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Biomarkers of dietary intake of micronutrients modulate DNA adduct levels in healthy adults

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DNA adducts, a reliable indicator of internal dose exposure to genotoxic agents and, possibly, of cancer risk, have been shown to be modulated by diet, particularly by the consumption of fresh fruit and vegetables, and by the intake of antioxidants (Palli *et al.*, 2000, *Int. J. Cancer*, 87, 444–451). We have therefore investigated the association between DNA adducts in peripheral leukocytes and plasma levels of selected micronutrients, also taking into account the role of metabolic polymorphisms and smoking history, in a large independent random sample of volunteers enrolled in the prospective study EPIC–Italy (~110 subjects from each of the three main geographical study areas, Northern, Central and Southern Italy). DNA adducts and five polymorphic metabolic genotypes were determined in peripheral leukocytes using the ^{32}P-post-labelling technique and PCR methods. Plasma levels of six carotenoids, retinol and α- and γ-tocopherol were determined in the same blood sample. Among 331 subjects, 78.3% had detectable levels of DNA adducts (mean 7.46 ± 0.48 per 10^9 nucleotides). Vitamin supplementation was reported by only a few subjects (3.9%). Strong inverse associations emerged between levels of DNA adducts and plasma retinol ($P = 0.02$), α-tocopherol ($P = 0.04$) and γ-tocopherol ($P = 0.03$), but not carotenoids (except a borderline inverse association with β-carotene, $P = 0.08$). An inverse significant association with plasma levels of retinol and γ-tocopherol persisted in the subgroup of non-smokers, whereas a negative association with α-tocopherol emerged only in smokers. DNA adduct levels did not show any significant variation according to analyzed genotypes. Stratification by *GSTM1* genotype, however, showed a significant negative association between DNA adduct levels and plasma levels of α- ($P = 0.02$) and β-carotene ($P = 0.02$) in subjects with the *GSTM1* null genotype. Our results confirm that biomarkers of dietary intake of antioxidants significantly modulate DNA adducts and suggest specific inverse associations between DNA adduct levels and antioxidant concentrations among *GSTM1* null subjects and smokers.

Introduction

Several epidemiological studies have indicated that a large proportion of human cancer cases result from a complex interaction of environmental factors, i.e. urban and industrial air pollution, chemical compounds derived from cigarette smoking, specific dietary habits and genetic factors (1–3). Dietary habits characterized by frequent consumption of fresh fruit and vegetables have been shown to reduce the risk of cancer (4–6). Similar inverse associations also emerged between the incidence rates of various cancers and estimated intakes or plasma concentrations of some micronutrients, of which fruit and vegetables are the main sources (1,7–9).

DNA adducts have been widely used in order to identify health hazards and to evaluate the dose–response relationship in humans exposed to carcinogens and mutagenic compounds and are considered a biomarker of internal dose. DNA adducts tend to be higher among subjects heavily exposed to air pollutants, such as police officers and bus drivers (10,11), and it has been suggested that high levels of DNA adducts might be predictive of cancer risk, reflecting both the exposure to environmental xenobiotics and susceptibility to carcinogens (2,12,13).

Individual susceptibility to cancer can be partly explained by variability in specific enzyme metabolic pathways. Polymorphisms in these metabolic genes have been linked to increased risk of cancer in several case–control studies (14). A genetic deficiency in the detoxifying enzyme glutathione S-transferase (GST) M1 (*GSTM1* null genotype) has been associated with increased risk of lung (15,16) and bladder cancer (17) and increased polycyclic aromatic hydrocarbon (PAH)–DNA adducts (18,19).

Dietary constituents in fresh fruit and vegetables might play a relevant role in DNA adduct formation by inducing or inhibiting enzymatic activities (20,21). Antioxidant micronutrients have been shown experimentally to inhibit DNA damage by PAH and other carcinogens and to alter expression of metabolic enzymes (22,23).

A few human studies evaluated the effects of micronutrients on DNA adducts. Grinberg-Funes *et al*. (24) reported an inverse association between blood levels of α-tocopherol and vitamin C and PAH–DNA adducts in lymphocytes: the relationship was found only in those subjects with the *GSTM1* null genotype. Mooney *et al*. (25) reported a similar inverse association between plasma levels of retinol, β-carotene and α-tocopherol and PAH–DNA adducts in subjects lacking the *GSTM1* detoxification gene. Another study (26) did not report any association between the plasma levels of β-carotene and α-tocopherol and level of DNA adducts in lymphocytes. However, it has also been suggested (27) that polymorphisms

Abbreviations: B[*a*]P, benzo[*a*]pyrene; BMI, body mass index; GST, glutathione *S*-transferase; PAH, polycyclic aromatic hydrocarbon.

of selected metabolic genes and plasma β-carotene may modulate the level of DNA adducts. We have recently published a cross-sectional study on the association of DNA adduct levels with diet and other individual characteristics in 300 healthy adults randomly selected in the Italian EPIC cohorts (28), in which strong inverse associations emerged with the reported frequency of consumption of fresh fruit and vegetables, olive oil and the intake of all major antioxidants, particularly β-carotene and vitamins C and E.

The epidemiological evidence suggests, therefore, that DNA damage may be modulated by genetic and nutritional risk factors. In this study we aimed to investigate the association between DNA adduct levels and plasma levels of selected micronutrients (six carotenoids, retinol and α- and γ-tocopherol), measured in the same blood sample, also taking into account the role of metabolic polymorphisms and smoking history in a large series of healthy adults randomly sampled among volunteers enrolled in the Italian EPIC cohort.

Materials and methods

Subjects

EPIC–Italy is the Italian section of the large prospective European project EPIC (European Prospective Investigation on Cancer and Nutrition) (29); the recruitment of 47 749 volunteers, in the age interval 35–64 years, was carried out in the period January 1993–March 1998 in five participating centres, across different areas of the country. A random sample of ~110 subjects, stratified by age, sex and centre, was selected from each of the three main geographical study areas (Northern, Central and Southern Italy), in order to obtain a series of about 330 participants representative of the national cohort. This sample is independent of that used in the previous study which included another group of 300 volunteers (28).

Diet and lifestyle questionnaires

Dietary information on the frequency of consumption of >120 foods and drinks in a 12 month period prior to enrollment was obtained by a self-administered Food Frequency Questionnaire, validated in a pilot phase (30). All individual questionnaires were checked and coded by trained dieticians, computerized and then transformed into estimates of intake for a series of over 30 nutrients according to specifically developed Italian Food Tables (31). The consumption of vitamin supplements was specifically investigated.

A standardized lifestyle questionnaire, representing the Italian translation of a common English model adopted by all European centres (in two separate versions for men and women), was also filled in by each participant. All subjects were originally classified according to smoking history reported at enrollment in three categories: never, former and current smokers. Since DNA adduct levels in smokers have been reported still to be elevated 14 months after quitting smoking (32), we decided *a priori* to combine former smokers with one of the other categories (never or current) according to a cut-off value of 4 years since cessation. We thus used a dichotomous classification to define 'smokers' (as subjects classified as current smokers at enrollment plus former smokers who reported to have quit smoking in the last 4 years before enrollment) and 'non smokers' (as subjects classified as never smokers at enrollment plus subjects who reported to have quit smoking at least 4 years before enrollment).

Blood collection and storage

Informed consent was obtained from all subjects prior to enrollment in the study. The project has been approved by the local Ethical Committee in Florence. Blood samples were collected in citrate tubes and were processed by centrifugation in a dedicated laboratory in each centre, on the same day as collection, divided into 28 aliquots of 0.5 ml each (12 plasma, eight serum, four concentrated red blood cells and four buffy coat), using an automatic aliquoting and sealing machine specifically developed by BICEF, France (Cryo-Bio Straw). The aliquots were stored in liquid nitrogen tanks at −196°C in a local biological bank in each centre in Italy and centrally at IARC, Lyon. Straws were retrieved and shipped in dry ice to laboratories for DNA extraction and DNA adduct analyses and to detect metabolic polymorphisms. Plasma levels of micronutrients were determined at the Nutrition and Cancer Unit of IARC (Lyon).

DNA ^{32}P-post-labelling technique

Leukocyte DNA was isolated and purified from stored buffy coats by enzymatic digestion of RNA and proteins followed by phenol–chloroform extractions (28). DNA samples (5 μg) were digested with 0.21 U micrococcal nuclease and 0.174 U spleen phosphodiesterase at 37°C for 4.5 h. After treatment of DNA samples with 5 μg nuclease P1 for 30 min at 37°C, the hydrolysate enriched in adducted nucleotides was then labelled by incubation with 24 μCi carrier-free [γ-^{32}P]ATP (3000 Ci/mM) and 10 U T4 polynucleotide kinase at 37°C for 30 min in 25 μl of bicine buffer mixture. Resolution of ^{32}P-labelled DNA digests treated with nuclease P1 was carried out on PEI–cellulose TLC plates using the contact transfer technique (28). The solvent systems selected were: (D1) 1 M sodium phosphate, pH 6.8; (D3) 4 M lithium formate, 7.5 M urea, pH 3.5; (D4) 0.65 M LiCl, 0.45 M Tris–HCl, 7.7 M urea, pH 8.0; (D5) 1.7 M sodium phosphate, pH 5.0. The adduct spots were detected by autoradiography from 72 to 96 h at −80°C using Kodak XAR-5 films and intensifying screens. DNA adduct levels were determined by excising areas of chromatograms and measuring the levels of radioactivity present by Cerenkov counting. The results were expressed as relative adduct labelling = c.p.m. in adduct nucleotides/c.p.m. in total nucleotides. The detection limit of the nuclease P1 modification of the DNA ^{32}P-post-labelling technique was 0.1 adduct per 10^9 nucleotides, as previously reported (28). The reproducibility of the DNA ^{32}P-post-labelling technique was verified by analysing ~20% of DNA samples in a second independent experiment, and the results of the two analyses were in perfect agreement ($r = 0.98$). All the analyses were carried out blind prior to decoding. One standard was routinely included in the analysis, i.e. benzo[*a*]pyrene (B[*a*]P)–DNA adducts, from liver of mice treated i.p. with 0.06 mg/kg B[*a*]P for 24 h. The average level of B[*a*]P–DNA adducts was 5.1 ± 0.1 per 10^8 nucleotides.

Polymorphism analysis

A multiple PCR method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes and polymorphic alleles at the *CYP1A1 MspI*, *NAT2* and *MTHFR* loci in genomic DNA samples (obtained from stored buffy coats as described above).

GST. This method had both *GST* primer sets (*GSTM1*, 5′-AACTCCCTGAAAAGCTAAAGC and 5′-GTTGGGCTCAAATATACGGTGG; *GSTT1*, 5′-TCCTTACTGGTCCTCACATCTC and 5′-TCACCGGATCATGGCCAGCA) in the same PCR which included a third primer set for albumin (5′-GCCCTCTGCTAACAAGTCCTAC and 5′-CCCTAAAAAGAAAATCGCCAATC) and used 30 cycles with denaturing at 94°C for 1 min, annealing at 64°C for 1 min and extension at 72°C for 1 min.

CYP1A1. DNA was amplified in a total reaction volume of 50 μl containing 1.2 mM dNTP, 1.2 μM oligonucleotide primers and 2.5 U *Taq* polymerase (AmpliTaq; Perkin-Elmer). DNA samples were amplified using the primers 5′-CTGACTGGCTTCAGCAAGTT and 3′-TAGGAGTCTTGTCTCATGCCT. PCR was performed for 45 cycles with denaturing at 95°C for 1 min, annealing at 56°C for 1 min and extension at 65°C for 2 min. PCR products were digested with excess *MspI* (New England Biolabs) for 3 h and then electrophoresed through 1.8% agarose and visualized by ethidium bromide staining.

NAT2. Three known slow acetylator alleles (*NAT2*5*, *NAT2*6* and *NAT2*7*) were identified. PCR was carried out in a total volume of 50 μl using primers 5′-TGACGGCAGGAATTACATTGTC and 3′-ACACAAGGGTTTATTTTGTTCC. The PCR mixture contained 5 μl DNA, 50 pM each primer, 200 μM dNTPs, 1.5 U *Taq* polymerase (AmpliTaq), 10 mM Tris–HCl buffer, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$. PCR was performed for 35 cycles with denaturing at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min. PCR products were incubated with restriction enzymes *Kpn*I, *Taq*I and *Bam*HI from Gibco. Rapid acetylator genotypes are wild-type (*4) allele homo- or heterozygotes, slow acetylator genotypes are those with any two or the three slow acetylator alleles (*5, *6 or *7).

MTHFR (677 C → T). Primer sequences were 5′-TGAAGGAGAAGGTGTTCTGCGGGA and 5′-AGGACGGTGCGGTCAGAGTG. Amplification was performed using initial denaturation at 95°C for 2 min followed by 29 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. Amplified product was digested with *Hin*f1 before electrophoresis.

Plasma levels of micronutrients

Aliquots of plasma were extracted from the central EPIC biological bank in Lyon. Manipulation and second storage were done in liquid nitrogen (−196°C). On the day of analysis, aliquots were rapidly thawed at room temperature, protected from light. Samples were analysed for six carotenoids (α- and β-carotene, zeaxanthin, β-cryptoxanthin, lutein and lycopene), retinol

and α- and γ-tocopherol by reversed phase high performance liquid chromatography (HPLC 1100 system; Hewlett Packard, Les Ullis, France) following a method based on that of Steghens *et al*. (33). Samples were extracted with 800 µl of hexane after 30 min and the mobile phases were enriched with 1 ml/l triethylamine. One aliquot from a standard quality control sample was analysed and injected at the beginning, middle and end of each series. Chromatograms were integrated automatically by the system (Chemstation v.6.4; Hewlett Packard) but controlled one by one by three different laboratory technicians. Peaks for carotenoids that were under the detection limits were set at 0.

Statistical methods

The correlation between the micronutrients measured in the same blood sample was investigated by Spearman rank correlation coefficient.

To investigate the relationship between DNA adduct levels and plasma concentration of selected micronutrients, we compared adduct values across tertiles of plasma levels. Negative samples (those below 0.1 adduct per 10^9 normal nucleotides, the threshold of detection of the DNA ^{32}P-post-labelling method) were assigned a value of 0.1. In order to carry out statistical analyses, we used log transformed data for micronutrients and first divided all available subjects into tertiles of plasma levels of each nutrient, and the mean levels of DNA adducts across tertiles were compared by analysis of covariance, introducing into each model terms for age, sex, centre, smoking habit (never, former and current), body mass index (BMI), year and period of blood drawing and total caloric intake. The covariance analysis was also performed separately for 'non-smokers' and 'smokers' and separately for each genotype according to selected polymorphic metabolic genes. *Post hoc* Dunnett tests were performed for multiple comparisons among tertiles of plasma levels for each carotenoid and selected micronutrient. All analyses were performed on log transformed data. The tests for linear trends were calculated by including ordered variables in each covariance model with log transformed adduct values.

A P value $\leq 5\%$ was considered significant. All the analyses were performed by the statistical package SAS.

Results

Study subjects characteristics

Results were available for 331 subjects (163 men) included in this cross-sectional sample of EPIC–Italy (Table I): 111 from Turin and Varese, the two centres in Northern Italy, 109 from Florence in Central Italy and 111 from Southern Italy (28 females from Naples, where only women were enrolled, and 83 subjects from Ragusa). The mean age was 54.4 years (54.2 years for males and 54.5 for females). The mean values for weight, height and BMI were 77.6 kg, 171 cm and 26.6 kg/m^2 for males and 65.6 kg, 158 cm and 26.4 kg/m^2 for females.

Blood samples were obtained at enrollment in the period 1993–1998: the distribution was in agreement with that of the total cohort: 132 samples were drawn in 1993–1994, 152 in 1995–1996 and 47 in 1997 and in the first two months of 1998. The samples were obtained all the year round (except August) in all centres, usually with a peak in the Spring months.

Among participants, never smokers represented the largest group (127/331 or 38.4%), whereas 34.7% (115/331) reported at enrollment to be former smokers and 26.9% (89/331) current smokers. According to our dichotomous classification, non-smokers and smokers were 69.8 and 30.2%, respectively.

The consumption of vitamin supplements in our sample was reported at blood drawing by only a few subjects (13/331, 3.9%).

DNA adducts

DNA adducts were detected in 78.3% of the samples (259 subjects), with some variation between centres (lowest in Ragusa, 68.7%, and highest in Naples, 100%), but not between genders (79.1% in males and 77.4% in females).

The crude mean of the DNA adduct level was 7.46 (±0.48) per 10^9 nucleotides, with relevant differences between subjects from the five participating centres: crude mean values were 14.8, 9.2, 6.8, 5.1 and 4.8 for volunteers from Naples, Florence, Varese, Turin and Ragusa, respectively (data not shown).

Table I. Adjusted geometric means[a] of DNA adducts per 10^9 normal nucleotides, according to selected sociodemographic characteristics (EPIC Italy, 331 subjects 1993–1998)

Characteristic	*n*	DNA adducts (adjusted geometric mean)	Percent of positive[b]
Area/EPIC centre			
Northern Italy			
Turin	56	1.70	71.4
Varese	55	3.47	83.6
Central Italy			
Florence	109	3.20	80.7
Southern Italy			
Naples	28	11.99	100.0
Ragusa	83	1.34	68.7
Sex			
Male	163	3.70	79.1
Female	168	2.66	77.4
Smoking history[c]			
Current smokers	89	3.69	79.8
Former smokers	115	2.57	76.5
Never smokers	127	3.25	78.7
Smoking history[d]			
Smokers	100	3.21	77.0
Non-smokers	231	3.05	78.8

[a]From a covariance analysis including terms for age, sex, centre, period of blood drawing, year of blood drawing, BMI and smoking history.
[b] >0.1 DNA adducts per 10^9 nucleotides.
[c]As reported at enrollment.
[d]According to two categories: 'smokers' (current smokers at enrollment plus former smokers who reported to have quit smoking in the last 4 years before enrollment) and 'non-smokers' (never smokers at enrollment plus subjects who reported to have quit smoking at least 4 years before enrollment).

Overall, differences between centres persisted after adjustment for age, sex, smoking history, BMI, period and year of blood drawing, with samples from Ragusa showing lower mean levels than those from all other centres (Table I). No difference emerged according to smoking history [either in three (current, ex and never smoker) or two categories (smokers and non-smokers)].

Plasma levels of selected micronutrients

Table II shows the plasma levels of the micronutrients measured. Crude mean values together with adjusted mean values from a covariance analysis according to geographical area are reported for six carotenoids, retinol and α- and γ-tocopherol. Among the six carotenoids, β-carotene, lutein and lycopene showed the highest concentrations. Mean plasma levels were significantly higher in Southern Italy for total carotenoids ($P = 0.009$) and particularly for lycopene and β-cryptoxanthin ($P = 0.0001$ for both micronutrients), whereas mean levels of α-carotene were higher in Northern Italy ($P = 0.0001$). In contrast, β-carotene and lutein levels did not show any significant geographical variation. Differences between geographical areas also emerged for retinol and α- and γ-tocopherol, with lower mean levels in Southern Italy ($P = 0.0003$, $P = 0.008$ and $P = 0.006$, respectively).

The mean values of micronutrients measured in the 13 subjects reporting the use of vitamin supplements (mostly vitamin C, selenium and vitamin D) did not differ from those measured in all the other subjects.

Table II. Overall crude and adjusted means[a] of plasma levels of selected micronutrients according to geographical area (EPIC Italy, 331 subjects 1993–1998)

Micronutrient	Crude mean (±SE) (μmol/l)	Adjusted mean (±SE) (μmol/l)			*P* value for trend
		Geographical area			
		North	Center	South	
α-Carotene	0.15 ± 0.01	0.19 ± 0.02	0.14 ± 0.02	0.13 ± 0.02	0.0001
β-Carotene	0.51 ± 0.02	0.55 ± 0.04	0.50 ± 0.04	0.54 ± 0.04	0.53
β-Cryptoxanthin	0.38 ± 0.02	0.43 ± 0.04	0.31 ± 0.04	0.45 ± 0.04	0.0001
Lutein	0.61 ± 0.02	0.61 ± 0.03	0.59 ± 0.03	0.67 ± 0.04	0.16
Lycopene	1.06 ± 0.02	0.94 ± 0.05	1.03 ± 0.05	1.28 ± 0.05	0.0001
Zeaxanthin	0.11 ± 0.01	0.13 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.003
Total carotenoids	2.84 ± 0.06	2.88 ± 0.12	2.70 ± 0.12	3.19 ± 0.13	0.009
Retinol	1.71 ± 0.02	1.80 ± 0.05	1.66 ± 0.05	1.56 ± 0.05	0.0003
α-Tocopherol	24.27 ± 0.40	26.10 ± 0.91	24.38 ± 0.90	23.08 ± 0.95	0.008
γ-Tocopherol	1.29 ± 0.04	1.48 ± 0.10	1.20 ± 0.10	1.10 ± 0.10	0.006

[a]From a covariance analysis including terms for age, sex, period of blood drawing, smoking habit (never, ex and current), year, total caloric intake (kcal) and BMI.

The Spearman correlation coefficients between selected micronutrients measured in the same blood sample are available on request.

DNA adducts and plasmatic micronutrients

In the whole series, our multivariate analyses found strong inverse associations between DNA adduct levels and plasma concentrations of retinol ($P = 0.02$), α-tocopherol ($P = 0.04$) and γ-tocopherol ($P = 0.03$). No association emerged with the plasma levels of carotenoids, except a borderline inverse association ($P = 0.08$) with β-carotene (Table III).

When the analyses were stratified by smoking status, a significant inverse association with plasma levels of retinol and γ-tocopherol ($P = 0.01$ and $P = 0.04$, respectively) persisted in the group of non-smokers. On the other hand, an inverse association ($P = 0.007$) with plasma levels of α-tocopherol emerged only among smokers (Table IV). Analyses were also repeated in the group of 89 current smokers and the results were not materially different. β-Cryptoxanthin showed divergent patterns of association with DNA adduct levels in the two groups of smokers and non-smokers.

DNA adducts and metabolic polymorphisms

The frequencies of *GSTM1* and *GSTT1* null genotypes in this series were 50.2 and 20.4%, respectively (Table V). The frequencies of *CYP1A1* polymorphic, *NAT2* slow and *MTHFR* TT homozygous genotypes were 22.5, 56.9 and 23.4%, respectively, in agreement with expected values. DNA adducts did not show any significant variation according to all these genotypes.

DNA adducts, plasma micronutrients and GSTM1 polymorphisms

Strong negative associations between DNA adduct levels and plasma levels of selected micronutrients emerged in subjects with the *GSTM1* null genotype (Table VI). DNA adducts tended to be lower in subjects classified in the highest tertiles of plasma levels of α-carotene ($P = 0.02$), β-carotene ($P = 0.02$) and retinol ($P = 0.002$) among volunteers with the *GSTM1* null genotype ($n = 160$). A borderline negative association with plasma level of α- and γ-tocopherol also emerged in this subgroup ($P = 0.07$ and $P = 0.08$, respectively). These associations were not evident among subjects with a *GSTM1* wild-type genotype.

Table III. Adjusted geometric means[a] of DNA adducts per 10^9 normal nucleotides according to tertiles of plasma levels of micronutrients measured on the same blood sample (EPIC Italy, 331 subjects 1993–1998)

Micronutrient	DNA adducts (adjusted geometric mean)			*P*-value for trend
	Tertile of plasma levels			
	I (low)	II	III	
α-Carotene	3.73	3.27	2.57	0.18
β-Carotene	3.93	3.46	2.37	0.08
β-Cryptoxanthin	2.68	3.17	3.55	0.30
Lutein	3.34	3.17	2.93	0.61
Lycopene	3.69	2.92	2.99	0.46
Zeaxanthin	3.35	3.71	2.48	0.24
Total carotenoids	3.12	4.30	2.40	0.37
Retinol	4.03	3.58	2.16	0.02
α-Tocopherol	4.02	3.16	2.36	0.04
γ-Tocopherol	4.05	3.12	2.33	0.03

[a]From a covariance analysis including terms for age, sex, centre, period of blood drawing, total caloric intake (kcal), year, BMI and smoking history.

Discussion

In the current study we evaluated the association between DNA adduct levels and plasma concentrations of selected micronutrients, taking into account the effect of metabolic polymorphisms and smoking history in a large sample of healthy adults enrolled in the Italian EPIC cohort. Our results show a strong inverse association between DNA adduct level and plasma concentrations of retinol and α- and γ-tocopherol: leukocyte DNA adduct levels tended to be lower in subjects classified in the highest tertile of plasma levels of these micronutrients. Overall, no association emerged with plasma levels of total or specific carotenoids, although we found a borderline inverse association with β-carotene. Specific patterns of associations between antioxidant levels and DNA adducts emerged when analyses were carried out stratifying according to smoking history and *GSTM1* genotype, with α-tocopherol showing an effect only among smokers and α- and β-carotene only among *GSTM1* null subjects.

Our main results are in agreement with previous studies (24,25) showing that plasma levels of selected micronutrients (retinol, β-carotene and α-tocopherol) were inversely

Table IV. Adjusted geometric means[a] of DNA adducts per 10^9 normal nucleotides according to tertiles of plasma levels of micronutrients measured on the same blood sample by smoking history (EPIC Italy, 1993–1998)

Micronutrient	Non-smokers[b] ($n = 231$)				Smokers[c] ($n = 100$)			
	DNA adducts (adjusted geometric mean)			*P* value for trend	DNA adducts (adjusted geometric mean)			*P* value for trend
	Tertile of plasma levels				Tertile of plasma levels			
	I (low)	II	III		I (low)	II	III	
α-Carotene	2.87	3.43	2.41	0.51	3.37	1.59	1.81	0.17
β-Carotene	3.85	3.05	2.27	0.12	2.78	2.40	1.70	0.42
β-Cryptoxanthin	1.77	2.91	3.76	0.02	3.15	2.54	0.97	0.06
Lutein	2.87	2.79	2.82	0.96	2.94	2.56	1.67	0.28
Lycopene	3.29	2.43	2.94	0.75	3.56	2.07	1.49	0.10
Zeaxanthin	3.03	3.20	2.36	0.40	2.48	3.31	1.43	0.32
Total carotenoids	2.56	3.76	2.40	0.78	3.01	2.77	1.33	0.15
Retinol	3.83	3.32	1.74	0.01	2.40	2.34	2.27	0.92
α-Tocopherol	2.99	3.07	2.43	0.50	4.09	1.37	1.08	0.007
γ-Tocopherol	4.13	2.40	2.23	0.04	2.11	3.54	1.91	0.89

[a]From a covariance analysis including terms for age, sex, centre, period of blood drawing, total caloric intake (kcal), year and BMI.
[b]127 never smokers + 104 former smokers who reported to have quit smoking at least 4 years before enrollment.
[c]89 current smokers + 11 former smokers who reported to have quit smoking <4 years before enrollment.

Table V. Adjusted geometric means[a] of DNA adducts per 10^9 normal nucleotides according to genotype for selected metabolic polymorphisms (EPIC Italy, 331 subjects 1993–1998)

Polymorphism	*n*[b]	Percent of positive[c]	DNA adducts (adjusted geometric mean)	*P*[d]
GSTM				
Null	160 (50.2)	76.3	3.22	
Wild-type	159 (49.8)	80.5	3.17	0.94
GSTT				
Null	66 (20.4)	83.3	3.56	
Wild-type	258 (79.6)	77.5	3.16	0.63
CYP1A1				
Wild-type	251 (77.5)	78.1	3.09	
Polymorphic	73 (22.5)	80.8	3.56	0.56
NAT2				
Slow	186 (56.9)	76.3	3.04	
Fast	141 (43.1)	81.6	3.46	0.54
MTHFR (677 C → T)				
CC	98 (29.8)	78.6	3.43	
CT	154 (46.8)	76.6	2.96	
TT	77 (23.4)	81.8	3.49	0.99

[a]From analysis for covariance model including terms for age, sex, centre, period of blood drawing, year, BMI and smoking history.
[b]Some figures do not add up to the total because of some missing values.
[c]>0.1 DNA adducts/10^9 nucleotides.
[d]*P* value from a Wilcoxon Rank Sum test or Kruskal–Wallis test, as appropriate.

associated with DNA adduct levels. Our study is the first to report an inverse association between DNA adduct levels and plasma concentration of γ-tocopherol. Several human and animal studies have suggested that plasma concentration of γ-tocopherol may be inversely associated with cancer risk, particularly prostate cancer (34,35). We conducted several analyses (typically including comparisons for several nutrients in separate strata) and thus some of the statistically significant results may be due to chance, although our *a priori* hypothesis was clearly defined. A possible example of a chance finding is the divergent association of β-cryptoxanthin with DNA adduct levels in the two groups of smokers and non-smokers.

We have recently shown in a cross-sectional study (28) a strong inverse association between DNA adduct levels and reported frequency of consumption of fresh fruit and vegetables, the main sources of micronutrients with antioxidant properties (β-carotene and vitamins C and E). This strong inverse relationship between fresh fruit and vegetable consumption and DNA adduct formation was also reported in a recent case–control study on bladder cancer (36). Antioxidant vitamins (vitamin C, β-carotene and α-tocopherol) derived from fresh fruit and vegetables were significantly inversely correlated with DNA adducts: subjects with higher levels of intake of these foods had lower levels of DNA damage. Several dietary components (flavonoids, polyphenols and other natural compounds) have been shown to inhibit DNA adduct formation *in vitro* and *in vivo* (20). These dietary components might modulate DNA adduct formation by antioxidant activity or by interfering with the metabolic pathways of carcinogen activation/detoxification (20). This effect might explain the capacity of reduced cancer risk specifically associated with a dietary pattern rich in fruit and vegetables.

Levels of micronutrients measured in our series are quite similar to those evaluated in other recent studies in Mediterranean populations (37–39), and the consumption of vitamin supplements was reported quite rarely by our study participants. Among the six carotenoids measured, β-carotene, lutein and lycopene showed the highest concentrations, in agreement with series collected in Spain and Sweden (37) and in Italy (38). In our study population, strong geographical trends emerged, confirming different dietary patterns in the three major areas of the country, with higher levels of lycopene (and total carotenoids) in Southern Italy and higher levels of α-carotene, retinol and tocopherols in Northern Italy. Samples from Central Italy (Florence) showed intermediate levels for most of the biomarkers of dietary intake of the antioxidants we have measured. This variability, however, did not explain differences in adduct levels between centres, which are probably more related to environmental exposures (11,28).

Table VI. Adjusted geometric means[a] of DNA adducts per 10^9 normal nucleotides according to tertiles of plasma levels of carotenoids and other micronutrients measured on the same blood sample and by *GSTM* genotype (EPIC Italy, 1993–1998)

Micronutrient	*GSTM* null ($n = 160$)				*GSTM* wild-type ($n = 159$)			
	DNA adducts (adjusted geometric mean)			*P*-value for trend	DNA adducts (adjusted geometric mean)			*P*-value for trend
	Tertile of plasma levels				Tertile of plasma levels			
	I (low)	II	III		I (low)	II	III	
α-Carotene	5.12	3.53	1.92	0.02	3.33	3.43	3.39	0.97
β-Carotene	5.37	3.89	1.85	0.02	3.39	3.55	3.24	0.92
β-Cryptoxanthin	3.10	2.96	3.62	0.66	2.47	3.78	3.96	0.22
Lutein	2.94	4.12	3.00	0.92	4.86	2.92	2.85	0.14
Lycopene	3.17	2.70	3.64	0.68	3.42	3.38	3.36	0.96
Zeaxanthin	3.70	2.77	3.15	0.71	3.82	4.94	2.13	0.08
Total carotenoids	3.50	3.49	2.93	0.71	2.79	5.20	2.79	0.97
Retinol	6.06	3.74	1.60	0.002	3.11	3.46	3.64	0.66
α-Tocopherol	4.99	3.03	2.42	0.07	3.93	3.45	2.75	0.31
γ-Tocopherol	4.51	2.94	2.37	0.08	3.83	3.56	2.77	0.37

[a]From a covariance analysis including terms for age, sex, centre, period of blood drawing, total caloric intake (kcal), year, BMI and smoking history.

Overall, no clear association was found between cigarette smoking and DNA adducts, in agreement with the results of our previous study (28). However, the association between DNA adducts and plasma level of selected micronutrients was modulated by smoking history. Non-smokers with higher plasma levels of retinol and γ-tocopherol (but not α-tocopherol) showed lower levels of DNA adducts. A significant inverse association with α-tocopherol emerged only in smokers, suggesting a possible modulation of the relationship between micronutrients and adduct formation. The total levels of ^{32}P-post-labelled leukocyte DNA adducts may be reflective of different environmental carcinogen exposures, e.g. cigarette smoke, air pollution and dietary carcinogens (2,10). Thus, modulation by cigarette smoking of the relation between micronutrients and formation of DNA adducts may be due to the fact that antioxidant micronutrients play an important role in the antioxidant defense system against DNA damage induced by cigarette smoke, i.e. by quenching oxidants, including PAHs. Alternatively, this may be due to the fact that cigarette smoking has been shown to independently influence the levels of circulating antioxidant micronutrients (40).

Several studies (including cohorts and case–control studies) have reported an inverse association between vitamin E intake and lung cancer risk, particularly in smokers (41). Likewise, some studies showed that high intake of vitamin C was significantly associated with reduced risk of lung cancer (42,43), but a modest protective effect or no association was found in other studies (44–46). We could not evaluate the effects of plasma concentrations of vitamin C on DNA adduct levels because, according to our original protocol, measurements of this vitamin's levels were not carried out. With regard to vitamin A, several studies have shown contrasting results (45,46). Large intervention trials have also been carried out to test the hypothesis that antioxidant vitamin supplements could reduce lung cancer risk. Two randomized intervention trials aimed to reduce lung cancer risk in heavy smokers were terminated early because preliminary analyses indicated that supplementation with β-carotene and α-tocopherol (ATBC study) or β-carotene and vitamin A (CARET study) did not protect from lung cancer and might even be harmful (47,48).

Although the negative results of these intervention trials exclude a protective effect of these compounds when used as supplements, epidemiological evidence, including a recent report from the same ATBC study (8), consistently suggests that several dietary components may block carcinogenic pathways by antioxidant activity or by interfering with the binding of carcinogens to DNA. Estimates of intake of α-tocopherol, either as dietary intake (measured by a questionnaire) or as plasma level, probably correlate with other natural compounds present in foods. It is interesting that, recently, a positive association between higher DNA adduct levels and increased lung cancer risk in smokers was reported in a prospective study (13), whereas here we report an inverse association between α-tocopherol and DNA adduct levels only among smokers. This provides additional evidence for a possible protective role of α-tocopherol from lung cancer in smokers, although it cannot be excluded that the effects observed in epidemiological studies and attributed to α-tocopherol are actually due to some other natural compound present at high concentantion in the same foods. Possible candidates include phenols, flavonoids, isothiocyanates and other compounds. Residual confounding (due to low accuracy of self-reported smoking histories for both intensity and duration) has also been suggested as a reasonable explanation of the apparent protective effects of dietary antioxidants in epidemiological studies of lung cancer (49), but this is not applicable to our results in smokers versus non-smokers.

The role of genetic polymorphisms of several enzymes involved in the metabolism of chemical compounds was also explored, but differences only emerged on stratifying the analysis by *GSTM1* genotype. Frequencies of genotypes were in agreement with expected values. The inverse association between DNA adducts and plasma levels of some micronutrients (α- and β-carotene and retinol) were found only in those subjects with the null genotype. Borderline negative associations also emerged with plasma level of α- and γ-tocopherol. These results suggest that plasma levels of antioxidant vitamins may be very important in inhibiting DNA adduct formation in subjects who do not have the capacity to detoxify carcinogens via the *GSTM1* pathway. Both antioxidant

vitamins and *GSTM1* polymorphisms appear to protect against DNA damage (23). Antioxidant compounds such as vitamins C and E and β-carotene may act directly to inhibit oxidants and hence reduce DNA damage, whereas GST can catalyse the conjugation reaction between glutathione and substrates with electrophilic sites, increasing the detoxification of several procarcinogens. Other studies have already observed a relationship between DNA adducts and blood levels of vitamins in subjects with the *GSTM1* null genotype (24,25). On the other hand, an association between DNA adducts and *GSTM1* null genotype has been found by some studies (18,19,50).

In conclusion, our results show that DNA damage may be modulated by interactions between genetic, environmental and nutritional risk factors: overall, high plasma levels of selected micronutrients with antioxidant activity are significantly associated with reduced levels of DNA adducts in peripheral white blood cells. Some specific effects are evident only in subjects lacking the ability to detoxify carcinogens via a specific metabolic pathway (*GSTM1* null) or among smokers. This could contribute to better explaining the complex interaction between nutritional status, inherited genetic susceptibility to DNA damage and environmental exposures, including smoking.

Further studies are needed to evaluate the effects of dietary antioxidant compounds on human DNA damage and the effect of genetic polymorphisms on these associations.

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