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Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B_{12} concentrations and methylenetetrahydrofolate reductase polymorphisms. A pilot study

Maria Kapiszewska[a,*], Malgorzata Kalemba[a], Urszula Wojciech[a], Tomasz Milewicz[b]

[a]*Department of General Biochemistry, Faculty of Biotechnology, Jagiellonian University, Krakow, 30-387 Poland*

[b]*Collegium Medicum, Jagiellonian University, Krakow, 30-387 Poland*

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Abstract

Changes in the folate and vitamin B_{12} status in the body influence the extent of uracil misincorporation (UrMis) into DNA, which is one of the biomarkers of genomic stability and, thus, portends a risk of cancer. In our study, the level of UrMis into DNA was evaluated by the comet assay (using the specific DNA repair enzyme, uracil DNA glycosylase) in leukocytes from blood donated by healthy young women with positive folate balance achieved by 4 weeks of folic acid supplementation (400 μg/day). The nutritional status was evaluated on the basis of nine food diaries recorded by the subjects during two winter months. The data were computerized, and the intake of nutrients and micronutrients was estimated using the DIETA 2 program (Food and Nutrition Institute, Warsaw, Poland) linked to recently updated Polish food tables. The plasma folate and vitamin B_{12} concentration, as well as methylenetetrahydrofolate reductase (MTHFR) polymorphisms, were evaluated to determine their influence on the level of UrMis into DNA. The mean value of B_{12} intake for all subjects reached 100% of the Polish recommended dietary allowances (RDA), whereas the mean value of folate intake, before folate supplementation, was 50%, suggesting moderate deficiency. Folic acid supplementation brought the folate intake way above the RDA, and plasma folate concentration in each individual was above the deficient range (mean value 14.67 ng/ml). The UrMis did not correlate with the plasma folate concentration, but the level of UrMis was significantly lower in subjects with plasma vitamin B_{12} concentration above 400 pg/ml ($P=.02$) only after folic acid supplementation. The concentration of folate in plasma correlated ($P\leq.05$) with the wild-type MTHFR homozygote 1298 AA but not with the MTHFR 677 genotype. When subjects were grouped according to genotype, the mean concentration of folate in plasma was significantly lower in subjects with the MTHFR 677 (CT+TT) polymorphism, which was accompanied by a lower UrMis, compared to individuals with the CC genotype. The significantly higher concentrations of folate in serum, accompanied by increased UrMis, were seen in subjects with the combined MTHFR 1298 (AC+CC) genotype, as compared to the 1298 AA wild type. Our results suggest that more than 400 pg/ml of vitamin B_{12} in plasma in subjects with a positive folate balance is critical for genomic stability and indicate that the amount of UrMis into DNA is related to the MTHFR genotype.

Keywords: Comet assay; Folate; MTHFR 677 and 1298 genotype; Uracil incorporation into DNA; Vitamin B_{12}

1. Introduction

Inadequate folate intake emerged recently as an important nutritional factor that, in addition to causing a risk of neural tube defect, appears to play a role in cardiovascular disease [1–4] and in some malignancies [5–7]. The precise nature, however, of the inverse correlation between folate status and the risk of both degenerative diseases has not been clearly established yet [8]. The increased risk of certain cancers seems to be linked to an imbalance in deoxyribonucleotide pools, due to the deficiency in folate, which results in DNA instability [9–12]. Inadequate folate intake is accompanied by 5,10-methylenetetrahydrofolate (5,10-methylene-THF) deficiency, and because of that a blockade of the methylation of deoxyuridyne monophosphate (dUMP) to deoxythymidine monophosphate leads to uracil misincorporation (UrMis) into DNA in place of thymine [13]. During the normal repair processes, the uracil is removed by uracil DNA glycosylase, and

* Corresponding author.
E-mail address: mkapisz@if.uj.edu.pl (M. Kapiszewska).

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DNA breaks that ultimately occur may lead to mutagenesis as well as malignant transformation [13,14]. The amount of 5,10-methylene-THF depends not only on folate intake but also on the activity of the methyltetrahydrofolate reductase (MTHFR; EC 1.5.1.20), an enzyme that reduces 5,10-methylene-THF to 5-methyltetrahydrofolate (5-methyl-THF), the principal circulating form of folate in serum [4,15]. The gene for MTHFR exists as genotypic variants at nucleotide 677 (C/T) and at nucleotide 1298 (A/C). The wild-type 677 CC variant gene product is the more active form, which is associated with higher plasma folate concentrations [16]. The importance of the 1298 polymorphism in determining MTHFR activity is less well understood [17].The activity of the enzyme also depends on its inhibitor, *S*-adenosylomethionine (SAM), which is a main donor of methyl groups for the DNA methylation [8]. The SAM synthesis is enhanced when the concentration of B_{12} and methionine is sufficiently high, and because of that the MTHFR activity may decrease independently of MTHFR polymorphisms. Thus, adequate vitamin B_{12} and methionine intake may increase the amount of 5,10-methylene-THF, favoring purine and thymidylate syntheses, which are essential for the synthesis and repair of DNA. In such a case, a decrease in the dUMP and reduced UrMis into DNA should be observed under adequate folate intake, but opposite results should occur at the deficient folate level. This working hypothesis was tested by comet assay which assessed the extent of endogenous DNA alkaline-labile lesions and the number of DNA breaks produced by uracil DNA glycosylase in the DNA sites where uracil was incorporated in the white blood cells taken from young women before and after 4 weeks of folic acid (400 μg/day) supplementation. The influence of MTHFR genetic polymorphisms and both plasma folate and B_{12} concentration on these DNA lesions was also assessed.

Our results strongly suggest that, in addition to adequate folate intake, (1) plasma B_{12} concentration above 400 pg/ml is necessary to minimize the UrMis into DNA; (2) the extent of UrMis into DNA depends on the plasma folate concentration, which is related to the MTHFR genotype; and (3) folate recommended dietary allowances are not easy to achieve by young Polish women living in a big city during the winter months.

2. Materials and methods

2.1. Chemicals

Agarose Electrophoresis Grade Ultra Pure and Agarose Low Melting Point Ultra Pure were purchased from Gibco BRL (UK); Agarose Top Vision, dNTPs (deoxynucleotides), Pfu DNA polymerase, *Hin*fI restriction enzyme and *Mbo*II restrictase were purchased from Fermentas (Lithuania); dimethyl sulfoxide, uracil DNA glycosylase, ethydium bromide, propidium iodide, Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) were from Sigma (USA); DNA Extraction Kit was delivered by A&A (Poland); ethylenediaminetetraacetic acid (EDTA) and Triton X-100 were from MP Biomedicals; primers were from IBB PAN (Warsaw, Poland); salts and hydroxides were purchased from POC (Poland); the Bayer Diagnostics ADVIA Centaur folate and B_{12} assay were supplied by Bayer Corporation Diagnostics Division (Tarrytown, NY).

2.2. Study design and subjects

This work was designed as a pilot study for a larger project investigating the relationship between genotype polymorphism and DNA damage in women in the reproductive age. Nineteen female, healthy, nonsmoking volunteers, aged 20–23, were recruited from students in southern Poland. The data for DNA UrMis were obtained from 14 subjects as five missed the second blood withdrawal. Subjects were asked not to make any changes to their diet or lifestyle during the study period and to maintain a 1 day/week food diary and give a drop of blood from their fingers for DNA lesion assessment. At the beginning of the second month, the subjects were asked to begin taking 400 μg of folic acid per day (Puritan's Pride, Bohemia, USA) for 4 weeks. The blood sample from a finger was taken the next day after folate supplementation. Additionally, fasting venous blood samples were collected into 10-ml EDTA-coated vacutainers for DNA and plasma isolation. Subjects' body weight, body fat percentage (measured by bioimpedence) and circumference of waist and hips were measured. Waist-to-hip ratio (WHR) was assessed. Body mass index (BMI) was calculated, dividing body weight in kilograms by square height in meters.

2.3. Dietary assessment

Subjects were trained how to estimate food portions using food photographs (album of food; Food and Nutrition Institute, Warsaw, Poland), how to fill the food diary and record as much information as possible about the food consumed. Data from all individual questionnaires were entered into a computer data base, and the amount of dietary compounds was estimated with a specifically developed program DIETA 2 (Food and Nutrition Institute, Warsaw, Poland) linked to recently updated Polish food tables. Each food product is characterized by the contents of 77 nutrients, its energy value and percentage of energy derived from protein, fats and carbohydrates.

2.4. Analysis of DNA damage

The alkaline single-cell gel electrophoresis assay (comet assay) with enzyme modification was used for the detection of endogenous DNA damage in individual cells. Five microliters of fresh whole-blood samples was taken for evaluation of DNA alkaline-labile damage and of

UrMis into DNA, and mixed with 75 μl of 0.5% melted agarose (low melting point) and layered on 1% agarose (normal melting point) precoated microscopic slides. The electrophoresis was performed according to the original laboratory protocol for the detection of DNA damage in mammalian cells prepared by Tice et al. [18], slightly modified. The slides were briefly subjected to lysis (for 1 h at 4°C in 2.5 M NaCl, 100 M Na_2 EDTA, 1% Triton X-100, 10 mM Tris, 10% dimethyl sulfoxide, pH 10). After lysis, the slides were washed three times in Tris buffer (0.4 M, pH 7.5) and then incubated at 37°C for 45 min only with the buffer (60 mM Tris/HCl, 1 mM EDTA, 0.1 mg/ml BSA, pH 8) or with 50 μl of uracil glycosylase (0.1 U/slide) for detection of UrMis into DNA. Then, the slides were washed again. After 40 min of unwinding in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA) at 4°C, the electrophoresis was conducted at 0.74 V/cm for 20 min in a cold room. The current was adjusted to 300 mA. After electrophoresis, the slides were neutralized and exposed to cold 100% methanol for 5 min and allowed to dry. The slides were stored in a dry place until analysis (performed within 1 month). Before viewing, the slides were placed in distilled water for 5 min and stained with propidium iodide (2.5 μg/ml). Each experimental point was run in duplicate. Two slides per experiment, with 50 randomly selected cells per slide, were analyzed.

2.5. Evaluation of DNA damage

For visualization of the DNA damage, observations were made using a 20× objective (the final magnification was 200×) on an epifluorescence microscope (Olympus IX-50) equipped with appropriate filters. The microscope was linked to a computer through a CCD camera (Videotronic) for transporting images to the computer software. Komet 5, a comet analysis system developed by Kinetic Imaging (Liverpool, UK), was used for the quantification of DNA damage. The DNA damage parameters were automatically generated by the image analysis system. The results were presented taking into account the tail length (TL) of the comet. However, the analysis of the percentage of DNA in the tail or tail moment parameters led to the same conclusion, with a similar degree of statistical signification. The net UrMis (TL UrMis) into DNA was obtained by subtracting the tail length values reflecting alkaline-labile single strand breaks (TL SSB) from slides covered only with Tris buffer from those incubated with uracil DNA glycosylase.

Statistical evaluation of the relationship between DNA damage and other investigated factors (e.g., dietary compounds, anthropometric measurement, B_{12} and folate concentration in serum) was performed.

2.6. Vitamin B_{12} and folate concentration in plasma

Fasting blood samples were drawn from all subjects by venipuncture on the first day after supplementation with folic acid. Plasma aliquots were stored at −80°C in cryogenic tubes until analysis. Plasma folate and B_{12} concentrations were determined using the Bayer Diagnostics ADVIA Centaur folate assay, which is a competitive immunoassay using direct chemiluminescence technology (Department of Clinical Biochemistry, Jagiellonian University Hospital, Krakow, Poland).

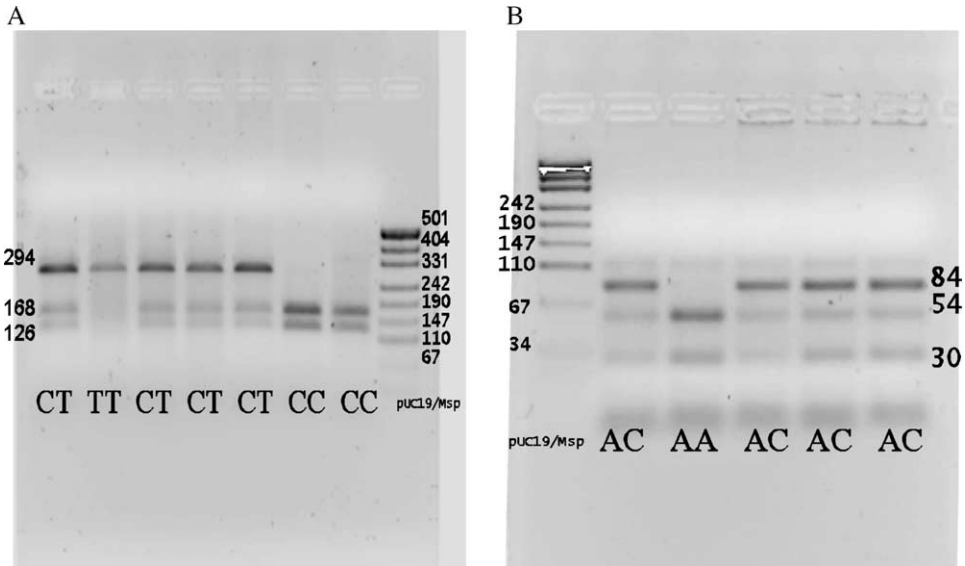

Fig. 1. (A) PCR-based RFLP analysis of the MTHFR (codon 222) polymorphism. The CC lines represent a homozygote sample (MTHFR 677 CC, wild-type) where both alleles contain a C at position 677; whereas the CT lines represent a heterozygote where one allele contains a C and the second allele contains a T at position 677; the TT line represents a homozygote where both alleles contain a T at position 677. (B) PCR-based RFLP analysis of the MTHFR (codon 429) polymorphism. The AA line represents a homozygote sample (MTHFR 1298 AA) where both alleles contain an A at position 1298, whereas the AC lines represent a heterozygote where one allele contains an A and the second allele contains a C at position 1298.

2.7. Analysis of the MTHFR 677 and 1298 genotype

2.7.1. DNA isolation

Immediately after withdrawal, the blood samples were stored at −80°C until extraction. The DNA was extracted using the A&A Blood Mini Kit (A&A, Poland), according to the manufacturer's protocol. The MTHFR genotype was determined by a polymerase chain reaction-based procedure [19].

2.7.2. MTHFR C677 T

MTHFR gene polymorphism was determined from a single blood sample. The polymorphism in human MTHFR has been attributed to the 677 position mutation (C→T), which causes alanine to valine substitution that results in thermolability and reduction of enzyme activity. Allelic discrimination of the 667 C→T MTHFR gene was determined using the restriction fragment length polymorphism method. Primers used for PCR were as follows: upstream — 5′ CCTTGAACAGGTGGAGGCCAG3′ and downstream — 5′ GCGGTGAGAGTGGGGTGGAG3′ . With 5-min predenaturation at 95°C, amplification was performed using Pfu-polymerase during 35 cycles (95°C for 1 min, 65°C for 1 min, 72°C for 1 min) followed by a 5-min extension at 72°C. PCR products were digested for 1 h at 37°C with 5 U of *Hin*fI restriction enzyme. The enzyme recognizes the palindrome sequence G↓ANTC. The presence of a cleavage site in the 294-bp fragment (168 and 126 bp) differentiates the variant (677 TT) from the wild-type allele (677 CC), which is not cut by *Hin*fI. In the heterozygote samples, all three bands (294, 168 and 126 bp) were present. The digestion products were separated on 2% Top-Vision agarose gel stained with ethidium bromide (Fig. 1A).

2.7.3. MTHFR A1298C

The second, less common polymorphism in the MTHFR gene at the 1298 position (A→C) results in glutamate to alanine substitution and causes mildly decreased MTHFR activity. Determination of the 1298 A→C MTHFR polymorphism was carried out by the PCR/RFLP method. For DNA amplification, the following primers were used: upstr — 5′ CTTTGGGGAGCTGAAGGACTACTAC3′ and downstream — 5′ CACTTTGTGACCATTCCGGTTTG3′ . With 5-min predenaturation at 95°C, PCR was performed using Pfu-polymerase during 35 cycles (95°C for 1 min, 62°C for 1 min, 72°C for 1 min), followed by a 5 min extension at 72°C. Enzyme digestion of PCR products was performed for 1 h at 37°C with 2 U of *Mbo*II restrictase. The digestion products were visualized on 4% Top-Vision agarose gel stained with ethidium bromide. The 1298 CC variant homozygote, which is not cut by *Mbo*II enzyme, produced four fragments (84, 31, 30 and 18 bp), whereas the 1298 AA wild-type allele, which contains the sequence recognized by *Mbo*II, produced five (54, 31, 30, 28 and 18 bp) fragments. In the heterozygote samples, all six fragments were present (84, 54, 31, 30, 28 and 18 bp). The 1298 AC allele identification relied on the presence/absence of an 84/56-bp band (Fig. 1B).

2.8. Statistical analysis

Descriptive statistics in the text and tables are given as mean ± S.D. (unless indicated otherwise). Statistical analysis was conducted using the STATISTICA version 6 software. The Pearson correlation coefficient was calculated between DNA damage end points (TL SSB or TL UrMis) and all nutrient, vitamin and mineral intake. Only those results significant at the $P=.05$ level were included for further statistical analysis. Correlation between folate and vitamin B_{12} concentration in plasma and intake of all nutrients was also examined by using Pearson correlation coefficient. Nonparametric Mann–Whitney *U* test was applied to determine the relationship between (1) amount of DNA alkaline-labile sensitive DNA sites (TL SSB), (2) extent of UrMis into DNA (TL UrMis) and plasma vitamin B_{12} concentration. Additionally, the same analysis was performed to calculate the association between MTHFR polymorphism and plasma folate concentration. Data are shown as box-and-whisker plot. Unpaired *t* test was used to analyze the association between plasma folate concentration and mean values of UrMis into DNA within two groups of the MTHFR gene polymorphisms (C677 and A1298): "low function (heterozygote+homozygote mutated alleles)" and "high function (wild-type genotype)". Allele frequencies in the study groups were determined by counting alleles and calculating simple proportion.

2.9. Ethical consideration

The study protocol was reviewed and approved by the Jagiellonian University Medical College Ethics Committee.

3. Results

3.1. Subject characteristics

The anthropological characteristics of the investigated group are presented in Table 1. Body weight positively correlated with fat percentage ($r=.57$; $P<.033$) and with circumference of hip ($r=.8$; $P<.0006$). Moreover, WHR associated positively with energy intake ($r=.6$; $P<.023$), fat intake ($r=.63$; $P<.016$) and starch intake ($r=.7$; $P<.006$) but not protein intake. Body mass index was correlated with

Table 1
Selected anthropometric parameters of the subjects (19 subjects)

Parameters	Mean±S.D.	Min	Max
Age (years)	23.1±0.76	22	25
Height (cm)	168.7±8.7	154.5	182
Weight (kg)	60.13±7.9	45	71
BMI (kg/m^2)	21.13±2.4	17.1	27.23
WHR	0.74±0.05	0.64	0.82
Body fat percentages	20±6.1	11.5	32.8

Table 2
Vitamin B_{12} and folate intake during 2 months of studies based on nine dietary diary and analyzed by a specifically developed program (DIETA 2) linked to recently updated Polish food tables

	Mean ± S.D.	Median	Min	Max
B_{12} intake (μg/day)	3.12±1.44	2.87	1.48	6.85
B_{12} in plasma (pg/ml)	402.68±114.4	377.20	229.60	660.40
Folate intake (μg/day)	224.98±87.83	216.85	104.02	467.75
Folate in plasma (ng/ml)	14.67±3.87	13.30	10.50	23.20

Vitamin B_{12} and folate concentrations in plasma after 4 weeks of folic acid supplementation were determined using the Bayer Diagnostics ADVIA Centaur folate and B_{12} assay (a competitive immunoassay using direct chemiluminescence technology).

percentage of body fat (r=.57; P<.0001) and with circumference of waist (r=.49; P<.0001).

3.2. Dietary assessment and correlation with vitamin B_{12} and folate concentrations

The folate and B_{12} dietary intake and the plasma concentrations of both vitamins following 4 weeks of folic acid supplementation are shown in Table 2. The mean value of vitamin B_{12} intake reached 100% of RDA, despite the fact that 50% of the individuals consumed vitamin B_{12} below the RDA level. Only one subject within the investigated group achieved 100% of folate RDA, and the mean intake level was 52% of RDA. The vegetable and fruit consumption strongly correlated (P=.0062) with folate intake. Folic acid supplementation (400 μg/day for 4 weeks) brought the folate intake way above the RDA level. After folate supplementation, the plasma folate concentrations for each individual were way above the deficiency range, and in 32% of cases they were at intermediate level (up to 12.1 ng/ml). In 26% of subjects, folate level exceeded 20 ng/ml. Plasma folate concentration did not significantly correlate with folate dietary intake. However, as plasma folate concentrations were only measured after supplementation, it cannot be excluded that plasma folate level might have correlated with folate dietary intake.

The data presented in Tables 3–5 show the significant correlation of vitamin B_{12} and folate dietary intake with several macro- and micronutrients consumed by the subjects during the study period.

None of the macro- and micronutrients correlated with plasma vitamin B_{12} level. However, plasma folate concentration positively correlated with vitamin E (r=.75; P=.0019), flavonoids (r=.4; P=.015) and PUFA (r=.59; P=.028).

3.3. DNA base damage and food consumption

A statistically significant negative relationship was observed between UrMis and percentage flavonoid intake from other sources than fruit and vegetables (r=−.55; P=.04), consumption of black tea (r=−.56; P=.036) and quercetin level (r=−.56; P=.03) before folate supplementation. This association was not seen after a 4-week-long folate supplementation. A statistically significant association was observed (P=.028) between plasma vitamin B_{12} concentrations, with group division below and above 400 pg/ml as an independent (grouping) variable and the UrMis as a dependent variable, when tested by Mann–Whitney U test (Fig. 2).

3.4. MTHFR polymorphism frequencies in study groups related to plasma folate concentration

The MTHFR variant alleles were determined by the PCR restriction fragment length polymorphism assay (Fig. 1). The frequency of the three MTHFR 677 CT polymorphisms was 26% for homozygous wild type (677 CC), 58% for heterozygous (677 CT) and 16% for homozygous mutant (677 TT). The frequency of the three MTHFR 1298 AC polymorphisms was 57% for homozygous wild type (1298 AA), 32% for heterozygous (1298 AC) and 10% for homozygous mutant (1298 CC). The 677 T allele frequency was 0.45, and the carrier frequency for the 1298 C allele was 0.26. There were no significant differences in the anthropometric parameters between the genotypes. Because only a small number of subjects participated in the study, it seemed to be reasonable to investigate the correlation between homozygous wild-type and combined variant homozygous and heterozygous groups. Thus, subjects were divided into four groups based on the wild type, the MTHFR 677 CC and 1298 AA, and on the non-wild type, the MTHFR 677 (CT+CC) and 1298 (AC+CC), respectively. The mean value of the plasma folate concentration and UrMis was calculated for each genotype group. No significant differences (P>.05) in plasma folate and DNA damage were detected between all four group combinations when the Mann–Whitney U test

Table 3
The relationship between vitamin B_{12} and folate dietary intake and dietary macronutrients based on nine dietary diaries (19 subjects) and analyzed by a specifically developed program (DIETA 2) linked to recently updated Polish food tables

	Energy	Proteins total	Animal proteins	Plant proteins	Carbohydrate	Sucrose	Fiber	Fats total	Saturated fatty acids
B_{12} intake									
r	.68	.81	.75	–	.055	–	–	.65	.64
P	.008	.0005	.002	–	.04	–	–	.01	.01
Folate intake									
r	–	.54	–	.55	–	.6	.8	–	–
P	–	.05	–	.04	–	.02	.0005	–	–

r indicates Pearson correlation coefficient; "–" means lack of significant correlation (above P=.05).

Table 4
The relationship between vitamin B_{12} and folate intake and amino acids in the food consumed by the subjects ($N=19$) evaluated based on nine dietary diaries (19 subjects) and analyzed by a specifically developed program (DIETA 2) linked to recently updated Polish food tables

	B_{12} intake		Folate intake	
	r	P	r	P
Isoleucine	.82	.0003	–	–
Leucine	.84	.0001	–	–
Lysine	.78	.001	–	–
Methionine	.8	.0006	–	–
Cysteine	.73	.0029	–	–
Phenylalanine	.84	.0002	–	–
Tyrosine	.84	.0002	–	–
Threonine	.8	.0006	–	–
Tryptophan	.8	.00070	–	–
Valine	.83	.0002	–	–
Arginine	.69	.0065	.71	.004
Histidine	–	–	.82	.0003
Alanine	.67	.009	.68	.0067
Asparagine	.74	.003	.63	.01
Glycine	.63	.01	.58	.03
Proline	.86	.0001	–	–
Serine	.84	.0002		–

"–" means lack of significant correlation ($P>.05$).

was used. However, when one-sided test was performed to compare the mean values of plasma folate concentration in the individuals with a wild-type genotype and with combined MTHFR mutated variants, a significantly lower ($P<.05$) (Fig. 3) plasma folate concentration was observed for the combined 677 CT genotype, as compared to wild-type 677 CC. Contrary to the MTHFR C677 genotype, the plasma folate levels were significantly higher ($P<.05$) in

Table 5
The relationship between vitamin B_{12} and folate intake and microelements and other vitamins in food consumed by individuals in the study group ($N=19$) based on nine dietary diaries and analyzed by a specifically developed program (DIETA 2) linked to recently updated Polish food tables

Microelements and vitamins	B_{12} intake		Folate intake	
	r	P	r	P
Na	.59	.02	–	–
K	–	–	.79	.0006
Ca	.74	.0025	.61	.02
P	.74	.0023	.64	.012
Mg	–	–	.75	.001
Fe	.73	.0025	–	–
Zn	.57	.013	.55	.037
Cu	–	–	.68	.007
Mn	–	–	.77	.0013
Vitamin A	.66	.01	–	–
Retinol	.82	.0003	–	–
Beta-carotene	–	–	.72	.0032
Vitamin E	–	–	.51	.063
Thiamine	.76	.001	–	–
Riboflavin	.83	.0003	–	–
Niacin	.78	.0009	–	–
Vitamin B6	–	–	–	–
Vitamin C	.71	.004	–	–
Vitamin D	.8	.0006	–	–
Flavonoids	–	–	.73	.0026

"–" means lack of significant correlation ($P>.05$).

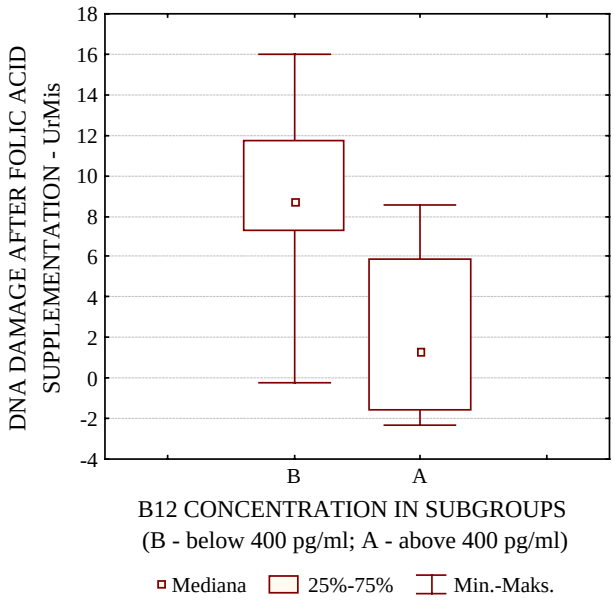

Fig. 2. Box-and-whisker plots showing a relationship between vitamin B_{12} concentration in plasma (with group division: below and above 400 pg/ml of vitamin B_{12} level) and the DNA damage analyzed by comet assay and represented by TL induced by the uracil DNA glycosylase in the DNA sites where uracil was misincorporated into DNA. Presented statistical analysis was performed using the Mann–Whitney *U* test. Solid bar indicates median; upper and lower limits of box, 75th and 25th percentiles; upper and lower bars, maximum and minimum values.

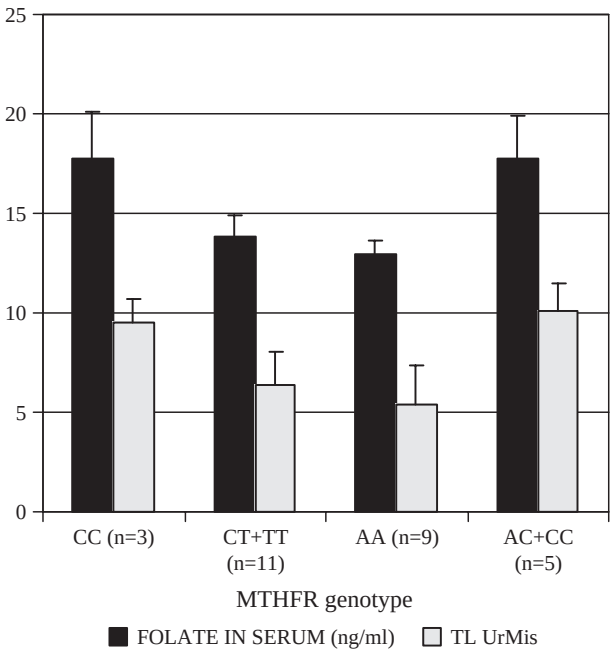

Fig. 3. Mean values (±S.D.) of TL, representing the UrMis into DNA determined by comet assay (modified with uracil DNA glycosylase treatment) (TL UrMis) and the mean folate concentration in plasma (±S.D.) within the group of subjects stratified by the MTHFR polymorphisms: CC, which represents the wild-type, and CT+TT, the combined mutated genotype of the MTHFR 677 CT genotype; AA, which represents the wild-type, and AC+CC, the combined mutated genotype of the MTHFR 1298 AC genotype; *n*=number of subjects.

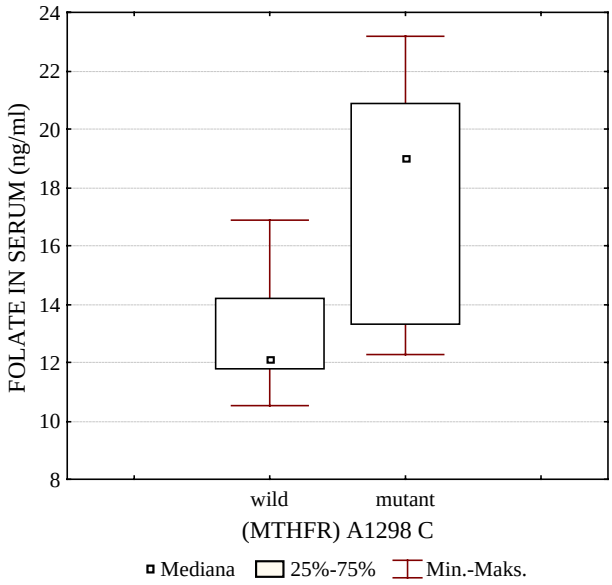

Fig. 4. Box-and-whisker plots showing the relationship between plasma folate concentration within a group stratified by the MTHFR 1298 genotype: the wild-type group represents homozygote sample (MTHFR 1298 AA), where both alleles contain an A at position 1298, whereas the "mutant group" represents individuals with combined genotype: heterozygotes, where one allele contains an A and the second allele contains a C at position 1298, and homozygote, where both alleles contain a C at position 1298.

individuals with the 1298 AC mutation, as compared to those with wild-type 1298 AA. The plasma folate level was much lower in the group with the MTHFR 1298 AA genotype, as compared to the group with the MTHFR 677 CC. Each individual with the 677 CC wild type bore the mutated allele of the 1298 A. The wild-type MTHFR 1298 was associated with the mutated 677 variants. When Mann–Whitney *U* test was performed, a significant association was found between folate serum concentrations and MTHFR 1298 AC genotype ($P<.05$) (Fig. 4), but the MTHFR 677 polymorphism variants showed only a trend toward such association ($P=.1$). The mean values of UrMis into DNA were always lower in the group with lower plasma folate concentration, and the differences were significant within the particular genotype (wild-type vs. mutated variants) (Fig. 4).

The mean value of plasma vitamin B_{12} concentration was significantly lower (325±46.9 pg/ml) in subjects with the wild-type genotype (MTHFR 677 CC), as compared to the combined mutated [MTHFR 677 (CT+TT)] genotype (445.01±59.16 pg/ml). The plasma vitamin B_{12} concentration within the group with the MTHFR 1298 AA wild-type genotype was significantly higher (435±69.8 pg/ml), as compared to the combined genotype AC+CC (344±36.83).

4. Discussion

In order to study the effect of positive folate balance on DNA stability, we analyzed the amount of alkaline-labile endogenous DNA sites and the amount of UrMis into DNA and converted into DNA breaks by uracil DNA glycosylase in lysed leukocytes after a 4-week-long supplementation with folic acid. DNA breaks were analyzed by comet assay, the most widely used technique at present [20]. Its modified version was applied to analyze not only the level of endogenous DNA damage but also that of a more specific lesions, such as oxidative DNA damage and UrMis into DNA due to folate deficiency [21–24].

We examined the DNA damage in leukocytes instead of lymphocytes, despite the latter being frequently used in this type of research [22,24–27]. The reason for that was that the half-life of lymphocytes (several months) is probably too long a period to reflect changes in DNA molecules caused by 4 weeks of intervention. As the leukocyte population includes more than 60% of granulocytes, which have sufficiently short half-life (7 days), we expected that leukocytes will reflect the folate-induced changes much better. For the same reason, folate concentration in plasma, not in red blood cells, was measured [13,14]. Basically, the red blood folate concentration reflects the tissue folate storage, but plasma folate concentration is much more sensitive to short-lasting alterations in diet [28,29].

The individuals chosen for the study were a very homogeneous group, as far as gender, age, weight, lifestyle and smoking habits were concerned. This allowed to minimize the impact of these confounding features. A significant positive association between body weight and BMI and percentage of body fat ($r=.56$; $P=.01$ and $r=.82$; $P=.0001$, respectively) existed within that group.

The rationale for conducting our research was the recently published study on Polish women [30]. It showed that the mean daily folate intake in more than 50% of them was below the recommended level (400 μg) for prevention of fetal neural tube defect at the childbearing age. The incidence of neural tube defects in Poland in the 1990s was 2.68 per 1000 births and did not change much in the previous 20 years. Mortality caused by it is much higher in Poland than in many other European countries. In addition to the well-known effect of folate deficiency on neural tube defect in newborns [31–33], the role of folate and vitamin B_{12} in cardiovascular diseases [34,35] is also under intensive investigation. Studies on the involvement of folate and B_{12} status in carcinogenesis [36–41] are a relatively new research area, despite the fact that both vitamins play a crucial role in DNA methylation [38,42] and nucleotide synthesis [43].

In our study, the folate intake was strongly associated ($P=.02$) with fruit and vegetable consumption. These results were confirmed by strong correlation with intake of plant protein and flavonoids, fibers, vitamin E and a group of amino acids, different from that of proteins of animal origin (Tables 3 and 5). A lower than optimal plasma folate concentration in human subjects was likely to create a mild deficiency, as the mean value of folate intake reached only 52% of Polish RDA (the mean folate intake for the studied group was 225 μg/day).

Folic acid supplementation (400 μg/day during 4 weeks) was provided to the study participants to achieve an optimal folate balance. After that time, the plasma folate concentration in each individual reached the level above the deficiency range, and the mean value was 14.67±3.87 ng/ml (33.3 nmol/L), similar to values recently reported in European countries [44–46]. It was shown by Fenech [47] that plasma folate concentration greater than 34 nmol/L was only achievable when the folate intake levels were more than 400 μg of folic acid per day, whereas in studies carried out by Brouwer et al. [48], 4 weeks of supplementation with folic acid at 250 μg/day were sufficient to maintain a high plasma concentration level. The World Health Organization has proposed 13.6 nmol/L for folate concentration [not regarding the genetic defect 677 (TT)] to avoid health risk. The physiological range of folate in the serum of individuals on unsupplemented diet was reported to range usually between 8 and 35 nmol/L [49–52].

In Poland, the nationwide governmental program concerning preconception folic acid supplementation ("Primary Prophylaxis of Neural Tube Defects") has been operating since 1997. It was shown that between 1999 and 2001 folic acid supplementation increased from 15% to 19% among women aged 18–45, from 11% to 13% among nonpregnant women between those ages and from 9% to 16% among women under 20 years. The percentages refer to the proportion of women taking supplements.

The food preferences of the investigated young Polish women during the winter months resulted in them having an adequate amount of vitamin B_{12}, and the RDA was achieved by eating a typical Polish winter cuisine. The mean value of B_{12} consumption reached 100% of the Polish RDA. The median plasma vitamin B_{12} concentrations (377.2 pg/ml) were comparable with those considered as optimal in healthy individuals (382 pg/ml — the median value given by the ADVIA Centaur Assay manual); such a level was achieved when the intake was higher than 2 μg of vitamin B_{12} per day [51,53]. The lack of statistically significant correlation between B_{12} intake and the concentration of B_{12} in plasma indicates that B_{12} availability is also influenced by other physiological and biochemical processes [54]. The new RDA, higher than 400 pg/ml, for vitamin B_{12} was proposed by Fenech [55]. He summarized the data from in vitro and in vivo experiments with human cells and human subjects and showed that the RDA for vitamin B_{12}, valid at that time, was not sufficient to minimize DNA damage.

The amount of vitamin B_{12} consumed with food was significantly associated with animal protein intake (Table 2). That was also confirmed by its correlation with total amount of fat, saturated fatty acids, some minerals and amino acids different from those which correlate with folate intake (listed in Tables 3–5). The food of animal origin, such as meat, poultry, eggs, milk and dairy products, was the main source of vitamin B_{12} in the studied group.

In conclusion, the recommended folate intake was very difficult to reach, especially with food traditionally eaten in Poland during the winter months. Fresh dark green leafy vegetables, especially spinach, asparagus, broccoli, lettuce, or even liver, which are the best sources of folate, were eaten sporadically by the subjects taking part in our studies.

The most significant finding in our study was that folate supplementation (400 μg/day for 4 weeks) minimized the UrMis into DNA only when serum vitamin B_{12} concentration was higher than 400 pg/ml (296 pmol/L). The effect was independent of the MTHFR genotype. That finding not only strongly confirms the results obtained by others, but also shows that UrMis into DNA analyzed by comet assay can be compared with the results obtained using the micronucleus index, another commonly used biomarker of chromosome damage. Using the latter method, Fenech [47] found a significant negative correlation between the micronucleus frequency in lymphocytes and plasma B_{12} status in young men. In another study, Fenech et al. [50] showed that micronucleus index was not significantly correlated with folate intake, but there was significant negative correlation with serum vitamin B_{12} level. The cross-sectional study also showed that in females the micronucleus frequency was negatively correlated with serum B_{12}, but there was no significant correlation between micronucleus index and red blood cell folate status [51]. Similarly, we did not find any significant association between endogenous DNA damage and plasma folate concentration. Our results suggest that plasma vitamin B_{12} concentration, in addition to plasma folate concentration, is an important risk factor influencing chromosome damage in leukocytes. Moreover, the data from intervention studies carried out by Fenech et al. [50,51] indicate that the current sufficiency level of vitamin B_{12} in plasma, which is based on prevention of anemia (200 pg/ml), is lower than the average concentration level at which DNA damage is minimized. A three-and-a-half times higher intake of the current Australian RDA is required to achieve the plasma concentration (406 pg/ml), which minimizes the micronucleus index in lymphocytes.

The association between adequate folate and vitamin B_{12} intake and health protection has recently been a topic of research [56–64]. The inadequate amount of folate and/or vitamin B_{12} intake can disrupt the balance between two metabolite intermediates in one-carbon (methyl group) metabolism, namely, 5-methyl-THF and 5,10-methylene-THF. The balance between these two forms depends also on the MTHFR activity, which is associated with common genetic polymorphisms [8].

The most common genetic variant of MTHFR occurs when cytosine is replaced by thymine at position 677 C/T, causing the reduced activity of MTHFR. This can lower the folate level in serum [65]. The population frequency of 677 TT homozygosity ranges from 1% in Africa to 20% in the United States and to even higher percentage among Italians and US Hispanics [66]. European Caucasians exhibit substantial variations, and the frequency of the T

allele MTHFR 677 T ranges between 33% and 37% but is significantly lower in the Baltic countries (23%) [16,54,67,68].

In our studies, the MTHFR variant alleles were determined by a PCR restriction fragment length polymorphism assay. The calculated T allele frequency was 0.45, which was comparable with data obtained by Domagala et al. [69,70] in studies of the Polish population.

Recently, another polymorphism in MTHFR 1298 AC was discovered [71]. It involves A→C substitution at bp 1298 in exon 7, resulting in Glu→Ala substitution in the MTHFR protein. The initial observations suggest that the 1298 AC polymorphism is associated with lower enzyme activity, but not with thermolability. The effect of the 1298 AC or 1298 CC mutations is not as profound in impairing MTHFR enzyme activity as that of 677 CT or 677 TT [72]. The frequency of the MTHFR 1298 CC homozygous mutant, when calculated for our group of subjects, was 10%, and the frequency for the 1298 C allele was 0.26. The obtained results were very much the same as those obtained by Szczeklik et al. [73] in the population sample also from southern Poland. We did not observe either MTHFR 677 CC/1298 AA or 677 TT/1298 CC combined genotype, whereas double heterozygotes 677 CT/1298 AC were represented in 16% of subjects, similarly to results obtained in studies carried out on Polish population by Domagala et al [69,70]. In another study [17,74] 15% of individuals were double heterozygotes. We did not find individuals with 677 TT/1298 AC and 677 CT/1298 CC mutations, and they were not found in other studies either [17,44].

It was shown that the reduced MTHFR activity due to 677 CT polymorphism lowered the plasma folate level and minimized UrMis into DNA, whereas the 1298 AC polymorphism did not influence the plasma folate concentration [75–77].

In our studies, the mean plasma folate concentration was significantly lower in subjects with the combined MTHFR 677 (CT+TT) genotype, as compared with the wild type. The extent of UrMis into DNA was also lower in the group with mutated alleles. All individuals with the MTHFR 677 CC genotype also bore mutation in the MTHFR 1298 allele. These findings support the hypothesis that the increased availability of 5,10-methylene-THF directs the reaction toward thymidine synthesis and markedly improves the quality of DNA synthesis. Bagley and Selhub [78] showed that the MTHFR 677 TT genotype significantly reduced the production of 5-methyl-THF, contrary to MTHFR 677 CC, where the entire 5-methyl-THF was a component of folate pool analyzed in erythrocytes.

In contrast to the MTHFR 677 CT polymorphism, the other polymorphism in the 1298 nucleotide of the MTHFR gene affects the regulation of enzyme activity through its inhibitor, SAM. The heterozygosity and homozygosity for the 1298 C allele, as shown by van der Put [71], are not associated with a lower plasma folate level.

In the group examined by us, the mean plasma folate concentration for individuals with 1298 AA genotype was significantly lowered, as compared with the cumulative (AC+CC) genotype. The statistically significant dependence between plasma folate level and the MTHFR 1298 genotype was also confirmed by results from the Mann–Whitney U test ($P<.05$), whereas the MTHFR 677 genotype showed a trend toward increasing plasma folate concentration ($P=.1$). The increased concentration of plasma folate in the MTHFR 1298 mutated variants was followed by the increase in UrMis into DNA. Our results are in agreement with the study of Crott et al. [79], who showed that lymphocytes from a heterozygote 1298 AC significantly incorporated more uracil into DNA than the wild-type 1298 AA.

The lower plasma folate concentration in wild-type MTHFR 1298 AA genotype, as compared with the MTHFR 677 CC, can be explained by the impact of the parallel existence of 677 T mutated alleles in individuals with the MTHFR AA genotype. The presence of the wild-type 677 CC alleles in the same individuals who had the 1298 AC genotype may result in high level of MTHFR activity and increased plasma folate level. We also observed that plasma vitamin B_{12} concentration coincided with the plasma folate level within a group stratified by the genotype. Higher B_{12} concentration was seen among individuals with the MTHFR 1298 AA (all are 677 CT) and 677 CT (80% are 1298 AA) genotype (435 ± 41.9 pg/ml and 445 ± 64.5 pg/ml, respectively), as compared with the MTHFR 677 CC and 1298 AC (325.26 ± 51.2 pg/ml and 344.5 ± 56.7 pg/ml). Because the mutated 1298 nucleotide is located within the SAM-regulatory domain, the inhibitory effect of SAM on the MTHFR activity may be more pronounced in the wild-type MTHFR 1298 AA genotype.

In addition, the concentration of plasma vitamin B_{12}, when folate and methionine intake are adequate, can be a very important determinant of genomic stability, in particular among subjects with the combined 677 CT and 1298 AA genotype. In particular, the disruption of the one-carbon metabolism due to MTHFR polymorphism, as well as to folate deficiency, seems to be involved in the risk of leukemia. As for the MTHFR 677 T genotype, its protective role against adult and childhood leukemia seems to be very consistent [80–82].

The role of MTHFR 1298 AC polymorphism is more controversial. Franco et al. [82] showed that the MTHFR 677 CT variant was linked to a significant decrease in the risk of developing childhood acute lymphoblastic leukemia. However, they also observed a trend toward an increased risk for MTHFR 1298 homozygosity. Krajinovic et al. [81] did not observe either any protective effect against childhood leukemia in individuals with 677 CT/1298 AC. Wiemels et al. [83] reported the existence of a significant association between MTHFR A1298 alleles and hyperdiploid leukemias. In their studies, the 1298 CC homozy-

gotes were fourfold less frequent than homozygote 1298 AA individuals among the hyperdiploid leukemia cases, as compared with the control group.

In conclusion, if the level of folate intake (including folic acid supplementation) is sufficient,

1. the amount of UrMis depends on the plasma vitamin B_{12} concentrations, and the concentration above 400 pg/ml minimizes UrMis into DNA, independently of the genotype
2. the amount of UrMis is lower when the plasma folate concentration is lower
3. higher activity of MTHFR 677 CC decreases the availability of 5,10-methylene-THF for DNA synthesis, resulting in more pronounced UrMis, and the influence of the MTHFR 1298 polymorphism on UrMis seems to depend on the presence of the allele altered at 677 and on the plasma vitamin B_{12} concentration

It appears from this preliminary study that the amount of UrMis into DNA, evaluated in leukocytes by comet assay, is a strong predictor of inadequate vitamin B_{12} concentration in plasma.

The findings have to be confirmed by a larger population study. Such research is already underway.

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