*Free Rad. Res.*, Vol. 32, pp. 355–362
Reprints available directly from the publisher
Photocopying permitted by license only





# The Steady-State Levels of Oxidative DNA Damage and of Lipid Peroxidation ($F_2$-Isoprostanes) are not Correlated in Healthy Human Subjects

TIMOTHY ENGLAND[a], EMILY BEATTY[b], ALMAS REHMAN[a], JAFFAR NOUROOZ-ZADEH[c], PAULO PEREIRA[d], JAMES O'REILLY[b], HELEN WISEMAN[b], CATHERINE GEISSLER[b] and BARRY HALLIWELL[a,e,*]

[a]*International Antioxidant Research Centre, King's College, Guys Campus, London Bridge, London SE1 9RT, UK;* [b]*Nutrition, Food and Health Research Centre, King's College, Franklin-Wilkins Building, 150 Stamford Street, London SE1 8WA, UK;* [c]*Centre for Clinical Pharmacology and Therapeutic Toxicology, Department of Medicine, University College, London WC1E 6JJ, UK;* [d]*Department of Ophthalmology and Visual Science, Biomedical Institute for Research in Light and Image, Coimbra, Portugal;* [e]*Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260*

Accepted by Dr. M. Dizdaroglu

*(Received 20 July 1999; In revised form 20 September 1999)*

Oxidative damage to DNA in human tissues can be determined by measuring multiple products of oxidative damage to the purine and pyrimidine bases using gas chromatography-mass spectrometry (GC-MS). Oxidative damage to lipids (lipid peroxidation) can be quantitated by the mass spectrometry-based determination of $F_2$-isoprostanes, specific end-products of the peroxidation of arachidonic acid residues in lipids. For both DNA base damage products and 8-*epi* prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), there is a wide variation in levels between different healthy human subjects. We measured multiple products of oxidative damage to DNA bases in white cells, and 8-*epi* $PGF_{2\alpha}$ in plasma, from blood samples obtained from healthy human subjects in the UK and in Portugal. No correlation of 8-*epi* $PGF_{2\alpha}$ levels with levels of any modified DNA base (including 8-hydroxyguanine) was observed. We conclude that no single parameter can be measured as an index of "oxidative stress" or "oxidative damage" *in vivo*.

*Keywords:* Oxidative DNA damage, lipid peroxidation, isoprostanes, $F_2$-isoprostanes, 8-hydroxyguanine, antioxidant

*Abbreviations:* GC-MS, gas chromatography-mass spectrometry; 8-*epi* $PGF_{2\alpha}$, 8-*epi* prostaglandin $F_{2\alpha}$; 8OHG, 8-hydroxyguanine; 8OHdG, 8-hydroxy-2′-deoxyguanosine; BSTFA, *N*, *O*-bis(trimethylsilyl) trifluoroacetamide; BHT, butylated hydroxytoluene; PFB-ester, pentafluorobenzyl-ester; PFB-Br, pentafluorobenzyl bromide; DIPEA, di-iso-propylethylamine; NICI, negative ion chemical ionization; SIM, selected ion monitoring; GSH, reduced glutathione; HPLC, high performance liquid chromatography; 5-OHMe-hydantoin, 5-hydroxy-5-methylhydantoin; 5-OH hydantoin, 5-hydroxydantoin; 5-OH uracil, 5-hydroxyuracil; 5-OHMe uracil, 5-(hydroxymethyl) uracil; 5-OH cytosine, 5-hydroxycytosine; FAPy adenine, 4,6-diamino-5-formamidopyrimidine; 8-OH adenine, 8-hydroxyadenine; 2-OH adenine, 2-hydroxyadenine; FAPy guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH guanine, 8-hydroxyguanine

*Corresponding author. Department of Biochemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260. E-mail: bchbh@nus.edu.sg.

## INTRODUCTION

It is increasingly recognised that oxidative damage to DNA contributes to the age-related development of cancer[1–6] and that oxidative damage to lipids (lipid peroxidation) is a major contributor to atherosclerosis, and to cardiovascular disease generally.[7–10] Both lipid peroxidation and oxidative DNA damage can be modulated by diet to some extent.[11–15] Hence the use of "biomarkers" of oxidative DNA damage and of lipid peroxidation has been proposed as a mechanism for identifying which diets, and other lifestyle factors,[16–18] could minimize oxidative damage and thus could putatively delay the onset of cardiovascular disease and of the major cancers.[19,20] There has been considerable debate over the applicability, sensitivity, reproducibility and validity of various biomarkers of oxidative damage, but there seems to be an increasing consensus that the measurement of isoprostanes by mass spectrometry may be the best currently-available biomarker of lipid peroxidation in the human body.[14,20–30] Plasma levels of $F_2$-isoprostanes, specific end-products of the peroxidation of arachidonic acid residues,[21] are elevated in smokers[25] and other situations of oxidative stress,[21–24] and decreased by antioxidant administration.[14,21]

Even apparently-healthy subjects show wide variations in the $F_2$-isoprostane levels in their body fluids.[14,21,25,31] In part this could be explained by differences in diet, but other factors may also be involved, e.g. levels of endogenous antioxidant defences (which may be in part genetically determined), and rate of metabolism of isoprostanes.[14,26] In other words, some healthy human subjects appear to show higher extents of lipid peroxidation than others, even on comparable diets. These subjects could conceivably be more predisposed to the later development of diseases involving lipid peroxidation, such as atherosclerosis and perhaps diabetes.[32–34]

There is less agreement about which (if any) is the "best" biomarker to use when measuring oxidative DNA damage.[35] Measurement of 8-hydroxylated guanine (either as the base 8-hydroxyguanine [8OHG] or more often as the nucleoside 8-hydroxy-2′-deoxyguanosine [8OHdG]) is the most popular technique, largely because of the availability of an HPLC-based assay,[1,2] but other products of oxidative damage to DNA bases can also be measured.[35–37] In principle, mass spectrometric methods for simultaneously determining multiple DNA base oxidation products should be more chemically-rigorous than HPLC techniques, but, as with all methods, artifactual oxidation of DNA bases during isolation, hydrolysis and analysis of DNA can sometimes occur to an extent variable between laboratories. Several protocols that claim to eliminate such artifacts have been described.[35,37–39]

Levels of 8OHdG (or 8OHG), like those of isoprostanes, vary widely between individuals, even in subjects with comparable plasma levels of antioxidants.[11,40] In one study,[41] levels of 8OHdG in lymphocytes were found to be positively correlated with mortality rates from coronary heart disease and colorectal cancer. An important question is whether subjects who show high levels of base oxidation products in their DNA also show high levels of lipid peroxidation, i.e. are they under a general state of "oxidative stress"? In the present paper we have measured levels of a plasma $F_2$-isoprostane (8-*epi* $PGF_{2\alpha}$) as an index of lipid peroxidation, and a wide range of DNA base oxidation products in white blood cell DNA, both plasma and cells being from the same blood samples. Two separate groups of healthy human volunteers were used, one in the UK and one in Portugal, in order to examine possible correlations in two countries with different diets.[41] All analyses of isoprostanes and oxidative DNA damage were conducted in the UK to avoid methodological variations.

## MATERIALS AND METHODS

### Reagents

Unless otherwise stated, chemicals were of the highest quality available from Sigma Chemical

Co. (Poole, Dorset, UK), BDH Chemical Co. (Gillingham, Dorset, UK) or Aldrich Chemical Co. (Milwaukee, WI, USA). Ribonucleases A (bovine pancreas, molecular biology grade) and $T_1$ (*Aspergillus oryzae*) were from Sigma. 8-Hydroxyadenine, thymine glycol and FAPy guanine were synthesised as described previously.[42] 2-Hydroxyadenine, 5-hydroxycytosine and 5-(hydroxymethyl) uracil were gifts from Dr. Miral Dizdaroglu (NIST, Gaithersburg, USA). Other reagents needed for GC-MS analysis of oxidative DNA damage were obtained as described in Refs. [39,40] Prostaglandin $F_2$ (9,11,15-trihydroxy-5,11-eicosadienoic acid) standards including 9$\alpha$,11$\alpha$-, 9$\alpha$,11$\beta$-, 9$\beta$,11$\alpha$-, 8-*epi*- and 3,3′,4,4′-tetradeuterated 9$\alpha$,11$\alpha$-$PGF_2$ ($PGF_2$-$d_4$) were obtained from Cascade Biochemicals (Reading, UK). Sep-Pak $C_{18}$ (500 mg) cartridges were obtained from Millipore Incorporation (Millford, MA, USA). Aminopropyl ($NH_2$) cartridges (Supelclean LC-$NH_2$, 500 mg) were supplied by Supelco (Bellefonte, PA, USA). *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA).

## Subjects

### *Group A*

Twenty volunteers (11 males, 9 females) age range 22–45, mean age 29.7 ± 6.7 years were recruited by advertising within King's College and the local community by means of poster advertisements, to take part in a study comparing the effects of high-flavonoid and low-flavonoid diets on parameters of oxidative stress.

All subjects were non-smokers and had normal haematology and liver function tests. Exclusion criteria were the regular consumption of antioxidant supplements or of medication, acute illness, or changed dietary habit or contraception within four weeks prior to starting the study. Female subjects were not pregnant or lactating according to a medical questionnaire. The study was approved by King's College Research Ethics Committee and all subjects supplied written, informed consent. The high-flavonoid diet used (details to be published elsewhere) did not decrease levels of plasma $F_2$-isoprostanes, or of oxidized DNA bases in white cells when compared with the low-flavonoid diet, and so the data for subjects have been pooled, giving 40 samples for analysis (20 on the low-flavonoid and 20 on the high-flavonoid diet).

### *Group B*

Thirty-three volunteers (7 males, 26 females), mean age 50.7 ± 15.2 years (range 27–76 years) were recruited from the staff of the Biomedical Institute for Research in Light and Image and from the University for the Elderly, Coimbra, Portugal. The exclusion criteria were as listed above. The study was approved by the Ethics Committee of Coimbra University Hospitals and all subjects supplied written, informed consent.

## Statistics

Results were analysed using SPSS version 6. Two extreme outliers (greater than ±2SD) were observed in the 8-OH guanine data set of the UK subjects and were excluded. Normality of distribution was tested using Kolmogorov–Smirnov Goodness of Fit Test. As all data were normally distributed, correlations between sets of data were determined using Pearson's method.

## Sample Collection

Blood was collected by venepuncture into sampling vials (10 ml) containing lithium-heparin (Portugal) or ETDA (UK) for cell collection, and indomethacin-stabilized citrated plasma for isoprostane analysis.[31] Blood samples were centrifuged at 2000× *g* for 10 min at room temperature. Blood cells including the buffy layer were transferred into new containers and stored at −70°C until analysis, a protocol which does not increase oxidative DNA damage.[40] Aliquots (1 ml) of the plasma were transferred to Eppendorf tubes and

20 µl of butylated hydroxytoluene [BHT] (5 mM in methanol) was added. The samples were stored at −70°C until analysis. Samples were stored for a maximum time of 6 months, a period which does not alter isoprostane levels (if BHT has been added).

## Analysis of Oxidative DNA Damage

DNA isolation and analysis of DNA base damage products by GC-MS were carried out as described in Rehman *et al.*[40] Derivatisation was carried out at room temperature in the presence of ethanethiol.[39]

## Analysis of Isoprostanes

### *Solid-Phase Extraction Procedure*[31]

Plasma (1 ml) was incubated with 1 ml of aqueous potassium hydroxide (1.0 M) at 45°C for 30 min to release esterified lipids. Water (1 ml) was added and the pH adjusted to 2 by sequentially adding 1 M HCl (1 ml) and 0.1 M HCl (3 ml) while vortexing. $PGF_2$-$d_4$ (5 mg in 50 ethanol) was added as an internal standard and the sample was centrifuged at 2400× *g* for 5 min. The supernatant was applied onto a $C_{18}$ cartridge preconditioned with methanol and water (pH 3.0). The cartridge was sequentially washed with 10 ml of water (pH 3.0) and acetonitrile–water (15 : 85, v/v) to remove non-lipid materials.

Lipids were eluted by washing the cartridge with 5 ml of hexane–ethyl acetate–propan-2-ol (30 : 65 : 5, v/v). This eluate was then applied to an $NH_2$ cartridge, preconditioned with hexane (5 ml). The $NH_2$ cartridge was sequentially washed with 10 ml of hexane–ethyl acetate (30 : 70, v/v), acetonitrile–water (9 : 1, v/v) and pure acetonitrile. $F_2$-isoprostanes were eluted from the $NH_2$ cartridge with 5 ml of ethyl acetate–methanol–acetic acid (10 : 85 : 5, v/v). The sample was immediately transferred into a screw-cap vial and the solvent evaporated under nitrogen at room temperature.

### *Derivatization*

The pentafluorobenzyl (PFB)-ester was prepared by adding 40 µl of pentafluorobenzyl bromide (10% in acetonitrile) and 20 µl of di-iso-propylethylamine [DIPEA] (10% in acetonitrile) to the dried sample following extraction as above. The vials were sealed with a Teflon-lined cap, kept at 40°C for 45 min and the solvent was removed under a stream of nitrogen.

Fifty µl of *N,O*-bis(trimethylsilyl) trifluoroacetamide [BSTFA] followed by 5 µl DIPEA (10% in acetonitrile) were added. The vials were sealed and kept at 4°C for 12 h. The solvent was removed under nitrogen and the residue was reconstituted in iso-octane (20 µl) containing 10% BSTFA. All the samples were stored at −20°C until GC-MS analysis.

### *Gas Chromatography-Mass Spectrometry*

GC-MS/NICI analysis was carried out on a Hewlett Packard 5890 GC linked to a VG70SEQ mass spectrometer (Fisons Instruments, Manchester, UK) using ammonia as reagent gas. $F_2$-isoprostanes were separated on an SPB-1701 column (30 m × 0.25 mm ID; 0.25 µm film thickness, Supelco Incorporation, Bellefonte, PA). Samples (2 µl) were injected into a temperature programmed Gerstel injector (Thames Chromatography, Maidenhead, UK). The GC oven was programmed from a temperature of 175°C to 270°C at a rate of 30°C/min. Quantitative analysis was performed using selected ion monitoring (SIM) of the carboxylate anion $[M\text{-}181]^-$ at $m/z$ 569 for the $F_2$-isoprostanes and $m/z$ 573 for $PGF_2$-$d_4$ as the internal standard.

## RESULTS

Two groups of healthy human volunteers, non-smokers, were involved in this study, one from London and one from Portugal. The Portuguese group was older (50.7 ± 15.2 versus 29.7 ± 6.7 years). Levels of the $F_2$-isoprostane 8-*epi* $PGF_{2\alpha}$

in the London group were 107 ± 37 pg/ml plasma (mean ± SD), range 57–197 pg/ml. Levels of oxidative DNA damage in white blood cell DNA are summarized in Table I. As observed previously,[11,40] there was a wide variation between subjects. There was no correlation between any product of oxidative damage to the DNA bases and lipid peroxidation, as assessed by 8-*epi* $PGF_{2\alpha}$ levels. For illustration, Figure 1 shows this lack of correlation with the commonly used "biomarker" 8OHG, and Figure 2 shows the lack of correlation with the total of all DNA base oxidation products.

For the Portuguese group levels of 8-*epi* $PGF_{2\alpha}$ were 81 ± 55 pg/ml plasma. Levels of DNA base damage products are summarized in Table II. Again, levels of 8-*epi* $PGF_{2\alpha}$ were not correlated with any DNA base damage product. For example, for total DNA damage $r$ was −0.178 ($p = 0.301$) and for 8OHG $r$ was −0.037 ($p = 0.429$).

TABLE I Levels of modified bases in white cell DNA from healthy English subjects. The range of levels of base products (expressed as nmol/mg DNA) in 40 blood samples from 20 healthy subjects

| Base product measured | Range (nmol/mg DNA) |
|---|---|
| 5-OHMe hydantoin | 0.014–1.145 |
| 5-OH hydantoin | 0.005–0.086 |
| 5-OH uracil | 0.002–0.039 |
| 5-OHMe uracil | 0.002–0.018 |
| 5-OH cytosine | 0.028–0.151 |
| Thymine glycol | 0.046–0.624 |
| FAPy adenine | 0.026–0.240 |
| 8-OH adenine | 0.002–0.114 |
| 2-OH adenine | 0.018–0.309 |
| FAPy guanine | 0.020–0.844 |
| 8-OH guanine | 0.006–0.082 |
| Total of base products measured | 0.350–2.790 |

1 nmol of a lesion per mg DNA corresponds to approximately 32 lesions/$10^5$ DNA bases.

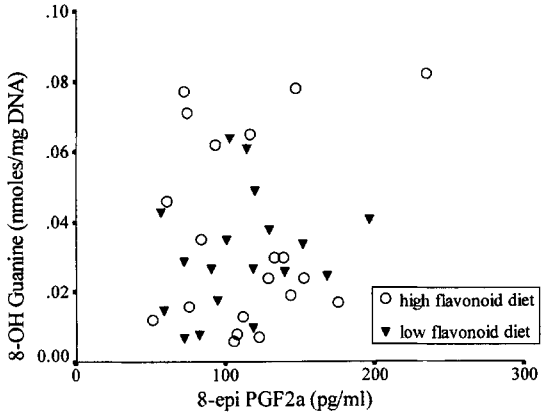


FIGURE 1 Lack of correlation between levels of plasma 8-*epi* $PGF_{2\alpha}$ and white cell 8-hydroxyguanine levels in blood samples from healthy English volunteers. The correlation coefficient was 0.169, $p = 0.311$. A diet low or high in flavonoids did not alter levels of oxidative DNA base damage, so the data have been combined.

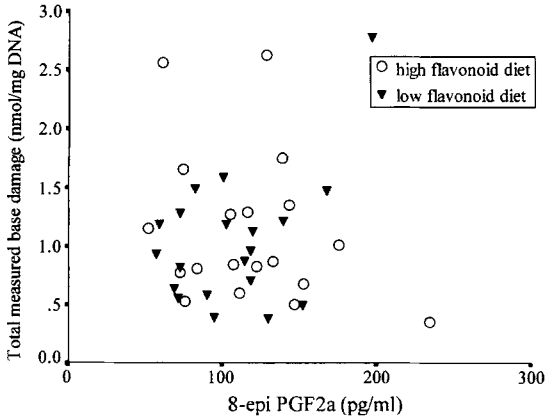


FIGURE 2 Lack of correlation between levels of plasma 8-*epi* $PGF_{2\alpha}$ and levels of all oxidized DNA bases (i.e. the sum of 5-OHMe hydantoin, 5-OH hydantoin, 5-OH uracil, 5-OHMe uracil, 5-OH cytosine, thymine glycol, FAPy adenine, 8-OH adenine, 2-OH adenine, FAPy guanine and 8-OH guanine) in blood samples from healthy English volunteers. The correlation coefficient was −0.01, $p = 0.966$.

TABLE II Levels of modified bases in white cell DNA from healthy Portuguese subjects. The range of levels of base products (expressed as nmol/mg DNA) in 33 healthy subjects

| Base product measured | Range (nmol/mg DNA) |
|---|---|
| 5-OHMe hydantoin | 0.062–0.153 |
| 5-OH hydantoin | 0.034–0.139 |
| 5-OH uracil | 0.024–0.065 |
| 5-OHMe uracil | 0.017–0.029 |
| 5-OH cytosine | 0.052–0.145 |
| Thymine glycol | 0.306–0.626 |
| FAPy adenine | 0.561–1.260 |
| 8-OH adenine | 0.062–0.147 |
| 2-OH adenine | 0.441–1.081 |
| FAPy guanine | 0.684–1.361 |
| 8-OH guanine | 0.113–0.182 |
| Total of base products measured | 3.127–4.127 |

1 nmol of a lesion per mg DNA corresponds to approximately 32 lesions/$10^5$ DNA bases.

## DISCUSSION

Our data show that, in healthy adult volunteers from two different countries, there was no correlation between plasma levels of 8-*epi* $PGF_{2\alpha}$, and DNA base oxidation products in white cell DNA from the same blood samples. Indeed, whereas mean levels of 8-*epi* $PGF_{2\alpha}$ tended to be slightly lower in the Portuguese subjects, levels of 8OHG and other oxidized bases were somewhat higher. Extensive variations in 8OHdG levels in lymphocytes between subjects from different countries have been reported elsewhere.[41] The Portuguese subjects were older ($50.7 \pm 15.2$ versus $29.7 \pm 6.7$ years) than the London ones. Since oxidative DNA damage levels may increase with age,[43] this might contribute to the difference.

Of course, as we have used only a single biomarker of lipid peroxidation (levels of the $F_2$-isoprostane $PGF_{2\alpha}$), it is possible that other indices could give different answers. Nevertheless, our data show that measurement of neither $PGF_{2\alpha}$, nor 8OHG, nor any other product of DNA base oxidation is by itself an adequate overall index of "oxidative damage" or "oxidative stress". Hence, to evaluate the effects of diet or other lifestyle parameters on oxidative damage in the human body, separate parameters of oxidative damage to DNA, lipids (and probably proteins) must be measured.[19,20]

To some extent our conclusions are not unexpected. Steady-state levels of oxidative DNA damage could be affected by variations (including genetically-determined ones) in levels of antioxidant defence and DNA repair enzymes, the effectiveness of the sequestration of transition metal ions into forms that will not catalyze formation of $OH^{\bullet}$ and other radicals that can attack DNA[10] and the rate at which cells with oxidatively-damaged DNA are eliminated, e.g. by apoptosis. Levels of low-molecular-mass antioxidants, both endogenous (such as GSH and histidine-containing dipeptides) and diet-derived (ascorbate, tocopherols, flavonoids, carotenoids etc.) may also be important. Plasma levels of $F_2$-isoprostanes are determined both by their rates of formation and their rates of metabolism[26] and urinary excretion, all of which could vary between subjects. Rates of formation could be affected by levels of exogenous and endogenous antioxidant defences and "catalytic" transition metal ions, and rates of clearance by variations in levels of enzymes metabolizing isoprostanes and in renal function.

Another important point that has been much debated in the literature[37–40,44] is whether the GC-MS technique we have used to assess oxidative DNA damage generates artifacts. Our measured mean levels of 8OHG (nmol/mg DNA) were 0.034 for the UK subjects and 0.135 for the Portuguese subjects, or 4.9 and 19.5 8OHG/$10^5$ guanines respectively. However, in two recent inter-laboratory comparisons of levels of 8OHG/8OHdG in "standard" calf thymus DNA, the values determined by our methodology were comparable to those measured on the same sample by several laboratories using HPLC methodology or GC-MS with prepurification.[45,46] Mecocci *et al.*[43] found levels of about 3 8OHdG per $10^5$ guanines in muscle DNA from subjects $< 40$ years old, but $> 5$ for subjects aged 40–50, and $\sim 8$ for subjects aged 51–60, the mean age range of the Portuguese subjects examined in the present paper. Hence we do not think that GC-MS analysis of oxidative DNA base damage generates significant artifacts if proper analytical conditions are used.

### *Acknowledgements*

We are grateful to the Ministry of Agriculture, Fisheries and Food (MAFF), the World Cancer Research Fund and Asta Medica for research support.

### *References*

[1] R.A. Floyd (1990) The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis* **11**, 1447–1450.
[2] H. Kasai (1997) Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of

cellular oxidative stress during carcinogenesis. *Mutation Research* **387**, 147–163.

[3] B.N. Ames (1989) Endogenous oxidative DNA damage, aging and cancer. *Free Radical Research Communications* **7**, 121–128.

[4] J.R. Totter (1980) Spontaneous cancer and its possible relationship to oxygen metabolism. *Proceedings of the National Academy of Sciences of the USA* **77**, 1763–1767.

[5] B.N. Ames, M.K. Shigenaga and T.M. Hagen (1993) Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the USA* **90**, 7915–7922.

[6] K.B. Beckman and B.N. Ames (1998) Oxidative DNA damage: assessing its role in cancer and aging. In: O.I. Aruoma and B. Halliwell (Eds.), *DNA and Free Radicals. Techniques, Mechanisms and Applications*. OICA International Press, St. Lucia.

[7] D. Steinberg and A. Lewis (1997) Oxidative modification of LDL and atherogenesis. *Circulation* **95**, 1062–1071.

[8] H. Esterbauer, R. Schmidt and M. Hayn (1997) Relationships among oxidation of low-density lipoprotein, antioxidant protection, and atherosclerosis. *Advances in Pharmacology* **38**, 425–456.

[9] S. Yla-Herttuala (1998) Is oxidized LDL present *in vivo*? *Current Opinions in Lipidology* **9**, 337–344.

[10] B. Halliwell and J.M.C. Gutteridge (1999) *Free Radicals in Biology and Medicine*, Chapter 9, 3rd edn., Clarendon Press, Oxford, UK.

[11] B. Halliwell (1998) Can oxidative damage be used as a biomarker of cancer risk in humans? *Free Radical Research* **29**, 469–486.

[12] H. Verhagen, H.E. Poulsen, S. Loft, G. van Poppel, M.I. Willems and P.J. van Bladeren (1995) Reduction of oxidative DNA damage in humans by Brussels sprouts. *Carcinogenesis* **16**, 969–970.

[13] S.J. Duthie, A. Ma, M.A. Ross and A.R. Collins (1996) Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Research* **56**, 1291–1295.

[14] D. Pratico, R.K. Tangirala, D.J. Rader, J. Rokach and G.A. FitzGerald (1998) Vitamin E suppresses isoprostane generation *in vivo* and reduces atherosclerosis in Apo E-deficient mice. *Nature Medicine* **4**, 1189–1192.

[15] S. Loft, E.B. Thorling and H.E. Poulsen (1998) High fat diet induced oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion in rats. *Free Radical Research* **29**, 595–600.

[16] S. Asami, T. Hirano, R. Yamaguchi, H. Ito and H. Kasai (1998) Reduction of 8-hydroxyguanine in human leukocyte DNA by physical exercise. *Free Radical Research* **29**, 581–584.

[17] S. Loft, K. Vistisen, M. Ewertz, A. Tjonneland, K. Overvad and H.E. Poulsen (1992) Oxidative DNA damage estimated by 8-hydroxyguanosine excretion in man: influence of smoking, gender and body mass index. *Carcinogenesis* **13**, 2241–2247.

[18] J.A. Drury, G. Jeffers and R.W.I. Cooke (1998) Urinary 8-hydroxydeoxyguanosine in infants and children. *Free Radical Research* **28**, 423–428.

[19] B. Halliwell (1996) Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radical Research* **25**, 57–74.

[20] B. Halliwell (1999) Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutrition Reviews* **57**, 104–113.

[21] L.J. Roberts II and J.D. Morrow (1997) The generation and actions of isoprostanes. *Biochimica et Biophysica Acta* **1345**, 121–135.

[22] G. Pratico, L. Iuliano, A. Mauriello, L. Spagnoli, J.A. Lawson, J. Rokach, J. Maclouf, F. Violi and G.A. FitzGerald (1997) Localization of distinct $F_2$-isoprostanes in human atherosclerotic lesions. *Journal of Clinical Investigation* **100**, 2028–2034.

[23] G. Davi, P. Alessandrini, A. Mezzetti, G. Minotti, T. Bucciarelli, F. Costantini, F. Cipollone, G.B. Bon, G. Ciabattoni and C. Patrono (1997) *In vivo* formation of 8-epiprostaglandin $F_{2\alpha}$ is increased in hypercholesterolaemia. *Arteriosclerosis Thrombosis and Vascular Biology* **17**, 3230–3235.

[24] Z. Mallat, I. Philip, M. Lebret, D. Chatel, J. Maclouf and A. Tedgui (1998) Elevated levels of 8-iso-prostaglandin $F_{2\alpha}$ in pericardial fluid of patients with heart failure. *Circulation* **97**, 1536–1539.

[25] J.D. Morrow, B. Frei, A.W. Longmire, J.M. Gaziano, S.M. Lynch, Y. Shyr, W.E. Strauss and J.A. Oates (1995) Increase in circulating products of lipid peroxidation ($F_2$-isoprostanes) in smokers. *New England Journal of Medicine* **332**, 1198–1203.

[26] S. Basu (1998) Metabolism of 8-isoprostaglandin $F_{2\alpha}$. *FEBS Letters* **428**, 32–36.

[27] J. Nourooz-Zadeh, B. Halliwell and E.E. Anggard (1997) Evidence for the formation of $F_3$-isoprostanes during peroxidation of eicosapentaenoic acid. *Biochemical and Biophysical Research Communications* **236**, 467–472.

[28] J. Nourooz-Zadeh, E.H.C. Liu, E.E. Anggard and B. Halliwell (1998) $F_4$-isoprostanes: a novel class of prostanoids formed during peroxidation of docosahexaenoic acid. *Biochemical and Biophysical Research Communications* **242**, 338–344.

[29] J.L. Roberts II, T.J. Montine, W.R. Markesbery, A.R. Tapper, P. Hardy, S. Chemtob, W.D. Dettbarn and J.D. Morrow (1998) Formation of isoprostane-like compounds (neuroprostanes) *in vivo* from docosahexaenoic acid. *Journal of Biological Chemistry* **273**, 13 605–13 612.

[30] J. Nourooz-Zadeh, E.H.C. Liu, B. Yhlen, E.E. Anggard and B. Halliwell (1999) $F_4$-isoprostanes as a specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *Journal of Neurochemistry* **72**, 734–740.

[31] J. Nourooz-Zadeh, N.K. Gopaul, S. Barrow, A.I. Mallet and E.E. Anggard (1995) Analysis of $F_2$-isoprostanes as indicators of non-enzymatic lipid peroxidation *in vivo* by gas chromatography-mass spectrometry: development of a solid-phase extraction procedure. *Journal of Chromatography B* **667**, 119–208.

[32] G. Davi, G. Ciabattoni, A. Consoli, A. Mezzetti, A. Falco, S. Santarone, E. Pennese, E. Vitacolonna, T. Bucciarelli, F. Costantini, F. Capani and C. Patrono (1999) *In vivo* formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. *Circulation* **99**, 224–229.

[33] N.K. Gopaul, E.E. Anggard, A.I. Mallet, D.J. Betteridge, S.P. Wolff and J. Nourooz-Zadeh (1995) Plasma 8-epiPGF$_{2\alpha}$ levels are elevated in individuals with non-insulin dependent diabetes mellitus. *FEBS Letters* **368**, 225–229.

[34] J. Nourooz-Zadeh, J. Tajaddini-Sarmadi, S. McCarthy, D.J. Betteridge and S.P. Wolff (1995) Elevated levels of authentic plasma hydroperoxides in non-insulin dependent diabetes mellitus. *Diabetes* **44**, 1054–1058.

[35] Special issue. Measurement and mechanism of oxidative DNA damage (1998) *Free Radical Research* **29**, 461–624.

[36] M. Dizdaroglu (1990) Gas-chromatography mass-spectrometry of free radical-induced products of pyrimidines and purines in DNA. *Methods in Enzymology* **193**, 842–857.
[37] S. Senturker and M. Dizdaroglu (1999) The effects of experimental conditions on the levels of oxidatively modified bases in DNA as measured by gas chromatography-mass spectrometry. How many modified bases are involved? Prepurification or not? *Free Radical Biology and Medicine* **27**, 370–380.
[38] T. Douki, T. Delatour, F. Bianchini and J. Cadet (1996) Observation and prevention of an artifactual formation of oxidized DNA bases and nucleosides in the GC-EIMS method. *Carcinogenesis* **17**, 347–353.
[39] A. Jenner, T.G. England, O.I. Aruoma and B. Halliwell (1998) Measurement of oxidative DNA damage by gas chromatography-mass spectrometry: ethanethiol prevents artifactual generation of oxidized DNA bases. *Biochemical Journal* **331**, 365–369.
[40] A. Rehman, A. Jenner and B. Halliwell (2000) Gas chromatography-mass spectrometry analysis of DNA: optimisation of protocols for isolation and analysis of DNA from human blood. *Methods in Enzymology* (in press).
[41] A.R. Collins, C.M. Gedik, B. Olmedilla, S. Southon and M. Bellizzi (1998) Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates. *FASEB Journal* **12**, 1397–1400.
[42] M. Dizdaroglu and D.S. Bertgold (1986) Characterization of free-radical induced base damage in DNA at biologically relevant levels. *Analytical Biochemistry* **156**, 182–188.
[43] P. Mecocci, G. Fano, S. Fulle, U. MacGarvey, L. Shinobu, M.C. Polidori, A. Cherubini, J. Vecchet, U. Senin and M.F. Beal (1999) Age-dependent increases in oxidative damage to DNA, lipids and proteins in human skeletal muscle. *Free Radical Biology and Medicine* **26**, 303–308.
[44] M. Dizdaroglu (1998) Facts about the artifacts in the measurement of oxidative DNA base damage by gas chromatography-mass spectrometry. *Free Radical Research* **29**, 551–563.
[45] J. Lunec (1998) ESCODD: European standards committee on oxidative DNA damage. *Free Radical Research* **29**, 601–608.
[46] ESCODD, European standards committee on oxidative DNA damage. (2000) Comparison of different methods of measuring oxidative DNA damage. *Free Radical Research* (in press).