Role of Macronutrients and Micronutrients in DNA Damage: Results From a Food Frequency Questionnaire

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ABSTRACT: The links between diet and genomic instability have been under investigation for several decades, and evidence suggests a significant causal or preventive role for various dietary factors. This study investigates the influence of macronutrients (calories, protein, and carbohydrates) and micronutrients, such as vitamins and minerals, as assessed by a food frequency questionnaire, on genotoxicity biomarkers measured by cytokinesis-blocked micronucleus assay and comet assay. The results found significant positive and negative correlations. Macronutrient frequency tends to increase with higher intake of caffeine, calcium, magnesium, zinc, and protein (P<.05, Spearman correlation). Calorie and omega-6 intakes are negatively correlated with DNA damage measured by the comet assay. These results are somewhat controversial because some of the correlations found are contrary to dominant views in the literature; however, we suggest that unraveling the association between diet and genetic instability requires a much better understanding of the modulating role of macronutrients and micronutrients.

KEYWORDS: macronutrients, micronutrients, food frequency questionnaire, genetic instability, CBMN assay, comet assay

Introduction

Dietary habits are recognized to be an important modifiable environmental factor influencing cancer risk, depending on the foods consumed and the specific type of cancer.¹⁻³ Nutrition science consists in the application of molecular biology technology in epidemiologic investigations that, starting from an individual health perspective, upgrades to the population.⁴ Nutritional genomics studies the functional interaction between nutrition (macronutrients and micronutrients) and an individual's genome at the molecular, cellular, and systemic level.⁵,⁶ Integrating the information regarding nutritional intake, effect biomarkers, susceptibility biomarkers (gene polymorphisms and expression), diseases, and phenotypes may lead to the development of nutritional biomarkers.⁷ Understanding the influence of nutrition on metabolic pathways and homeostatic control and the mechanisms of its (de) regulation could lead to evidence-based dietary intervention strategies aimed at restoring health and fitness for preventing diet-related diseases.⁸

Dietary patterns involve complex interactions of food and nutrients, being important to study not only each individual nutrient but also the diet as a whole. An important example is the high consumption of vegetables, legumes, fruits, nuts, minimally processed cereals, monounsaturated lipids, moderately high consumption of fish, low consumption of dairy and meat products, and regular but moderate intake of alcohol of the well-characterized Mediterranean diet, which is linked with cancer prevention.⁹,¹⁰

Important epidemiologic studies postulate the role of carcinogens present in food and how its exposure is linked to specific cancers and how incidence varies among countries.¹ The classical evidence of the relationship between diet and cancer is shown by the change in the incidence of certain types of cancers occurring in populations that migrate to a different geographic area and culture,¹¹ suggesting that international differences in cancer incidence can be attributed primarily to environmental or lifestyle factors rather than genetic ones.¹,¹¹

There are several known mechanisms by which diet can influence cancer development: food carcinogens that act on DNA provoking its damage, blockage, or induction of enzymes involved in the activation or deactivation of carcinogenic substances, inadequate intake—promoting alterations in DNA synthesis, repair, or methylation, thereby influencing mutation rate and/or gene expression.¹² The impact of energy balance and growth rates—hormone levels and growth factors—should also be taken into account because it influences cell division, cell cycle, DNA repair, and replication rates.¹²

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Tools to assess markers of general DNA damage include cytokinesis-blocked micronucleus cytome (CBMN) assay, a comprehensive system for measuring DNA damage which measures end points such as micronuclei (MN), a biomarker of chromosome breakage and/or whole chromosome loss; nucleoplasmic bridges (NPB), a biomarker of DNA misrepair and/or telomere end-fusions; and nuclear buds (NBUD), a biomarker of elimination of amplified DNA and/or DNA repair complexes.13,14

MN are small, extranuclear bodies, originate in dividing cells from acentric chromosome/chromatid fragments or whole chromosome/chromatid that lag behind in anaphase, and are not included in the daughter nuclei in telophase.15–19

NPB occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. In the absence of breakage of the anaphase bridge, the nuclear membrane eventually surrounds the daughter nuclei and the anaphase bridge, and a nucleoplasmic bridge is formed.20 NBUD have been observed in cultures grown under strong selective conditions that induce gene amplification, as well as under moderate folic acid deficiency.14,17 Gene amplification plays a crucial role in the malignant transformation of human cells as it mediates the activation of oncogenes or the acquisition of drug resistance.21

Another useful tool is the comet assay, a simple and sensitive method for detecting DNA-break strands that originated from many sources, such as through oxidative DNA damage.22,23

The comet assay has become one of the standard methods for assessing DNA damage, with a wide range of applications, namely, in genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as fundamental research in DNA damage and repair24,25; studying the mechanisms of action of genotoxic chemicals; investigating oxidative damage as a factor in disease; monitoring oxidative stress in animals or human subjects resulting from exercise, diet, or exposure to environmental agents; studying the effects of dietary antioxidants; and monitoring environmental pollution by studying sentinel organisms.6,27

Although there is a general recognition that diet influences cancer risk, information regarding precise dietary factors that determine human cancer is an ongoing debate.9,11,28,29 Diet is usually a complex of both nutritive (macronutrients and micronutrients) and non-nutritive food constituents; thus, the search for specific factors is bound to involve a long gathering of epidemiologic information regarding food habits. Epidemiologic studies based on food frequency questionnaires (FFQs) are tools aimed at such goals and can be very important to develop a hypothesis regarding diet and cancer.

The aim of this study was to investigate the potential influence of macronutrients (calories, protein, and glucides) and micronutrients (vitamins and minerals) by a FFQ on genotoxicity biomarkers measured by the CBMN assay and the comet assay.

Methods

Subjects and personal questionnaire

The sample studied consisted of 44 healthy subjects from the Lisbon and Tagus Valley Region, mainly whites, in Portugal with administrative job descriptions that assigned to participate in the study.

The study participants filled in a questionnaire on individual characteristics and working practices. These included questions regarding age, gender, tobacco and alcohol consumption habits, medication, hereditary diseases, cancer history, exposure to chemical agents, and characterization of professional activity. The questionnaire was validated by application of a pretest in a group of subjects, which was used as a pilot sample at the beginning of the study.

Food frequency questionnaire

Dietary intake was assessed using a self-administered FFQ30,31 The FFQ included type and quantity of food intake, namely, some food items, which allowed for the quantification of different macronutrients and micronutrients. The FFQ is a 3-page booklet with a list of 92 common food and beverage items and questions relating to food preparation and dietary habits. Participants were required to indicate how often each food and beverage was usually consumed per month, week, or day. Average daily consumption was based on the participants’ reports on how often a specified serving size of each food or beverage item was consumed. This information, along with the nutrient composition of the food item/unit weight taken from 92 selected items, allowed participants’ daily micronutrient and macronutrient intake to be calculated using the FREQUAN dietary analysis program.32

Laboratory Proceedings

Cytokinesis-blocked micronucleus assay

Evaluation of genotoxic effects was conducted by applying the CBMN assay in peripheral blood lymphocytes as described in Ladeira et al.33,34 All blood samples were collected from subjects by venipuncture with heparin between 10 and 12 AM, and coded and analyzed under blind conditions. Briefly, lymphocytes were isolated using Ficoll–Paque gradient and placed in RPMI 1640 culture medium with l-glutamine and red phenol added with 10% inactivated fetal calf serum, 50 µg/mL streptomycin + 50 U/mL penicillin, and 10 µg/mL phytohemagglutinin. Duplicate cultures from each subject were incubated at 37°C in a humidified 5% CO₂ incubator for 44 hours, and cytochalasin B 6 µg/mL was added to the cultures to prevent cytokinesis. After 28 hours of incubation, cells were spun onto microscope slides using a cytocentrifuge. Smears were air-dried and double-stained with May–Grünwald-Giemsa and mounted with Entellan. The criteria for scoring the nuclear abnormalities in lymphocytes
were the ones established and validated by the HUman MicroNucleus International Collaborative Project available in http://www.humn.org and in Fenech et al. 35

Comet assay
Isolated lymphocytes were cryopreserved following the protocols of Duthe et al. 36 and Singh and Lai. 37 Briefly, isolated lymphocytes suspended in RPMI medium with l-glutamine were centrifuged (600 g, 10 minutes) and resuspended in freezing mix (90% v/v heat-inactivated fetal calf serum and 10% v/v dimethyl sulfoxide [DMSO]), frozen at −1°C/min in polystyrene, and stored at −80°C.

For analysis of DNA damage and oxidative damage by alkaline comet assay, a modification of the originally described technique by Singh et al. 38 was used to measure the basal level of DNA oxidation in lymphocytes. 39 Briefly, cell suspension (2.0 × 104 cells/mL) was mixed with 140 µL of 1% low melting point agarose (LM Pronadisa) in a microcentrifuge tube and added to a slide previously precoated with 1% agarose (SeaKem), 2 gels per slide, and covered with a cover slip (22 mm × 22 mm × 1.0 mm) and allowed to set on a cold plate. The cover slips were removed and the slides immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM TRIS, 1% Triton X-100 [pH 10]) for 60 minutes. Following lysis, the slides were immersed in 2 changes of electrophoresis buffer (1 mM Na2EDTA, 0.3 M NaOH [pH 13]), and DNA was allowed to unwind for 20 minutes before electrophoresis at 1.14 V/cm, 300 mA for a further 20 minutes. DNA unwinding and electrophoresis were performed in a cold unit at 4°C. The slides were transferred to a Coplin jar and immersed in PBS and then in distilled water, both for 10 minutes at 4°C. After that, the slides were dehydrated in increasing ethanol concentrations (70%, 96%, and 100%), 5 minutes each. The slides dried at room temperature were stained with DAPI (1 µg/mL) and then visualized and scored by one single observer using Zeiss AxioScope.A1 fluorescence microscope according to the criteria of scoring comets from each gel described by Collins. 40 and the Comet Assay IV software from Perceptive Instruments (Bury St Edmunds, UK) was used.

Statistical analysis
Variables were compared with the normal distribution using the Shapiro-Wilk test. The association between the macronutrients and micronutrients and genotoxicity biomarkers was investigated by Spearman correlation coefficient. Statistical analyses were performed using the SPSS package for Windows, version 21.0.

Results
Individual characteristics such as gender distribution, age, tobacco habits, and alcohol consumption were analyzed for the sample of 44 individuals. In the sample, 75% were women (n = 33) and 25% men (n = 11), with a mean age of 39.26 ± 1.46 years. Concerning tobacco habits, nonsmokers comprised 77.3% of the sample (n = 34), whereas 22.7% were smokers (n = 10).

Twenty-three items were selected from the FFQ based on the possible influence on genomic stability. Therefore, the following nutritional items were selected from the FFQ for analysis: total calories; protein; glucides; fat; niacin; calcium; copper; magnesium; manganese; zinc; iron; selenium; vitamins B12, C, D, E, and K; retinol; folate; omega-3 and omega-6; fibers; and caffeine. A statistical description of the amounts consumed daily by item in the sample, as well as their reference intake values, is shown in Table 1. All the studied items presented values between the minimum and maximum reference values, meaning no deficiencies or excesses existed.

To investigate the possible correlations between genotoxicity biomarkers and nutritional items, bivariate Spearman correlations were computed. There were 2 significant positive correlations, between genotoxicity biomarkers (MN and NBUD) and protein, and a negative correlation between calories and % DNA (Table 2).

Regarding significant correlations with micronutrients, MN were positively correlated with calcium, magnesium, zinc, and caffeine and niacin with NBUD, and none of the vitamins assessed by the FFQ exhibited a statistically significant correlation with the genotoxicity biomarkers studied.

Other positive correlations were found, however, without statistical significance, namely, niacin, copper, and caffeine with all the genotoxicity biomarkers measured by CBMN and with oxidative DNA damage. Vitamin C was also positively correlated with the CBMN genotoxicity biomarkers under study (MN, NPB, and NBUD) but not with the biomarkers measured by the comet assay (DNA damage and oxidative DNA damage).

Two statistically significant negative correlations were found between omega-6 and calorie intake and DNA damage measured by the comet assay.

Several other negative correlations were found without reaching statistical significance, namely, between vitamins B12 and D and all the genotoxicity biomarkers studied; folate was correlated with MN, NPB, NBUD, and DNA damage, and omega-3 and omega-6 were negatively correlated with NPB, NBUD, DNA damage, and oxidative DNA damage.

Discussion
Determining the intake levels of macronutrients and micronutrients required to maintain genome stability is an essential step in the definition of optimal diets for the prevention of
Nutrition and Metabolic Insights

We have conducted an FFQ inquiry of 44 subjects, followed by a detailed assessment of DNA damage markers (MN, NPB, NBUD, and DNA tail by the comet assay), which found 7 significant positive correlations and 2 significant negative ones. We have found correlations between calorie and protein intake and genotoxicity biomarkers. The average amount of calories ingested daily was negatively correlated with % DNA in tail, an indicator of DNA damage. This was somewhat unexpected, as calorie restriction reduces metabolic rate and oxidative stress, meaning lower calorie ingestion is expected to decrease DNA damage, as well as damage to other molecules, such as protein and lipids. Regardless of the source and nature of DNA damage, DNA repair is better preserved and/or enhanced when caloric consumption decreases. The possible mechanisms associating calorie restriction with cancer prevention involve the regulation of cellular proliferation and apoptosis (decrease in DNA replication) and reduction in metabolic rate, oxidative damage, and inflammation mediators (reduction in reactive oxygen species [ROS] and consequent reduction in DNA damage). Therefore, it is possible that because of the self-reported nature of the FFQ, some dietary items become subjectively overrated, in a way that cannot be easily corrected.

A positive correlation was found between protein intake and micronucleus, meaning people with higher protein consumption would on average have higher micronucleus frequency. In general, high-protein diets show a protective effect that could be due to an increased plasma concentration of sulfhydryl amino acids, such as cysteine and methionine, which have

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Table 1. Dietary parameters (macronutrients, micronutrients, and others) collected by FFQ (mean intake per day ± SD) and respective dietary reference intakes.

| Dietary Parameters | Mean ± SD (Range) | Dietary Reference Intakes (Food and Nutrition Board, Institute of Medicine, National Academies) |
|--------------------|-------------------|--------------------------------------------------------------------------------------------------|
| Macronutrients     |                   |                                                                                                 |
| Calories           | 2527.40 ± 123.07  | Variable by age and gender (kcal)                                                                |
| Protein            | 115.36 ± 37.02 (57.89-211.31) | Variable by age and gender, g/d                                                                |
| Glucides           | 315.00 ± 125.56 (127.54-621.198) | Variable by age and gender, g/d                                                                |
| Fat                | 95.75 ± 28.49 (41.45-150.72) | Variable by age and gender                                                                      |
| Niacin             | 27.75 ± 9.27 (12.53-50.17) | 35 mg/d                                                                                            |
| Calcium            | 1227.97 ± 582.02 (538.96-3584.74) | 1000 mg/d                                                                                      |
| Copper             | 2.10 ± 0.84 (0.98-4.25) | 700 µg/d                                                                                          |
| Magnesium          | 417.03 ± 159.16 (161.72-805.18) | Female: 265 mg/d Male: 350 mg/d                                                                     |
| Manganese          | 4.82 ± 2.30 (1.24-10.79) | Female: 1.8 mg/d Male: 2.3 mg/d                                                                    |
| Zinc               | 14.89 ± 4.69 (8.02-24.96) | Female: 6.8 mg/d Male: 9.4 mg/d                                                                  |
| Iron               | 18.82 ± 1.07 (1.13-6.66) | Female: 8.1 mg/d Male: 6 mg/d                                                                    |
| Selenium           | 138.67 ± 8.58 (1.13-6.66) | 45 µg/d                                                                                           |
| Vitamin B12        | 12.31 ± 0.78 (1.13-6.66) | 2.0 µg/d                                                                                          |
| Vitamin C          | 163.65 ± 97.61 (41.37-440.86) | Female: 60 mg/d Male: 75 mg/d                                                                    |
| Vitamin D          | 4.67 ± 0.35 (1.13-6.66) | 10 µg/d                                                                                           |
| Vitamin E          | 11.80 ± 0.67 (1.13-6.66) | 12 µg/d                                                                                           |
| Vitamin K          | 18.40 ± 15.13 (2.46-81.63) | Female: 35 µg/d Male: 45 µg/d                                                                    |
| Retinol            | 776.51 ± 70.10 (1.13-6.66) | Female: 500 µg/d Male: 625 µg/d                                                                  |
| Folate             | 401.21 ± 26.21 (1.13-6.66) | 320 µg/d                                                                                          |
| Omega-3            | 1.64 ± 0.59 (0.67-3.63) | Variable by age and gender                                                                      |
| Omega-6            | 12.30 ± 5.38 (5.44-27.10) | Variable by age and gender                                                                      |
| Others             |                   |                                                                                                 |
| Fibers             | 30.01 ± 14.05 (9.32-61.28) | Female: 25 g/d Male: 38 g/d                                                                     |
| Caffeine           | 83.22 ± 59.16 (4.13-350.25) | 200-400 mg/d                                                                                     |

Abbreviation: FFQ, Food Frequency Questionnaire.
considerable antioxidant activity, or via increased glutamine, which increases tissue antioxidant glutathione.46,47 Many studies associate increased cancer risk with diets rich in starches, such as sugar and sucrose.12 In contrast, a diet rich in dietary fibers has been associated with low risk of cancer because fibers can have the potential to dilute carcinogens, speed up bulk transition, reduce time for carcinogen absorption, and also serve as a substrate to generate short-chain fatty acids used by colonic epithelial cells.48,49 In this sense, a diet with a high proportion of simple glucides that is high in dietary fibers might increase the risk of cancer development. The study of the relation between fiber and glucide consumption with biomarkers detected statistically significant correlations, in a positive sense with low intensity, between fiber consumption and NBUD ($r = .343$, $P = .023$) and glucide consumption and NBUD ($r = .308$, $P = .042$). Regarding the interaction between fiber and glucide consumption, a positive statistically significant correlation, with low intensity, with NBUD ($r = .315$, $P = .037$) was detected. These results indicate that higher intakes of fibers and glucides can lead to higher NBUD values.

We have also examined the correlation between fiber/glucide consumption and genotoxicity biomarkers and found a statistically significant positive correlation between them ($r = .871$, $P < 0.0001$).

Niacin, or nicotinic acid, is one of the few vitamins that has an intimate role in DNA synthesis, DNA repair, and cell death.50–52 Niacin is required as a substrate for poly(ADP-ribose) polymerase (PARP), which is involved in cleavage and rejoining of DNA and in telomere length maintenance. The consequence of its deficiency is increased DNA oxidation, DNA breaks, and an elevated chromosome damage rate.51–53 According to the Expert Group on Vitamins and Minerals (2003), there are no genotoxicity and carcinogenicity data available for nicotinic acid, and the impact of niacin on human carcinogenesis is therefore confounded by the effect of other micronutrients.53 Our results show a positive correlation with NBUD. As already mentioned, NBUD are biomarkers of gene amplification, and they play a crucial role in the malignant transformation of human cells as they mediate the activation of oncogenes or the acquisition of drug resistance.21

Fenech and Bonassi54 showed a decrease in micronucleus frequency related to niacin intake using the CBMN assay, and no results for NBUD were reported.
Calcium has not been considered of etiological importance in cancer for many years. However, several studies have suggested that dietary calcium may be associated with a reduced risk of colon cancer.\textsuperscript{55} Calcium may bind with fats in the intestine, making fats less accessible to metabolism by bile salts and the subsequent formation of carcinogenic breakdown products.\textsuperscript{56} Fenech and Bonassi\textsuperscript{54} showed a decrease in MN frequency related to calcium intake. Our results are not consistent with most epidemiologic literature as we have found a positive correlation between calcium and MN, but we corroborate Smith et al\textsuperscript{56} who also found that high calcium intake was associated with higher levels of MN.

Magnesium by itself is not genotoxic, being highly required to maintain genomic stability. Adding to its stabilizing effect on DNA and chromatin structure, magnesium is an essential cofactor in almost all enzymatic systems involved in DNA processing. As an essential cofactor in nucleotide excision repair, base excision repair, and mismatch repair, magnesium is required for the removal of DNA damage. Its relation with tumor formation is more complex; magnesium appears to be protective at early stages but promotes the growth of existing tumors at latter stages.\textsuperscript{57,58} Any decrease in magnesium below protective at early stages but promotes the growth of existing tumors at latter stages.\textsuperscript{57,58} Any decrease in magnesium below physiological levels should trigger Mg-deficiency-related diseases, such as cardiovascular diseases, accelerated aging, and cell cycle control apoptosis and carcinogenesis.\textsuperscript{58} Our results showed a positive correlation between magnesium and micronucleus frequency. Fenech et al\textsuperscript{46} showed that a mitogen response significantly and positively correlated with plasma zinc, magnesium, selenium, and folate, thus corroborating our results insofar as magnesium is concerned.

Zinc is an important element for numerous proteins, playing a pivotal role in essential cell functions such as cell proliferation and apoptosis, defense against free radicals, and DNA damage repair.\textsuperscript{58,50,51,59-61} Alterations in zinc status would affect DNA integrity by altering oxidative stress, antioxidant defenses, and DNA repair functions.\textsuperscript{60,61} Our study found a positive correlation between zinc and MN, as higher zinc intake was associated with increased MN. This result is contrary to most of the literature; namely, Sharif et al\textsuperscript{62} showed that supplementation in an elderly population was beneficial for reducing the micronucleus frequency and DNA damage. Changes in dietary zinc intake affect DNA single-strand breaks, appearing to be a critical factor for maintaining DNA integrity in humans.\textsuperscript{65} Antioxidants, such as vitamins A, D, and E, are known to be reducing agents, and these molecules are capable of slowing or preventing the oxidation of other molecules being associated with reduced risk of several chronic diseases, particularly some cancers and heart diseases.\textsuperscript{64-67} They are at the end of oxidative chain reactions, removing free radicals and preventing the oxidation of unsaturated fats, and are clearly documented antigenotoxic and antimutagenic potential antioxidants.\textsuperscript{67} Epidemiologic evidence indicates that intake of foods that are naturally rich in vitamin C is associated with reduced risk of cardiovascular and neurodegenerative diseases and various cancers, but the extent to which vitamin C contributes to this effect remains unclear.\textsuperscript{51} Folate plays a key role in a number of processes related to DNA integrity, such as DNA synthesis and methylation. In vitro studies have shown that folic acid deficiency causes a dose-dependent increase in uracil incorporation into human lymphocyte DNA. Folate administration reduces DNA uracil incorporation and the occurrence of chromosome breaks in human cells.\textsuperscript{65,68,69} A low folate concentration has been implicated as a potential promoter of carcinogenesis, for example, in colorectal cancer, lung, breast, pancreatic, gastric, esophageal, and prostate malignancies.\textsuperscript{3,28,50,65,70,71}

The results achieved by the FFQ regarding these items were not significant despite the majority of negative correlations, meaning an increase in the intake of vitamins would decrease genotoxic damage; however, statistical significance was not reached.

Omega-6 polyunsaturated fatty acids (PUFAs) are considered to increase lipid peroxidation because of lipid peroxidation products, whereas omega-3 PUFAs exert a chemopreventive effect.\textsuperscript{72} In our study, a significant negative correlation was found between omega-6 and % DNA in tail, meaning higher intake of omega-6 will decrease DNA damage. This result is contrary to most of the literature, given omega-6 fatty acid’s reputation for promoting cancer, as shown by the study of Bishop et al\textsuperscript{73} on DNA damage and dietary fatty acids, where whole blood omega-6 PUFAs were positively correlated with DNA damage, also measured by the comet assay. Along with omega-3, omega-6 plays a crucial role in brain function and normal growth and development. Besides some omega-6 fatty acids promoting inflammation (linoleic acid and arachidonic acid), γ-linolenic acid actually reduces inflammation and even protects DNA.\textsuperscript{73}

Epidemiologic studies have suggested that people who consume a diet high in omega-3 fatty acids may experience a lower prevalence of cancer, and many small trials have attempted to assess the effects of omega-3 fatty acid in the diet, either as omega-3 fatty acid–rich foods or as dietary supplements. A systematic review made by MacLean et al\textsuperscript{74} regarding the effects of omega-3 fatty acids on cancer risk, which compiled a large body of literature spanning numerous cohorts from many countries and with different demographic characteristics, did not provide evidence of a significant association between omega-3 fatty acids and cancer incidence. Our study did not lead to any statistically significant correlation associating omega-3 and biomarkers.

We have found a positive correlation between caffeine consumption and micronucleus, in line with Kiefer and Wiebel\textsuperscript{75} who showed that caffeine increases the number of micronuclei in test cell lines. Also, Smith et al\textsuperscript{46} showed a statistically significant association between MN frequency and coffee consumption. This study also reported that intake of decaffeinated...
coffee was not associated with a similar increase in MN. There is no unequivocal epidemiologic evidence indicating that caffeine is carcinogenic to humans, although it has been a suspected carcinogen, primarily for cancers of the pancreas, bladder, kidney, and ovary. Caffeine is involved in the inhibition of p53, homologous recombination pathway, namely, Rad-51, inhibition of DNA synthesis, delays in cell proliferation, reverse cell cycle checkpoint function, enhanced genotoxicity after DNA damage by ultraviolet radiation, and others.

Conclusions
Nowadays, the link between environment, where diet is included, and cancer is gaining even more evidence, indicative of a significant causal or preventive role for various dietary factors. Therefore, our study deemed to investigate the influence that macronutrients and micronutrients could have on DNA damage, as a known precursor of mutations that can lead to disease. The use of CBMN technique and comet assay to study human nutrition and cancer is an accepted method, and positive and negative correlations were found.

In general, investigations support the hypothesis that dietary antioxidants may protect against cancer as a moderate effect of long-term antioxidant supplementation on oxidative DNA damage, but significant associations are difficult to find for many reasons. On one hand, studies are usually based on relatively small samples of healthy subjects; on the other hand, synergistic effects involving more than one antioxidant, not seen for each one, should be taken into account, but that requires much larger samples. To unravel the association between diet and cancer, the modulating role of macronutrients and micronutrients needs to be more clearly understood. It is possible that other technical tools for measuring DNA damage might allow for capturing more accurate associations involving the antioxidants examined. It is also possible that the range of antioxidant concentrations and/or oxidative DNA damage in this study was not wide enough to detect associations or it can be concluded that associations simply do not exist. Finally, our study was based on a self-administered FFQ, allowing for the subjectivity to which questionnaires are usu-
