ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 1, 2007

News & Views

Higher Leukocyte 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine and Lower Plasma Ascorbate in Aging Humans?

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ABSTRACT

Is oxidative damage of DNA responsible for physiological changes associated with aging? The authors note a positive correlation between the age of human subjects with the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in leukocyte DNA. The levels of urinary 8-oxo-7,8-dihydroguanine and 8-oxodG followed the same pattern of correlation. Age-dependent decline in the concentration of plasma vitamin C was also evident. These interesting observations in humans point towards the need to scrutinize in detail the role of oxidative DNA damage and compromised antioxidant defense systems in age-related physiological disorders. *Antioxid. Redox Signal.* 9, 143–150.

THE NEED FOR EVIDENCE IN HUMANS FOR THE FREE RADICAL THEORY OF AGING

The oxidative stress hypothesis of aging (or free radical hypothesis) is one of the most popular explanations of how aging occurs at the molecular level. In many cases, oxidative stress leads to an age-dependent increase in the cellular level of oxidatively-modified macromolecules, including DNA (35). Damage to DNA is particularly harmful since it may be fixed into mutation, if not repaired in a proper time, and passed into a daughter cell. Oxidatively-generated damage to DNA has often been blamed as a possible basis for the physiological changes associated with aging and degenerative diseases related to aging such as cancer (3, 26).

A number of research groups have reported on the effect of aging on DNA oxidation in animal models (10, 14). However, a summary of these kinds of studies shows no clear effect. It is difficult to explain why in some studies age-related increase in oxidatively-damaged DNA was observed (17), whereas in others no effect was described (1). It is possible that one reason for the discrepancies may be the reliability of the biomarkers used. Moreover, to date, no comprehensive studies concerning age-related oxidatively-damaged DNA in humans have been conducted. Therefore, the purpose of this work was to assess age-related changes in oxidative DNA damage in humans. For the first time, the broad spectrum of oxidative DNA damage biomarkers was analyzed: urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanine (8-oxoGua), as well as the level of oxidatively-damaged DNA in leukocytes. The most popular method of exploring oxidatively-damaged DNA on the level of the whole organism is determination of 8-oxoGua in DNA of surrogate tissues such as white blood cells. The whole body burden of oxidative stress may be assessed by the measurement of urinary excretion of 8-oxoGua and its 2′-deoxyribonucleoside equivalent 8-oxodG (33). One of the most reliable methods of this analysis involves HPLC prepurification, followed by gas chromatography with isotope dilution MS detection (30). In addition to unequivocal identification of the analyzed compounds and high sensitivity, the use of isotopically-labeled internal standards compensates for potential losses of the analytes during sample work-up (12).

Antioxidant vitamins (A, C, and E) and uric acid are effective free radical scavengers; therefore they should protect biomolecules such as DNA. In addition to the aforementioned

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analyses, the concentration of antioxidant vitamins A, C, and E and uric acid was determined in blood serum.

AGE-RELATED OXIDATIVE DNA DAMAGE IN HUMANS

To our best knowledge, to date there has only been one *in vivo* study concerning humans, where the age-dependent increase in 8-oxodG in DNA isolated from human skeletal muscle was investigated (23). In the aforementioned work, about eight times higher values of 8-oxodG were found in the oldest group (70 years or more) than in the youngest one (below 40 years) and no correlation between the damage and age was reported. However, the lowest value of 8-oxodG detected in those studies (3.28 8-oxodG/10^5 dG) was about one order of magnitude higher than that found in our study (4.82/10^6 dG) and those recommended by ESCODD (8). In any case, the study by Meccoci *et al*. (23) did not include the group of children (group A) that is included in our study.

We found a highly significant rise in the background level of 8-oxodG in leukocyte DNA in elderly (D) and middle age (C) groups in comparison with adults (B); respective values are: 5.79 ± 2.22, 4.82 ± 1.62, and 3.59 ± 1.60 per 10^6 dG molecules (Fig. 1), and a statistically significant, positive correlation between age and 8-oxodG levels in leukocyte DNA. However, a steady increase of 8-oxodG levels in DNA isolated from leukocytes together with age was seen only when the youngest group (A) was excluded, with a respective value of 4.63 ± 1.11 8-oxodG per 10^6 dG molecules (Fig. 1), since the level was significantly elevated in the group of youngest subjects when compared with the group representing "adults" (group B). It is likely that the unexpected high level of oxidative DNA damage in group A may reflect the higher metabolic rate of children. Children who are growing fast have a higher metabolic rate than adults. A high metabolic rate, in turn, requires a high level of mitochondrial respiration and subsequent elevated production of reactive oxygen species (ROS), which are responsible for the formation of DNA modifications analyzed in our work. Indeed, in our previous study, highly significant, positive correlations between specific metabolic rates and excretion rates for 8-oxodG and 8-oxoGua were found (9). Our results were recently confirmed by the H. Kasai group (36). However, it would be of interest in forthcoming studies to delineate more precisely whether the levels of 8-oxodG may fluctuate with age over the age range of 15–30 years.

WHY DOES OXIDATIVE DNA DAMAGE INCREASE WITH AGE?

We hypothesized that the observed age-related increase is a result of deficiency in the ability of the cells from older subjects to remove the damage, or it may mirror an intensification of processes responsible for the damage formation or both.

Involvement of repair processes

An age-related decrease in DNA repair capacity has been demonstrated mostly for nucleotide excision repair (NER) (13, 15). However, base excision repair (BER) is primarily responsible for the removal of oxidative DNA base damage, and age-dependent reduction of hOGG1 glycosylase, the major enzyme involved in the removal of 8-oxo-7,8-dihydroguanine, was also reported (4).

Urinary excretion rate, especially that measuring the level of 8-oxoGua, is the most sensitive marker of the average oxidative stress to DNA of all body cells (6, 27, 33). Therefore, besides analyses of the background level of 8-oxodG in leukocyte DNA, urinary excretion of the modified base and nucleosides was also determined. Since both parameters showed a similar age-related pattern, it is likely that their changes reflect, at least in part, age-dependent intensification of oxidative stress that resulted in DNA damage. However, since urinary excretion rates may also represent repair processes (see our earlier works, Refs. 12, 27, 30), we cannot entirely exclude the possibility that the observed less distinct changes in age-dependent urinary excretion rates than of the

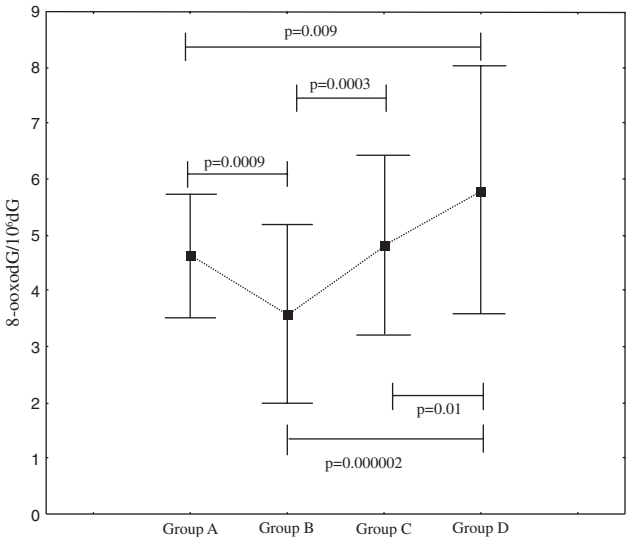

FIG. 1. Mean levels of 8-oxodG in leukocyte DNA in different age groups. Levels of 8-oxodG was determined using HPLC with electrochemical and UV detectors. Statistical analysis was performed using U Mann–Whitney's test.

background level of 8-oxodG in DNA (compare Figs. 1, 2A, and 2B) may also reflect some deterioration of the repair mechanism(s). Hence, age-related increase of oxidative stress appears to elevate oxidative DNA damage and the rate of repair represented by 8-oxoGua excretion, although the activation of the repair process does not prevent accumulation of 8-oxodG in cellular DNA.

Impaired mitochondrial function

Impaired mitochondrial function is a factor that may predispose to oxidative stress and DNA damage in the aged subjects. This in turn may be responsible for increased ROS production. Indeed, several studies of the mitochondrial respiratory chain function in humans and animals have demonstrated an age-related decrease in respiration and increased production of ROS during aging (21, 37). Another support for the age-related decline in mitochondrial function is provided by the demonstration that the amount of cytochrome c oxidase (COX)-deficient muscle fiber increase in healthy aging humans (19).

Decline of antioxidant defense

Another source of age-related increase of oxidative DNA damage may be decline of antioxidant defense. Therefore, we

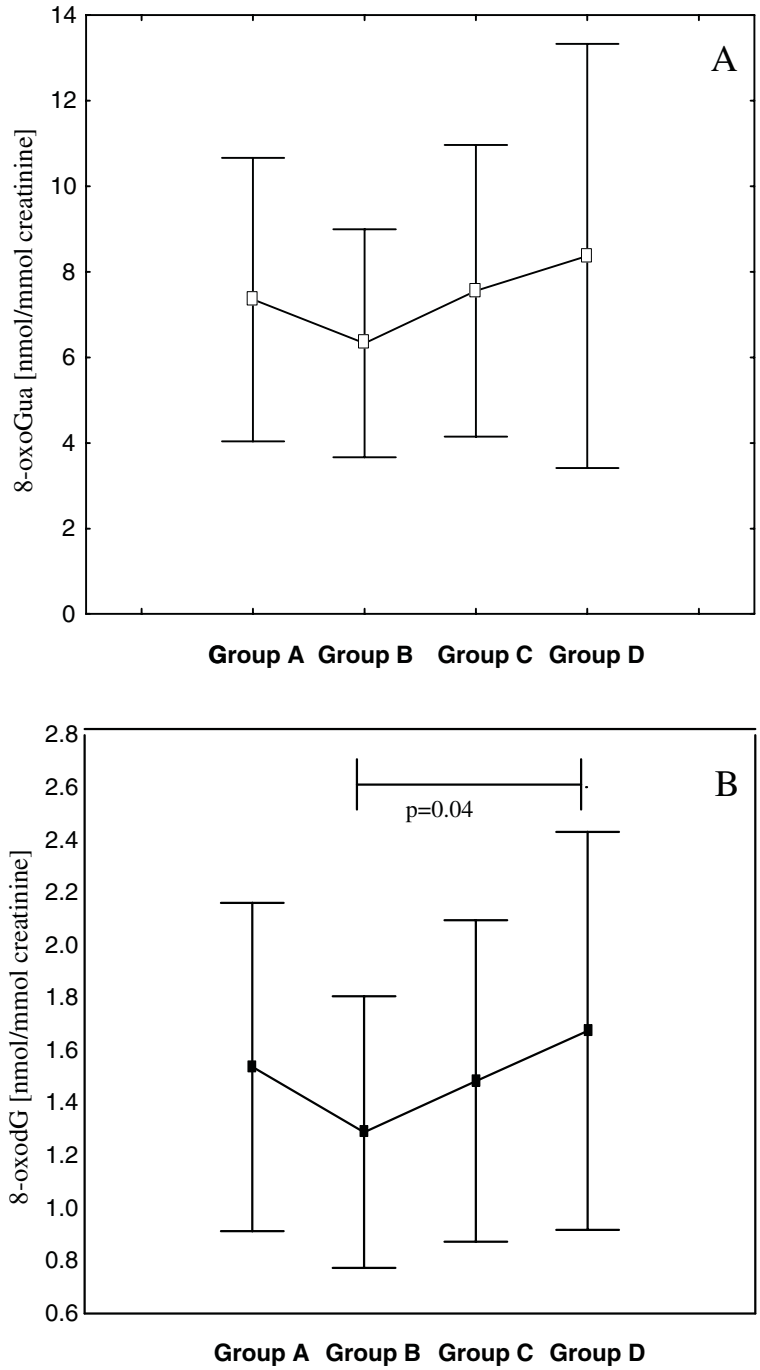

FIG. 2. The mean levels of 8-oxoGua (A) and 8-oxodG (B) in urine in the different age groups. The levels were analyzed with HPLC/GC/MS methodology. Statistical analyses were performed using U Mann–Whitney's test.

analyzed the endogenous level of antioxidant vitamins, as well as uric acid, which are effective free radical scavengers. Age-dependent decline in the concentration of vitamin C with the highest mean value in group A (71.44 ± 17.73 μmol/L) and the lowest in group D (46.76 ± 21.67 μmol/L) and a small but statistically significant increase in the level of vitamin E, the lowest value in group A (25.31 ± 4.73 μmol/L) and the highest in group D (29.94 ± 13.97 μmol/L)/ were observed (Fig. 3A and B). Vitamin C is a major aqueous phase antioxidant. It should also be remembered that vitamin C acts in synergy with tocopherol by regenerating tocopheroxyl radical to tocopherol. One of the plausible explanations of the above-presented changes in vitamin concentrations is the sequential consumption of these antioxidants as a result of age-dependent intensification of oxidative stress. It was shown that during free radical-mediated oxidation, a decrease in vitamin E concentration can only be seen after the complete consumption of vitamin C. The sequential consumption of these antioxidants was also shown by the use of electron spin resonance (ESR) spectroscopy (32).

Vitamin E is a potent nutrient for promoting health in the aged population via the improvement of cellular immunity decreased with aging (24). Therefore, the observed significant positive correlation with age of vitamin E concentration (Fig. 3B) may be a form of compensation and maintenance of health during aging. It should also be remembered that antioxidant vitamins may have biological activities that are separate from their direct antioxidant effect (*i.e.*, they can regulate changes in gene expression) (38). Therefore, age-dependent changes in their concentrations may also affect genome functions in this way.

A similar compensation in antioxidant defense of aging organism may be the gradual increase of uric acid with age (Fig. 3C). The level of uric acid showed statistically significant positive correlation with the age of the subjects. Uric acid in physiological concentration is regarded as a main antioxidant. Not only does it efficiently scavenge free radicals, but it has also been shown to stabilize ascorbic acid in human serum (34) and to reduce consumption of α-tocopherol and β-carotene (31). However, it is also possible that increased levels of uric acid in the elderly may be a sign of accelerated cell death in this age range (16). Further research will be necessary to determine the role of the antioxidants in human aging.

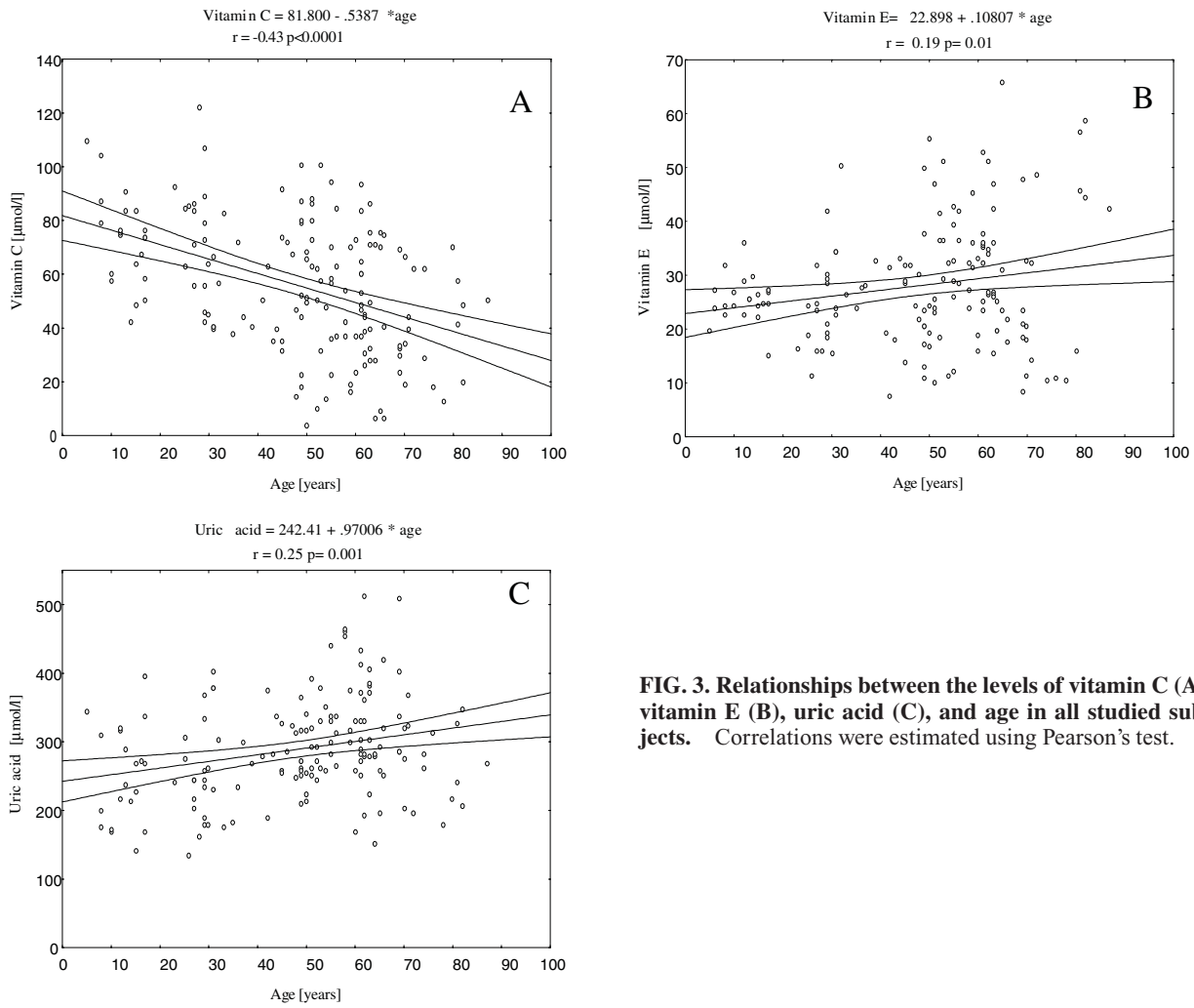

FIG. 3. Relationships between the levels of vitamin C (A), vitamin E (B), uric acid (C), and age in all studied subjects. Correlations were estimated using Pearson's test.

IMPLICATIONS

As can be seen in Fig. 1, the "adult" group exhibits the lowest values of oxidative DNA damage. Evolution theory assumes that organisms are not programmed to age, instead evolution selects for survival and reproduction (18). Therefore, it is possible that the lowest values of this harmful, potentially mutagenic, oxidative DNA modification in group B may constitute a proof of "specific concern" of evolution for humans of reproductive age. Interestingly, the concentration of vitamin A (retinol) was significantly higher in the "adult" group (2.27 ± 0.68 μmol/L) than in any other group: group A (1.55 ± 0.54 μmol/L), C (1.68 ± 0.65 μmol/L), and D (1.55 ± 0.67 μmol/L). In addition to playing a fundamental role as antioxidant and in the growth and differentiation of numerous types of cells, vitamin A (retinol) and its principal biologically active derivative, retinoic acid, may be involved in the regulation of testicular functions in rodents (20) and may play an essential role in female reproduction (5).

Individuals differ greatly in their rate of aging. There are also quite substantial interindividual differences in the levels of 8-oxodG in DNA (Fig. 4). These differences can be also seen within the "adult" group with a subgroup where the values are around two modifications per 10^6 unmodified bases and the second subpopulation where the values are much higher than the mean level (Fig. 4). It has been postulated that different factors that may influence the genome in adult life may influence the rate of subsequent functional decline of the organism (22). Therefore, it is possible that one of these factors is oxidative DNA damage with genome destabilizing properties.

On the basis of the presented data and literature reports, it seems reasonable to postulate that oxidative DNA damage/oxidative stress is probably a contributing factor in human aging. However, mechanisms that underline aging are highly complex and may depend on different factors such as genetic background, dietary behavior, and life style, to name a few. Therefore, oxidative stress may contribute in a limited extent to the aging of some individuals and could be a major factor in others. It should also be remembered that association between oxidative stress and aging is complicated by the consideration that there is no general agreement as to what aging is, when aging begins, what triggers its onset, and that oxidative stress occurs by multiple mechanisms. Therefore, there is clearly a need for further development and testing of the oxidative stress hypothesis of aging, using, for example, microarray analyses.

ABBREVIATIONS

BER, base excision repair; dG, 2′-deoxyguanosine; ESCODD, European Standards Committee on Oxidative DNA Damage; GC/MS, gas chromatography with isotope dilution mass spectrometric detection; Gua, guanine; hOGG1 glycosylase, human 8-oxo-7, 8-dihydroguanine glycosylase; HPLC, high performance liquid chromatography; MPA, methaphosphoric acid; NER, nucleotide excision repair; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; ROS, reactive oxygen species; SD, standard deviation; WBC, white blood cells;

APPENDIX

Subjects

All parameters were determined in 255 healthy subjects divided into four age-related groups: group A: children (mean age 13 years), group B: adults (mean age 31 years), group C: middle age (mean age 50 years), and group D: elderly (mean age 67 years).The study groups

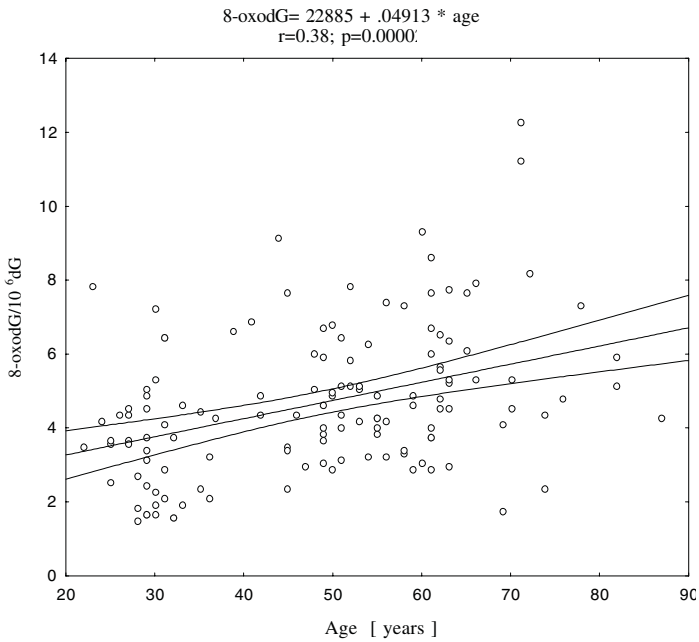

FIG. 4. Correlation between level of 8-oxodG in leukocyte DNA and age with exclusion of the group A. Relationship was estimated using Spearman's test.

were chosen so that the following criteria were matched**:** eating habits, body weight (with the exception of the children), and sex. None of the subjects had a history of chronic disease. All the subjects were non-smokers. The exclusion criteria were presence of known disease, intake of drugs, and antioxidants supplementation. The study was approved by the medical ethics committee of The L. Rydygier Medical University, Bydgoszcz, Poland, (in accordance with Good Clinical Practice, Warsaw 1998) and all the patients (or their parents) gave informed consent.

Isolation of leukocytes from venous blood

Venous blood samples from the patients were collected. The blood was carefully applied on top of Histopaque 1119 solution (Sigma, St. Louis, MO), and leukocytes were isolated by centrifugation according to the procedure described by the manufacturer.

DNA isolation and 8-oxodG determination in DNA isolates

DNA from leukocytes was isolated using the method described by Miller *et al.* (25) with some modifications (11). Briefly; the pellet of the cells was dispersed by vortexing in ice-cold buffer B (10 m*M* Tris, 5 m*M* Na_2EDTA, 0.15 m*M* deferoxamine mesylate, pH 8.0). Solution of SDS was added (to the final concentration of 0.5%), and vortexing repeated, RNase in 10 m*M* Tris pH 8.0 was added, and the mixture gently vortexed. After incubation for 30 min. at 37°C, a protease was added, the mixture gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C and transferred to a centrifuge tube with chloroform/3-methyl-1-butanol and vortexed vigorously. After centrifugation, the supernatant containing DNA was treated with 2 volumes of cold absolute ethanol to precipitate high molecular weight DNA. The precipitate was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation dissolved in nuclease P1 buffer (40 m*M* sodium acetate, 0.1 m*M* $ZnCl_2$, pH 5.1).

DNA hydrolysis to nucleosides. DNA samples in nuclease P1 buffer (200 µl) were mixed with nuclease P1 solution (20 µg protein). Samples were incubated for 1 h at 37°C. Thereafter, 30 µl of 1 *M* Tris-HCl, pH 8.5, and 5 µl of alkaline phosphatase solution containing 1.5 units of the enzyme were added to each sample following 1 h incubation at 37°C. All DNA hydrolysates were ultrafiltered using cut off 5000 Da filter units. 8-OxodG and dG in hydrolysates were determined using HPLC with electrochemical and UV detector. The HPLC system (Dionex Corporation, Sunnyvale, CA) consisted of a M480 pump, Gina 50 autosampler, 250 × 4.6 mm LC18S column (5 µm grain) equipped with 20 mm precolumn, and two detectors working in series: UV-VIS (UVD 340S) and Coulochem II 5200A electrochemical detector (ESA, Inc., Chelmsford, MA). DNA hydrolysates were chromatographed isocratically using 25 m*M* sodium acetate, 12.5 m*M* citrate, pH 5.0/MeOH (89:11). Detection of dG was performed at 254 nm. 8-OxodG was determined by the electrochemical detector: guard cell: +750 mV, detector 1: +130 mV (as a screening electrode), detector 2: +450 mV (as a measuring electrode set to sensitivity of 50 nA/V). Acquisition and quantitative analysis of the chromatograms were carried out using Chromeleon 4.3 software (Dionex Corporation). The amount of 8-oxodG in DNA was calculated as the number of 8-oxodG molecules per 10^6 unmodified dG molecules.

Urine analysis

In the present study, urine was collected as spot samples, and the concentrations of 8-oxodG and 8-oxoGua had to be adjusted by the creatinine concentration. A slow decrease in creatinine concentration was observed with age (28). However, age-associated differences in the excretion of the modifications are not likely to affect our results, because urinary creatinine concentrations were similar in the study groups with mean values: 10.25 mmol/L (group A), 11.50 mmol/L (group B), 11.28 mmol/L (group C), and 10.77 mmol/L (group D).

0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoGua, 0.05 nmol of [$^{15}N_5$] 8-oxodG, and 10 µl of acetic acid (HPLC grade, Sigma) were added to 2 ml of human urine. Isotopic purity of the applied standards was 97.65% and 95%, respectively. An isotopically labeled standard of 8-oxoGua was the kind gift of Dr. M. Dizdaroglu from the National Institute of Standard and Technology, Gaithersburg, MD. The standard of 8-oxodG was prepared from [$^{15}N_5$] dGTP (Silantes, GmbH, Munchen, Germany), according to the procedure described by Bialkowski and Kasprzak (2) with addition of an alkaline phosphatase digestion step. After centrifugation (2,000 *g*, 10 min), supernatant was filtered through a Millipore GV13 0.22 µm syringe filter, and 500 µl of this solution was injected onto an HPLC system. In the pilot study, isotopically labeled internal standards of unmodified compounds (1 nmol of [$^{13}C_3$] Gua and 1 nmol of [$^{15}N_5$] dG) were added to the urine samples to monitor fractions containing both these compounds and to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside. Isotopic purity of the applied standards was 96.4% and 98.0%, respectively.

Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to the method described by Ravanat *et al.* (29). In brief, the HPLC system consisted of a P580 gradient pump, a Gina 50 autosampler (both from Dionex), an SPD M10 AVP diode array detector from Shimadzu (Duisburg, Germany), and a Foxy 200 fraction collector from Isco Inc. (Columbus, OH). Urine samples enriched in labeled compounds were injected onto a Supelcosil LC (Supelco, Bellefonte, PA) 18 column (250 × 10 mm) equipped with a Supelguard LC18 guard column (20 × 4.6mm), both from Supelco. A 30 min linear gradient elution was performed (0.5% acetic acid at start to 0.5% acetic acid and 10% of acetonitrile), at a flow rate of 3 ml/min. After this time, the column was washed with 70% of acetonitrile for 10 min and equilibrated with 0.5% acetic acid for 20 min prior to a further injection. The effluent was monitored with a UV detector at 220–360 nm. Using standards for 8-oxoGua, 8-oxodG, Gua, and dG, we were able to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside. The collected fractions were dried by evaporation under reduced pressure in a Speed-Vac system (Savant, Ramsey, MN). The 8-oxodG was treated with 400 µl of 60% formic acid (Sigma) for 30 min. at 130°C.

GC/MS analysis was performed according to the method described by M. Dizdaroglu (7), adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored; these ions represent the masses of characteristic ions of the base shifted in the mass spectra according to the extent of labeling).

Determination of plasma vitamins A and E by HPLC

Sample preparation. To precipitate proteins, 200 µl aliquots of freshly prepared or freshly thawed plasma samples were mixed with 200 µl of HPLC-grade water and 400 µl ethanol. For the vitamin extractions, 800 µl of hexane was added and mixed for 30 min. Then, 600 µl of upper layers (hexane) was collected, dried in a Speed-Vac system, and dissolved in 150 µl of mobile phase with 85:15% (vol/vol) acetonitryl:methanol for the vitamin stabilization. Twenty µl of this solution was injected into the HPLC system.

Standard and control serum samples, with known vitamin E (α-tocopherol) and vitamin A (retinol) concentrations, were purchased from Chromsystems (Munchen, Germany) and prepared as plasma samples.

Chromatography

The HPLC system, consisting of a GP 40 gradient pump, a GINA 50 autosampler (both from Dionex), and a fluorimetric detector (Jasco FP-920, Jasco-Europe, Cremella, Italy), were used for vitamin E (α-tocopherol) and vitamin A (retinol) quantification. Samples were separated in an isocratic system C18 reversed phase column Atlantis DC 18 (3 mm × 150 mm × 5 µm) with a guard column. The mobile phase, containing acetonitrile and methanol (85:15) (vol/vol), at a flow rate 1.5 ml/min was used. The effluent was monitored with fluorimetric detection (ex. = 340nm, em. = 472 nm for retinol, and ex. = 290 nm, em. = 330 nm for α-tocopherol) and analyzed by Dionex PeakNet 4.3 software.

Determination of plasma vitamin C (ascorbic acid) and uric acid by HPLC

Standard solutions. A standard stock solution (1 m*M* of ascorbic acid) was made by dissolving ascorbic acid in 5% methaphos-

phoric acid (MPA). Aliquots of this solution were immediately frozen at −85°C and stored for no longer than 1 month. Working standards (in the range 1–40 μ*M*) were freshly prepared for each analysis. All solutions were carefully protected from light during preparation and analysis.

Sample preparation. Aliquots (200 μl) of freshly prepared or freshly thawed plasma samples were mixed with 200 μl of 20% MPA for protein precipitation and ascorbic acid stabilization. After centrifugation (10 min, 3,000 *g*, 4°C), the supernatants were collected and filtered through Millipore microcentrifuge filters (Milliport, Millipore, Bedford, MA). Twenty microliters of these filtrates was injected into the HPLC system.

Chromatography. The HPLC system consisted of HPLC pump, 717 PLUS autosampler (both from Waters) and a Photodiode Array Detector 2996 (Waters, Milford, MA) was used for ascorbic acid quantification. Samples were separated in the isocratic system C18 reversed phase column Spherisorb (Waters) 5 μmm ODS2 250 mm × 4.6 mm with C18 guard column, at a flow rate 1 ml/min. The mobile phase containing 5 m*M* KH_2PO_4, 1 m*M* Na_2EDTA, adjusted to pH 3.0 by phosphoric acid, was prepared from deionized water and filtrated through a 0.22 μm membrane before use. The effluent was monitored with UV detector at 245 nm (ascorbic acid detection), 280 nm (uric acid detection), and analyzed by Empower software (Waters).

Statistical analysis

All results are expressed as means ± SD. The STATISTICA (version 6.0) computer software (StatSoft, Inc, Tulsa, OK) was used for the statistical analysis. Student *t* test (for variables with normal distribution) and U Mann–Whitney's test (for variables with nonparametric distribution) were carried out. For normal distribution, variables were analyzed by the Kolmogorov–Smirnov test with Lillefor's correction. Statistical significance was considered at $p < 0.05$.

Correlation between analyzed parameters and age was estimated by Pearson's test (for variables with normal distribution), and Spearman's test (for variables with nonparametric distribution).

ACKNOWLEDGMENTS

This work was partly supported by ECNIS (European Cancer Risk, Nutrition, and Individual Susceptibility), a network of excellence operating within the European Union 6th Framework Program, Priority 5: "Food Quality and Safety" (Contract No 513943), and by grants from the Ministry of Science and Information Society Technologies 2P05D062 27 and PBZ-KBN-091/P05/55. RR, DG, RO, AS, TD, ASz, and JG were supported by a Foundation for Polish Science fellowship.

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Date of first submission to ARS Central, July 21, 2006; date of final revised submission, August 22, 2006; date of acceptance, August 22, 2006.

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