47	2.12±3.19	0.20±0.10
Smoking	NS (23)	437.17±125.14	7.17±3.28	9.00±7.32	1.74±2.36	0.91±2.00	2.10±0.32	14.63±1.11	2.06±3.12	0.19±0.08
	S (13)	474.92±131.88	6.92±1.85	6.77±5.37	2.15±2.51	1.08±1.61	2.00±0.30	14.72±1.50	1.51±0.85	0.20±0.12
B_{12}	<400 (12)	307.75±52.16	6.60±1.88	12.83±7.06	2.75±2.83	0.83±1.99	1.99±0.43	14.25±0.95	1.52±0.54	0.20±0.07
	400–500 (12)	452.33±28.60	7.40±1.86	5.33±5.18	1.25±2.45	0.83±1.75	2.13±0.28	15.22±1.74	1.80±0.99	0.24±0.13
	501–600 (7)	549.14±14.33	7.43±5.59	6.57±4.86	1.71±1.89	1.71±2.43	2.09±0.25	14.69±0.70	3.19±5.68	0.15±0.04
	>601 (5)	652.80±43.65	6.97±1.46	6.20±6.87	1.60±1.52	0.60±0.55	2.04±0.13	14.29±0.56	0.98±0.16	0.13±0.02
Folic acid	<5 (6)	505.33±84.58	3.92±0.77	4.33±2.94	1.83±1.94	0.50±0.55	2.13±0.21	15.28±1.44	3.84±6.00	0.19±0.06
	5-7 (15)	415.00±146.56	5.94±0.61	10.53±6.46	1.20±2.14	0.67±1.05	2.11±0.31	14.53±0.84	1.53±0.73	0.20±0.09
	7.1–9 (8)	467.00±138.68	7.90±0.47	4.75±5.06	2.75±3.24	0.63±1.77	2.00±0.33	13.92±1.05	1.31±0.43	0.17±0.05
	>9 (7)	462.29±94.78	11.27±3.18	10.43±8.75	2.43±2.15	2.43±3.21	1.99±0.39	15.27±1.68	1.52±1.13	0.21±0.17
Mean ±S.D.		450.81±127.06	7.08±2.82	8.19±6.69	1.89±2.39	0.97±1.84	2.07±0.31	14.66±1.24	1.86±2.54	0.19±0.10
Range of the group		216–708	2.6–18.3	0–28	0–8	0–7	1.23–2.51	12.44–18.86	0.51–16.05	0.07–0.54

for MN and SCGE parameters, as well as for vitamin B_{12} and FA concentrations.

Micronucleus test

The results of MN assay are shown in Table 2. Total number of MN increased linearly with age. Unexpectedly, somewhat higher values were observed in non-smokers than in smokers. Also, higher values of MN were found in group of alcohol consumers compared to non-consumers. The highest MN frequency was in the group with the lowest vitamin B_{12} concentration ($F=3.59$; $P=0.024$). The same was not observed for FA concentration, as the second and the fourth group, ranging 5.0–7.7 μg/l and >9 μg/l respectively, showed two times higher mean values of MN when compared with the others. The total number of BT increased with age, alcohol consumption, smoking, decrease in vitamin B_{12}, and increase in FA. BRT values correlated with alcohol consumption and smoking. Unexpectedly, the highest BRT values were observed in the group with the highest FA concentration. NDI (mean value: 2.07±0.31; range: 1.23–2.51) did not show significant variation between the subgroups for any of the grouping variable.

Comet assay

The results of comet assay are shown in Table 2. None of the studied covariates affected TL, TI or TM averages.

Smokers had an average tail length of 14.72±1.50 μm and non-smokers, 14.63±1.11 (see Table 2).

As regards vitamin B_{12}, the largest TL was observed in the second group in which vitamin B_{12} range was 400-500 ng/l, and dropped linearly to the fourth group.

When the FA subgroups were observed, the TL and TM values were the highest in the first group with FA concentration <5 μg/l. The highest TM values were observed in the oldest group.

Coefficient of variation and general regression model

Figure 1. shows that the MN assay parameters had a higher coefficient of variation than the SCGE parameters. The lowest coefficient was found for TL, followed by NDI, while the highest was observed for BRT and BT. Generally, the coefficient of variation decreased with age and the concentration of vitamin B_{12}.

In order to predict which covariate or group of covariates had the highest influence on each dependent variable, we used the general linear model. The results are presented as Pareto charts for each dependent variable (Fig. 2).

From the t values it is obvious that of the five grouping variables, vitamin B_{12} concentration had the highest (statistically significant) influence on the total number of MN. Age and the combination of alcohol and smoking were risk factors as well. Vitamin B_{12} concentration also had the highest influence on the BT, while the FA concentration was the most important risk factor for the BRT and NDI. It seems that the combination of alcohol, smoking, and low vitamin B_{12} concentration had the greatest effect on the TI and TM.

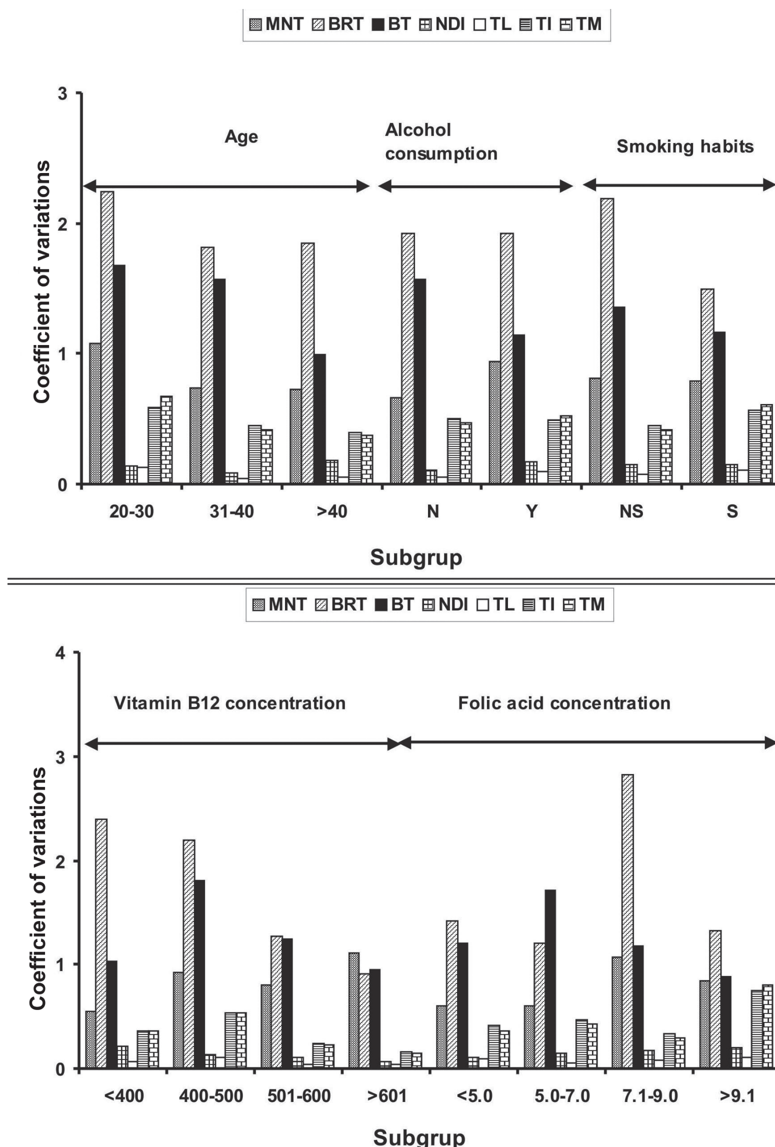

Figure 1. Variation coefficients of micronucleus assay (MNT, BT, BRT and NDI) and comet assay parameters (TL, TI, TM)
Male individuals were grouped by age, smoking, alcohol consumption, and concentrations of vitamin B_{12} and folic acid. MNT, total number of micronuclei; RT, total number of bridges; BT, total number of buds; BNDI, mitotic index; TL, tail length; TI, tail intensity; TM, tail moment.

Canonical correlation analyses

Canonical correlation analyses showed a positive insignificant correlation (canonical R = 0.61; $\chi^2 = 28.97$; $P = 0.253$) between the MN and SCGE parameters and the five covariates (age, smoking, alcohol consumption, vitamin B_{12} and FA) in the healthy males.

Vitamin B_{12} concentration had the highest influence on the MN and BT and on TI and TM. BRT and the NDI depended on the concentration of FA, while the TL mostly depended on age.

DISCUSSION

In this study, we used two different biomarkers, one of exposure (SCGE assay), and the other of effect (MN assay) to evaluate background DNA damage in white blood cells of healthy blood donors randomly selected from the general Croatian population. The main aim was to investigate the association between the two biomarkers and several external and internal factors, as well as the relationship between those factors.

The study shows a positive association between an increased number of MN and lower plasma vitamin B_{12} concentrations in healthy population, implying an involvement of vitamin B_{12} levels in maintaining DNA integrity, not just in vitamin B_{12} deficient population, but also in a healthy one. There is a small number of case studies that link vitamin B_{12} deficiency with increased levels of chromosome damage (Fenech, 2001). Glória *et al.* (1997) and Fenech *et al.* (1997) have shown significant negative correlation between the serum vitamin B_{12} baseline levels and MN frequency. A study of MacGregor *et al.* (1997) has shown that elevated MN index is strongly associated only with low levels of serum folate (less than 4 ng/l) and low levels of plasma vitamin B_{12} (less than 200 pg/l). None of our study subjects had so drastically low values of either, and our results could not be compared.

Our study has shown that the impact of vitamin B_{12} and folate can be also seen in healthy, non-deficient population. Our results had comparable mean values of FA and B_{12} to those of the study of Odagiri and Uchida (1998) on 33 non-folic and non-vitamin B_{12} deficient individuals (FA = 7 ng/l, vitamin B_{12} = 544 pg/l). However, they are a little higher than in the case control study on the healthy Italian population by Cafolla *et al.* (2000) or in the study by Zijno (2003), suggesting that the Croatian diet may be somewhat richer in folate.

Although there is evidence that recommended daily intake of micronutrients is suboptimal for the protection from genotoxic damage, our results, and also the results of Zijno *et al.* (2003), indicate that the relationship between genetic integrity and FA and vitamin B_{12} status could be more complex and influenced by other factors such as tobacco smoke and alcohol consumption.

Data on possible age-related increase in primary DNA damage in white blood cells are contradictory (Singh *et al.*, 1991; Mendoza-Núñez *et al.*, 2001). According to some authors, age appears to have no significant effect on the mean basal level of DNA damage (Wojewódzka *et al.*, 1999; Zhu *et al.*, 1999), as was also observed in our study.

Numerous studies have shown an increase in MN frequency with age, confirming MN as a sensitive marker of ageing (Zijno *et al.*, 2003; Kažimirova *et al.*, 2006). Fenech and Rinaldi (1994) found that age was the most important factor influencing the variance of MN in

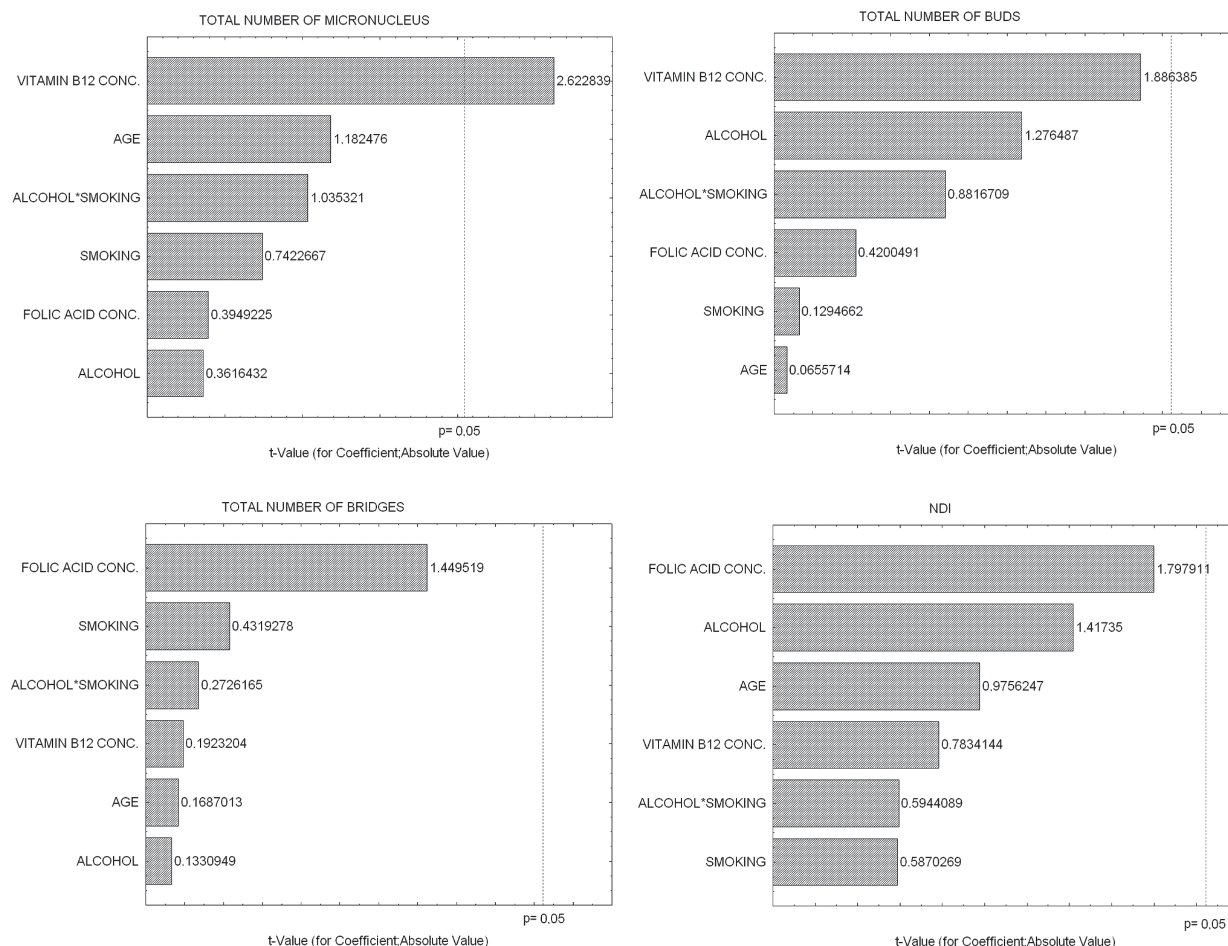

Figure 2. Pareto chart of *t*-values for coefficients produced by the general regression model
Micronucleus assay parameters were compared on the basis of two categorical factors (smoking and alcohol consumption) and three continuous factors (age, vitamin B_{12} concentration, folic acid concentration); df = 29; Sigma-restricted parametrization. Symbols are as in Fig. 1.

women and men, while micronutrient level had no apparent significant effects on genetic damage. While our results suggest that vitamin B_{12} concentration may have more influence than FA, Odagiri and Uchida (1998) found, by adjusting the MN index for age, that FA indeed had a protective effect against DNA damage.

The differences in vitamin B_{12} plasma concentrations between smokers and non-smokers are inconsistent from study to study. Although Gabriel *et al.* (2006) have shown lower plasma vitamin B_{12} levels in smokers than in non smokers, we found higher vitamin B_{12} concentrations and lower MN frequency and FA level in smokers. The explanation for this finding can be that B_{12}, as a methyl donor, has camouflaged the real effect. On the other hand, smokers had higher frequency of BT and BRT, which may be explained by the fact that different mechanisms are involved in the origin of MN and BT. Lindberg *et al.* (2007) have shown different mechanisms of origin of BT and MN in normal and folate-deprived human binucleate lymphocytes. They have shown an increase of MN and BT in FA-deprived cultures, with terminal acentric fragments.

Zijno *et al.* (2003) suggested that vitamin B_{12} may interact with genotoxic components of tobacco smoke. Some studies have clearly shown genotoxic effects of tobacco smoke, as well as its modifying effect on genome damage induced by other agents (Palus *et al.*, 1999; Zhu *et al.*, 1999; Hininger *et al.*, 2004; Milić *et al.*, 2008). Other studies however have reported no significant increase in the values of biomarkers evaluated (Šrám *et al.*, 1998; Wojewódzka *et al.*, 1999; Hoffman & Speit, 2005). A correlation between micronucleated cells and age, with an increase of 0.2 to 0.3 micronuclei per thousand cells per year, has been reported by Trkova *et al.* (2000). It is believed that this effect is either because of accumulated exposure to mutagens over the lifetime or because of the aging of the spindle apparatus (Burgaz *et al.,* 1999).

Gabriel *et al.* (2006) showed that when age, sex, alcohol consumption and number of cigarettes were tested to identify the strongest prediction model for folate concentration, smoking was the predominant predictor of MN formation. Regular alcohol consumption was also a significant determinant of MN frequency.

A combination of cytogenetic tests and the SCGE assay in biomonitoring studies makes it possible to compare the relative sensitivities of the two test systems and, therefore, gives us a possible clue about the fraction of the DNA damage detected by the SCGE assay that will lead to fixed mutations (Kassie *et al.,* 2000).

As a biomarker, the comet assay, which detects reparable DNA lesions or alkali-labile sites, reflects current exposure (over the previous few weeks) and actual levels of DNA damage present in white blood cells at the moment of blood sampling. It is known that most of the primary DNA lesions are successfully repaired in a few minutes (4–15 min) (Tice, 1995) to a couple of hours (2–3 h) (Singh *et al.,* 1988). Cytogenetic biomonitoring, on the other hand, detects fixed mutations which persist for at least one mitotic cycle (Kassie *et al.,* 2000) and provides additional information on the DNA damage levels, especially on past exposures. Humans are exposed to a variety of natural or synthetic genotoxic substances which can modify the baseline levels of DNA damage. During the lifetime everyone "accumulates" some level of radiation exposure, mainly due to the background, environmental radiation, diagnostic exposures, or small amounts of radioisotopes ingested with food.

The results obtained in this preliminary study bring a lot of useful background data that could be of value in future genotoxicological monitoring. They also confirm that the SCGE assay and MN assay are sensitive biomarkers and that they need to be further evaluated and standardized for the assessment of DNA damage in human biomonitoring studies and evaluation of accidental exposures.

Acknowledgements

This study was partly supported by the Ministry of Science, Education and Sports of the Republic of Croatia (grant number 0022-0222148-2137).

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