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Chapter 3

Biomarkers

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1. Introduction – Why do we need 'biomarkers?

The term 'biomarker' has been adapted from molecular epidemiology by free radical biologists to describe a molecular change in a biological molecule that has arisen from attack by reactive oxygen, nitrogen or halide species (Offord et al., 2000). It is applied equally to products derived from lipids, DNA, proteins and antioxidant consumption, where the chemical nature of the reaction may be proton abstraction, electron transfer or direct addition. Rates of reaction of these molecules with the hydroxyl radical show that this reaction is governed by diffusion, and thus by proximity to the site of generation (Anbar and Neta, 1967). However, peroxy

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radicals are more stable with a corresponding half-life of seconds, thereby allowing diffusion to a distant site (Gee et al., 1985). Information regarding the nature of the denaturing radical, as well as the localisation of oxidative stress, may be gleaned from the analysis of discrete biomarkers isolated from tissues/organelles/fluids (Griffiths et al., 1988). Biomarkers have, therefore, been used to evaluate the efficacy of many antioxidants in vitro, ex vivo and in vivo with mixed results (Branca et al., 2001; Crews et al., 2001a,b; Wild et al., 2001).

The mixed results may reflect expectations we have regarding the information that oxidative biomarkers can yield. Biomarkers may yield information on three progressive levels to disease outcome: (i) as measurable endpoints of damage to proteins/amino acids, oxidised lipids, oxidised DNA bases, (ii) as functional markers of, for example, blood flow, platelet aggregation, or cognitive function, and (iii) as endpoints related to specific disease (e.g. lens opacity).

While the clinical symptoms of a disease are endpoints in themselves, they are not suitable – in many cases – for early detection and, therefore, prevention of disease. A series of biomarkers would be preferred, each validated in sequence. To this end, the association between a biomarker and a disease should be defined. Fig. 1 illustrates the concept of biomarkers at various levels of exposure, functional effect and pathological endpoint, and examples that meet these criteria to different degrees. Whilst there is no known biomarker that bridges the gap between exposure and endpoint, our increasing knowledge regarding functional consequences of biomolecule oxidation is bringing this possibility even closer (see Fig. 1).

Currently used 'first level' biomarkers may ultimately be proven to relate directly to functional changes, and ultimately disease. In the meantime, they can yield important information on the nature of radical damage and antioxidant action in vivo, particularly regarding the nature of pro-oxidant effects, compartmentalisation and bioavailability.

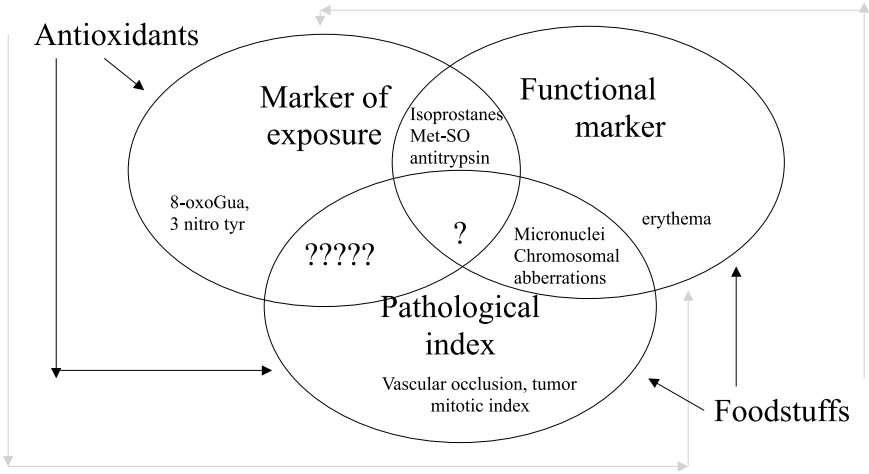


Fig. 1. The concept of biomarkers: biomarkers as possible targets for dietary antioxidants and supplements. Met-SO—methione sulfoxide; 8-oxoGua—8-oxoguanine; 3 nitrotyr—3 nitrotyrosine.

A valid biomarker should be:

- a major product of oxidative modification that may be implicated directly in the development of disease;
- a stable product, not susceptible to artefactual induction or loss during storage;
- representative of the balance between oxidative damage generation and clearance (i.e. the steady state, but also possibly applicable to the measurement of cumulative oxidative damage);
- determined by an assay that is specific, sensitive, reproducible and robust;
- free of confounding factors from dietary intake;
- accessible in a target tissue or a valid surrogate tissue such as a leucocyte; and
- measurable within the limits of detection of a reliable analytical procedure.

One of the key issues faced in the analysis of any oxidation product is the validity of the assay. The following criteria should be met: (i) identity, (ii) linearity, (iii) accuracy, (iii) selectivity, (iv) reproducibility and (v) an appropriate limit of detection (LOD). These issues have been addressed in the Appendices to this report. In addition, normal ranges should be established for each assay, reflecting age and sex of the subject group. Physiological changes, such as oxidation occurring during exercise, can then be defined in relation to the normal range.

This review will consider the current status of biomarkers in the context of the desirable properties listed above. The chemistry of formation of each biomarker will be considered along with an overview of the analytical procedures, highlighting the limitations and validity of each biomarker where possible. Finally, their application as endpoint analytes in supplementation studies will be discussed. This will reflect the state of the art in biomarker determination, and enable the emerging antioxidant paradox to be better understood.

2. Biomarkers of protein oxidation

2.1. Introduction

The earliest work on oxidation of a biologically important protein was on the effects of selected radicals generated by radiolysis on the enzyme, lysozyme (Adams et al., 1969). Both the thiocyanate radical (a selective modifier of tryptophan) and the hydroxyl radical (·OH) were found to inactivate the enzyme, implying that tryptophan residues are essential for biological activity, now well established from classical enzymology. Similarly, for $\alpha-1$-antitrypsin, modification of a single methionine residue at position 358 rendered the protein inactive (Carp and Janoff, 1979). Subsequent hydrolysis and amino acid analysis revealed the presence of methionine sulphoxide. This was one of the first pieces of evidence linking amino acid oxidation to denaturation of proteins and loss of function. The importance of protein oxidation in respect of altered function is exemplified by oxidative modifications to histidine and lysine in low density lipoproteins (LDL), which cause altered receptor

recognition; LDL modified in this way is preferentially taken up by scavenger receptors in a non-regulated process (Steinberg et al., 1989).

The process of protein oxidation frequently introduces new functional groups, such as hydroxyls and carbonyls, which contribute to altered function and turnover. Improved characterisation of the effects of protein oxidation has identified a spectrum of secondary effects including fragmentation, cross-linking and unfolding, which may accelerate or hinder proteolytic and proteosome-mediated turnover, according to the severity of oxidative damage (Dean et al., 1997).

The complexity of protein structure, arising from the primary sequence and involvement of carbohydrate moieties in structure stabilisation, together with a lack of specific and sensitive methodologies, has hindered the development of oxidative biomarkers (Davies et al., 1999; Griffiths, 2000). Generally, those amino acids capable of delocalising charge, such as amino acids containing aromatic and thiol side chains, are more susceptible to oxidative attack. However, a large number of aliphatic residues are subject to oxidation with the generation of protein carbonyl moieties.

Interest has also focused on the analysis of specific protein-bound oxidised amino acids. Of the 22 amino acids, aromatic and sulphydryl containing residues have been regarded as being particularly susceptible to oxidative modification, forming L-DOPA from tyrosine, ortho-tyrosine from phenylalanine, sulphoxides and disulphides from methionine and cysteine, respectively, and kynurenines from tryptophan. Latterly, the identification of valine and leucine hydroxides, reduced from hydroperoxide intermediates, has been described. The following sections will examine these potential biomarkers against the criteria laid out above, through examination of methodology and applications in antioxidant studies.

2.2. Major issues/questions

In the analysis of proteins and their oxidation products, there are many possible sources to examine – urine, plasma, cell, nucleus, mitochondria, and cytoplasm. In considering cellular oxidation, what is the relationship between target tissue (disease) and surrogate cells such as lymphocytes? For mitochondrial and nuclear proteins, the cell density required to achieve sufficient sensitivity is orders of magnitude greater. Within the plasma, several proteins are present at high concentrations; which protein should be selected? Will all proteins show the same response?

The presence of oxidised metabolites in the urine is indicative of effective repair/degradation of a modified protein. However, the degree of degradation is governed by the degree of oxidation, where highly oxidised proteins accumulate, and also spatial location with respect to degradative enzymes; the lens, for example, is spatially removed from proteosomal apparatus and hence accumulates oxidised proteins.

It is important to examine how a particular modification affects both structure and function. Is oxidation that has no effect on function, important? This depends on the question being addressed. Whilst it is possible to consider generic markers of protein oxidation, such as protein carbonyls, which may arise from several different amino acids, in order to undertake a more detailed analysis of protein oxidation and, in particular, to study susceptibility of distinct amino acids to oxidation in vivo, it is

Table 1
Hydrolysis of proteins for amino acid analysis

Hydrolysis procedure	Analyte
Reduction with sodium borohydride (1 mg/ml), acid precipitated and freeze dried prior to gas-phase hydrolysis; 5% mercaptoacetic acid, 1% w/v phenol in 6 mol/l HCl	Leucine and valine hydroxide (Fu and Dean, 1997)
Pronase E, 24 h	Tryptophan metabolites (Griffiths *et al.*, 1992)
Pronase E or alkaline hydrolysis, 24 h	Nitrotyrosine (Whiteman and Halliwell, 1999)

necessary to undertake protein isolation and hydrolysis prior to analysis. The greater the number of manipulations that oxidised proteins are exposed to, the greater the likelihood of further oxidation. Precautions should therefore be taken to minimise in vitro oxidation, and these will be alluded to below. Table 1 summarises the procedures required for sample preparation, illustrating the uses of both enzymic and acid hydrolyses.

Appropriate quality assurance material with known amounts of oxidised analyte can be incorporated to calculate sample recovery. In the absence of such material, the assumption is made that loss of oxidised residue occurs at the same rate as loss of native residue, although there is no proof of this at present. It is likely that the adoption of quadropole time of flight (Q-TOF) MS systems will obviate the need for such a large degree of sample processing, as it should be possible to determine each residue without prior hydrolysis. The hydrolysis step is an area of concern with regard to the artefactual oxidation of amino acids. Steps should be undertaken to minimise oxidation by, for example, the addition of mannitol. The presence of lipids in plasma samples can contribute to significant secondary modification, and delipidation should be undertaken in the early stages of sample preparation.

2.3. Protein carbonyl biomarkers

2.3.1. Biochemistry

Carbonyls are ubiquitous products of oxidation arising on amino acid side chains as well as sugars and lipids (Berlett and Stadtman, 1997). Whilst glucose addition is a simple condensation reaction between the carbohydrate aldehyde group and protein amino group, the resultant Schiff's base can undergo further oxidative reactions, which are collectively referred to as glycoxidation reactions. Both oxo-acids and aldehydes are formed following oxidative attack, having either the same number of carbon atoms or one less than the original amino acid (Stadtman and Berlett, 1991).

Carbonyls can be generated in response to a wide variety of oxidising stimuli including alkoxy and peroxy radicals as shown in Fig. 2.

2.3.2. Methods of measurement

The methods applied to the measurement of physiological protein oxidation vary from immunodetection by ELISA or Western blot to analytical HPLC. A specific

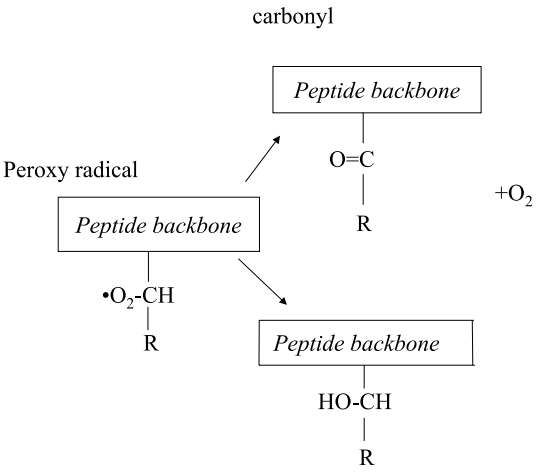


Fig. 2. Carbonyl formation.

reaction between protein carbonyls and dinitrophenyl hydrazine generates the hydrazone chromophore with an Absorbance$_{max}$ (A_{max}) at 360 nm, with a molar absorption coefficient of 22,000 M^{-1} cm^{-1}. This requires a large sample size for spectrophotometric determination, and gives little information on the protein affected. Products can be separated by HPLC. Several antibodies are available for the detection of the dinitrophenyl hydrazine carbonyl product, which has been used in a semi-quantitative manner in Western blot analysis of both SDS–PAGE and 2-D proteomic gels. These methods have been reviewed elsewhere (Griffiths et al., 1999a; Schacter et al., 1996). An ELISA procedure has been developed for quantitative analysis of carbonyl content on specific proteins (Buss et al., 1997; Carty et al., 2000). This offers the advantages of sensitivity, small sample volume, reproducibility and large sample throughput.

Although protein carbonyls are a generic marker of oxidation, they appear to be yielding useful information. From a histochemical point of view, the appearance of carbonyl functions represents the single major change resulting from the oxidation of proteins. Histochemical detection of such protein-associated carbonyl functions (Pompella et al., 1996a), an adaptation of the method originally developed by Levine et al. (1994), can be used. Protein carbonyls are first derivatised by 2,4-dinitrophenyl hydrazine (2,4-DNPH) to yield the corresponding 2,4-dinitrophenyl hydrazones. In the second step, the dinitrophenyl (DNP) groups, which become associated with proteins in this way, are detected immunochemically by means of a commercial anti-DNP antiserum; finally, bound antibodies are identified with a conventional biotin–avidin system or equivalent (Pompella et al., 1996a). In principle, the 2,4-DNPH/anti-DNP procedures should reveal all kinds of protein carbonyls, irrespective of their origin. With this method, oxidised proteins have been visualised in several conditions (e.g. in activated neutrophil phagocytes (Cambiaggi et al., 1997; Pompella et al., 1996a), in brain tissue from Alzheimer patients (Smith et al., 1996) and in

sarcoma cells exposed to pro-oxidant treatments (Frank et al., 1998)). On the other hand,the usefulness of the 2,4-DNPH/anti-DNP procedures for the histochemical detection of protein glycoxidation products has not yet been evaluated.

2.3.3. Storage, stability and limitations in use

Protein carbonyls have been demonstrated to be stable for three months at −80 °C. The carbonyl assay measures generic oxidation products, however, and does not differentiate between those arising directly from protein oxidation and those formed by adduction of another oxidised product (e.g. glucose, malondialdehyde (Burcham and Kuhan, 1996)). In addition, proteins form carbonyls at different rates when oxidised in vitro, where changes seen in one protein may be of a different order of magnitude in another protein. Cao and Cutler have extensively investigated the determination of protein carbonyls in tissue, and conclude that the method of DNPH derivatisation is valid only for the study of purified protein (Cao and Cutler, 1995), indicating that sample cleanup is essential, and histochemical analysis must be interpreted with caution.

2.4. Protein thiol biomarkers

2.4.1. Biochemistry

Examination of the percentage native amino acid remaining following oxidation in vitro shows that for many proteins cysteine/cystine is a highly susceptible moiety (Table 2). The free thiol group of cysteine readily undergoes reversible oxidation to form a disulphide, which can be 'repaired' in the presence of a thiol donor such as glutathione. Further oxidation leads irreversibly to cysteic acid (Creed, 1984). Oxidation of the single amino acid cysteine is complicated by the observation that the product may be a composite mixed disulphide. Indeed, in biological systems, the levels of such a product are also indirectly under the influence of inducible enzymes of the glutathione cycle, and thus inherently related to changes in gene expression, conditioning and priming.

Another sulphur-containing amino acid, methionine, is also considered to be highly susceptible to oxidative change; end products include the sulphoxide and the sulphone. Hypochlorous acid, nitric oxide and singlet oxygen are all capable of eliciting this change. It is also subject to an enzymic repair process via methionine sulphoxide reductase (PMSR), the activity of which declines with age (Petropoulos et al., 2001). Recently, Biewenga has described that dihydrolipoate can function as a co-factor for PMSR in the repair of alpha-1-antitrypsin (Biewenga et al., 1998). This

Table 2
Susceptibility of individual amino acids within IgG to oxidation radiolytically generated radical species

Amino acid	Cysteine	Methionine	Histidine	Tryptophan	Tyrosine
$OH^{\cdot}$	78	100	100	93	100
$O_2^{-\cdot}$	76	100	100	100	100
$ROO^{\cdot}$	97	98	100	78	100

Results are expressed as a percentage of their original concentration.

enzyme restores the oxidised residue to its native form on the protein backbone, without any detectable product, and so methionine oxidation may give misleading information on the degree of oxidation if analysed alone.

2.4.2. Methods of measurement

Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) readily forms a mixed disulphide with thiols, liberating the chromophore 5-mercapto-2-nitrobenzoic acid (A_{max} 410 nm, $\sim 13,600\ M^{-1}\ cm^{-1}$) (Ellman, 1959). Only protein thiols that are accessible to this water-soluble reagent are modified. Carrying out the titration in the presence of 6 M guanidinium chloride usually enables quantification of inaccessible thiols. DTNB conjugates of glutathione and other thiols can be separated by HPLC.

Ultrasensitive colorimetric quantitation of both protein and non-protein thiols is now possible using a method reported by Singh et al. (1993). Thiols or sulphides reduce a disulphide-inhibited derivative of papain, stoichiometrically releasing the active enzyme. Activity of the enzyme is then measured using the chromogenic papain substrate L-3-*N*-alpha-benzoyl-DL-arginine *p*-nitroanilide (BAPNA). The enzymic amplification step has a sensitivity for detection of thiols or sulphides of approximately 0.2 nanomoles or about 100-fold better than that obtained with DTNB. Thiols in proteins can be detected indirectly by incorporating the disulphide cystamine into the reaction mixture. Cystamine undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain.

Maleimides are excellent reagents for thiol-selective modification, quantitation and analysis in cellular systems, where attachment of fluorescent tags aids their visualisation (Ohmori et al., 2001). The reaction involves addition of the thiol across the double bond of the maleimide to yield a thioether. Several of the maleimides have very low fluorescence until they react with thiols to form fluorescent adducts. In some cases this permits their use for both the quantitation of thiols and their semi-quantitative localisation in cellular organelles. Monobromobimane (Chou et al., 2001) is also essentially non-fluorescent until it reacts with thiols, and it can be used for their quantitation.

Nitrosothiols appear to play an important role in the signalling function of nitric oxide (NO). This can be detected in biological samples by the *Griess reagent* following HPLC and post-column metal-catalysed release of NO (Akaike et al., 1997).

The loss of reduced sulphydryl (–SH) groups can be measured histochemically. Methods have been developed and optimised (Nohammer, 1982; Nohammer et al., 1981) and have found application in the evaluation of protein thiol redox status in neoplastic and pre-neoplastic cells (Nohammer et al., 1986; Pompella et al., 1996b). Fluorescent labelling procedures have been developed for the visualisation of both total and cell surface protein reduced thiols, at the single cell level by laser-scanning confocal microscopy (Pompella et al., 1996a).

2.4.3. Storage, stability and limitations on use

Cysteine residues are notoriously labile in an isolated system, which explains the inactivation of proteins during isolation. Great care must be taken to minimise ar-

tefactual oxidation, through incorporation of chelating agents (e.g. EDTA) in buffers, maintenance of ice-cold temperatures throughout and addition of dithiothreitol.

The major limitation of this approach is that only changes in reduced thiol content can be measured. Without any measure of oxidised product, thiol analysis should be considered a poor marker. At present, there are no adequate procedures available for assaying disulphide formation, and it is likely that the only adequate methodology may arise from the development of immunological reagents.

2.5. Aliphatic amino acid biomarkers

2.5.1. Biochemistry

One of the longer-lived reactive species produced by oxygen radical attack on proteins is hydroperoxide. In time, these oxidising species decay to form stable, long-lived hydroxides, and both can be measured by HPLC (Fig. 3).

The amino acid residue most susceptible to hydroperoxide formation is valine, but leucine and lysine are also prone to this modification. Within proteins, however, the hydrophobic residues are protected from bulk aqueous radicals, and lysine hydroperoxides are the more sensitive indicators. Hydroperoxides are rapidly broken down in the presence of metal ions (Fu et al., 1995a; Robinson et al., 1998), and are also subject to breakdown to hydroxides by glutathione peroxidase. Hydroperoxides can be effectively reduced to hydroxides post-extraction, which are poorly susceptible to further oxidative modification.

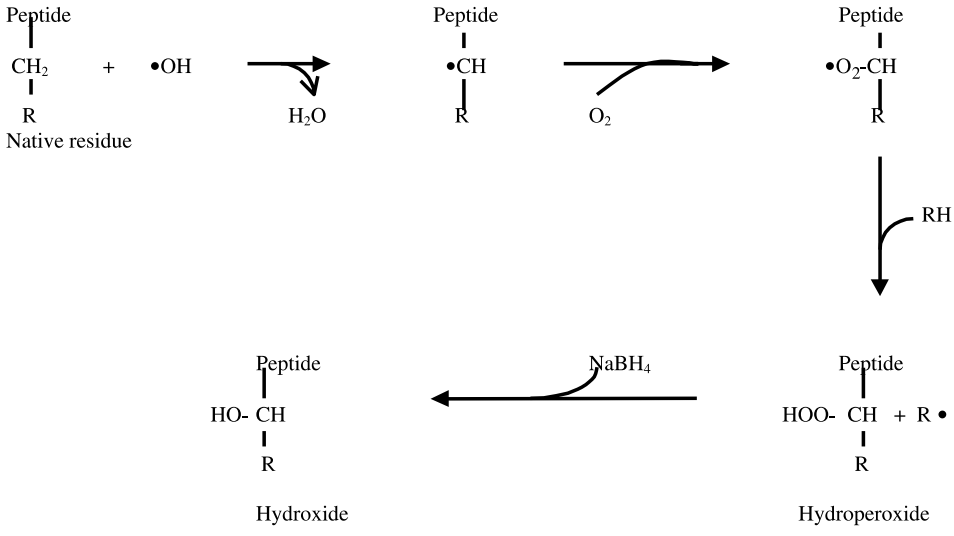


Fig. 3. Hydroperoxide and hydroxide formation.

The formation of hydroxides, by the addition of ·OH in the presence of oxygen, across the side chains of amino acids is relatively specific to aliphatic amino acid side chains. Extensive characterisation of the three isomeric forms of valine and leucine hydroxides has been undertaken by Fu et al. (1995b); Fu and Dean (1997); Morin et al. (1998).

Lysine can also undergo limited attack at the C6 atom to yield the corresponding aldehyde, adipic semi-aldehyde. Alpha semi-adipic aldehyde is also formed enzymatically, and this may confound some determination of this product. Nevertheless, researchers in Denmark have adopted this biomarker (Young et al., 2000).

Histidine is frequently involved in the co-ordination of metal ions, and thus is a target for metal catalysed oxidation (Stadtman, 1993). Little is known about the chemistry of oxidation of histidine by the range of biologically relevant reactive species, and hence it is too early to determine whether the oxidation products 2 and/or 8-oxohistidine are useful biomarkers.

2.5.2. Methods of measurement

Hydroperoxides can be detected following HPLC separation of amino acids by chemiluminescence, following post-column reaction between microperoxidase and hydrogen peroxide. Hydroperoxides in plasma are degraded by either enzymic or acid hydrolysis, and can only be successfully measured in low molecular weight (MW) plasma filtrates, where the quenching effect of larger proteins is removed (Griffiths et al., 1999a). Following reduction of hydroperoxides by borohydride, subsequent analysis of hydroxides has largely been restricted to measurement of leucine, valine and more recently the hydrophilic surface amino acid, lysine (Fu et al., 1995b; Fu and Dean, 1997; Morin et al., 1998). HPLC analysis requires a two-step procedure; initially samples are fractionated on an amino column, which is followed by further chromatography of the ortho-phthalaldehyde (OPA) derivatised products using a Zorbax ODS column with fluorescence detection. At present, measurement of these analytes has been undertaken in 'disease' versus 'normal' tissue, and it remains to be seen whether such species may be helpful in following the effects of dietary antioxidants. The present method using a two-step HPLC procedure, together with the low concentration of oxidised product found in normal subjects, however, suggests that these analytes may have limited usefulness.

Oxidative modification of lysine residues in the apolipoprotein component of LDL, either by inter-chain cross-linking, or following adduct formation with lipid peroxidation products, alters the electrophoretic mobility of LDL, causing it to move further towards the positive electrode in agarose gel. Associated with this loss of positive charge, receptor–ligand interactions are also modified and an increase in cytotoxicity is observed (Asmis and Jelk, 2000).

Lysine modification by lipid peroxidation products (linoleic hydroperoxide) can yield neoantigenic determinants such as *N*-epsilon-hexanoyl lysine (HEL). An antibody has been successfully raised to this epitope (Kato et al., 2000).

2.5.3. Storage, stability and limitations on use

Observations that these analytes are not stable on storage or hydrolysis mean that hydroperoxides do not fulfil the criteria required for biomarkers.

2.6. Oxidised Tryptophan Biomarkers

2.6.1. Biochemistry

After cysteine loss, aromatic amino acids are the next most sensitive residues, owing to their capacity to delocalise radical intermediates around their ring structures. The most frequent changes are ring addition reactions and introduction of oxygen, which in the case of tryptophan causes ring cleavage to yield *N*-formyl kynurenine (Fig. 4). Many of these changes are also associated with generation of a novel autofluorescence of a longer wavelength, which confers increased sensitivity and specificity in analysis.

The light energy emitted by UV radiation can be directly absorbed by tryptophan, producing an excited intermediate. The tryptophanyl radical can be dissipated though transfer to dissolved oxygen (to yield $O_2^{\cdot-}$) by antioxidants or through charge transfer – i.e. the movement of an unpaired electron along a peptide or protein backbone to a susceptible amino acid of lower redox potential.

Thus, in any given protein, the target of attack may not be the ultimate site of damage. In the absence of charge transfer or repair, tryptophan undergoes a ring opening following ·OH attack to yield predominantly *N*-formyl kynurenine and kynurenine (Griffiths et al., 1992; Singh et al., 1984). Kynurenines are also synthesised enzymically, and whilst these compounds are not incorporated during protein synthesis, which makes them potential markers in proteins, they are excreted in urine and therefore it is not possible to use these analytes in urine analysis.

2.6.2. Method of measurement

Several groups have undertaken the measurement of oxidised tryptophan metabolites over the past ten years (Giessauf et al., 1996; Griffiths et al., 1992; Pirie, 1971). All have utilised reverse-phase chromatography, eluting the oxidised residues with increasing acetonitrile concentration. Eluent is monitored with native UV absorption and autofluorescence, and peak identity confirmed by spiking with authentic standard, and UV and fluorescence spectra.

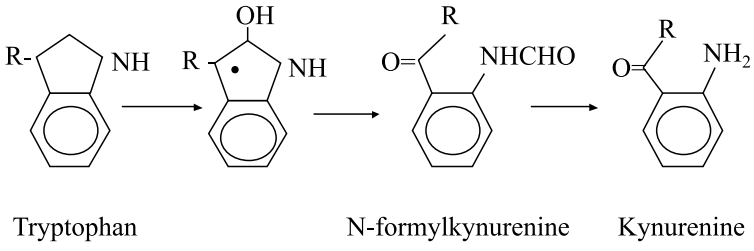


Fig. 4. Oxidation of tryptophan.

2.6.3. Storage, stability and limitations on use

All aromatic amino acids are inherently light-sensitive, and so should be stored in light-tight containers. Urinary analysis of kynurenines as oxidative products cannot be undertaken, since these moieties can also arise directly through enzymic processes.

2.7. Oxidised tyrosine biomarkers

2.7.1. Biochemistry

Tyrosine dimerisation has been proposed to contribute to the aggregation of proteins frequently observed on oxidation. However, since oxygen and antioxidants rapidly repair the phenoxyl radical intermediate, it is only likely to be a significant product in metalloproteins, where the intermediate is stabilised through change in redox status of the associated transition metal (Wilks and Ortiz de Montellano, 1992). L-DOPA is formed from the electrophilic addition of hydroxyl radical to the unsaturated/ring of tyrosine, where its presence within a protein structure is indicative of post-synthetic oxidation (Fig. 5).

Hydroxylation of phenylalanine is a typical reaction of hydroxyl radicals, in which the resultant tyrosine is hydroxylated at the *ortho*- or *meta*-position, and is therefore distinct from native tyrosine (Fig. 6) The debate as to the physiological relevance of bityrosine is set to rage with a number of contradictory reports in the literature (Daneshvar et al., 1997; Kato et al., 1998). However, this may reflect the dependence of bityrosine formation on the structure of the protein under attack.

The nucleophilic non-radical oxidant, hypochlorous acid (HOCl), is particularly reactive with tyrosine, inducing the characteristic chlorotyrosine. Furthermore, bromotyrosine may also be generated under the action of eosinophil peroxidase. However, it has been reported that both chloro- and bromotyrosine can undergo a second halogenation step, which interferes with their measurement. A further ring addition product of tyrosine, nitrotyrosine, is formed from attack by nitrosylating

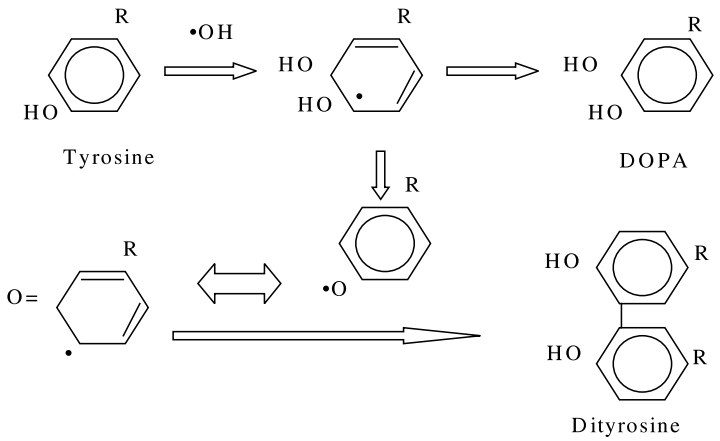


Fig. 5. Oxidation of tyrosine.

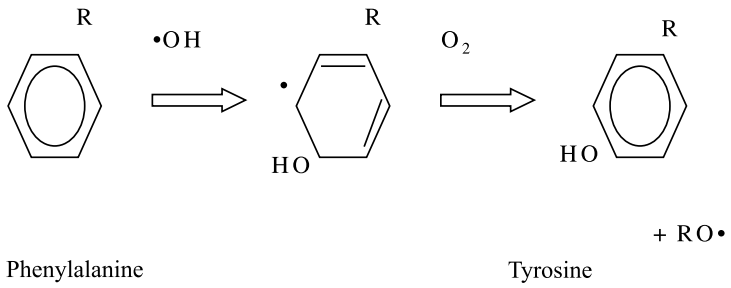


Fig. 6. Oxidation of phenylalanine.

species including nitric oxide, peroxynitrite and reactions between hypochlorite and nitrogen-containing species [reviewed in (Crow, 1999)].

Phenylalanine reacts with hydroxyl radicals to form three hydroxylated products, *para*-, *meta*- and *ortho*-tyrosine. The latter two are not normal products of any enzymic process.

2.7.2. Methods of measurement

HPLC has been used to analyse phenylalanine oxidation products. However, caution must be employed in the detection system used following HPLC separation, as (Reddy et al., 1999) report co-elution of an unknown compound that interferes with the determination of *m*-tyrosine. As with any of these detection systems, the use of diode array or coulometric detection can overcome these problems.

Detection of the oxidised tyrosine moiety, L-DOPA, following HPLC also relies on native UV absorption (280 nm) and autofluorescence (excitation wavelength [E_x] 280 nm, emission wavelength [E_m] 320 nm). The formation of *o*- and *m*-tyrosine from phenylalanine can be observed under the same analytical conditions. In contrast, dityrosine is followed using longer wavelength fluorescence emission at 410 nm. 3-Nitrotyrosine can be quantified by ELISA or HPLC using either UV or electrochemical detection (Crow, 1999; Shigenaga, 1999; ter Steege et al., 1998).

2.7.3. Storage, stability and limitations on use

L-DOPA is a relatively long-lived species that can confer reducing activity in its environment.

Bityrosine may only be detectable in proteins where the intermediate phenoxyl radical can be stabilised through a co-ordinated metal ion such as within globins and peroxidases. Whiteman and Halliwell have demonstrated the reduction of nitrotyrosine by hypohalous acid, thus questioning the validity of nitrotyrosine as a biomarker (Whiteman and Halliwell, 1999). This possibility for destruction by other oxidants may account for some of the ambiguities existing in the determination of nitrotyrosine in atherosclerotic plaques. In addition, acid hydrolysis of proteins has

been shown to cause artefactual induction of nitrotyrosine, and therefore alkaline or enzymic hydrolysis must be employed. The significance of tyrosine modification has been extensively reviewed by Crow (1999).

2.8. Formation of neoepitopes on oxidised proteins

Following the structural alterations introduced by an oxidant insult, proteins can acquire new antigenic properties, owing to the formation of new epitopes on the polypeptide chain. This is primarily the case with reactive aldehydes derived from lipid peroxidation, which are able to bind to several amino acid residues. By means of specific polyclonal or monoclonal antibodies, the occurrence of malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) bound to cellular protein has been documented under a number of experimental and clinical conditions. Protein-bound lipid aldehydes have been demonstrated in collagen-producing fibroblasts (Bedossa et al., 1994; Chojkier et al., 1989); in the liver of human alcoholics (Niemela et al., 1994), hepatitis C patients and other chronic liver diseases (Paradis et al., 1997a,b); in the arterial wall during experimentally induced atherosclerosis (Palinski et al., 1989); in activated neutrophils (Cambiaggi et al., 1997; Quinn et al., 1995); in nigral neurons of Parkinson patients (Yoritaka et al., 1996); in ferric-nitrilotriacetate-induced renal carcinogenesis; (Uchida et al., 1995) and in human renal carcinoma (Okamoto et al., 1994). Most of these studies were made possible by the availability of thoroughly characterised monoclonal antibodies (Toyokuni et al., 1995; Waeg et al., 1996).

Specific epitopes are also present in oxidised LDL (ox-LDL), a distinctive class of oxidised proteins probably involved in the pathogenesis of atherosclerosis. The exact nature of such epitopes is a matter of debate, although it seems certain that the antigenicity of ox-LDL can be at least partially accounted for by the binding of lipid peroxide-derived aldehydes, such as MDA and 4-HNE, to the LDL apoprotein moiety (Requena et al., 1997). By means of poly- and monoclonal antibodies raised against in vitro-oxidised LDL, the immunohistochemical visualisation of ox-LDL has been repeatedly reported in atherosclerotic lesions (Palinski et al., 1989; Paolicchi et al., 1999; Yla-Herttuala et al., 1990).

With respect to 'advanced glycation end products' (AGEs), immunohistochemical studies aimed at determining the sites of accumulation of AGE-modified proteins have generally employed antibodies specific for *N*-ε-(carboxymethyl) lysine, the main antigenic structure produced during protein glycoxidation (Ikeda et al., 1998). In this way, AGEs have recently been detected in several disease conditions (Matsuse et al., 1998; Sasaki et al., 1998; Sun et al., 1998; Takayama et al., 1998).

Besides protein oxidation resulting in increased levels of protein carbonyls, the oxidative attack brought on protein by reactive nitrogen species has also received attention by histochemical research. The reaction of peroxynitrite anion ($ONOO^-$) with aromatic amino acids results primarily in the addition of nitrate groups to the *ortho*-position of tyrosine, a process referred to as 'protein nitration'. Histochemically, protein nitration has been documented by means of immunomethods employing anti-nitrotyrosine antibodies (Beckman et al., 1994; Good et al., 1996; Virag et al., 1998).

2.9. Validation of assays for protein oxidation biomarkers

There is a real need for standardisation of the many protocols used to measure protein oxidation that are currently adopted in different laboratories worldwide. In the case of carbonyls, this can be provided through the preparation of a quality assurance material containing defined numbers of carbonyl derivatives, which is of a size to behave like a small protein. Such a material could be processed through protein extraction procedures and used to confirm minimal oxidation in vitro. In addition, however, different protein hydrolysis methods must be examined to assess efficiency, yield and the potential for artefactual induction of oxidised residues. There is also a need for appropriate external standard materials, which are not subject to oxidative variation. Again, this may be addressed through the quality assurance procedures. Table 21 gives a summary of validation status of the various assays.

2.10. Relationship of protein oxidation to disease

Protein carbonyls have been most strongly linked with ageing and associated phenomena, as reviewed by Chevion et al. (2000). In a study of low birth weight babies, Winterbourn et al. observed raised levels of protein carbonyls in cord blood compared to full-term babies; there was no relation to chronic lung disease and retinopathy (Winterbourn et al., 2000). Systemic markers are less sensitive and may not reflect the changes at target tissues. In light of this, Chen et al. examined levels of protein carbonyls in seminal fluid from men with varicocele (Chen et al., 2001), and observed higher levels in the disease group and subclinical varicocele compared to controls.

Analysis of thiol status has been undertaken immunohistochemically in a semi-quantative manner in cancer cells (Wardman and von Sonntag, 1995). Thiol status of plasma decreases with ageing but this effect may be explained, in part, through reduced albumin production from ageing liver. However, if the focus is directed to target tissue, such as seminal fluid in patients with varicocele (Chen et al., 2001), a clear reduction in thiols is observed, which supports the hypothesis of increased oxidative stress in this tissue.

Both 3-hydroxy valine and 5-hydroxy leucine have been detected in normal tissues, including plasma, intimal artery and lenses. Concentrations are described in Table 3, and can increase seven- to tenfold in diseased tissue, depending on the severity of disease. (Fu et al., 1998a,b).

Elevated levels of *N*-formyl kynurenine have been observed in cataractous lenses, IgG from patients with rheumatoid arthritis and in LDL from atherosclerosis patients (Giessauf et al., 1996; Griffiths et al., 1992; Pirie, 1971). Limited analysis of kynurenines has been done in normal healthy subjects, and they are detectable in plasma IgG but not albumin. Porphyrin enhanced photo-oxidation of bovine serum albumin in vitro causes the formation of oxidised tryptophan metabolites (Silvester et al., 1998).

Table 3
Antioxidant supplementation effects on protein oxidation

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C	500 mg/d	5 smokers/gp	20 days		Plasma	Globin carbonyl	No change	Colorimetric	Lee et al. (1998)
Vitamin E	200 IU/d						44% decrease		
β-carotene	9 mg/d						No change		
Red ginseng	1.8 g/d						21% decrease		
Vitamin C	400 mg/d	40	105–119 days	Crossover placebo	Plasma	Immuno-globulin carbonyl	Decrease ≫ in those with low baseline ascorbate	ELISA	Carty et al. (2000)
						Plasma thiol	No effect	Colorimetric	
Vitamin C	250 mg	32	35 days	Placebo controlled	Plasma	carbonyl	Reduced ($p < 0.05$)	Spectrophotometric	Peng et al. (2000)
β-carotene	3 mg								
α-tocopherol	100 IU								
Folic acid	250 μg								
Grape seed extract	3.89 mg catechin								
α-tocopherol	400 mg/d			+fish oil or DHA	Plasma	Carbonyl	No effect		Wander and Du (2000)
Lycopene	39–150 mg/d	19	7 days		Serum	Thiols	NS trend towards protection	Colorimetric	Rao and Agarwal (1998)
Grape seed extract	200 ml containing 31.3 phenolics	15	7 days	Crossover	Plasma albumin	2-adipic semi-aldehyde	No effect	HPLC + fluorescence	Young et al. (2000)
Blackcurrant and apple juice	750 mg/d 1000 mg/d 1500 ml/d	5	7 days	Crossover	Plasma albumin	2-adipic semi-aldehyde	Increased with time and dose	HPLC + fluorescence	Young et al. (1999)
Parsley	3.73–4.49 apigenein/MJ/d	14	7 days	Crossover	Plasma oxidation	2-adipic semi-aldehyde	No effect	HPLC + fluorescence	Nielsen et al. (1999)
Diet rich in antioxidants		Smokers (22)	21 days	Placebo controlled crossover	Plasma	Total protein carbonyl	No change		Van den Berg et al. (2001)

There are a number of reports demonstrating the generation of protein-bound L-DOPA, from tyrosine, in a dose-dependent fashion following ·OH attack (Dean et al., 1997; Fu et al., 1998a). There is confusion in the literature, however, as to the presence of nitrotyrosine in atheromatous plaques, with some workers describing elevated levels and others not observing any difference between normal and diseased tissue. Nonetheless, L-DOPA, dityrosine, 3-chlorotyrosine and 3-nitrotyrosine are all detectable in normal *intima*, but only L-DOPA and dityrosine are present in normal lenses, demonstrating the lack of inflammatory processes in oxidation of lens tissue. In contrast, studies on the vascular *intima* suggest that levels of dityrosine present are greater than would be predicted from hydroxyl radical alone, which would suggest the involvement of myeloperoxidase-catalysed oxidation reactions (Fu et al., 1998a). Absolute levels are reported in Table 21.

2.11. Modulation of protein oxidation biomarkers by antioxidants

Supplementation of the rat diet with the flavonoid rutin for 18 days caused a reduction in protein carbonyl content (Funabiki et al., 1999). Supplementation with α-tocopherol or a combination of α-tocopherol (40 mg/day) and ascorbic acid (24 mg/day) for 15 days protected against protein carbonyl formation in iron-deficient rats during iron repletion (Srigiridhar and Nair, 2000). Tocotrienols reduced the protein carbonyl levels in the ageing nematode, *Caenorhabditis elegans* (Adachi and Ishii, 2000). These limited studies are consistent in demonstrating a protective effect against plasma protein oxidation by dietary intervention.

Long-term vitamin C supplementation has been observed to protect against protein oxidation in human subjects (Carty et al., 2000). In subjects with low baseline antioxidant status, vitamin C supplementation significantly reduced the degree of protein oxidation after ten but not five weeks. An overall negative correlation between ascorbate and IgG carbonyl content was observed ($r = -0.145$, $p = 0.019$). Partial analysis of samples from a study of long-term supplementation (Porkkala-Sarataho et al., 2000) demonstrated that individuals receiving both vitamins C and E show a 30% decrease in protein carbonyl levels after one year (unpublished findings, Griffiths et al., 2002). Again, there is a significant inverse correlation between vitamin C and protein carbonyl status ($r = -0.575$, $p < 0.001$).

Wander and Du (2000) have examined the antioxidant effect of vitamin E in supplementation with eicosapentaenoic and docosahexaenoic acids. The fish oil had no effect on protein carbonyl content nor was the presence of 400 mg α-tocopherol in the diet a modulator of plasma protein carbonyl formation. These studies are summarised in Table 3.

Studies in vitro of HDL oxidation have confirmed the early formation of methionine sulphoxide, which is enhanced by α-tocopherol (Garner et al., 1998). This lesion is also subject to active repair by methionine sulphoxide reductase, where reduced activity of the enzyme is associated with ageing (Petropoulos et al., 2001).

Analysis of thiols has been undertaken in antioxidant supplementation studies in humans (Carty et al., 2000). However, as may be expected from the limitations of this method (see Section 2.4.3) it has not yielded any consistent or strong observations. In plasma, measurements are confounded by the high thiol content of albumin and its associated function in transport of small molecules, including drugs such as D-penicillamine. Cellular thiol analysis is confounded by the interrelationship between glutathione and its associated enzymes. Histochemical analysis has not been used in the context of dietary antioxidant status/supplementation.

Generation of 2-oxohistidine is observed during LDL oxidation in vitro, which can be inhibited by vitamin C (Retsky et al., 1999). Nitrogen-centred radicals of lysine, formed by HOCl, are protected against by the presence of ascorbic acid, glutathione and Trolox® in vitro.

A recent study in Watanabe heritable hyperlipidaemic rabbits, using an antibody against lysine-malondialdehyde, examined uptake of radiolabelled antibody into plaques in animals fed diets supplemented with dietary vitamins C and E, for six months. Those on normal diets developed lesions rich in modified lysine, whereas those fed diets supplemented with vitamins C and E showed fewer oxidation-specific epitopes (Tsimikas et al., 2000).

Grape seed extract rich in catechins and phenolics was given as a supplement for one week to 15 subjects; no effect was seen on protein oxidation (Young et al., 2000). A second study looked at fruit juice in five subjects again for one week, with three doses. In this study, plasma 2-amino-adipic semi-aldehyde increased with time and dose, indicating a pro-oxidant effect (Young et al., 1999). A third study examined parsley, rich in flavone, for one week in 14 subjects. Again, no significant changes were observed in plasma protein 2-adipic semi-aldehyde residues (Nielsen et al., 1999). The rather inconclusive nature of these studies may be simply a reflection of the lack of statistical power, combined with short duration of supplementation.

A pilot study on ten subjects, receiving ascorbic acid supplementation for 10 weeks, has observed significant protection against formation of *N*-formyl kynurenine (Griffiths et al., 2002).

There are no published reports describing the effects of dietary antioxidants on tyrosine oxidation products.

2.12. Future perspectives

There are several techniques and markers with the requisite sensitivity and specificity to be applied in the evaluation of effects of dietary antioxidants on protein oxidation. But, it is important that any protein selected should be of relevance to the outcome of an altered function. A recent study in SOD2 knockout mice clearly showed increased oxidation and inactivation of aconitase and NADPH oxidase, and adverse effects on mitochondrial energy production (Williams et al., 1998).

Other issues that should be considered are: (1) Is there a difference between the ability of different classes of antioxidant to protect plasma proteins, compared to

cellular proteins? Is this a direct function of their hydrophobicity indices? (2) What is the subcellular distribution of protein oxidation, and how is the breakdown of oxidised proteins via the proteosome affected by antioxidants? Protein-L-isoaspartate *O*-methyl transferase (PCMT) catalyses methylation of oxidised aspartate residues, and thereby signals repair or degradation of age-damaged proteins. Antioxidants can inhibit appropriate methylation (Ingrosso et al., 2000), and this may impair the removal of oxidised proteins, suggesting a negative benefit of antioxidants. This merits further study.

Protein sequence information on databases such as *SwissProt* may ultimately allow us to predict susceptible proteins and residues, based on our knowledge of tertiary structural and secondary sequence susceptibility patterns. Together with the advances in proteomics, understanding of the differential susceptibility of proteins to oxidative insult will be further enhanced.

2.13. Conclusions

It is evident from the body of evidence presented that a 'fingerprint' of the oxidising species present in vivo should be attainable by adopting a broad spectrum of protein-based biomarkers. The levels of these different biomarkers from different studies are summarised in Appendix A.

Based on the criteria outlined in the introduction of this report, and the observations of unequivocal elevation in disease, the likely candidates for biomarkers are isolated protein carbonyls (accepting that these may arise from secondary processes), bityrosine, L-DOPA, and *ortho*-tyrosine.

It is also possible to examine LDL in plasma and its propensity for uptake by monocytes and endothelial cells, where modified LDL is taken up by the scavenger receptor. Specific modification to one lysine residue is sufficient to dramatically increase uptake of LDL (Griffiths et al., 1999a,b). This validated procedure could be applied to evaluate the functional consequences of antioxidant supplementation in a human intervention study.

This chapter has highlighted the advantages of adopting several biomarkers for studying in vivo oxidation of proteins, and these methods should now be carefully applied, using quality assurance material, to controlled studies of antioxidant intake to evaluate the significance of dietary antioxidants in preventing physiological oxidative changes.

3. Introduction to lipid peroxidation biomarkers

3.1. Introduction: biochemistry of lipid peroxidation

The principal lipid-rich sites in vivo are the lipid-carrying lipoproteins and cell membranes. Increased concentrations of lipid peroxidation end products are found in most, if not all, human diseases. However, they play a significant pathological role

in only some of them. For example, peroxidation appears to be important in atherosclerosis, and in worsening the initial tissue injury caused by ischemic or traumatic brain damage. Lipid peroxidation often occurs late in the injury process. Many assays are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole process.

Triglycerides in LDL and phospholipids in membranes are highly susceptible to free radical attacks. The first step in the lipid peroxidation (LPO) process in biological membranes is the abstraction of a hydrogen atom, from a methylene group next to a double bond (Fig. 7).

This produces a carbon-centred radical (2). The carbon radical is stabilised by a rearrangement of the double bond to form a conjugated diene (3). Since oxygen is present at high concentration in the membrane of the cell, it can easily react with the carbon-centred radical, forming the peroxyl radical (4). The peroxyl radical reacts further with another phospholipid/triglyceride-linked fatty acid forming a hydroperoxy group (5) and a new carbon-centred radical (2). The lipid hydroperoxide will react further to form cyclic peroxide, cyclic endoperoxide, and finally aldehydes including malondialdehyde.

During the peroxidative pathway, via reactive intermediates, several end products are formed such as aldehydes (MDA and 4-HNE), pentane and ethane, 2,3 *trans*-conjugated dienes, isoprostanes and cholesteroloxides.

The biological activities of MDA, and other aldehydes like 4-HNE, include cross-linking with DNA and proteins, which alters the function/activity of these molecules. MDA and 4-HNE have shown tissue toxicity. MDA, for instance, can react with amino and thiol groups. The aldehydes are more diffusible than free radicals, which means damage is exported to distant sites. Aldehydes are quickly removed from cells as several enzymes control their metabolism.

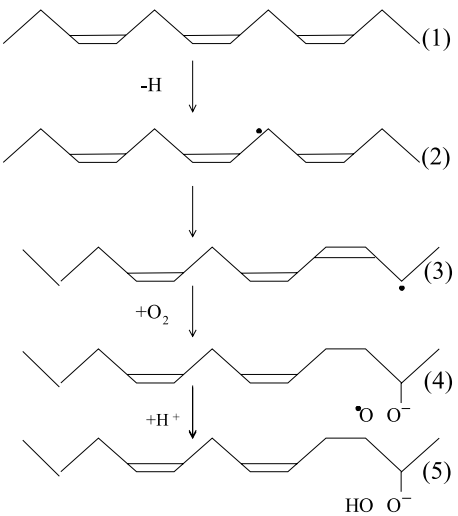


Fig. 7. The lipid peroxidation process.

3.2. Malondialdehyde

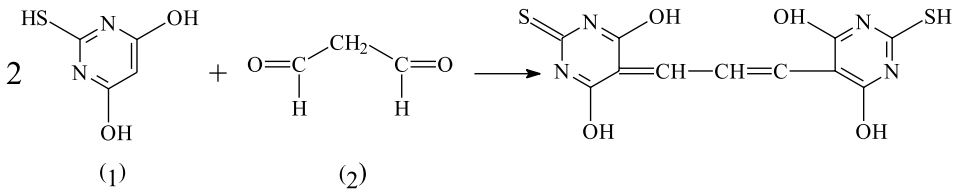
3.2.1. Methods of measurement

One of the mostly frequently used methods in free radical research is the determination of the thiobarbituric acid (TBA) adduct and MDA in plasma and/or urine. MDA is one of the free radical breakdown products of lipid peroxidation. The test for TBA-reactive substances (TBARS) was developed to determine the degree of peroxidation of fatty acids in foods as a measure for rancidity (Yu and Sinnhuber, 1957). In most peroxidation processes, small amounts of malondialdehyde are formed. In the TBA test, MDA is heated with TBA under acidic conditions. Malondialdehyde will then react with TBA, forming an adduct that absorbs at 532 nm (Fig. 8). An improved method uses the fluorescence of TBA–MDA adduct, which has an excitation wavelength of 531 nm and emits at 553 nm.

Unfortunately, TBA reacts with various other substances too. Measuring the fluorescence of the malondialdehyde adduct gives some improvement in specificity. A popular method present is to separate the malondialdehyde product, and the interfering components, using HPLC. The exact intensity of colour that is formed during the TBA reaction depends on the type and strength of the acid involved in the reaction.

The direct Schiff's reaction has long been employed for the histochemical identification of aldehydes in tissues, and was used for the first visualisation of lipid peroxidation in cryostat sections of rat liver (Benedetti et al., 1984). The same procedure was applied, with success, to the detection of lipid peroxidation induced in vivo in the whole animal, by glutathione-depleting agents (Pompella et al., 1987), as well as to other experimental conditions, such as the selective involvement of rat *substantia nigra* during iron-induced lipid peroxidation in vitro (Tanaka et al., 1992) and of rat tubular proximal epithelium during lipid peroxidation in vivo induced by the nephrocarcinogen iron nitrilotriacetate (Toyokuni et al., 1990).

The distribution of LPO in relation to cell injury in rat livers subjected to anterograde versus retrograde perfusion with different pro-oxidant toxins and under different oxygen tensions has been studied (Masuda and Yamamori, 1991a,b). However, the use of Schiff's reaction is limited by poor reproducibility, and the strong acidity of the reagent can induce false-positive results in tissues rich in plasmalogens, such as myocardium, where the so-called 'pseudoplasmal' reaction can be seen. These difficulties have stimulated the development of alternative procedures.



(1) (2)

Fig. 8. Reaction of TBA (1) and MDA (2) leading to the TBA–MDA adduct (3).

Good results have been obtained using a reaction based on 3-hydroxy-2-naphthoic acid hydrazide (NAH) followed by coupling with a tetrazolium salt; the reliability of the NAH reaction was assessed by means of microspectrophotometric analysis of tissue sections and comparison with data obtained by biochemical determination of LPO in the same specimens (Pompella and Comporti, 1991). The NAH reaction allowed visualisation of regions first affected by lipid peroxidation in vivo, following haloalkane toxicity and at a lower detection limit than found using the direct Schiff's reaction (Pompella and Comporti, 1991).

A further improvement in the histochemistry of lipid peroxidation was obtained with the employment of fluorescent reagents. Fluorochromes result in an appreciable increase in the sensitivity of detection if combined with analysis by confocal laser-scanning fluorescence microscopy with image video analysis. Interesting results with this procedure have been obtained by exploiting the fluorescence of the NAH reagent itself (Pompella and Comporti, 1993). An alternative approach to fluorescent derivatisation of cellular carbonyls uses a biotin-labelled hydrazide coupled with fluorescent-conjugated streptavidin in studies focused on cultured neural cells (Harris et al., 1994).

An additional tool for the detection of LPO is the naturally fluorescent fatty acid, *cis*-parinaric acid. Once pre-loaded in living cells, *cis*-parinaric acid is readily consumed during lipid peroxidation, thus allowing the monitoring of the lipid peroxidation process in the form of a fluorescence decrease (Hedley and Chow, 1992).

3.2.2. Storage, stability and limitations on use

Despite its simplicity, many errors can be made with the interpretation of the results of the TBA test. One reason for this is the instability of MDA. MDA that is produced in its free form in vivo will quickly be metabolised to carbon dioxide and acetic acid by aldehyde dehydrogenase. For this reason, it is also impossible to make an acceptable calibration curve with MDA itself. To create a calibration curve for MDA measurements, the adduct must be prepared from a derivative, by hydrolysing it in a solution that contains TBA in order to form a stable product. MDA can also be formed from arachidonic acid during the derivatisation reaction of MDA with TBA at a temperature of 95 °C. During this procedure, degradation of biomolecules, like fatty acids, can occur leading to false information about the amount of MDA. Another disadvantage of the TBA test is its cross-reactivity with other materials. Many products such as biliverdin, acetaldehyde-sucrose, and reducing sugars – including deoxyribose, deoxyglucose, methionine, glutamic acid and many others – react with TBA forming chromogens identical to a product that is formed when MDA reacts with TBA (Gutteridge, 1981; Halliwell and Gutteridge, 1981, 1989). The application of the TBA assay to human body fluids will also measure MDA as a breakdown product of endoperoxides, which are enzymically formed by cyclo-oxygenase in the prostaglandin synthesis pathway (Watanabe et al., 1979). The amount of dietary fatty acids affects the determination of the MDA.

The determination of MDA with the TBA test leads to an overestimation of free radical damage. The simple TBA test, which picks up all substances reacting with TBA to form adducts absorbing at 532 nm, is still widely used as a measure of

oxidative stress but it is dramatically flawed (Halliwell and Chirico, 1993). The HPLC assay accurately measures MDA–TBA, but there is no guarantee that the MDA present has arisen from oxidative processes. Finally the exact intensity of colour that is formed during the TBA reaction depends on the type and strength of the acid that is used. Since different laboratories use different TBA assays, it is difficult to compare results from the literature.

3.3. Conjugated dienes

3.3.1. Method of measurement

Conjugated dienes are primary products of the breakdown of fatty acids. Since conjugated dienes normally do not occur in living cells, they are considered to be specific products of free radical reactions. Their structure and mechanism of formation are described in Fig. 7.

Conjugated dienes can be measured spectrophotometrically in the UV-region, at 230–235 nm. A disadvantage of the diene assay is that many other biological substances, even the polyunsaturated fatty acids, absorb in the same UV-region. In addition, no suitable reference material is available. This is absolutely necessary because of the background absorption of the polyunsaturated fatty acids. Corongiu et al. achieved a greater sensitivity for the diene assay by applying second-derivative spectroscopy (Corongiu et al., 1986), and improved analytical methods like HPLC and GC–MS (Iversen et al., 1984, 1985) have been developed.

3.3.2. Storage, stability and limitations of use

The validity of the methods to measure lipid peroxidation is still questionable (Thompson and Smith, 1985). For the reasons mentioned above, the conjugated diene assay is not suitable for determination of lipid peroxidation in vivo. The conjugated diene assay can be useful when applied to studies that use only pure lipids. It is imperative to state that this assay should not be used on human tissues or body fluids. The plasma diene conjugate is >90% derived from 9,11 diene-conjugated linoleic acid, derived from dietary dairy products.

3.4. LDL lag phase

3.4.1. Method of measurement

Essentially a way of measuring the oxidisability of plasma LDL, the lag phase assay gives an estimate of the state of antioxidant protection of lipids in the plasma. The induction of conjugated dienes over time, following incubation with copper or the radical initiator AAPH, is recorded in order to calculate the 'lag time' before the exponential rise in conjugated diene formation. The method is as for conjugated dienes (Section 3.3) using isolated LDL in this case rather than total plasma.

3.4.2. Storage, stability and limitations of use

LDL is very susceptible to artefactual oxidation ex vivo, and isolated LDL should be prepared rapidly in the presence of antioxidants at 4 °C, with the headspace flushed with nitrogen.

The relevance of copper to the physiological oxidation of LDL remains unanswered, and this assay is perhaps better viewed as a bioassay of vitamin E status. It is worthwhile to note that use of copper as an initiating catalyst in this process may, ultimately, lead to α-tocopherol enhanced oxidation of LDL.

3.5. Hydrocarbon gases

3.5.1. Biochemistry

During lipid peroxidation hydrocarbon gases, such as pentane and ethane, are formed (Riely et al., 1974). The concentrations that are formed are rather low, and depend on the presence of transition metals to decompose peroxides. Another pathway, in which alkanes are formed, is the reaction of a carbon-centred radical with an oxygen molecule, which leads to the formation of an alkoxy radical. The alkoxy radical can undergo a β-scission leading to an alkyl radical and an aldehyde. In many studies, the formation of alkanes in the C2–C5 range is analysed, though ethane and pentane are most frequently used. Since pentane can be metabolised in vivo (Wade and van Rij, 1985), ethane is a better marker for lipid peroxidation.

3.5.2. Method of measurement

Currently, the analytical method that is used most frequently is a capillary GC method, with an adsorption/desorption sample-handling method. The analytical methods used to determine alkanes have been reviewed (Schaffer, 1989). Advances in adsorption technology trace gas analysis are permitting more progress in this field (Andreoni et al., 1999).

3.5.3. Storage, stability and limitations on use

Several disadvantages exist when using alkanes as markers for lipid peroxidation. A high oxygen tension can lead to the formation of peroxides, by the alkyl radicals, instead of a hydrogen abstraction. It is important to know the concentration of oxygen in the target organ at the time of alkane measurement. Also, the blood flow through the target organ plays an important role (Allerheiligen et al., 1987). During heavy exercise, the blood flow to the muscles increases leading to a higher release of the alkanes stored in the membrane. Different target organs metabolise alkanes at different rates. This will lead to a so-called 'first pass effect', where alkanes in the venous blood flow will first pass the lung prior to other organs. Another problem that arises is the background signal. Pathways other than lipid peroxidation can form alkanes, but they can also originate from air pollution. Careful sampling and interpretation of the results should take place, in order to prevent misinterpretations of the results. Another issue of concern is the background level of isoprene and pentane in human breath since the two are difficult to separate by gas chromato-

graphy (GC). Finally, hydrocarbon gases are produced by bacteria, and can be influenced by variation in levels of air pollution.

3.6. Lipofuscin

3.6.1. Biochemistry

The term lipofuscin refers to autofluorescent lysosomal storage bodies that accumulate in cells during ageing. The molecular composition and the mechanisms of formation of these cellular inclusions are not known in great detail.

It is well established that deficiencies in antioxidant micronutrients result in massive accumulation of autofluorescent lysosomal storage bodies in many cell types of a variety of organisms. This observation has been interpreted to indicate that tissue oxidative damage is involved in lipofuscin formation. However, there is evidence that the composition of the storage bodies, which accumulate as a consequence of antioxidant deficiencies, differs significantly from that of lipofuscin.

3.6.2. Method of measurement

An HPLC assay for A2-E, a primary fluorophore, has been developed for human RPE lipofuscin, which is both precise and rapid (Sundelin and Nilsson, 2001). Fluorescence activated cell sorting (FACS) has been used previously to quantify age-related changes in the total autofluorescence of whole cells such as neurones, fibroblasts and macrophages in vitro (Sheehy, 2002).

3.6.3. Storage, stability and limitation on use

The assay for A2-E is both precise and rapid, but unlikely to be applicable to lipofuscin in other tissues. While the sample-processing rate is at least an order of magnitude greater using FACS, quantitative microscopy yields a much more precise estimate of lipofuscin concentration but is labour-intensive. The new FACS approach may be ideal for applications requiring only semi-quantitative results, but it is unlikely to be suitable for studies where lipofuscin measurements need to be as precise as possible.

3.7. Lipid peroxides

3.7.1. Biochemistry

Lipid peroxides are formed when free radical reactions occur in the cell membranes or in LDL. MDA, measured after hydrolysis of the lipid peroxides, is determined as an indirect quantitative measurement. However, there are several disadvantages (see Section 3.2.1).

7-Oxygenated sterols are the major oxysterols associated with lipid peroxidation in biological membranes and lipoproteins. The two hydroperoxides are the primary labile products, which are rapidly reduced to corresponding hydroxy or dehydrated to oxo-derivatives. There is strong epidemiological evidence linking atherosclerotic progression with these cholesterol oxidation products (COPS).

Stimulated neutrophils produce HOCl, via the myeloperoxidase-catalysed reaction of hydrogen peroxide with chloride ions. Cholesterol when exposed to HOCl gives rise to several products (Van den Berg et al., 1993) notably the epimeric 5,6-epoxides, with hydroxy derivatives also formed. The formation of chlorohydrin products of cholesterol has been reported although the maximum formation of chlorohydrins occurred between pH 4 and 5 (Hazen et al., 1996). Eosinophil peroxidase and myeloperoxidase catalyse the oxidation of bromide by hydrogen peroxide to produce hypobromous acid (HOBr). Carr et al. (1998) showed that the reaction of HOBr with oleic, linoleic and arachidonic acids leads to the formation of bromohydrins. Other oxidation products, not containing bromine, such as dihydroxy derivatives are also detected (Carr et al., 1996). Experiments with purified *Escherichia coli* phospholipids exposed to HOCl and HOBr showed the formation of haloamines and halohydrins (Carr et al., 1998). Bromohydrins were formed directly or through the action of bromamines. Bromohydrins and chlorohydrins can be used as lipid oxidation markers originating from neutrophils and eosinophils.

3.7.2. Method of measurement

Lipid peroxides can be determined by means of an iodimetric method. In this method, iodide is oxidised to iodine, which absorbs light at 360 nm (Hicks and Gebicki, 1979; Pryor and Castle, 1984). The I_2 can also be determined by means of a titration with sodium thiosulphate. However, a disadvantage of this method is that it is not applicable to biological fluids. In biological fluids many other chemicals are able to oxidise iodide, leading to an erroneous estimation of the lipid peroxidation.

MDA, hydrocarbon gases, conjugated dienes and 4-hydroxy alkenals (Esterbauer et al., 1991) are all indirect measures of lipid peroxidation. A better and direct method has been developed using HPLC with UV detection (Piretti and Pagliuca, 1989; Terao and Matsushita, 1987); however, the disadvantage of UV detection is that it cannot distinguish between the original lipid, the formed peroxide and the hydroxide. To overcome this problem, detectors with a higher specificity, like electrochemical detection (Funk and Baker, 1985), chemiluminescence (Frei et al., 1988) or mass spectrometry, can be used instead. Another direct method to determine the degree of lipid peroxides is gas chromatography combined with an MS detector. This method is highly sensitive (10 pg), but it has the disadvantage that the lipid peroxide has to be reduced to the hydroxide followed by a derivatisation step during which further oxidation may occur.

3.7.3. Storage, stability and limitations on use

Several sensitive, and selective, techniques exist to determine lipid peroxides in biological samples, although most involve sample pre-treatments that are sensitive to lipid peroxide formation owing to the presence of molecular oxygen. Addition of antioxidants like butylated hydroxyl toluene (BHT), or glutathione, can prevent this problem.

3.8. Isoprostanes

3.8.1. Biochemistry

Isoprostanes are free radical oxidation products of arachidonic acid. Two separate routes of peroxidation, an endoperoxide route and a dioxethane/endoperoxide mechanism, can form the isoprostanes. The free radical product that is used as a marker for oxidative stress is 8-iso-IPF2α. Diets rich in fruit and vegetables diminish the excretion of urinary 8-epi-PGF2a (Thompson et al., 1999).

The isoprostanes in plasma have a short half time, approximately 18 minutes, and are excreted rapidly, which means that they must be formed constantly to maintain a steady-state concentration. They appear to be a good marker for oxidative stress and isoprostanes have potent biological activity in the vasculature, where they are believed to interact with a novel class of prostaglandin receptors exerting discrete effects on platelets and the endothelium (Minuz et al., 1998).

3.8.2. Method of measurement

F2-isoprostanes from biological sources can only be measured as free compounds using GC–MS, and therefore extraction from tissues requires an alkaline hydrolysis step, which removes the esterified form from phospholipid. Following derivatisation using BSTFA, the isoprostanes are carried for negative ion chemical isolation by methane, from 190 to 300 °C, where the ion monitored is the carboxylate anion m/z 569. LC-MS-MS has been adopted as the method of choice for multiple small molecule product analysis (Li et al., 1999).

3.8.3. Storage, stability and limitations on use

Most of the isoprostanes are present in plasma in the esterified form, the rest being present in the free form. The normal concentration of isoprostanes in tissues and body fluids is low, compared to a situation where lipid peroxidation plays an important role. The disadvantage of measuring isoprostanes in plasma is that it is not possible to measure them over a period of time, since the half-life of isoprostanes is too short. In order to measure isoprostanes formed during a given time period, levels have to be measured in urine. However, local kidney peroxidation presents a problem. This is overcome by measuring the metabolite of iso-PFG2α, 2,3-dinor 8-iso PGF1a in urine (Morrow et al., 1990; Morrow et al., 1999). The formation of iso-PFG2α in vivo involves a cyclisation step, where a carbon-centred radical is involved. The formation of the isoprostane 5-ring requires that the carbon radical does not react with oxygen, since this will lead to a different class of compounds, the isofuranes.

Post-prandial levels of 8-epiPGF2a in lipoproteins rise following a fatty meal, demonstrating the confounding effect of diet (Gopaul et al., 2000). However, if measurements are corrected for levels of arachidonic acid, there is no post-prandial difference, which emphasises the importance of relating an oxidised metabolite to the parent compound.

Whilst artefactual formation of isoprostane products does occur at −20 °C, storage at −70 °C prevents this, for up to 6 months. Addition of BHT or TPP to the sample assists in stability (see Fig. 9).

3.9. Possible new biomarkers of lipid oxidation

Another possible new marker is the recently described isolevuglandin adduct (Brame et al., 1999; Roberts et al., 1999a,b). Isolevuglandins are highly reactive γ-ketoaldehydes, which are formed via a non-enzymic rearrangement in the isoprostane pathway. Isolevuglandins bind covalently with proteins, and cause protein–protein as well as protein–DNA cross-linking (Salomon et al., 2000b). Levuglandins form oxidised pyrole adducts (lactams and hydroxylactams) with the ε-amino group

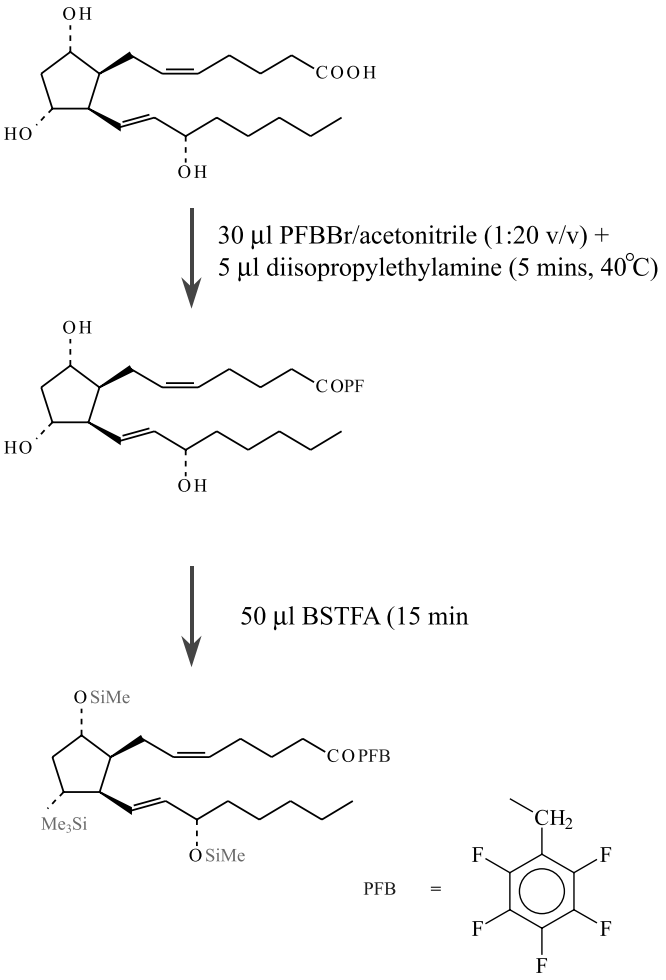


Fig. 9. Derivatisation of 8-iso-PGF2α for mass spectrometry.

of lysine (Roberts et al., 1999b). Isolevuglandin–protein adducts in human plasma are more closely correlated with cardiovascular disease than are the classical risk factors LDL or total cholesterol (Salomon et al., 2000a,b). HPLC Tandem Mass Spectrometry and Western Blot analysis of polyacrylamide gels have been developed (Brame et al., 1999; Roberts et al., 1999b; Salomon et al., 2000a,b).

Nitration of unsaturated fatty acids by NO-derived reactive species can also be determined by GC–MS (O'Donnell et al., 1999).

3.10. Relationship of lipid peroxidation to disease

Peroxidation appears to be important in atherosclerosis, and in worsening the initial tissue injury caused by ischaemic or traumatic brain damage. However, oxidative stress can damage many biological molecules; indeed, proteins and DNA may be more significant targets of injury than lipids, and lipid peroxidation often occurs late in the injury process. Many assays are available to measure lipid peroxidation, but no single assay is an accurate measure of the whole process.

Pentane and ethane exhalation have also been demonstrated in a variety of clinical conditions associated with oxidative stress, e.g. rheumatoid arthritis, myocardial infarction, multiple sclerosis, smoking, infection with the human immunodeficiency virus (HIV), inflammatory bowel disease and the respiratory distress syndrome (Aghdassi and Allard, 2000).

Whilst there are many old reports in the literature describing conjugated diene levels in different disease states, they cannot be relied on owing to the possible interferences from diet. Similarly, the ability to measure lipofuscin accumulation accurately is essential before any understanding of its role in physiological ageing, and human disease, can be extended. HPLC and GC/MS analyses of hydroperoxides in normal plasma gives levels lower than those reported by TBA-based tests. However, this may reflect the lack of specificity of the latter. Levels are elevated in cardiovascular disease (Salonen et al., 1997). Certain F2-isoprostanes have been shown to be elevated in conditions that pre-dispose to accelerated development of cardiovascular diseases such as diabetes, asthma, hypercholesterolaemia and cigarette smoking (Wood et al., 2000a). They are also suggested to evoke important biological effects in smooth muscle and platelets (Minuz et al., 1998) promoting thrombotic events. Table 24 summarises the normal ranges and LOD for several common LPO products.

3.11. Modulation of lipid peroxidation biomarkers by antioxidants

Diene conjugate measurement has not been applied to supplementation studies for several reasons (Section 3.3.1) nor is there a suitable reference material. A simple method for isolation of LDL from plasma to allow baseline determination of conjugated dienes is reported (Ahotupa and Asankari, 1999). In these studies the absorption can be followed, and can be seen as an early stage of lipid peroxidation. No reports have evaluated effects of antioxidant intake on lipofuscin levels in tissue. However, in a randomised trial, 123 healthy individuals were fed a control diet – low in fruits, vegetables, and dairy products – with 37% of calories from fat. Participants

were then randomised to consume for eight weeks: (i) the control diet, (ii) a diet rich in fruits and vegetables but otherwise similar to the control diet, and (iii) a combination diet rich in fruits, vegetables, and low-fat dairy products and reduced in fat. Alkane exhalation was determined to be significantly reduced by the diets rich in the fruits and vegetables ($p = 0.04$ compared to control) and the combination diet ($p = 0.005$ compared to control) (Miller III et al., 1998).

The popularity of the TBARS assay is exemplified by its use in antioxidant intervention studies both in humans and in animals. During a fish oil supplementation study in 46 menopausal women, the addition of α-tocopherol (400 mg/day) for 15 days was able to block the rise in TBARS seen with fish oil supplements alone (Wander and Du, 2000). The effects of β-carotene supplementation on TBARS in 24 patients with cystic fibrosis during twelve weeks supplementation, at a dose of 1 mg/kg (max 50 mg/day), showed a significant decrease in plasma levels (Rust et al., 1998). Again, it is reported that flavonoids caused a reduction in plasma TBARS in five subjects taking increasing doses (as juice) for up to three weeks (Young et al., 1999). These data are reviewed in Tables 4–7.

Several studies that have used LDL oxidation lag time to assess the benefits of antioxidant supplementation (Table 7). The effects of short-term (four weeks) dietary supplementation with tomato juice (500 ml/day), vitamin C (500 mg/day) and vitamin E (800 U/day) in 57 well-controlled type II diabetics have been examined (Upritchard et al., 2000). Supplementation with tomato juice, which increased plasma lycopene levels threefold, caused a 42% increase in lag time ($p = 0.001$). An independent study in 42 type I diabetics supplemented with vitamin E only (400 IU/day) found no evidence of increased lag time in the disease group; however, control subjects did demonstrate an increase in resistance to LDL oxidation (Astley et al., 1999). In another study of the effects of non-alcoholic red wine extract or quercetin for two weeks in 21 healthy males, the lag time for copper-initiated oxidation of LDL ex vivo was increased significantly for both groups (Chopra et al., 2000). Portkaala has also successfully used this assay to demonstrate positive effects of tocopherol supplementation in the ASAP study (Porkkala-Sarataho et al., 2000).

Cholesterol hydroperoxides have been examined in the ASAP study, in which vitamins C (500 mg/day) and E (182 mg/day) were given as supplements, either independently or together, over a minimum of a year and up to three years (Porkkala-Sarataho et al., 2000). Whilst vitamin C alone had no significant effect, both vitamin E and also combined vitamins C and E caused a significant reduction in serum 7-betahydroxycholesterol (Table 4).

The apoE−/− knockout mouse is a good model system in which to test the role of antioxidants in the preservation of health. Letters et al., 1999 report that progressive atherosclerosis in apo E −/− mice is associated with aortic lipid peroxidation as assessed by the concentrations of hydroperoxides and hydroxides of cholesteryl esters, measured by HPLC.

Recently it was reported that the formation of isoprostanes is dependent on oxygen tension, a higher oxygen tension leads to less isoprostanes (Anon, 2000). A suggestion was made that to prevent the influence of oxygen tension both the isoprostanes and the isofuranes should be measured. Studies using knockout animals

Table 4
Antioxidant supplementation effects on lipid peroxidation as lipid peroxides

Dietary supplement(s)	Dose	Sample no	Duration	Format	Tissue	Analyte	Effect observed	Method	Reference
Vitamin C Vitamin E β-carotene Selenium Cu Zn	60 mg/day 30 IU/day 3 mg/day 40 ug/day 80 mg/day 2 mg/day	30 passive smokers	60 days		Plasma	Lipid peroxide	decrease		Howard et al. (1998)
Vitamin C+ Vitamin E+ β-carotene + Selenium + Zinc +	500 mg/day 400 IU/day 20,000 IU/day 100 mg/day 30 mg/day	30 marines	14 days	Placebo controlled winter training	Plasma Urine	Lipid peroxides TBARS HNE	Exercise-induced increase (30%) is prevented with supplement	HPLC Both placebo and supplement increased oxidation	Pfeiffer et al. (1999)
Vitamin C+ Vitamin E	500 mg/day 182 mg/day	48	1115 days	Placebo controlled	Plasma	7 β-hydroxy cholesterol	Decrease with E alone and C combined	HPLC	Porkkala-Sarataho et al. (2000)

have provided evidence of the sources of isoprostanes, and their association with atherosclerosis; 12,15 lipooxygenase knockout animals crossed with ApoE knockout animals showed a reduction in lesion development and urinary isoprostanes compared to ApoE knockout animals (Cyrus et al., 2001). Using the streptozotocin model of diabetes in the rat, a reduction in levels of 8 epiPGF2a was observed on supplementation with vitamin E. However, this was not mimicked by changes in the degree of endothelial impairment (Palmer et al., 1998).

Several groups have investigated the effects of vitamin E supplementation, in humans, on levels of urinary isoprostane metabolites. The studies have varied in duration of supplement, dose, number of subjects and population. Fourfold higher levels of urinary isoprostane metabolite in normal subjects were observed in one study (Marangon et al., 1999) compared with another (Meagher et al., 2001). A 27% reduction in metabolite excreted was measured following a dose of 596 mg vitamin E per day for 48 days. No reduction across a dose range of 200–2000 mg vitamin E per day was observed in the latter study (Meagher et al., 2001).

No protection was observed in smokers, at doses over 5–21 days, although heavy smokers excreted higher levels of urinary isoprostane metabolites (Pratico, 1999; Reilly et al., 1996). In contrast, four studies have examined vitamin E supplementation in disease. Significant reductions in subjects with hypercholesterolaemia, diabetes and prothrombotic states were observed, but no benefit was observed in cystic fibrosis patients (Ciabattoni et al., 2000). Whilst dietary intake of fatty foods has been shown not to influence the levels of plasma isoprostanes provided correction for arachidonic acid is made, a study directed at determining the effects of an antioxidant-rich diet in normal subjects again had no effects on isoprostane levels.

Table 5 describes some supplementation studies that have been undertaken using F2α isoprostane as an endpoint in vivo.

3.12. Functional consequences of lipid peroxidation

Levels of phosphatidylcholine fragments are increased in plasma during various forms of oxidative stress. The concentration of phosphatidylcholine fragments is higher in elderly people; after smoking one cigarette in healthy adults; during reperfusion after treatment with cardiopulmonary bypass; and in vitamin E-deficient rats that were exposed to high oxygen concentration.

The biological activity of phosphatidylcholine fragments is the initiation of the platelet activating factor (PAF) receptor. In the normal biological pathway, one of the components that initiates the PAF-inflammatory receptor is PAF. PAF-like fragments can also be formed via the free radical oxidation of cellular and lipoprotein phosphatidylcholine. The fragments that are formed after the oxidation of phosphatidylcholine can be divided into two different types, the PAF-like phospholipids and the phospholipid fragments, which react according to an alternative mechanism. The biological synthesis of PAF is a two-step process that is heavily controlled by the intracellular free calcium. The first step in the process is the removal of an *sn*-2 fragment after which an acyl fragment is transferred via acetyl coenzyme A (acetyl CoA). This process is regulated by phosphorylation and an increase in free cellular

Table 5
Antioxidant supplementation effects on lipid peroxidation as urinary isoprostanes

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin E	596 mg/day	15	48 days		Urine	Isoprostane III metabolite	27% decrease	RIA	Marangon et al. (1999)
Vitamin E	0 mg/day	12 smokers	21 days		Urine	Isoprostane III metabolite	No effects	RIA	Patrignani et al. (2000)
	201 mg/day 402 mg/day 804 mg/day	15–30/ day							
Vitamin E	100 IU/day	5 smokers	5 days		Urine	Isoprostane III metabolite	No effect	GC/MS	Reilly et al. (1996)
	80 IU/day	15–30/day 5 smokers >30/day							
Vitamin E	900 IU/day	7 prothrombotic state	28 days	Placebo controlled	Urine	Isoprostanes metabolite	iPF2α-III decrease 45% iPF2α-VI decrease 30%	GC/MS	Pratico (1999)
Vitamin E	402 mg/day	10 NIDDM	14 days		Urine	Isoprostane III metabolite	43% decrease	RIA	Davi et al. (1999)
Vitamin E	67 mg	22	14 days		Urine	Isoprostane III metabolite	34% decrease	RIA	Davi et al. (1997)
	472 mg	Hypercholesterolaemia					58% decrease		
Vitamin E	300 mg, twice daily	Liver cirrhosis	30 days	Placebo	Urine	Iso F2aIII	51% drop	RIA	Ferro et al. (1999)
Vitamin E		36 cystic fibrosis			Urine	Isoprostane III metabolite	No effect	GC/MS	Ciabattoni et al. (2000)

Table 5 (*continued*)

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin E	200 mg/day	5	48 days	Placebo	Urine	Isoprostane III metabolite	No effect	GC/MS	Meagher et al. (2001)
	400 mg/day	5							
	800 mg/day	5							
	1200 mg/day	5							
	2000 mg/day	5							
Vitamin C	4 g/day	Healthy subjects	14 days				37% fall in F2		
Vitamin C	2 g/day	Smokers	5 days	Placebo controlled crossover	Urine	8-epiPGF2a	22% drop	GC/MS	Reilly et al. (1996)
Diet rich in antioxidants		Smokers (22)	21 days	Placebo controlled crossover	Urine	8-epiPGF2a	No change	GC–MS	Van den Berg et al. (2001)

Table 6
Antioxidant supplementation effects on lipid peroxidation as TBARS or MDA

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Alpha tocopherol	400 mg/day	46 menopausalwomen	15 days	Fish oils	Plasma	TBARS	No change	HPLC	Wander and Du (2000)
Grape seed extract	200 ml contaminating 31.3 mg phenolics	15	7 days	Crossover	Plasma total lipid or LDL	MDA	No effect		Young et al. (2000)
β-carotene	1 mg/kg to a max dose of 50 mg/day	24 cystic fibrosis patients	84 days	14 healthy controls	Plasma	TBARS	Decrease	HPLC	Rust et al. (1998)
Blackcurrant and apple Juice	750 ml 1000 ml 1500 ml/day	5	7 days/dose	Crossover	Plasma	TBARS	Decrease	HPLC	Young et al. (1999)
Lycopene	39–150 mg/day	19	7 days		Plasma	TBARS	NS trend towards protection	HPLC	Young et al. (1999)

Table 7
Antioxidant supplementation effects on lipid peroxidation as LDL lag time

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Tomato juice Vitamin C Vitamin E	500 ml/day 500 mg/day 800 IU/day	57 well controlled diabetics	28 days	Placebo	Plasma	LDL lag time	42% increase in lag time with tomato juice	Spectroscopy	(Upritchard et al., 2000)
Folic acid Alpha tocopherol Ascorbic acid β-carotene Vitamin B12	0.65 mg 150 mg 150 mg 12.5 mg 0.4 mg	23 patients with coronary artery disease	15 days		Plasma	In vitro LDL oxidation	Decrease in LDL oxidation of Hcy	Spectrophotometric	(Bunout et al., 2000)
Vitamin E	400 IU/day	42 diabetics (type 1)	56 days	31 Normal controls	Plasma	LDL lag time	Positive effect observed in normal only	Spectroscopy	Astley et al. (1999)
Red wine extract Quercetin	1 g/day 30 mg/day	21 healthy males	14 days		Plasma	LDL lag time	Positive effect in both supplemented groups	Spectroscopy	Chopra et al. (2000)

calcium concentration. The functional groups in PAF that interact with the PAF receptor are the choline group, the *sn*-1 ether bond and the *sn*-2 acyl group. Considering the free radical oxidation process of phosphatidylcholine, a variety of oxidation products are formed not all of which mimic the PAF function. Most of the PAF-like products are oxidised phosphatidylcholines, with four acyl *sn*-2 residues.

As mentioned before, the free radical formation of oxidised phosphatidylcholines, which have PAF-like properties, is uncontrolled. Therefore, this process can lead to a high concentration of molecules that can induce inflammatory agents.

Oxidation of phosphatidylcholine generates other non-PAF-like components, which also act as inflammatory mediaters (but not via the PAF receptor). The exact mechanism of the latter process is not yet fully understood.

3.13. Contribution of dietary intake to lipid peroxidation products

Food lipids can undergo lipid peroxidation creating potentially toxic compounds with subsequent damage to surrounding tissues. Cholesterol can autoxidise in the presence of oxygen, particularly in dehydrated foods stored in air, and at least 30 oxidation products have been detected.

The toxic effect of lipid peroxidation products only occurs if they are absorbed and incorporated into body tissues. A study with labelled compounds in animals showed that little if any lipid peroxide is absorbed intact, so no tissue incorporation occurs (Gurr, 1999). Other animal studies, however, showed an increase in the relative weight of the liver, increased MDA, peroxide and carbonyl concentrations in tissue, and a decrease in α-tocopherol and linoleic acid contents (Gurr, 1999). Since the labelling study showed negligible absorption of lipid hydroperoxides, the question remains: what is the mechanism of this toxicity? The answer may be that breakdown products, such as hydroperoxy alkenals, with a lower molecular weight are absorbed, and even more damaging than the lipid hydroperoxides. More research is needed to see if intact lipid peroxides, hydroperoxides and hydroxy alkenals are absorbed in humans. Preliminary results show already that this might be the case.

4. Biomarkers of DNA oxidation

4.1. Introduction

Free radical attack upon DNA generates a range of DNA lesions, including strand breaks and modified bases. Hydroxyl radical (·OH) attack on DNA leads to a large number of pyrimidine- and purine-derived base changes. Some of these modified DNA bases have considerable potential to damage the integrity of the genome (Dizdaroglu, 1991; Floyd, 1990).

8-oxo-2′-deoxyguanosine (8-oxo-dGuo) is one of the most critical lesions. The presence of 8-oxo-dGuo residues in DNA can lead to GC to TA transversion, unless repaired prior to DNA replication (Cheng et al., 1992). The presence of 8-oxo-dGuo may, therefore, lead to mutagenesis. Furthermore, many observations indicate a

direct correlation between 8-oxo-dGuo formation and carcinogenesis in vivo (Feig et al., 1994; Floyd et al., 1986). Oxyradicals induce mutagenesis of hotspot codons of the human p53 and Ha-Ras genes (Hussain et al., 1994; Le Page et al., 1995). In agreement with this finding GC to TA transversions are frequently detected in p53 gene and in ras protooncogene, in the case of lung carcinomas and primary liver cancer (Cheng et al., 1992; Hussain et al., 1994; Le Page et al., 1995). However, this change is not a unique marker for oxidative damage, as other DNA-damaging agents cause the same base change.

The mutagenic and carcinogenic potential of any modified DNA base is reflected in its miscoding properties. It has been demonstrated that several other bases have miscoding potential. The presence of 2-hydroxyadenine (2-OH-Ade) in DNA may induce A to C and A to T transversions, and an A to G transition (Kamiya and Kasai, 1995). 2-Hydroxy-deoxyadenosine-triphosphate (2-OH-dATP) is a substrate for DNA polymerase, and may be incorrectly incorporated by DNA polymerase (Kamiya and Kasai, 1995). 8-hydroxy-adenine (8-oxo-Ade) also has miscoding properties and induces mutation in mammalian cells (Kamiya et al., 1995). 5-Hydroxycytosine (5-OH-Cyt) has been shown to be a potentially pre-mutagenic lesion, leading to a GC to AT transition and a GC to CG transversion. 5-OH-Cyt appears to be more mutagenic than any other product of oxidative DNA damage (Feig et al., 1994). It is also possible that derivatives of guanine other than 8-oxo-Gua have miscoding properties (Ono et al., 1995). On the other hand, the biological consequences of other base modifications, like 4,6-diamino-5-formamido-pyrimidine (FapyAde), 5,6-dihydroxyuracil (5,6-diOH-Ura) and 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), have not been investigated. It is conceivable that these lesions may be pre-mutagenic as well.

Different approaches to measuring the extent of base oxidation in DNA, detecting oxidised bases/nucleosides in urine, and investigating other kinds of oxidative damage as well as the consequences of this damage are described.

4.1.1. Confounding factors

One of the confounding factors in the application of DNA oxidation biomarkers has been the lack of consensus on basal levels, when different research groups undertook similar analyses. This is being directly addressed through the EU funded ESCODD programme. Furthermore, there has been difficulty in comparing data between groups, arising from application of various methods for quantification.

4.1.2. Units and terminology

It is natural that different laboratories should have preferred ways of expressing their results, but this can lead to mutual incomprehension, misconceptions and alarming ambiguities. In the field of DNA damage, there is a particularly wide choice of units. Results from GC–MS are often expressed as nmol (or pmol or fmol) of oxidation product (such as 8-oxoguanine) per mg (or μg) of DNA. To compare results in the literature, we have to juggle with factors of 10^3 and can easily lose our hold on reality. The preferred unit for HPLC has been the number of oxidised bases relative to unoxidised (e.g. n 8-oxo-dGuo per 10^5 dG). The choice of 10^5 rather than

10^6 seems irrational, since jumps of three orders of magnitude are the norm. Sometimes, results are present as 'n 8-oxo-dGuo per 10^6 bases', but does this mean per 10^6 guanines, or per 10^6 total DNA bases?

A group of methods – alkaline elution, alkaline unwinding and the comet assay – all depend on converting lesions to DNA breaks, with a lesion-specific endonuclease, and then measuring the break frequency. The assays are calibrated against the DNA-breaking ability of X-rays, which is known in terms of DNA breaks per 10^9 Da per Gy. This can be easily converted to breaks per 10^6 bases, or per 10^6 base pairs (another potential source of confusion; if there are n breaks per 10^6 bases of single-stranded DNA, how many breaks are there per 10^6 base-pairs of double-stranded DNA?) or even breaks per μm of DNA.

It is tempting to express results as a number of breaks per cell, if we know the DNA content, but this varies, of course, according to the phase of the cell cycle. To convert from DNA break frequency related in some way to DNA length, to relative frequency of the damaged compared to undamaged bases, requires knowledge of the relative frequency of the particular base; in humans, only about 20% of the bases is guanine. Based on the relative content of the four bases in human DNA, and their molecular weights, we can say that:

- 1 *8-oxo-Gua per* 10^9 Da is equivalent to 1 8-oxo-Gua per 3.06×10^6 bases (or per 1.53×10^6 base-pairs) – i.e. *0.33 8-oxo-Gua per* 10^6 *ases*.
- *1 8-oxo-Gua per* 10^9 Da is equivalent to 1 8-oxo-Gua per 0.61×10^6 Gua – i.e. *1.64 8-oxogua per* 10^6 *gua*.
- *1 fmol 8-oxo-Gua per* μg *DNA* is equivalent to *1.64 8-oxo-Gua per* 10^6 *Gua*.

In ESCODD (2000), it has been agreed that everyone will express results in terms of altered bases (or nucleosides) per 10^6 unaltered bases (nucleosides). It is hoped that this system will come to be used as a *lingua franca* in the literature, even if individual favourite units continue to be used as well.

As for the chemical name of the oxidised form of deoxyguanosine that we measure, this should strictly be 8-oxo-7,8-dihydro-2′-deoxyguanosine although it is commonly referred to as 8-oxo-deoxyguanosine or 8-hydroxydeoxyguanosine. 8-Oxo-dG or 8-oxo-dGuo are acceptable abbreviations (with 8-oxo-Gua for the oxidised base).

4.1.3. Nuclear and mitochondrial DNA damage

Mitochondrial DNA (MtDNA) genes have a mutation rate that is 10- to 17-fold greater than that for nuclear DNA genes, which is thought to be of particular importance in ageing. Characterisation of the forms of DNA base damage products within the two organelles highlights key differences with 5-oxo-Hyd, 5-oxo-MeHyd and 5-oxo-MeURA found in a much higher proportion in relation to 8-oxo-Gua in mitochondrial DNA (Zastawny et al., 1998). Even so, basal levels of 8-oxo-Gua were reported as 0.54 nmol/mg nuclear DNA compared to 2.38 nmol/mg mtDNA. Defective repair of mitochondrial DNA may be of greater relevance to ageing, but is unlikely to have an impact on the development of mutations associated with cancer,

suggesting that analysis of compartmentalised DNA rather than global DNA analysis may yield greater information on oxidative stress in disease. In addition, preferential partitioning of antioxidants between organelles may lead to differential effects on levels of base damage products.

4.1.4. Lymphocytes as surrogate tissues

Lymphocytes are often used as surrogate cells, which are supposed to inform about oxidative stress – measured at a certain level of 8-oxo-dGuo – in other tissues (Collins et al., 1998a; Lenton et al., 1999). However, to our knowledge to date, there have been no experimental data to support such a relationship. Factors responsible for the formation of 8-oxo-dGuo, and for oxidative stress, may vary in different tissues. For example, such factors as the activity of key antioxidant enzymes (e.g. SOD, catalase and glutathione peroxidase), which may influence oxidative stress, vary in different organs (Halliwell and Gutteridge, 1989). Differences in metabolic rate may influence reactive oxygen (ROS) or nitrogen species (RNS) production (Loft et al., 1994), and these differences may also depend on the tissue. Therefore, in a recent study, the question was asked, whether there is a relationship between the myometrial tissues, removed from uterine myoma patients, and lymphocytes regarding amounts of 8-oxo-dGuo (Foksinski et al., 2000). The level of 8-oxo-dGuo was analysed in DNA isolated from: (i) uterine myomas, (ii) marginal tissues free of pathological changes, and (iii) lymphocytes of patients with uterus myoma. Although the mean level of 8-oxo-dGuo in DNA isolated from lymphocytes was lower than that in the tissues, there was no correlation between the amounts of the modified base in lymphocytes and the normal or pathological tissues (Foksinski et al., 2000). Further studies are needed to test whether the absence of the relationship can be extended to other tissues.

4.2. Measurement of DNA damage with the comet assay

The comet assay, or single cell gel electrophoresis (SCGE), is a technique that can be applied to the measurement of oxidative DNA damage in individual cells. It is widely used, and has become refined in the past ten years, by those who appreciate its simplicity and speed (McKelvey-Martin et al., 1993). It can be applied to any cell population, provided they have been isolated from tissue without degradation (Lovell et al., 1999). Although it is common to isolate cells from various animal tissues, the availability of tissues from humans is limited, and at present human population studies tend to use lymphocytes as surrogate tissues (Tice et al., 2000).

The comet assay is essentially a sensitive method for detecting DNA strand breaks. It can also be used to measure oxidised bases, with the addition of a step in which DNA is incubated with bacterial repair endonucleases, which recognise and remove damaged bases and make nicks at the resulting abasic (AP) sites in the DNA. Endonuclease III detects oxidised pyrimidines; formamidopyrimidine DNA glycosylase (FPG) recognises altered purines including 8-oxo-Gua (Collins, 2000).

4.2.1. Practical details

Cells for analysis are embedded in agarose, the cells lysed in detergent with removal of cytoplasmic proteins and most nuclear proteins with high salt, leaving nucleoids, which are then subjected to alkaline electrophoresis. Negatively charged DNA is attracted to the anode, but only the loops of DNA possessing a break are free to migrate, presenting the image of a comet with a tail when subsequently viewed under the fluorescence microscope following fluorescent staining. Tail length increases rapidly at low levels of damage, soon reaching a maximum, but tail intensity continues to increase up to the level of about three breaks per 10^9 Da of DNA. The comet assay is one of the most sensitive measures of strand breakage.

Quantitation of the comet images is achieved by either computer image analysis or a visual scoring system, which has been adopted by many laboratories. Class 0 has no discernible tail and class 4 has almost all DNAs in the tail. Calibration can be achieved against X-ray irradiated cells, where the frequency of strand breaks introduced is known.

The repair endonuclease (endonuclease III or FPG) is introduced after the lysis step. Parallel gels are incubated with buffer, but no enzyme, to give the background level of strand breaks. After electrophoresis, subtracting background comet score from comet score following enzyme treatment calculates the degree of oxidative damage.

4.2.2. Storage, stability, and limitations of the assay

If lymphocytes are frozen according to protocols normally employed to preserve viability, they can be stored for at least one year without significant increase in damage. Because of the nature of the comet assay, it is not possible to include an internal standard. When analysing damage in DNA from lymphocyte samples collected in a population study, or intervention trial, it is recommended to run a standard lymphocyte sample alongside the experimental samples. The standard lymphocytes are from a single lymphocyte preparation (pooled from different volunteers if necessary), frozen as aliquots. Several intervention studies have evaluated the resistance to hydrogen peroxide-induced strand breaks in the comet assay. However, this is complex to interpret, where any resultant alteration to the comet score is a composite of both endogenous antioxidant defences as well as DNA repair capacity. It is unknown whether cell preparation affects the latter.

The great advantage of the comet assay, compared to chromatographic and other assays requiring extensive sample preparation, is that there is little chance for spurious oxidation of guanine to occur. However, there are potential problems:

- Calibration of the assay is indirect. It is done by comparison with the comets produced by a range of doses of ionising radiation, which induce strand breaks with a well-established dose relationship. Results can then be expressed in standard units (breaks per 10^9 Da, or – in the case of base oxidation- 8-oxo-dGuos per 10^6 dGs). It must be admitted that this calibration is rarely done; it is common for results to be expressed simply as % DNA in tail or arbitrary units.
- Calculation of the amount of oxidative damage is dependent on subtraction of background comet score from comet score following enzyme treatment. This is

valid only if the dose response is linear, which is the case at moderate levels of damage, but not when the assay approaches saturation (i.e. most of the DNA is in the comet tails).
- It is possible that FPG does not detect all 8-oxoguanines, though there is no evidence that this is the case. In fact, the DNA after lysis and removal of proteins is in an ideal naked, supercoiled state for enzyme attack.
- If lesions (breaks or damaged bases) occur in clusters, in close proximity within one DNA loop, the comet assay will register only a single lesion. The degree to which clusters occur with endogenous damage is not known.
- On the other hand, as FPG recognises not just 8-oxo-Gua but other altered purines, it may actually overestimate 8-oxo-Gua.

4.3. Measurement of DNA base oxidation by HPLC

HPLC is widely used for the measurement of 8-oxo-dGuo in biological samples of DNA such as animal tissue or white blood cells. The small extent of base oxidation requires the use of electrochemical detection (ECD). Coulometric ECD is more sensitive and is most commonly used, although amperometric detection has been applied successfully in ESCODD trials.

4.3.1. Practical details

Although mitochondrial DNA is a minor component of total cellular DNA, it may be more heavily oxidised, and it is advisable to remove it by isolating nuclei first and preparing the DNA from them. Nuclei are simply prepared from cells by lysis in buffer with non-ionic detergents; tissues must be homogenised. The nuclei are separated by centrifugation, and the DNA released by adding sodium dodecyl sulphate. RNA and protein are digested by adding ribonuclease, incubating at 37 °C for 30 min, then adding proteinase K and incubating at 37 °C for a further 30 min. Short periods of incubation are advised to minimise oxidation. Chloroform/isoamyl alcohol is added, the mixture shaken, and then after centrifugation, the DNA collected from the aqueous layer. After a further chloroform/isoamylalcohol extraction, sodium chloride is added to the aqueous layer to 1.4 M, and after a further centrifugation the DNA is precipitated with two volumes of cold ethanol (Wood et al., 2000b).

An alternative method employs sodium iodide in the DNA precipitation step; this method is claimed to minimise oxidation (Helbock et al., 1998).

Various regimens have been employed to hydrolyse the DNA. The two-enzyme (P1 nuclease and alkaline phosphatase) and four-enzyme (DNase I, phosphodiesterases I and II, and alkaline phosphatase) methods gave similar values for 8-oxo-dGuo in control experiments (Wood et al., 2000), and the two-enzyme method is now more common.

4.3.2. Storage, stability and limitations of the method

Samples can be stored frozen for many months. Cells are frozen using methods normally employed to preserve viability, and kept in liquid nitrogen or at −80 °C

under nitrogen; alternatively, samples are frozen after isolating the DNA again at −80 °C under nitrogen.

Oxidation of guanine, at any stage in sample preparation, is the most significant problem, alleviated by including antioxidants and by carrying out procedures in the absence of oxygen, if possible.

As problems of spurious oxidation of DNA are resolved, and the amount of 8-oxo-dGuo detected in the DNA of normal human cells becomes even smaller, it is increasingly difficult to detect it, even with highly sensitive coulometric electrochemical detection. Increasing the amount of DNA analysed is one option, but columns can be overloaded. In addition to the accurate detection of 8-oxo-dGuo, it is crucial to measure unoxidised dG accurately, since the content of 8-oxo-dGuo is expressed relative to dG.

HPLC does not give a definitive identification of the individual compounds in a mixture. Spiking with standards identifies peaks, and further confirmation can be obtained by carrying out a voltammogram – measuring the electrochemical signal over a range of voltages gives a characteristic profile for different substances; but this evidence is not conclusive.

4.4. Measurement of DNA base oxidation by GC–MS

4.4.1. Biochemistry of 8-oxoguanine, adenine and fapy derivatives

In order to understand the biological consequences of DNA damage there is a need to know the particular type of base modification found in the global DNA damage. The two methods that offer such a possibility are GC/MS and LC–MS–MS, which have been introduced to analyse several lesions of oxidative DNA damage. Enzymatic methods such as the comet assay, although very sensitive, detect the oxidative DNA damage recognised by the particular enzyme, which generally means more than one lesion.

4.4.2. Methods of measurement

Several techniques are currently used to analyse the base products, but GC/MS and LC–MS–MS are the only ones that can unequivocally identify a wide spectrum of oxidatively modified DNA bases. Using these techniques, quantification of samples is achieved by adding an appropriate internal standard to the DNA sample. Custom-synthesised, stable isotope-labelled analogues of a number of modified bases have been obtained for quantitative determination of oxidative base damage in DNA (Dizdaroglu, 1994), using isotope-dilution mass spectrometry.

GC–MS has been particularly prone to oxidation artefacts during sample preparation, and many published results are now open to question. However, current modifications to this technique give values of 8-oxo-Gua in cellular DNA quite comparable with those obtained by HPLC–ECD. To avoid possible artefacts, repair endonucleases (FPG and endonuclease III) can be used instead of acid hydrolysis to liberate the base products from unmodified DNA samples (Jaruga et al., 2000).

For evaluation it is absolutely necessary to use internal standards of the analysed modifications, labelled with stable isotopes. During all steps of the procedure,

appropriate antioxidants should be added, and even traces of oxygen eliminated. Room temperature, rather than high temperature derivatisation (above 100 °C), results in substantial reduction of artefactual formation of 8-oxo-dG. Although pre-purification with HPLC prevents artefactual formation of oxidatively modified DNA bases, this is a tedious and time-consuming procedure (see also (Frelon et al., 2000)). Applying the enzyme FPG instead of acid hydrolysis gives the lowest values of 8-oxo-Gua in cellular DNA. FPG and HPLC techniques have been used successfully to analyse 8-oxo-Gua. However, these approaches may underestimate the concentration of 8-oxo-Gua in cellular DNA. Also the large amounts of pure enzymes required for enzymic hydrolyses of DNA may make the cost of this procedure prohibitive for most laboratories. There were no significant differences between the levels of 8-oxo-Gua in DNA measured by GC/MS when FPG digestion, or formic acid, was used to release the base (Rodriguez et al., 2000). This observation suggests that 8-oxo-Gua is not formed as artefact during derivatisation, or formic acid hydrolysis, under the proper conditions. Artefacts of derivatisation may depend on experimental conditions, especially on the complete exclusion of the oxygen. There remains an urgent need for a consensus on the measurement techniques and conditions between laboratories. The ESCODD trials are an important step in this direction. LC-tandem MS is emerging as the method of choice for multiple DNA oxidation product analysis, bearing in mind the caveats of care taken during extraction and hydrolysis (Anon, 2001).

4.4.3. Storage, stability and limitations of the method

The GC–MS technique, combined with the use of repair enzymes, may be applied to analysis of the background level of individual oxidatively modified DNA bases. The level of damage is quite comparable with the lowest values obtained by the other methods. Taking into consideration the fact that measurement of single base product can give misleading interpretations (England et al., 1998; Podmore et al., 1998), it is reasonable to postulate that the above method is one of the techniques that should be used to measure oxidative DNA damage.

Use of FPG in this modified method means that it is subject to the caveats described in relation to the use of this enzyme in the comet assay (Section 4.2.1).

4.5. Analysis of guanine oxidation products in urine

An alternative approach to assess oxidative DNA damage is measurement of the damage products excreted into urine. It is generally believed that the products of oxidative DNA repair are excreted into the urine without further metabolism (Loft and Poulsen, 1999; Shigenaga et al., 1989; Suzuki et al., 1995), and that 8-oxo-dGuo in urine represents the primary product of repair in vivo. This compound – as a nucleoside – may indicate involvement of nucleotide excision repair pathway (NER) (Cooke et al., 2000; Loft and Poulsen, 1998). However, others report that oxidatively damaged DNA bases are mostly repaired by the base excision repair pathway (BER) although NER may play a minor role (Dianov et al., 1998; Girard and Boiteux, 1997). Several glycosylases, which specifically recognise and remove 8-oxo-Gua in

human cells, have been described (Hazra et al., 1998; Radicella et al., 1997). 8-oxo-dGuo appearing in urine may, in fact, represent oxidation of breakdown products from the DNA of dead cells, as suggested by Lindahl (2001), and assays of 8-oxo-dGuo in urine are best regarded as assays of oxidative stress rather than DNA repair.

4.5.1. Method of measurement

The analysis of 8-oxo-dGuo in urine by GC–MS presents particular difficulties (Helbock et al., 1998; Loft and Poulsen, 1998), and until now there has been no reliable assay for its detection. A new technique allows simultaneous determination of 8-oxo-dGuo and 8-oxo-Gua, in the same urine sample (Ravanat et al., 1999; Weimann et al., 2001, 2002). This method involves a HPLC pre-purification step, followed by gas chromatography with isotope dilution mass spectrometric detection. Since a previous study demonstrated that diet could influence the level of 8-oxo-Gua in rat urine (Park et al., 1992), it was essential to check whether a similar phenomenon is seen in humans. Two groups of subjects were recruited: (i) healthy non-smokers (five males and seven females, average age 32 years) were fed nucleic acid free diet (glucose and starch), and (ii) non-smoking, surgically treated patients (eight males and four females, average age 55 years) for three days were infused with two litres of 10% glucose. Urine samples (24 h output) were collected after three days on the nucleic acid free diet, and three to five days after returning to the normal, unrestricted diet.

These experiments showed that diet has no influence on the level of either product of oxidative DNA damage in human urine (Fig. 10). Possible reasons for the inconsistency between this work, and the earlier study that demonstrated an influence of diet on 8-hydroxyguanine in rat urine (Park et al., 1992), have been discussed elsewhere (Gackowski et al., 2001)

Thus, urinary excretion of 8-oxo-Gua and 8-oxo-dGuo does not depend on the diet, in the case of humans. The level of both the compounds may be a better indicator of formation of oxidative damage in cellular DNA, or of whole-body oxidative stress, than the level of 8-oxo-dGuo alone.

The LC-tandem MS method for analysis of urinary excretion of oxidised bases has been successfully applied (Weimann et al., 2002).

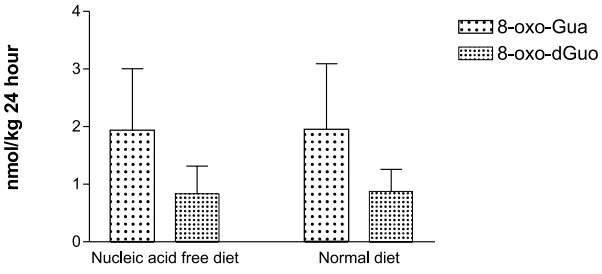


Fig. 10. Urinary excretion of 8-oxo-Gua and 8-oxo-dGuo.

4.5.2. Limitations and criticisms

The possibility that processes, other than repair processes, can contribute to 8-oxo-Gua and 8-oxo-dGuo level in human urine cannot be excluded. A further possible source of the modifications in urine may be the fact that they are derived from dead cells. The urinary level of 8-oxo-Gua may also include a contribution from oxidised RNA, particularly if mechanisms exist to maintain the integrity of RNA molecule although nobody has detected such a mechanism as yet. The oxidation of the cellular nucleotide pool is also a potential source of excreted adducts. All these possibilities remain to be tested.

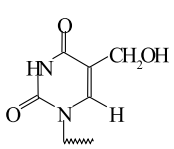
4.6. Immunochemical methods

The principle that DNA could become immunogenic if linked to a carrier molecule, such as methylated serum albumin, was the first step in the development of antibodies as probes for DNA. The next stage demonstrated that oligodeoxynucleotides could also act as haptens for the generation of DNA antibodies. The use of methylene blue to sensitise the photo-oxidation of guanine residues, specifically, generating unknown DNA adducts pre-dated the expansion of the field of free radical biochemistry by many years, and the potential use of these antisera to identify oxidative modification of DNA in vivo was not realised. In fact, it was work carried out on oxygen-radical attack on DNA that led to the characterisation of major products of methylene blue photo-oxidation in this reaction system. 8-oxo-dGuo appears to predominate with some ring opened purine (formamidopyrimidine) formation also occurring.

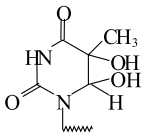
Several antibodies have now been produced, and some are available commercially. Some of the major lesions detected by these antibodies are shown in Fig. 11.

8-Oxo-dGuo was discovered in 1984 (Kasai and Nishimura, 1984), and almost immediately monoclonal antibodies were prepared using principles derived from earlier attempts to produce antibodies to oxidised DNA lesions. 8-Oxo-dGuo was linked to BSA, via a periodate linkage, before immunisation. The antibody generated lacked specificity, however, and no further use of this antibody has been reported.

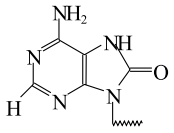
A monoclonal antibody to 8-oxo-dGuo was used in a 'cleanup' procedure for urinary 8-oxo-dGuo prior to HPLC–EC detection (Degan et al., 1991). The antibody was later reported to have three orders of magnitude greater affinity for 8-oxo-dGuo than those produced previously (Kasai and Nishimura, 1984). Both antibodies appeared to lack specificity, and clearly detected native as well as modified bases and related structures, such as uric acid. The use of antibodies in solid-phase extraction procedures to pre-purify analytes of interest in urine, or other complex biological matrices, is a standard technique used prior to HPLC–EC or GC–MS analysis. It is clear that the deoxynucleoside, 8-oxo-dGuo, is increasingly the focus of attention for measurement in urine since its presence is independent of dietary influence. Other groups have developed monoclonal antibodies with far greater specificity for 8-oxo-dGuo. N45.1 is highly specific for 8-oxo-dGuo (Toyokuni et al., 1997) although with



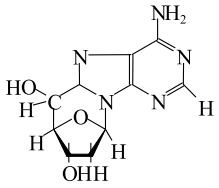
5 -Hydroxymethyl-2' -uracil (18.2.1)



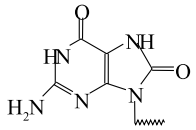
Thymine glycol (18.2.2)



8-Oxoadenine (18.2.3)



8,5'-cyclo-2'-deoxyadenosine (18.2.4)



8-Oxoguanine (18.2.5)

Fig. 11. Representation of structure of major oxidative DNA lesions to which antibodies have been raised.

some orders of magnitude less cross-reactivity with the corresponding RNA oxidation product, 8-oxo-guanosine (8-oxo-Gua).

4.6.1. Methods of measurement

N45.1 has been exploited commercially, in kit form, principally for measurement of 8-oxo-dGuo in urine, and to a lesser extent in serum and cell-culture supernatants. It has also been used successfully in the immunohistochemical localisation of 8-oxo-dGuo in paraffin-embedded sections.

In an ELISA-based assay, the immunogen is electrostatically attached to the 96-well ELISA plate. Urine or 8-oxo-dGuo standards compete with this solid-phase antigen for the antibody. Antibody remaining bound to the plate is localised with a peroxidase-labelled secondary antibody, and thus the lower the final absorbance the higher the concentration in the urine. Baseline measurements in healthy individuals are highly consistent between laboratories, supporting the robust and reproducible nature of this assay. The ELISA procedure can also be applied to DNA following enzymic digestion. However, as with measuring 8-oxo-dGuo by HPLC, the results must be related to the dGuo content of the DNA. The ELISA has comparable sensitivity to HPLC–EC of 8-oxo-dGuo.

Recent studies have explored the possibility of extending histochemical investigations to the detection of oxidised DNA. A quantitative immunohistochemical procedure for the determination of 8-oxo-dGuo, by means of a specific monoclonal antibody, has been developed (Toyokuni et al., 1997). Data have been obtained at the single cell level by Ahmad et al. (1999), employing an anti-8-oxo-dGuo monoclonal antibody as well as antisera specific for thymine dimers, and the oxidative lesions induced in DNA by hydrogen peroxide plus ascorbate.

4.6.2. Storage, stability, and limitations of the assay

As DNA extraction and hydrolysis are required prior to ELISA determination of 8-oxo-dGuo in cells, normal precautions apply including chelating agents and antioxidants during processing (Lunec et al., 1998) to prevent artefacts during processing. Samples are stable when stored as sterile DNA at −80 °C for 6 months.

Despite the ELISA being a sensitive and specific procedure, the antibody used exhibits some cross-reactivity with 8-oxoguanosine (8-oxo-Guo) from oxidised RNA. The latter is still a free radical product so its detection does not detract from the validity of the assay, in terms of monitoring oxidative stress in vivo. There is no cross-reactivity with the base product 8-oxo-Gua. The assumption that 8-oxo-dG exists only in its free monomeric form in urine is incorrect. It may also be present within oligomers of DNA, in urine as a putative excision – repair product, which may account for the discrepancies, and poor correlation, observed ($r = 0.4$) when comparing HPLC–EC and ELISA. The latter gives values four times higher than HPLC–EC (Ahmad et al., 1999). There may also be some cross-reactivity with 7-methyl-8-hydroxydeoxyguanosine, which is excreted from the urine in milligram amounts.

The detection of oxidative damage to DNA by immunochemical techniques has some clear advantages over more technically demanding, and complex, methods such as HPLC–EC, HPLC–MS/MS and GC–MS, particularly in terms of simplicity, reproducibility and versatility. However, its specificity, particularly for urine 8-oxo-dGuo estimation, is unclear.

4.7. ^{32}P post-labelling

The post-labelling assay to analyse aromatic DNA adducts is a very sensitive and reproducible method for DNA adduct analysis of animal and human tissues (Cui et al., 1995; Moller et al., 1996; Zeisig and Moller, 1995) and relies on enzymic incorporation of ^{32}P.

4.7.1. Method of measurement

The post-labelling method is combined with ^{32}P-HPLC analysis to enable analyses of complex mixtures (i.e. human tissues), and also to have a reproducible analytical system (Carlberg et al., 2000; Zeisig and Moller, 1997). The ^{32}P-HPLC method has not been applied to analysis of 8-oxo-dGuo since isotopes (^{32}P) oxidise DNA resulting in a high auto-oxidation giving false high levels (Moller and Hofer, 1997). By a pre-separation of dG and 8-oxo-dGuo, prior to ^{32}P exposure, the risk of oxidising dG is dramatically reduced. By doing this pre-separation, the ^{32}P-HPLC is able to

detect 8-oxo-dGuo in small amounts of DNA – as low as 200 ng, which is approximately 250 times less than what is normally used in HPLC–EC or MS analysis of 8-oxo-dGuo (Zeisig et al., 1999). The amount of work involved in the analysis of 8-oxo-dGuo is less with HPLC–EC but, if the amount of DNA is limited, ^{32}P-HPLC has great advantages, and in non-invasive human analyses, the amount of DNA often is a limiting factor.

4.7.2. Limitations and criticisms

Owing to the radiological hazard, the HPLC must be entirely dedicated to this procedure.

4.8. Validation of assays for DNA oxidation

4.8.1. Oxo-dGuo in lymphocyte DNA

For several years it has been evident that there is a very serious problem in attempting to measure DNA oxidation in biological tissue. Estimates of the basal level in human white blood cells range over orders of magnitude. Typical figures from the literature are 300 8-oxoguanines per 10^6 guanines by GC–MS (Podmore et al., 1998), between 40 (Degan et al., 1995) and two (Nakajima et al., 1996) per 10^6 by HPLC–ECD, and about 0.5 per 10^6 with the comet assay, or other methods, depending on the use of FPG to convert oxidised bases to DNA breaks. These discrepancies led to the setting up of the European Standards Committee on Oxidative DNA Damage (ESCODD) in 1997. The aim of ESCODD is to distribute standard samples to participating laboratories for analysis and, by comparing the results, to identify the problematic methods and develop optimal procedures for avoiding artefacts.

It was clear in 1997 that as good as GC–MS has been at identifying products of oxidative damage in heavily irradiated DNA solutions, it was prone to spurious oxidation of guanine during sample preparation, leading to exaggerated values for 8-oxoguanine. Processing of DNA for analysis by HPLC is also vulnerable, though less so. The first rounds of ESCODD demonstrated that even standard samples of 8-oxo-dGuo could not be measured accurately by most laboratories in the consortium (Lunec, 1998). There was a tenfold range in estimates of 8-oxo-dGuo in calf thymus DNA, and more than half the procedures tested failed to detect the dose–response in calf thymus DNA samples, doctored by treatment with photo-sensitiser and light to introduce additional 8-oxoguanine (ESCODD, 2000).

ESCODD is now a Concerted Action funded by the European Commission, with 27 members. In rounds of analysis of 8-oxo-dGuo solutions, calf thymus DNA, oligonucleotides containing 8-oxoguanine, and DNA isolated from liver and from HeLa cells, about half of the participating laboratories returned values close to the median while the remaining laboratories (not always the same ones) depart widely from the median. ESCODD has recommended protocols to protect samples from oxidation; but if the lowest estimates for normal cells 0.5 8-oxoguanines per 10^6 are correct, then oxidation of only one guanine per million will have a serious effect.

In the latest round, with HeLa cells, the comet assay, alkaline unwinding and alkaline elution (all depending on FPG-treatment of intact cellular DNA) have been

able to participate in inter-laboratory trials (ESCODD, in preparation). The three methods were very consistent, and gave a median value for the 8-oxoguanine content that was 2.6-fold less than the lowest values reported by chromatographic techniques. This is a lot better than a 1000× discrepancy, which was the starting point, but it remains true that some laboratories are still reporting values that are far higher.

As artefacts are eliminated, the peaks of 8-oxoguanine/8-oxo-dGuo to be measured become even smaller, and the problem of insufficient sensitivity becomes a serious one. Using a larger amount of DNA is not always an option, as biological material is often limited. In any case, heavy loading of a column with hydrolysed DNA can lead to saturation for (non-oxidised) guanine (dG), and consequently over-estimation of the ratio of 8-oxoguanine to guanine.

4.8.2. Urinary measurements

It is often recommended that when measuring any analyte in urine a 24-h excretion of the product should be estimated. This requires an accurately timed collection of urine, over a 24-h period, to be made by a subject, and the whole sample mixed to homogeneity, prior to analysis. This will generate a result, which is an average excretion of the product over that particular 24-h period. Under normal circumstances, because of variations in body size of individuals, correction factors have to be calculated for true comparisons of overall excretion rates. However, a simpler procedure is to reference the analyte, in the urine, against a substance present in the urine that is not reabsorbed and relates directly to the concentration of the urine. This is, ideally, carried out with the first void urine specimen, as it is the most concentrated. Measuring an analyte in urine, relative to the corresponding creatinine concentration, is a valid procedure for correcting variations in concentration of urines. It correlates with much more involved laborious 24-h estimations, although this has been contested in the literature. It is reported that creatinine production depends on muscle mass and diet. In cross-sectional studies of heterogeneous subjects, or in longitudinal studies of subjects undergoing some dietary interventions or catabolism, the creatinine production may show large variation. A young muscular man may have many times the muscle mass and creatinine production of an elderly women, and yet they may have the same number of cells and possible amount of DNA. Moreover, a poor correlation ($r = 0.5$) has previously been shown between the 8-oxo-dGuo ratio in a morning spot urine sample as compared to excretion in the complete urine collection from the preceding 24 h (Loft and Poulsen, 1999). Poor correlations were observed in cancer patients undergoing irradiation therapy (Olinski and Loft, unpublished data).

4.9. DNA–aldehyde adducts

4.9.1. Biochemistry

The molecular processes that induce DNA modifications are not yet fully elucidated. However, glucose, like many other aldehydes generated from the oxidation reaction of lipids, reacts in vitro and in vivo with primary amino groups of amino acids, phospholipids, proteins, and DNA. This non-enzymatic glycation is believed

to contribute to diabetes and several age-related complications such as atherosclerosis and cataract formation. It has been hypothesised that reducing sugars (including ascorbic acid), like glucose, form guanosine adducts (Lee et al., 1995). In addition, glucose and ascorbic acid can auto-oxidise to glyoxal, producing glyoxylated DNA adducts. The resulting modification induced in DNA by aldehydes gives rise to mutational events most probably through the generation of apurinic sites. Propano adducts are derived endogenously via α, β-unsaturated aldehydes, and characterised by a six-membered ring formation on the nucleobase.

MDA is a ketoaldehyde generated from ω-6 PUFA and can react with DNA bases to form stable adducts, which are possible promutagenic lesions. The monomeric cyclic adduct formed with guanine, M_1G, has been studied intensively (Marnett, 1999). In contrast, *trans*-4-HNE is uniquely generated by oxidation of ω-6 PUFA such as linoleic and arachidonic acids. HNE is a major LPO product, and among the most cytotoxic aldehydes formed (Esterbauer et al., 1991). HNE can generate an alkyl substituted exocyclic propane adduct through Michael addition of HNE to 2′-deoxyguanosine. This highly specific DNA lesion can be used as a fingerprint of HNE-exposure (Chung et al., 1999b). In addition, HNE can react through an epoxide intermediate to form unsubstituted etheno (ε) adducts, an unsaturated five-membered extended ring formation on adenine, cytosine and guanine (Nair et al., 1999).

Substituted ε-analogues have been characterised from reaction of epoxidised HNE with adenine and guanine (Chen et al., 1998), and epoxides of other α, β-unsaturated aldehydes such as acrolein, crotonaldehyde – together with the highly cytotoxic LPO-product 2,4-decadienal – may yield similar adducts (Carvalho et al., 2000; Chung et al., 1996; Loureiro et al., 2000). Alternatively, etheno adducts can be formed via metabolites of various carcinogens such as vinyl chloride and ethyl carbamate (Bartsch et al., 1994; Guengerich, 1992). Glyoxal, a two-carbon ketoaldehyde, generated via LPO and glycation, has been shown to interact with DNA in vitro, and form an ethano adduct with guanine residues (Kasai et al., 1998). Glyoxal is widely present in various foods and, possibly, a product of ascorbate oxidation (Mistry et al., 1999). At present, the biological implications of glyoxylated DNA are not clear. An overview of cyclic DNA-adduct formation, through LPO-mediated aldehydes, is shown in Fig. 12; exemplified with guanine nucleosides that typically form cyclic adducts between N^1 and N^2 of the nucleobase. Presently, it is evident that LPO is occurring in vivo and that a multitude of reactive products, particularly aldehydes and ketoaldehydes, can modify important macromolecules such as DNA. These modifications are mutagenic. However, their relative importance in human carcinogenesis is neither resolved nor quantified.

4.9.2. Method of measurement

Analysis of endogenous DNA adducts is a challenging task demanding high specificity and sensitivity for unequivocal identification and quantification. With advances in analytical techniques, basal levels of certain exocyclic DNA adducts have now been measured in unexposed humans and rodents. The MDA adduct on

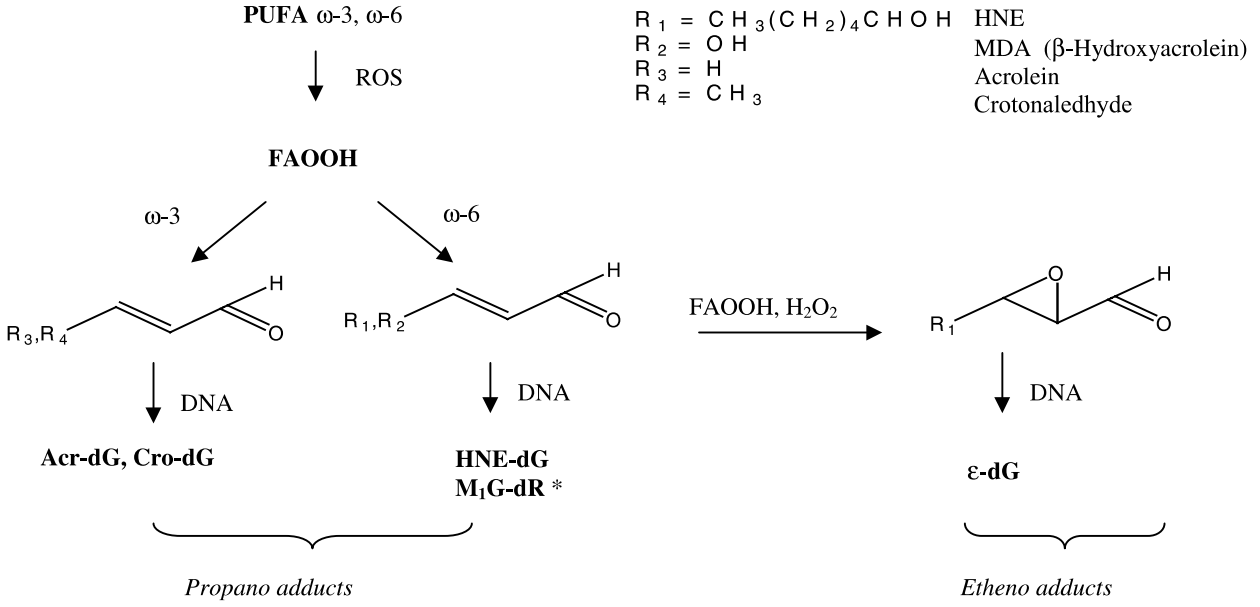


Fig. 12. Scheme for selected lipid peroxidation-derived aldehydes and consequential DNA damage exemplified by adduct formation on 2′-deoxyguanosine (dG). Stereoisomerism is not taken into account. ROS – reactive oxygen species. (*M_1G-dR has been characterised as a propano adduct albeit the exocyclic part is unsaturated).

deoxyguanosine, M_1 G-dR, has been detected in human liver by gas chromatography/electron capture negative-ion chemical ionisation mass spectrometry (GC/ECNCI–MS) and liquid chromatography in combination with electrospray and tandem mass spectrometry (LC/ES–MSMS), and in human leucocyte DNA by GC/ECNCI–MS combined with immunoaffinity purification (Chaudhary et al., 1994; Chaudhary et al., 1995; Rouzer et al., 1997). In addition ^{32}P-post-labelling/HPLC techniques, and an immunoblot assay, have been developed for quantification of MDA adducts in DNA of human tissue (Leuratti et al., 1999; Vaca et al., 1995; Yi et al., 1998). For analysis of etheno adducts with deoxyadenosine and deoxycytidine in untreated tissue, ^{32}P-post-labelling, combined with immunoaffinity purification, has been the principal method of detection (Nair, 1999; Varma, 1989; Watson et al., 1999). More recently, LC/ES–MSMS has been used to quantify various etheno adducts in human placenta (Chen et al., 1999; Doerge et al., 2000) and in human liver (Yen et al., 1996). Adducts measured in cellular DNA provide information about steady-state levels expressed as a concentration in the target tissue.

4.10. Products of reactive nitrogen species

RNS can induce various DNA lesions, including nitration, deamination and oxidation of bases as well as abasic sites and strand breaks (Halliwell, 1999; Nguyen et al., 1992; Szabo and Ohshima, 1997; Wink et al., 1991, 1998). The results from studies in vitro with isolated DNA, and deoxyguanosine showed that 8-nitro-guanine (8-NO_2-Gua) is the main type and isoform of nitrated bases from the reaction with exogenous nitrating agents (Wink et al., 1991, 1998; Yermilov et al., 1995a,b; Yermilov et al., 1996). More biologically related RNS, involving MPO or activated neutrophils, can also nitrate guanine in isolated DNA (Tuo et al., 2000). However, other types of DNA damage were generated simultaneously in abundance, and 8-NO_2 -Gua was not detected in nuclear DNA in activated neutrophils in vitro or in livers from mice with inflammation induced by injection of *Escherichia coli in vivo* (Tuo et al., 2000), although gaseous NO appeared to be able to induce nitroguanine in the DNA of lung fibroblasts in culture (Gupta and Lutz, 1999). Moreover, 8-NO_2-Gua decomposes spontaneously with a half-life of hours, and is not repaired by FPG (Tuo et al., 2000) suggesting that the biological relevance may be limited. These and other data suggest that other types of DNA damage rather than nitroguanine are important in the mutagenicity of RNS (Tretyakova et al., 2000; Tuo et al., 2000).

4.11. Endpoints arising from oxidative DNA damage

DNA damage is a marker of exposure to DNA damaging agents modulated by whatever defence mechanisms exist such as antioxidants in the case of oxidative damage. There are so many steps in the sequence of events between DNA damage and cancer that measuring DNA damage tells us rather little about cancer risk. Measuring markers of events downstream of the initial damage can provide more relevant information.

4.11.1. Mutations

Mutations result from the fixation of unrepaired DNA damage during replication. They are extremely rare events, and using traditional techniques only a few genes have been amenable to investigation. PCR combined with DNA microarray techniques should make analysis much easier. One of the genes commonly studied in stimulated human lymphocytes is the gene for hypoxanthine phosphoribosyltransferase (*hprt*), for which a simple selection system exists. It is X-linked, which means that a single mutational event in a cell, rather than the inactivation of two alleles, will render it HPRT-deficient and thus able to survive in a selective medium that kills wild-type cells. Smokers generally have elevated $hprt^-$ mutant frequencies compared to non-smokers (Duthie et al., 1995). It was also found that the $hprt^-$ mutant frequency was higher in individuals with low plasma vitamin C levels, but vitamin C and smoking are intimately linked making estimation of the relative importance of these variables difficult.

4.11.2. Chromosome aberrations

Chromosome aberrations are seen in metaphase preparations, after treatment of cells with DNA-damaging agents. They include breaks, deletions, translocations, duplications and dicentrics. In normal, untreated cells (such as lymphocytes stimulated to proliferate with mitogen), aberrations are seen at very low levels. Many aberrations lead to loss of genetic material or disruption of mitosis, and cell death; however, translocations are relatively stable, as no DNA is lost. The importance of chromosome aberrations as a marker of cancer risk was established in two prospective studies in which they were measured in several thousand individuals in the Nordic countries and Italy in the 1970s. Subsequent incidence of cancer was monitored, and found to be significantly higher in those with high chromosome aberration frequencies (Bonassi et al., 1999; Hagmar et al., 1994). In a recent intervention trial, supplementation for three months with vitamin C, vitamin E, β-carotene and selenium led to a decrease of greater than 50% in the frequency of chromosome aberrations in lymphocytes (Dusinska, Collins, in preparation).

4.11.3. Micronuclei

Micronuclei, like chromosome aberrations, are found in relatively few cells, but as they occur in interphase they are more easily monitored. They represent fragments or whole chromosomes excluded from the nucleus as it reforms at the end of mitosis. A decreased sensitivity of lymphocytes to hydrogen peroxide-induced micronuclei after consumption of white or red wine – possibly attributable to antioxidants such as vitamin C or hydroxycinnamates has been reported(Fenech et al., 1997). Supplementation with β-carotene reduced the frequency of micronuclei in cells recovered from sputum of smokers (van Poppel et al., 1992). Supplementation with vitamin C, vitamin E, β-carotene and selenium also led to a decrease in micronucleus frequency in lymphocytes from non-smokers (Dusinska, Collins in preparation).

4.12. Site-specific DNA damage

Since much of the DNA in cells is not essential, it is important to determine whether oxidation of bases occurs in the DNA that is vital for normal cell function, or in the remainder, commonly referred to as 'junk' DNA. It is assumed that only if the damage to DNA occurs in essential regions serious problems will occur.

Sites of ROS-induced DNA damage can be mapped by the ligation-mediated PCR (LMPCR) with resolution at the level of nucleotides (Rodriguez and Akman, 1998). This enables site-specific damage to be measured, which is likely to be of much greater relevance to the mechanisms leading to cancer. LMPCR is a genomic sequencing method for mapping rare single-stranded breaks, which result in 5′-phosphoryl ends. As with the comet assay, strand breaks are induced at sites of oxidative damage, following treatment with specific repair enzymes. A primer is extended to the breaks, using an annealed gene-specific oligonucleotide (upstream primer I), creating blunt double-stranded ends. After blunt end ligation of an asymmetric double-stranded linker, a population of molecules, whose size reflects the distance from the primer pairing site, is amplified by PCR, using a second gene-specific oligonucleotide (upstream primer 2), separated by sequencing polyacrylamide gel electrophoresis, transferred to a nylon membrane, and probed by hybridising with a radiolabelled probe, and the resulting autoradiogram is analysed. Control sequence analysis in the absence of FPG protein or endonuclease III (*N*th protein) identifies random strand break priming (Rodriguez et al., 1997).

The sensitivity of this assay for subtle changes, and responses to dietary intervention, remains to be determined. Indeed, treatments with hydrogen peroxide in vitro, at cytotoxic concentrations, are required in order to be able to generate sufficient damage to be mapped. Attempts to improve the sensitivity of the assay have been made by using genomic gene enrichment techniques. This has been achieved using size-fractionating restriction endonuclease-digested genomic DNA by continuous elution electrophoresis through preparative agarose gel, which can enhance LMPCR-derived base damage signal intensity by up to 24-fold (Rodriguez et al., 1999). Using this methodology, it is possible to identify background signals, but at this stage it is not possible to state whether these background signals are due to endogenous oxidative DNA damage or are a result of post-DNA artefacts. Studies designed to differentiate these possibilities are now of paramount importance. At present, LMPCR, combined with genomic gene enrichment by CEE, can detect two ROS-induced lesions per 10^5 bases. However, a large amount of DNA is required for the enrichment procedure, and current indications are that the sensitivity of LMPCR needs to increase further to a target detection sensitivity of one lesion per 10^6 bases, in order to bring it in line with assays for 8-oxoGua.

It is also possible to examine gene-specific damage and repair using fluorescent in situ hybridisation (with gene-specific oligonucleotides) combined with the comet assay (Collins, A.R. in preparation).

Gene-specific evidence of oxidative damage in p53 has been obtained by using the restriction site mutation assay, by the identification of mutants that confer resistance to the activity of specific restriction enzymes. Using a range of restriction enzymes,

five of the eight main p53 mutation hot spots [i.e. codons 175 (Hhai, GCGC), 213 (Taq1, TCGA), 248 (Msp1, CCGG), 249 (Hae111, GGCC) and 282 (MSP1, CCGG)] have been investigated (Jenkins et al., 2001). This procedure is more robust than LMPCR, and more suited to large-scale analyses.

To date analysis of base damage at the level of nucleotide resolution mapping has been restricted to the PGK1 promoter region and exons five and nine of p53 in fibroblasts. These data have confirmed that oxidation in vitro maps to a very similar pattern to that observed in vivo, and CGC triplets are particularly sensitive. Furthermore, the presence of zinc fingers may also confer some increased susceptibility to oxidation, where iron may substitute for zinc. Taken together, it should be possible to predict oxidative hotspot sites using GenBank and NIH databases. It will be important then to determine whether such changes are matched by oxidation in vivo.

LMPCR by its very methodology highlights analysis of pre-defined genes. Will such a narrow approach lead to oversights? The development of EST (expressed sequence tags)-based methods may allow us to look at a wider pool of genomic oxidative DNA damage. An excellent database in Swansea (http://lisntweb.swan.ac.uk/cmgt/index.htm) identifies sites of known oxidative damage, and therefore may facilitate the development of more closely allied disease-specific markers. However, it cannot be understated that the link between oxidative DNA damage and cancer is masked by the numerous potentially mutation sites, and the fact that, for the most part, cancer is a multi-hit process arising over the course of decades.

Another approach that offers considerable promise has been to use DNA repair enzymes as analytical tools to identify the sites of DNA oxidation. These enzymes are capable of identifying specific lesions in DNA The OGG1 protein as an analytical tool for isolation of sequences containing 8-oxoguanine from DNA (Iwanejko et al., unpublished observations). Preliminary data indicate that this oxidative lesion in DNA is confined to 'hot spots' in certain regions of the genome. Clearly, further development and application of such techniques may help identify key targets for oxidative DNA damage in vivo, leading to the identification of new and verified biomarkers of oxidative DNA damage for use in antioxidant intervention studies.

4.13. Relationship of DNA oxidation to disease

8-oxo-dGuo is a marker for oxidative lesions of DNA. It is the most prevalent oxidation product and it is a mutagenic event. Oxidation of DNA can be induced by chemicals, inflammation (oxidative stress) and gamma-irradiation. GC/MS with stable isotope dilution has successfully been applied in order to analyse the base products in DNA isolated from cancerous and non-cancerous tissues (Olinski et al., 1995, 1997) (see Table 8).

GC/MS has been used to identify oxidatively modified purines and pyrimidines (Jaruga et al., 2000) following specific endonuclease hydrolysis. The amount of 8-oxo-Gua in human lymphocytes was 3 per 10^7 bases, and it was quite comparable with those of other enzymatic assay estimates (comet assay, alkaline elution or alkaline unwinding) (Collins et al., 1996; Pflaum et al., 1997). However, in all the above-mentioned methods Fpg-sensitive sites were calculated instead of real

Table 8
Levels of oxidatively modified DNA bases (expressed as modified bases/10^6 bases) in different DNA samples

	5-oxo-Ura	5-oxo-Cyt	5,6-diHUra	FapyAde	8-oxo-Ade	FapyGua	8-oxo-Gua
CT DNA	4.2 ± 0.1	11.0 ± 1.3	2.9 ± 0.75	2.6 ± 0.4	1.4 ± 0.3	4.2 ± 0.9	0.8 ± 0.2
RL DNA	0.3 ± 0.06	3.3 ± 0.5	1.5 ± 0.5	1.0 ± 0.4	N/D	2.0 ± 0.2	0.2 ± 0.08
RN	N/D	2.9 ± 0.8	1.6 ± 0.5	N/D	N/D	1.8 ± 0.5	0.3 ± 0.1
HL DNA	N/D	0.2 ± 0.06	0.9 ± 0.4	0.9 ± 0.3	N/D	1.0 ± 0.3	0.3 ± 0.03

CT-DNA: calf thymus DNA, RT-DNA: rat liver DNA, RN: nuclei isolated from rat liver cells and HL: DNA from human lymphocytes.

8-oxo-Gua. This corresponded to the total amount of both Fapys, 8-oxo-Gua and 8-oxo-Ade, which comprises 21 damages per 10^7.

Basal levels of pro-mutagenic exocyclic DNA adducts, which arise from LPO-derived aldehydes, have been detected in tissues and body fluids from unexposed humans and animals (Bartsch, 1999; Chung et al., 1996; Uchida et al., 1995). This suggests that exocyclic DNA modifications, caused by endogenous sources of reactive aldehyde intermediates, are important lesions. Furthermore, exocyclic DNA adducts may serve as novel biomarkers of endogenous oxidative damage. Acrolein, crotonaldehyde and 4-hydroxy-2-hexenal, all generated from ω-3 PUFA, can interact with nucleophilic sites on DNA and produce chemically stable propano adducts (Chung et al., 1999a; Yi et al., 1997). Etheno bases are efficiently repaired by specific DNA glycosylases, confirming the toxicological significance of ε-lesions (Olinski et al., 1995). Excised adducts are excreted as free nucleobases or nucleosides as a consequence of BER or NER, respectively. Monitoring of repair products of specific endogenous DNA lesions in urine could be important in the non-invasive quantitative assessment of lipid peroxidation-related DNA damage for future clinical and public health studies.

4.14. Modulation of DNA oxidation biomarkers by antioxidants

The comet assay has been widely applied in biomonitoring and supplementation studies (Tables 11–13 and 15). Single antioxidants, mixtures of antioxidants, and foods rich in antioxidants have all been used, and protective effects (against endogenous or hydrogen peroxide-induced damage) observed after single or multiple doses.

The effects of antioxidants, or diets rich in such, on urinary excretion of 8-oxo-dGuo have been reported by several groups (Table 10). Most studies give negative results if a chromatographic method has been used. ELISA has been used to measure urinary 8-oxo-dGuo during supplementation studies (Tables 10 and 14).

Use of GC–MS to investigate effects of diet on oxidative DNA damage can offer the advantage of multiple analytes. However, the two reported studies to date have identified a complex phenomenon. Decreases in levels of 8-oxo-Gua with an associated increase in 8-oxo-Ade were reported in subjects who had received vitamin C (500 mg/day) for six weeks (Podmore et al., 1998). However, the technique used is

prone to artefactual oxidation. The pro-oxidant effects of ascorbate, when co-supplemented with iron, have been described in subjects who were already vitamin C replete, and an overall decrease in DNA oxidation in those subjects low in vitamin C (Rehman et al., 1998). These data suggest that it may be important to consider more than one lesion, where different bases may be protected to different extents by different antioxidant classes (Table 9 and 13).

There are no published reports on the effects of dietary antioxidants on DNA damage measured using ^{32}P-post-labellings.

Currently, the procedures for measuring DNA–aldehyde adducts have not been applied to any antioxidant intervention studies, although they have been used in biomonitoring studies. Table 22 summarises the normal range and limits of detection for common oxidised DNA bases.

5. Direct and indirect effects of oxidative stress: measures of total oxidant/antioxidant levels

5.1. Visualisation of cellular oxidants

5.1.1. Biochemistry: histochemical detection of ROS

Several of the biochemical changes induced by oxidant stress can be directly detected in tissue and cells by histochemical means. The determination of such 'histochemical biomarkers' has been used to discriminate areas, cellular types and, sometimes, subcellular sites affected by oxidant stress, in experimental models as well as in control tissue.

5.1.2. Method of measurement

The reduction of nitroblue tetrazolium to insoluble blue formazan has been widely used, especially in studies using activated phagocytes, to evaluate superoxide production at the level of the light microscope as well as with electron microscopy (Hirai et al., 1992). More specific procedures have been optimised for the detection of hydrogen peroxide (cerium chloride method) and superoxide (Mn^{2+}-diaminobenzidine method), and they are also capable of providing insights into the cellular production of singlet oxygen (reviewed by (Karnovsky, 1994)). Cytochemical studies on the detection of ROS currently exploit a method involving the pre-loading of living cells with 2′, 7′-dichlorofluorescin diacetate (DCF–DA), a compound whose fluorescence sharply increases in the presence of superoxide and other oxygen radical species.

5.1.3. Limitations, storage and stability

All samples must be analysed immediately on collection. The actual specificity of DCF–DA in detecting individual ROS is not known. It has been shown that DCF–DA can serve as a substrate for xanthine oxidase and other cellular peroxidases (Rota et al., 1999; Zhu et al., 1994). Since the latter process appears to be a source of superoxide, leading to artefactual amplification of DCF–DA fluorescence the use of the DCF–DA is questionable (Rota et al., 1999). It should be stressed that the

Table 9
Supplementation studies using lymphocyte DNA oxidation biomarker endpoints

Antioxidant supplement effects on white blood cell DNA damage by GC/MS or HPLC–EC									
Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C	500 mg/day	30	84 days	Plac cont	Lymphocyte total DNA	8-oxo-Gua 8-oxo-Ade	Decrease increase	GC–MS	Podmore et al. (1998)
Vitamin C+/– Iron Baseline ascorbate >70 uM	60 or 260 mg/day C and 14 mg/day Fe	23	42 and 84 days		Lymphocyte total DNA	8-oxo-Gua 8-oxo-Ade FapyGua	Increase at 6 weeks. Baseline at 12.	GC–MS	Rehman et al. (1998)
Vitamin C+/– Iron Baseline ascorbate <50 uM		17				5OhmU 8-oxoGua	Reduce on supplements		
Vitamin C Placebo	260 mg/day		42 and 84 days	Placebo		8-oxo-Gua 5HMU		GC–MS	Proteggente et al. (2000)
Vitamin C+ Iron	260 mg/day + 14 mg/day					5OH Cyt 5OHMe hydantoin			
Vitamin C depletion and supplementation	5–20 mg/day 60–250 mg/day	8 males	92 days		Sperm DNA WBC DNA	8OHdG	Increase Reverse on repletion No effect	HPLC–EC	Jacob et al. (1991)
Vitamin C	500 mg/day	30 male and female	84 days		Lymphocyte DNA	8-oxo-Gua	decrease	HPLC–EC (Guanase)	Cooke et al. (1998)

Table 9 (*continued*)

Antioxidant supplement effects on white blood cell DNA damage by GC/MS or HPLC–EC									
Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C Vitamin E β-carotene red ginseng	500 mg/day 200 IU/day 9 mg/day 1.8 g/day	5 smokers/gp	20 days		WBC	8-oxo-dGuo	No change 34% decrease No change 32% decrease	HPLC–EC	Lee et al. (1998)
Vitamin C Vitamin E β-carotene Selenium Cu Zn	60 mg/day 30 IU/day 3 mg/day 40 μg/day 80 mg/day 2 mg/day	30 passive smokers	60 days		WBC	8-oxo-dGuo	62% decrease	HPLC–EC	Howard et al. (1998)
Lycopene	39–150 mg/day	19	7 days		WBC	8-oxo-dGuo	NS trend towards protection	HPLC–EC	Rao and Agarwal (1998)
Vitamin C Alpha tocopherol β-carotene selenium	350 mg/day 250 mg/day 60 mg/day 80 μg/day	12 non-smokers 9 smokers	28 days	Placebo Double blind	WBC DNA	8-oxo-dGuo	Carotene caused increase in smokers and decrease in non-smokers	HPLC–EC	Welch et al. (1999)

Table 10
Antioxidant supplement effects on urinary excretion of DNA oxidation product by HPLC/EC or ELISA

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C	500 mg	20 smokers	60 days	Placebo	Urine	8-oxo-dGuo	No effect	HPLC–EC	Prieme et al. (1997)
Alpha tocopherol	200 mg								
CoQ	90 mg								
β-carotene	20 mg/day	122	98 days	Placebo	3 night pooled urine	8-oxo-dGuo	No effect	HPLC–EC	van Poppel et al. (1995)
β-carotene	30 mg/day	14	30 days	+/− exercise	Urine	8-oxo-dGuo	No effect	HPLC–EC	Sumida et al. (1997)
Vitamin C+	100 mg	15 HIV patients			Urine	8-oxo-dGuo	Protected against the increase in 8-oxo-dGuo observed during AZT treatment	HPLC–EC	de la Asuncion et al. (1998)
Alpha tocopherol	600 mg/day	8 + AZT							
		7 no AZT							
Vitamin C	500 mg/day	184	60 days		24 h urine	8-oxo-dGuo	No effect	HPLC–EC	Huang et al. (2000)
Alpha tocopherol	400 mg/day								
Vitamin C+	500 mg/day	30 marines	14 days	Placebo controlled Winter training	Urine	8-oxo-dGuo	No effect	HPLC–EC	Pfeiffer et al. (1999)
Vitamin E+	400 IU/day								
β-carotene +	20,000 IU/day								
Selenium+	100 mg/day								
Zinc +	30 mg/day								
Vitamin C+	500 mg/day	48	1115 days	Placebo	24 h Urine	8-oxo-dGuo	No effect	HPLC–EC	Porkkala-Sarataho et al. (2000)
Alpha tocopherol	400 mg/day								
Vitamin C	500 mg/day	30	84 days		Urine	8-oxo-dG to creatinine	Increase	ELISA	Cooke et al. (1998)

Table 11
Antioxidant supplement effects on white blood cell DNA damage by ELISA or comet

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C Vitamin E	1000 mg/day 800 mg/day	23	42 days	Challenge with H_2O_2 ex vivo	WBC	DNA damage	Decreased H_2O_2 induced damage but no effect on baseline	ELISA	Brennan et al. (2000)
Vitamin C+ Vitamin E+ β-carotene	250 mg/day 200 IU 6 mg	121 smokers	178 days	Placebo	Lymphocyte DNA Buccal DNA	8-oxo-dGuo	No effect	ELISA	Jacobson et al. (2000)
Vitamin C	1000 mg/day	8	One dose		WBC	Resistance to H_2O_2	Increase	Comet	Collins et al. (1997)
Vitamin C	6 or 60 mg/day	48	14 days	Placebo	WBC	SB Resistance to H_2O_2	No effect	Comet	Anderson et al. (1997)
Vitamin C β-carotene Vitamin E	100 mg/day 25 mg/day 280 mg/day	50 smokers 50 non-smokers	20 weeks	Placebo double blind	WBC DNA	Endo III sites Resistance to H_2O_2	Endogenous damage decreased and resistance increased	Comet	Duthie et al. (1996)
Rutin (quercetin glycoside)	500 mg/day	16	6 weeks	Placebo double blind	WBC DNA	Endo III site Resistance to H_2O_2	None	Comet	Boyle et al. (2000a)
Vitamin C	350 mg/day	12 non-smokers	28 days	Placebo double blind	WBC DNA	Strand breaks	No effect	Comet	Welch et al. (1999)
Alpha tocopherol β-carotene selenium	250 mg/day 60 mg/day 80 μg/day	9 smokers							
A/b carotene Lutein Lycopene	15 mg/day 15 mg/day 15 mg/day	40	84 days	Placebo double blind	WBC DNA	Endo III sites	No effect	Comet	Collins et al. (1998b)
A/b carotene Lutein Lycopene	15 mg/day 15 mg/day 15 mg/day	8	7 days	Subjects as own controls	WBC DNA	SB	Protection against atmospheric oxygen	Comet	Torbergsen and Collins (2000)

Table 12
Effects of single supplements on white blood cell DNA oxidation

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C	35 mg/kg	6	1 h	+ ionising radiation after isolation	WBC	Resistance to H_2O_2	Increased	Comet	(Green et al., 1994)
Vitamin C	1 g	12	Blood collected 2–4 h and 18–24 h		WBC	Resistance to H_2O_2	Increased at 2–4 h	Comet	Panayiotidis and Collins (1997)
Alpha tocopherol	1 g						Increased at 18–24 h		
β-carotene	45 mg								

Table 13
Effects of foodstuffs on white blood cell DNA damage

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Tomato serving	1 serving		1 day		Lymphocyte	8-OH-Gua	Decrease	GC–MS	Rehman et al. (1998)
						8OHAde	increase		
Fruit and veg servings	5–12/day	28 women	14 days		WBC DNA	8-oxo-dGuo	21% decrease NS for all subjects. 32% decrease for plasma carotene <56 ng/ml No effect	HPLC–EC	Thompson et al. (1999)
					URINE			ELISA	
Mediterranean diet + red wine 240 ml/day		21	92 days	Compare with high fat diet	WBC DNA	8-oxo-dGuo	High fat diet increases DNA damage. Levels decrease in both diets with wine	HPLC–EC	Leighton et al. (1999)
Spanish diet		52		Swedish diet	WBC DNA	8-oxo-dGuo	Increased levels in Spanish women correlated with AT and carotenoids	HPLC–EC	Bianchini et al. (2000)
Diet low in flavanols or supplemented	76–110 mg/day	10 stable diabetic	14 days		WBC	Strand breaks	protect	Comet	Lean et al. (1999)
Tomato puree	25 g/day = 7 mg lycopene, 0.3 mg β-carotene	9 women	14 days		WBC	H_2O_2 resistance	Increased	Comet	Porrini and Riso (2000)

Tomato juice Carrot juice Spinach powder		23	14 days	Compare with same subjects on diet low in carotenoid	WBC	SB	Increase in DNA repair enzyme-sensitive sites (tomato and carrot)	Comet	Pool-Zobel et al. (1998)
Soy milk	1 l/days	10	4 week	Cow/rice milk	WBC	SB Resistance to H_2O_2 Endo III sites	Decrease in endogenous damage, increase in H_2O_2 resistance	Comet	Mitchell and Collins (1999)
Flavanol diets	High low	36	14 days	Crossover	WBC	Oxidised bases	No change	GC–MS	Beatty et al. (2000)
Tomato juice Carrot juice Spinach powder		23	14 days		WBC	SB Endo III sites	Decrease after carrot juice	Comet	Pool-Zobel et al. (1997)
Rye crispbread	77 g/day	12	14 days		WBC	SB Resistance to H_2O_2 Endo III sites	No effect	Comet	Pool-Zobel et al. (2000)
High PUFA + alpha tocopherol	$15\% \pm 80$ mg	21 males	28 days	Low PUFA $5\% \pm 80$ mg cross-over	WBC	SB, H_2O_2 resistance, Endo III sites	High fat increases endo III sites, and reduces H_2O_2 resistance. α-tocopherol protects	Comet	(Jenkinson et al., 1999)
Vegan diet		$N = 20$ (vs 20 controls)		Vs controls	WBC	SB	Tail moment significantly lower in a subset (those that did not use vitamin or mineral supplements)	Comet	Verhagen et al. (1996)

Table 14
Effects of foodstuffs on urinary excretion of oxidised DNA

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Brussels sprouts	300 g/day	5 males 5 females	1 week	Vegetable control	Urine	8-oxo-dGuo	Decrease in 4/5 males No effect in females	HPLC–EC	Verhagen et al. (1997)
High vs low intake of fruits and vegetables	448 ± 111 g/day vs 224 ± 53 g/day	10 vs 10 males		High vs low intakers	Urine	8-oxo-dGuo	No effect	HPLC–EC	Hertog et al. (1997)
Brussel sprouts	300 g/day	10	21 days	Vegetable control	Urine	8-oxo-dGuo	28% decrease in urine excretion	HPLC–EC	Verhagen et al. (1995)

Table 15
Effects of single meal on white blood cell DNA oxidation

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Kiwi fruit juice	0.5 l		24 h	Placebo controlled crossover	WBC	Resistance to H_2O_2	Increase	Comet	Collins et al. (2001)
Onion meal		6 women	4 h		Urine	8-oxo-dGuo	Decrease	ELISA	Boyle et al. (2000b)
Onion + cherry tomato					WBC	strand breaks, endogenous and H_2O_2-induced Endo III sites	Decrease in endogenous damage and resistance to H_2O_2 increased Decreased	Comet	

specific detection of superoxide anions in biological samples is potentially biased due to several factors (Fridovich, 1997), and therefore requires accurate application of specificity controls.

5.2. Measurement of hydrogen peroxide

5.2.1. Biochemistry

Hydrogen peroxide is an important ROS generated in vivo by enzymatic processes. However, whether it is present in biological fluids, and if it can be measured accurately in blood, urine and exhaled breath, will determine if it is a valuable biomarker of oxidative stress in vivo.

5.2.2. Methods of measurement

Several assays have been adopted for determination of hydrogen peroxide in biological fluids, which are either direct or indirect. These include the peroxidase-catalysed oxidation of tetramethylbenzidine (Dekhuijzen et al., 1996), the release of $^{14}CO_2$ from ^{14}C-labelled 2-oxo-glutarate by hydrogen peroxide (Varma, 1989), the measurement of oxygen released by oxygen electrode after catalase addition (Jackson et al, 1978), HPLC determination with chemiluminescence detection (Frei et al., 1988), and a simple colurimetric method in which hydrogen peroxide oxidises Fe(II) to Fe(III) and forms a chromogen with xylenol orange (Nourooz-Zadeh, 1999). The specificity of the reaction should be confirmed in all cases through SOD inhibition studies, to demonstrate superoxide-independent hydrogen peroxide determination.

5.2.3. Storage, stability and limitations on use

Fresh samples should be used as superoxide plays a key role in many auto-oxidation reactions and can, over time, lead to elevated levels of peroxide in samples. Similarly, cell free fluids must be prepared to avoid any contribution by activated phagocytes.

5.3. Measurement of the ratio of antioxidant/oxidised antioxidant

5.3.1. Biochemistry

Total specific antioxidant measurement provides only limited information on levels of oxidative stress. This is exemplified in rheumatoid arthritis and diabetes, where overall levels of reduced ascorbate are lower than in normal subjects but the magnitude of the degree of oxidative stress is shown in measurement of dehydroascorbic acid as a ratio of total ascorbate. Other examples include GSSG/GSH, vitamin E/quinone, and ubiquinol/ubiquinone. For several of the active antioxidant components of the diet considered in *Bioavailability and metabolism*, there is insufficient knowledge about both the active form and the oxidised metabolite, and further work is necessary before such an approach can be adopted (e.g. in the analysis of flavonoids).

5.3.2. Method of measurement

Analytical methods are generally good, in terms of specificity for the analyte concerned. There is, however, contention about the true levels of oxidised form of ascorbate and glutathione. Following acid precipitation, using either metaphosphoric or trichloroacetic acid, the samples are considered stable for analysis of ascorbate for up to four weeks at temperatures of −20 °C or lower. Ascorbic acid and dehydroascorbate are measured by HPLC, with either direct UV- or fluorometric detection, following derivatisation with orthophthalaldehyde. Glutathione is frequently determined by either the colorimetric enzymic recycling assay or HPLC using either UV, fluorometric or electrochemical detection.

Glutathione reacts with *ortho*-phthalaldehyde to form a stable, highly fluorescent tricyclic derivative that is easily separated and quantified by HLPC. The glutathione adduct is separated and adducts are detected fluorometrically. Detection of 0.1 to 200 pmol glutathione produces a linear response, and the recovery of reduced and oxidised glutathione from rat liver homogenate, bile, and plasma is quantitative {Neuschwander-Tetri & Roll 1989 903/id}. Reduction of the oxidised form to the reduced form, for analysis by enzymic means (e.g. GSH reductase), is frequently adopted in the determination of oxidised glutathione. Trichloroacetic acid, but not perchloric acid, is recommended to deproteinise samples for analysis. Samples should also be pre-treated with *N*-ethylmaleimide to prevent oxidation of GSH to GSSG (Asensi et al., 1994).

5.3.3. Storage, stability and limitations on use

Both glutathione and vitamin C are particularly labile, and precautions must be taken during sampling and storage. Quantification of ascorbate and dehydroascorbate is confounded by their instability. The half-life of DHAA in heparinised plasma is approximately two minutes. However, the levels are also a function of the oxidation of ascorbate (Koshiishi and Imanari, 1997). DHAA appears to be generally absent in plasma samples, but both *meta*phosphoric and trichloroacetic acids lead to artefactual catalysis of DHAA formation, through promotion of metal ions release from haemoglobin and/or transferrin (Koshiishi et al., 1998).

Glutathione levels decrease within minutes of sample collection, but then remain stable for up to four weeks in acidified preparations at −80 °C. It is essential therefore that sample processing occur immediately on collection. A recent comparison between HPLC–UV and the enzymic recycling assay showed the HPLC assay to detect another substance that is co-eluted and derivatised with GSSG, leading to 200-fold higher results than obtained with the recycling assay (Sian et al., 1997).

5.4. Total antioxidant capacity

5.4.1. Biochemistry

Antioxidants form an intricate network in the protection against reactive species, and analysing only one antioxidant in isolation of others gives an incomplete overview. This provides the rationale for the simultaneous assessment of all antioxidants present in a sample. A decrease in the levels of antioxidants might be used

as a biomarker of oxidative stress. The Total Antioxidant Capacity (TAC) of body fluids (mainly blood plasma and serum) has been used as a measure of antioxidant status of the body. However, there are several points of concern with respect to the estimation and use of this parameter.

5.4.2. Terminology

The total antioxidant capacity gives the total amount of radicals that can be scavenged by an antioxidant or a biological sample. However, it may be more appropriate to refer to this assay as a measure of partial antioxidant activity, because of the nature of the scavenging against aqueous radicals. This is sometimes referred to as (total) antioxidant activity. It should be noted that an antioxidant is defined as a compound that, at a relative low concentration, prevents or delays the oxidation of another compound. So the term antioxidant activity comprises all processes to prevent or delay oxidation of another compound, including radical scavenging, prevention of radical formation and induction of antioxidant enzymes. Antioxidant activity can thus not be used to denote antioxidant capacity and therefore the term TAC, as the recommended one, will be used throughout this report. In the literature TAC is sometimes also denoted as TRAP and TAS.

The main determinants of the TAC of blood plasma are urate and protein thiols. This may be different in other body fluids, for example, in seminal plasma the concentration ratio of ascorbate to urate is about one (Gavella et al., 1997) and the relative contribution of ascorbate will be much more significant. Moreover, other components that have a higher contribution than ascorbate and urate to the TAC of seminal plasma have been identified (van Overveld et al., 2000).

5.4.3. Methods of measurement

Inhibition assays utilise the measurement of a delay or slowing down of an oxidation reaction in the presence of an antioxidant.

An example of an inhibition assay is the assay based on the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) by 2,2′-azobis(2-amidopropane) (AAPH). In this TAC assay, a coloured product (ABTS cation radical, $ABTS^+$ is formed. Antioxidants delay the onset of the reaction and the magnitude of this delay (induction time) is proportional to the concentration of antioxidants in the sample (Arnao et al., 1996; Bartosz et al., 1998a,b; Bartosz and Bartosz, 1999; Miller et al., 1993; Yu and Ong, 1999).

A drawback in using the induction time as the basis for the estimation of TAC is the difficulty in precise determination of induction time. Other parameters, which can be used for the evaluation of TAC, are: (i) absorbance of the product after fixed time under standardised conditions, and (ii) reaction rate after its commencement. However, not all antioxidants affect the reaction rate. Moreover, in some cases, biological samples increase rather than decrease the reaction rate of some assays (Valkonen and Kuusi, 1997; Janaszewska and Bartosz, submitted). Fixed-time assays are precise (as based on single measurements of absorbance value), but are influenced by both the induction time and the oxidation rate of the substrate. Therefore, their interpretation may be more complex (Bartosz and Bartosz, 1999).

Another type of inhibition assay is based on a spectrophotometric, or spectrofluorimetric, assay of the consumption of an oxidised substrate and protection of the substrate by antioxidants. In these assays, either rate of the substrate decay or area under the curve (AUC) of substrate concentration versus time is measured.

One of the first proposed TAC inhibition assays was that of TRAP, based on the measurement of induction time of oxygen consumption by diluted blood plasma, supplemented with linoleic acid (Valkonen and Kuusi, 1997; Wayner et al., 1985, 1987, 1986). A commercial kit for determination of 'Total Antioxidant Status' Randox® is based on the fixed-point assay of antioxidant inhibition of ABTS oxidation by hydrogen peroxide catalysed by metmyoglobin. Although the exact mechanism of the reaction is complex (see below) and the oxidant (the ferryl form of metmyoglobin) not very representative, the great advantage of this generic assay is the full standardisation of assay conditions and the comparability of results obtained in different laboratories. The related ORAC assay (Cao et al., 1995, 1993; Courderot-Masuyer et al., 1999) quantifies generation of fluorescence from AAPH, in a non-linear fashion, measured as AUC. Enhanced chemiluminescence and dye bleaching methods have also been described (Komosinska-Vassev et al., 2000; Kondo et al., 1999; Metsa-Ketela, 1991; Naguib, 2000; Popov and Lewin, 1999, 1994; Tubaro et al., 1998; Venditti et al., 1995; Visioli and Galli, 1997).

A naïve interpretation of the inhibition assays is that they are based on a competition between the indicator and the antioxidants for the oxidant. However, the real situation is usually more complex. In many systems in which oxidation of the indicator is an easily reversible reaction, a significant part of the reaction may be due to re-reduction of the oxidised indicator by antioxidants present in the sample. In such systems, the result of the reaction reflects, to a significant extent, the capacity of the antioxidants assayed to react with the oxidised form of the indicator rather than with the oxidant (e.g. employing ABTS since the ABTS cation radical is easily reduced (Bartosz and Bartosz, 1999)). This aspect is not necessarily a drawback of an assay – chemical repair reactions constitute an important mode of antioxidant action in vivo – but it may influence the TAC values measured in different assay systems.

Reduction assays are based on the assumption that antioxidants are reductants, and the amount of sample components able to reduce an indicator is equal to the amount of antioxidants present in the sample. Several reductive assays have been proposed including reduction of 1,1-diphenyl-2-picrazyl radical (Blois, 1958; Yagi et al., 1999) and the Ferric Reducing Activity of Plasma (FRAP), which measures the reduction of a colour ferric complex (Benzie and Strain, 1996; Benzie and Strain, 1999). The $ABTS^{+}$ decolourisation assay (Pellegrini et al., 1999; Re et al., 1999) makes use of the relative stability of $ABTS^{+}$, which can be pre-prepared by oxidation of ABTS with potassium persulphate, or by other means, and used as a substrate for reduction.

Reduction assays are precise, as they are based on a difference in absorbance values that can be measured exactly. The main problems with reductive assays of TAC concern their specificity and the kinetics of reduction. Reduction rate depends on the temperature, which seems in practice to be the main variable. However, various antioxidants react at different rates. Times to reach completion may differ for

different compounds, and for complex samples like blood plasma, may exceed those suggested in the protocol (Janaszewska and Bartosz, submitted). Thiol compounds show very little activity at the low pH used in the FRAP assay. On the other hand, because $ABTS^+$ is promiscuous, the $ABTS^+$ decolourisation assay does not show saturation for complex biological samples and the values obtained depend strongly on the time and temperature. Therefore, values reported without precise details of the assay cannot be compared. However, refinements made to the method have made the TEAC more robust; pre-generation of the ABTS in the presence of thermolabile 2,2′-azobis(2-amidinepropane) dihydrochloride (ABAP) before the antioxidants are added avoids the interference from compounds that prevent radical formation (Van den Berg et al., 1999). Evaluation of lipid soluble antioxidants using TEAC can be diffiult (Van den Berg et al., 1999) unless the antioxidants are pre-solubilised in appropriate solvents.

5.4.4. Storage, stability and limitations on use

Ascorbate is a major contributor to TAC assays, and decays rapidly on storage, influencing the measurement of TAC in stored samples.

Table 16 highlights the discrepancies observed in determination of TAC by different groups. One reason for these discrepancies is the diversity of assay methods, employing different oxidants (in the inhibition assays) and indicators. Activity of antioxidants with respect to various oxidants also differs (Regoli and Winston, 1999), as it does with the indicator. Moreover, the TACs measured with blood plasma and serum differ (Ghiselli et al., 2000). The anticoagulant used may also affect the results (Goode et al., 1995) as does sample storage. Usually, the samples are collected over some time period and stored at −80 °C, under the assumption that the storage does not affect the valued measured. A considerable drop of TAC (assayed with AAPH and R-phycoerythrin) during the first three days of storage (down to ca. 60% of the initial values) has recently been reported (Ghiselli et al., 2000). Similarly, a 30% decrease in plasma TRAP assay results was reported after three weeks of storage (Jozwik et al., 1997). More attention must be paid to the stability of TAC during sample storage.

Attempts have been made to assess the TACs of cell and tissue homogenates (Evelson et al., 2001; Jaruga et al., 1995; Popov and Lewin, 1999). In this case, the procedures for the preparation and storage of the material can be expected to be of even greater importance.

A more general reservation concerns the biological relevance of TAC. One might expect that high TAC values would be associated with biological fluids of crucial importance such as seminal plasma. Instead the TACs of blood plasma and seminal plasma are comparable with that of urine and much lower than that of faeces (Table 16).

There are several observations contradicting the view that TAC measurements in human plasma are an indicator of the antioxidant defences of target tissues. The TAC of blood plasma in rats fed an ethanol-supplemented diet increases although ethanol is known to induce oxidative stress. The effect is due to ethanol-induced purine degradation and an increase in the level of uric acid (Gasbarrini et al., 1998). TAC of blood plasma in critically ill patients, with renal dysfunction, is augmented, again due to an increase in uric acid level (MacKinnon et al., 1999). Caloric

Table 16
Total antioxidant capacity of human body fluids

Material	Value [Trolox equivalents, μM]	Method	References
Amniotic fluid	522 ± 87	$ABTS^{+\cdot}$ decoloration (Re et al., 1999)	Janaszewska and Bartosz (submitted)
Blood plasma	1120 ± 70	TRAP 22	Pellegrini et al. (2000)
Blood plasma	1440 ± 284	AAPH/Luminol (Metsa-Ketela, 1991)	Nyyssonen et al. (1997)
Blood plasma	2700 ± 500	$ABTS^{+\cdot}$ decoloration	Opara et al. (1999)
Blood plasma (22 ± 5y)	1720 ± 200		Kostka et al. (2000)
Blood plasma (71 ± 4y)	1770 ± 130	TAS (Randox)	
Blood plasma (EDTA)	1010 ± 130	FRAP (Benzie and Strain, 1999)	Castenmiller et al. (1999)
Blood plasma (EDTA)	1272 ± 199	ABAP/Luminol	Erhola et al. (1997)
Blood plasma (EDTA)	1155 ± 290	H_2-DCF-DA/AAPH	Valkonen and Kuusi (1997)
Blood plasma (heparin)	1103 ± 30	FRAP (Benzie and Strain, 1999)	Hamilton et al. (2000)
Blood plasma (heparin)	830 ± 4	TAS (Randox)	Komosinska-Vassev et al. (2000)
Blood plasma (heparin)	930 ± 150	FRAP (Benzie and Strain, 1996)	Lee et al. (2000)
Blood plasma (heparin)	1760 ± 190	TAS (Randox)	(56)
Blood plasma (heparin)	580 ± 79	TRAP (Wayner et al., 1985)	Langley et al. (1993)
Blood plasma (heparin)	1870 ± 10	ORAC (Cao et al., 1993)	Clermont et al. (2000)
Blood serum	382 ± 79	Enhanced chemiluminescence assay (Whitehead et al., 1992)	Ryan et al. (1997)
Feces	26 600–10 500 mol/kg	$ABTS^{+\cdot}$ decolorization	Garsetti et al. (2000)
Saliva	389 ± 190	DPPH reduction	Atsumi et al. (1999)
Saliva	59 ± 26	FRAP (Benzie & Strain 1999)	Janaszewska and Bartosz (submitted)
Seminal plasma	1443 ± 105	Enhanced chemiluminescence assay (Whitehead et al., 1992)	Hendin et al. (1999)
Seminal plasma	1654 ± 115	Enhanced chemiluminescence assay (63)	Pasqualotto et al. (2000)
Tear fluid	409 ± 162	FRAP (Benzie and Strain, 1999)	Choy et al. (2000)

restriction, a procedure known to prolong the lifespan of mammals, decreases TAC of rat serum (Cao et al., 1997). The contribution of uric acid to the TAC of blood plasma, obscuring changes in the concentrations of other antioxidants, limits its usefulness.

Attempts have been made to calculate, and use, the urate-independent fraction of TAC (Popov and Lewin, 1999).

Specific problems include the lack of linearity between antioxidant concentration and response (Van den Berg et al., 1999). Similarly, it has become apparent that

masking of antioxidant activity can be observed in clinical samples. For example, the interaction of flavonoids with proteins masks their measurement as antioxidants (Arts et al., 2001).

TAC is an easily, and increasingly employed biomarker in clinical studies. It can be useful in comparing the antioxidant content of body fluids, cell and tissue homogenates. However, in spite of the simplicity of the assays, execution of TAC requires caution and its interpretation poses questions that are still unresolved.

5.5. Validation of assays for direct oxidant and antioxidant biomarkers

There is a significant requirement for standardisation of the many protocols for measurement of hydrogen peroxide and TAC. For the TAC there is also a need for appropriate external standard materials, which are not subject to oxidative instability. In spite of the experimental simplicity, correct interpretation of the results of the TAC assays is complicated. There is a need to further develop and standardise the assay. Table 23 gives a summary of validation status of the various assays.

5.6. Relationship of oxidant/antioxidant measurement to disease

Using activated neutrophils, the cerium chloride method has been adapted for the detection of cellular sites of hydrogen peroxide production, with the laser-scanning confocal reflectance microscope (Robinson and Batten, 1990). The same methods have been successfully employed for demonstration of the production of ROS at the endothelial surface of cardiac vessels, during the first moments of reperfusion following a period of anoxia (Babbs et al., 1991; Shlafer et al., 1990). DCF–DA has been successfully used to investigate whole organs and isolated cells as well as in flow cytometry applications (Royall and Ischiropoulos, 1993; Tsuchiya et al., 1994).

Hydrogen peroxide can be detected in expired breath, and levels of peroxide increase in respiratory disorders (Dekhuijzen et al., 1996). However, it should be noted that pulmonary bacteria may contribute to this effect, and that saliva can contain high levels of hydrogen peroxide. Several groups report the concentration in urine of normal subjects with mean values of the order of 35 μmol/l, dependent on the analytical procedure (Halliwell et al., 2000).

Many groups have investigated post-mortem brain levels of glutathione, in both oxidised and reduced forms, from neurodegenerative disease patients. Levels of 50 μmol/l for GSSG have been reported, and should be treated with caution. By taking many precautions to ensure minimal post-mortem oxidation, Cooper et al. (1980) obtained a value for GSSG in normal brain of 2 μM, which is approximately 0.2% of total glutathione. There have been several other studies of GSH/GSSG in disease. The metabolism of glutathione in pre-mature infants has been shown to be impaired, which is explained by deficient cystathionase, rendering pre-mature infants at greater risk of oxidative stress (Vina et al., 1995). Cataractous lenses show differential tissue distribution of glutathione, relative to normal lenses, where levels in the cortex and nucleus of the lens were depleted with elevated levels of GSSG (Chakrapani et al., 1995). In an exercise study of GSH/GSSG, it was observed that

light exercise causes an oxidation of glutathione in COPD patients. This can be partially prevented by oxygen therapy (Vina et al., 1996).

Determination of the ratio of AA/DHAA in disease has also been undertaken as a biomarker of oxidative stress. In both active and passive smoking, the ratios of DHAA to AA were shown to be significantly elevated compared to non-exposed individuals at 11.2%, 10.3% and 7.1%, respectively (Ayaori et al., 2000).

5.7. Modulation of oxidant/antioxidant biomarkers by dietary antioxidants

Histochemical analysis of ROS has not been applied to the study of the benefits of dietary antioxidants. There are no reports in the literature on direct effects of antioxidant intake on levels of hydrogen peroxide in body fluids. However, the effects of supplementation with vitamin C (1 g per day) in diabetics have been reported by Sinclair et al., demonstrating that the antioxidant imbalance in type II diabetics relates to abnormal AA metabolism (Sinclair et al., 1991). Modification of guinea pig diets for five weeks, by varying levels of α-tocopherol from 15 to 1500 mg/kg basal diet, showed that the GSH/GSSG and GSH/total GSH ratios increased as a function of vitamin E (Rojas et al., 1996).

Several studies have examined the effects of supplementation with antioxidants on total antioxidant capacity (Table 17). Few show a significant beneficial effect of supplementation, possibly reflecting the lack of sensitivity to change of any single antioxidant within the plasma pool.

6. Induction of genes in response to oxidative stress

6.1. Background

The critical review of the role of oxidative stress in the regulation of gene expression in Chapter 4 raises the issue that the effects of ROS/RNS are not always deleterious, but play an important role in normal cellular signalling processes. This function appears likely to be both dose- and microenvironment-dependent. However, in circumstances of elevated oxidative stress, oxidants can elicit *de novo* synthesis and post-transcriptional activation of enzymes involved in detoxification, where gene expression changes are a function of redox-sensitive transcription factor activation.

6.2. Measurement of antioxidant responsive genes and proteins

Microarray technology has the power to resolve the change in expression of genes associated with dietary antioxidant intake. However, careful experimental design is essential. At best this is likely to be a semi-quantitative method, which will identify candidate genes for subsequent analysis by RT–PCR. It is important to note that any change in mRNA will not necessarily correlate with a change in measurable level of protein activity, and these final determinations will remain the most important analyses to be undertaken.

Table 17
Antioxidant effects on total antioxidant capacity

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Tomato puree	7 mg lycopene + 0.15 mg β-carotene	11	21 days		Plasma	TRAP	No effect	Fluorescence	Pellegrini et al. (2000)
Vitamin C	500 mg/day	5	14 days		Plasma and CSF	TRAP	No effect	Chemiluminescence	Lonnrot et al. (1996)
	1000 mg/day	5	14 days						
Encapsulated fish oil containing 205 mg alpha tocopherol	205 mg α-tocopherol/day		28 days	Placebo	Plasma	TAC	No effect		Yaqoob et al. (2000)
β-Carotene	1 mg/kg/day (max dose 50 mg/day) followed by 10 mg/day	24	84 days	Randomised placebo	Plasma	TAC	Non-significant trend to improvement		Renner et al. (2001)
Tocopherol and selenium	500 μg 150 mg	32	5 days	Placebo, double blind, randomised	Plasma	TAC	Decrease ($p < 0.002$) in TAC observed with selenium alone	Randox	Berger (2001)
Vitamin C	60 mg/day	16	14 days	Placebo controlled crossover	Plasma	TAC	Increase in TAC		Anderson et al. (1997)
	6 g/day	16							

6.3. Effects of antioxidant intake on the activity of antioxidant enzymes

The activity of several antioxidant enzymes and stress response genes such as haem oxygenase 1 is affected by the level of oxidative stress, as part of an adaptive response. Several studies of antioxidant supplementation have been undertaken using antioxidant enzyme levels and stress proteins as endpoints, and these are featured in Table 18. The evidence provided is inconsistent, and this may reflect differences in study design and study power.

7. Conclusions

Herein, we have identified strengths and weaknesses in the different methodologies available for biomarker measurement, and reviewed the use of these techniques in supplementation studies. Specifically, a set of criteria has been established that allow us to identify the validity of each methodology, and more importantly, identify areas for further study. These are defined in priority order as: (i) specificity (both biological in terms of source, and chemical in terms of analysis), (ii) stability (−80 °C for at least four weeks), (iii) reproducibility (CV <10%), (iv) association with disease, and (v) responsiveness (i.e. dose relationship between oxidant and formation, and antioxidant and protection) (see Table 19).

Following these guidelines, we recommend that:

- 8-oxoGua is suitable, but further work is required on other oxidised base analysis, enzyme sites in the comet assay, etheno sites, and 8-oxo-dGuo measurement in urine by chromatography or ELISA;
- strand break analysis alone is deemed to be inappropriate;
- for lipid peroxidation analysis, determination of lipid peroxides and isoprostanes is considered suitable with further work necessary on urinary isoprostane metabolites and hydrocarbon gases;
- measurement of TBARS, MDA, conjugated dienes and lipofuscin is considered inappropriate in this context;
- analysis of oxidant and antioxidant biomarkers indicated that further work is needed on peroxide determination but, with appropriate precautions, GSH:GSSG could be usefully applied;
- total antioxidant capacity analysis is considered inappropriate for investigating antioxidant supplementation effects, as with measures of LDL oxidisability or resistance of DNA to strand breaks;
- there are many likely suitable candidates in the determination of protein oxidation including nitrated amino acids, protein-bound tyrosine oxidation products, protein-bound tryptophan products, and methionine sulphoxide, but at present these biomarkers are in earlier stages of validation. A wider adoption of methodologies, through different laboratories, will support these early positive indicators; and
- thiol and hydroperoxide determinations are considered inappropriate.

Table 18
Antioxidant effects on antioxidant enzyme gene expression

Dietary supplement(s)	Dose	Sample no.	Duration	Format	Tissue	Analyte	Effect observed	Method	References
Vitamin C Vitamin E β-carotene Selenium Cu Zn	60 mg/day 30 IU/day 3 mg/day 40 μg/day 80 mg/day 2 mg/day	30 passive smokers	60 days		WBC	SOD GPX GR Catalase	18% decrease no effect	Enzyme assay	Howard et al. (1998)
Parsley	3.73–4.49 apigenein/MJ/day	14	7 days	Crossover	Erythrocyte	GR SOD GPX CAT	Increase No effect	Enzyme assay	Nielsen et al. (1999)
Blackcurrant and apple Juice	750 1000 1500 ml/day	5	7 days/dose	Crossover	Plasma	GPX	Increase	Enzymatic	Young et al. (1999)
Vitamin C	1000 mg/days	23	42 days		WBC	SOD	Decreased activities	Enzyme assay	Brennan et al. (2000)
Vitamin E	800 mg/day					GPX Catalase	No effect		
Vitamin C	250 mg	32	35 days	Placebo controled	Plasma	GPX	No effect	Enzyme assay	Peng et al. (2000)
β-carotene alpha tocopherol	3 mg 100 IU					SOD			
folic acid	250 μg				Lymphocytes	Hsp105	Enhanced synthesis		

Grape seed extract	3.89 mg catechin				Hsp90 Hsp70 Hsp40 Hsp32	Decreased synthesis	Scanning densitometry of SDS-gels	
Diet low in carotenoids +/– carrot juice		23	14 days	WBC	GST-P1	9/21 increase	ELISA	Pool-Zobel et al. (1998)

We believe that we are able to confidently use certain biomarkers, the most sensible approach being to adopt several validated and accepted biomarkers in any study, and take into account the ADME of the antioxidant under study (*Bioavailability and metabolism*).

Table 19
Recommendations for appropriate biomarkers (specific > stable > validity > disease > dose response)

	Specific	Stable	Validity	Disease	Dose response
DNA biomarkers					
8-oxo-Gua (DNA)	++	? possible artefactual oxidation	++	++	++
Other oxidised bases	++	?	?	?	?
Urinary 8-oxo-dGuo	?	++	++	++	++
Immunoassay	?	?	?	++	++
Etheno adducts	?	++	?	++	++
Strand breaks	–	? half-life = few minutes	++	++	++
Enzyme sites	?	++	++	++	++
Lipid oxidation biomarkers					
TBARS	–	–	–	++	++
MDA HPLC	+/–	–	?	++	++
Ethane, pentane	?	++	?	++	++
Lipofuscin	–	++	–	++	–
Lipid peroxides	++	–	?	++	?
Plasma Isoprostanes	+/–	?	++	++	++
Urinary iP Metabolites	++	++	?	?	?
Diene conjugates	–	–	?	NA	++
Oxidant/antioxidant biomarkers					
TEAC	-	++	++	++	++
ORAC	++	?	?	?	++
FRAP	–	++	++	?	++
Resistance to strand breaks	–	?	?	?	++
LDL lag time	–	?	-	++	++
GSH:GSSG	++	–	++	++	++
Protein oxidation biomarkers					
Carbonyls	+/–	++	++	++	++
Thiols	+/–	++	++	?	++
Nitrated amino acid	++	+	?	?	++
Protein Tyr oxidation products	++	+	?	?	? (bityrosine)
Protein Trp oxidation products	++	++	?	++	++
Methionine sulphoxide	++	++	?	?	++
Hydroxides/hydroperoxides	++	–	?	++	++
Protein 2-adipic semi aldehyde	?	++	?	?	?
Neoepitopes	?	++	?	++	++

Key: ++ Criterion met with confidence; + Criterion met; ? Criterion not previously fully evaluated; – Criterion not met.

The use of appropriate biomarkers is a vital addition to the protocol requirements. It is now possible to re-evaluate the intervention studies detailed in Tables 4–7, 9–15, 17, and 18 discounting supplementation studies that have adopted inappropriate biomarkers.
Table 20 shows that:

- out of 80 studies presented, less than 50% used appropriately validated methodology, and of these 66% showed evidence of benefit;
- in studies where the population under examination was under oxidative stress at baseline or with reduced antioxidant status, benefit was more frequently seen when compared to normal subjects; and
- other important features of protocol requirement are trial design and study power. The parallel group crossover design is the more robust (comparing the relative or absolute changes between groups). Van den Berg et al. (2001) have calculated the sample number required to show a 10% difference in the effect of supplement, demonstrating the power of the study undertaken.

Several important points emerge, which address the apparent discrepancies in results generated to date, and relate to the designs of the studies themselves and the hypothesis under investigation:

- Is the effect of an individual antioxidant on an oxidative biomarker under consideration or is the role of combinations of antioxidants, or foodstuffs, to be evaluated?
- Antioxidant effects should be evaluated in a dose-dependent manner, in order that any safe upper limit may be identified;
- What period of supplementation should be investigated, given that the proposed effects are lifelong in accumulation?
- The mechanism by which the component works may not be as an antioxidant per se (e.g. hydrogen donation), but via an indirect process such as modification of gene expression; and
- The subject groups must also be well thought out – it may be that only individuals with antioxidant levels lower than the normal range will show any significant benefit.

An appropriate biomarker for healthy individuals may not be suitable for individuals with a particular disease. Suitability of a biomarker should be determined separately for healthy and disease states, at each stage of the disease and, ideally, disease-specific. The 'normal' values for biomarkers in the apparently healthy, and

Table 20
Antioxidant intervention studies showing benefit

	Vitamin E	Vitamin C	Carotenoid	Flavonoid	Combined/diet
DNA oxidation	1 (1)	5 (8)	1 (4)	2 (4)	7 (10)
Lipid oxidation	6 (10)	2 (2)		2 (3)	3 (4)

(*n*) refers to the total number of studies using acceptable biomarkers; 80 studies examined.

those with chronic diseases, may be different at each stage of the disease. The effects of ageing (e.g. increase in nitrated proteins with age that is not associated with an increase in CVD) should also be observed. In healthy subjects, antioxidant intervention may be inappropriate where it suppresses an oxidative response, which is essential to normal metabolism (*Antioxidants, reactive oxygen and nitrogen species, gene induction and mitochondrial function*).

8. Future research needs

Key requirements arising from this work are:

- the establishment of an ESCODD-style validation study for all the favoured biomarkers described in Table 19;
- a re-examination of the kinetics of oxidative biomarker formation and their respective half-lives. This can then be superimposed on knowledge of the kinetics of antioxidant availability at the target site, as raised by *Bioavailability and metabolism*. Many of the discrepancies in studies to date may be explained by viewing biomarker changes in different 'time frames';
- an examination of the influence of genetic polymorphisms on biomarker kinetics through DNA repair or antioxidant uptake, for example; and
- the need to establish normal levels, intra-individual variation, and seasonal and diurnal variations for all biomarkers of interest, in order to design appropriately powered studies.

There are also several areas offering new horizons in our comprehension of antioxidant influence on biomarkers:

- identification of mutations and sites of oxidative damage, and how these are modulated by antioxidant intervention. It remains to be elucidated whether measurement of site-specific DNA damage can inform on disease outcome more effectively than markers of global DNA damage;
- determination of antioxidant effects on isoprostane modulation of flow-mediated dilatation, platelet aggregation and adhesion. Isoprostanes have biological function through prostaglandin receptors, which may impinge on disease outcome through effects within the vasculature. Therefore, analysis of the effects of antioxidants on physiological endpoints in association with oxidative biomarkers represents a move to closing the loop between primary biomarkers and disease endpoints;
- examination of antioxidant capacity to protect protein function through protection against critical amino acid oxidation;
- utilisation of knowledge from quantitative expression analysis and proteomics to identify sensitive markers of antioxidant responsiveness, to inform on the importance of antioxidant as simple hydrogen donating capacity and/or as molecules capable of complex interaction with signalling pathways; and

Table 21
Protein oxidation

Biomarker and method	Preferred units of measurement	Normal range/ tissue/age	Limit of detection	Inter-batch CV (%)	Applicability to tissues/limitations	Inter-individual variation
Carbonyl-spec	nmol/mg	0.2–4.0	0.02	8.9	Require isolated protein	30%
Carbonyl-ELISA	nmol/mg	0.2–4.0	0.02	4.7	Require isolated protein	30%
Kynurenines–HPLC	mmol/mol parent amino acid	0.2–0.5	50 pmol	9.3	Isolated, digested protein	2–3 fold
L-DOPA–HPLC	mmol/mol parent amino acid	0.4–1.0	20 pmol	8.9	Isolated, digested protein	25%
Bityrosine–HPLC	mmol/mol parent amino acid	0.002	50 pmol	8.9	Isolated, digested protein	Several fold
Hydroxides–HPLC	mmol/mol parent amino acid	0.01	2 pmol	13.2	Isolated, digested protein	
Chlorotyrosine–HPLC	mmol/mol parent amino acid	0.08			Isolated, digested protein	
Nitrotyrosine–HPLC (ECD)	mmol/mol parent amino acid	0.1	2 pmol	8.9	Isolated, digested protein	
Nitrotyrosine–ELISA	μM					

Table 22
DNA oxidation

Biomarker and method	Preferred units of measurement	Normal range/ tissue/age	Limit of detection	Inter-batch CV (%)	Applicability to tissues/ limitations	Inter-individual variation	References
8-oxo-dGuo–GC/MS	8-oxoG/10^6 DNA bases	Several lesions/10^6 DNA base[a]	Several lesions/10^7 DNA base	About 10	No limitations	Several fold[*1]	Olinski et al. (1992)
8-oxo-Ade–GC/MS	8-oxoA/10^6 DNA bases	Several lesions/10^6 DNA base[a]	Several lesions/10^7 DNA base	About 10	No limitations	Several fold[*1]	Jaruga et al. (1994)
5-OHMeU–GC/MS	5-OhmeU/10^6 DNA bases	Several lesions/10^6 DNA base[a]	Several lesions/10^7 DNA base	About 10	No limitations	Several fold[*1]	Olinski et al. (1995)
FapyGua–GC/MS	FapyG/10^6 DNA bases	Several lesions/10^6 DNA base[a]	Several lesions/10^7 DNA base	About 10	No limitations	Several fold[*1]	Olinski et al. (1997)

[a] The background levels strongly depend on the condition of the procedure. See TG1 Draft Report; ''My personal assessment of GC/MS method validity'', also (Jaruga et al., 2000; Rodriguez et al., 2000).

Table 23
Antioxidant and oxidant measures

Biomarker and method	Preferred units of measurement	Normal range/ tissue/age	Limit of detection	Interbatch CV	Applicability to tissues/Limitations	Inter-individual variation	References
FRAP	mmol/l	1.04 (0.64–1.63)	<2 μM	$<1\%$	Blood plasma	$<3\%$	Benzie and Strain (1999)
TAS	mmol/l	1.76 ± 0.19			Blood plasma		van Bakel et al. (2000),
ORAC	mmol/l	3.10 ± 0.20			Blood serum		Cao and Prior (1999)

Table 24
Lipid oxidation

Biomarker and method	Preferred units of measurement	Reference range/ tissue/age	Limit of detection	Interbatch CV	Applicability to tissues/limitations	Inter-individual variation	References
MDA–spec	μM	0–2.3	0.2	8.5%	Non-specific		▲
MDA–HPLC	μM	0.16–0.31	0.1	7.1%	Specific V.non-specific		⊕
Diene conjugates-spec	OD Units		0.02	4.5%	All tissues		
Exhaled gases-GC/MS	?	NO	?	?	DATA		
Ox-LDL antibodies	mU/l	26–117	< 1	4.0%	Specific Plasma only		Holvoet et al. (1995)
Antibodies to Ox LDL	ng equiv/ml	0–583	< 1	3.8%	Specific		Virella et al. (1993)
LDL lag time	?	NO	?	?	? DATA	?	
Isoprostanes–ELISA (urine)	PM	1965–3107		12%	Non-specific urine only		
Isoprostanes–GC?MS	Plasma pg/ml urine ng/mg creat	12–26 pg/ml 1.6–2.2 ng/mg creat	1 pg/ml	10.5%	Specific Urine/ plasma		
Cholesterol ester hydroperoxides-HPLC	NM	9.2–15.4	< 1.0	3.9%	Specific all tissues and fluids	?	Yamamoto et al. (1987); Yasuda and Narita (1997)
Phospholipid hydroperoxides HPLC	NM	22–108	20 nM	6.0%	Specific all tissues		⊕
Cholesterol hydroxides	?	?	?	?	?	?	

⊕ (unpublished data, Bevan, R. Griffiths, H.R. and Lunec, J.); ▲ J. Lunec Ph.D. Thesis (1980) University of London.

- analysis of plasma samples from prospective studies of disease outcome using validated biomarkers to establish the relationships between biochemical endpoint and ultimate disease development.

Appendix A

See Tables 21–24.

References

Adachi, H., Ishii, N., 2000. Effects of tocotrienols on life span and protein carbonylation in *Caenorhabditis elegans*. J. Gerontol. A Biol. Sci. Med. Sci. 55 (6), B280–B285.

Adams, G.E., Willson, R.L., Aldrich, J.E., Cundall, R.B., 1969. On the mechanism of radiation induced inactivation of lysozyme in dilute aqueous solutions. Int. J. Radiat. Biol. 16, 333–342.

Aghdassi, E., Allard, J.P., 2000. Breath alkanes as a marker of oxidative stress in different clinical conditions. Free Radic. Biol. Med. 28 (6), 880–886.

Ahmad, J., Cooke, M.S., Hussieni, A., Evans, M.D., Patel, K., Burd, R.M., Bleiker, T.O., Hutchinson, P.E., Lunec, J., 1999. Urinary thymine dimers and 8-oxo-2′-deoxyguanosine in psoriasis. FEBS Lett. 460 (3), 549–553.

Ahotupa, M., Asankari, T.J., 1999. Baseline diene conjugation in LDL lipids: an indicator of circulating oxidized LDL. Free Radic. Biol. Med. 27 (11–12), 1141–1150.

Akaike, T., Inoue, K., Okamoto, T., Nishino, H., Otagiri, M., Fujii, S., Maeda, H., 1997. Nanomolar quantification and identification of various nitrosothiols by high performance liquid chromatography coupled with flow reactors of metals and Griess reagent. J. Biochem. (Tokyo) 122 (2), 459–466.

Allerheiligen, S.R., Ludden, T.M., Burk, R.F., 1987. The pharmacokinetics of pentane, a by-product of lipid peroxidation. Drug Metab. Dispos. 15 (6), 794–800.

Anbar, M., Neta, P., 1967. A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic compounds in aqueous solution. Int. J. Appl. Radiat. Isot. 18, 493–523.

Anderson, D., Phillips, B.J., Yu, T.W., Edwards, A.J., Ayesh, R., Butterworth, K.R., 1997. The effects of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with "low" or "high" cholesterol levels. Environ. Mol. Mutagen. 30 (2), 161–174.

Andreoni, K.A., Kazui, M., Cameron, D.E., Nyhan, D., Sehnert, S.S., Rohde, C.A., Bulkley, G.B., Risby, T.H., 1999. Ethane: a marker of lipid peroxidation during cardiopulmonary bypass in humans. Free Radic. Biol. Med. 26 (3–4), 439–445.

Anon, 2000. 7th Annual Meeting of The Oxygen Society. November 16–20, 2000. San Diego, California, USA. Free Radic. Biol. Med. 29 (Suppl. 1), S1–S143.

Anon, 2001. Comparison of negative and positive ion electrospray tandem mass spectrometry for the liquid chromatography tandem mass spectrometry analysis of oxidized deoxynucleosides. J. Am. Soc. Mass Spectrom. 12 (1), 80–87.

Arnao, M.B., Cano, A., Hernandez-Ruiz, J., Garcia-Canovas, F., Acosta, M., 1996. Inhibition by L-ascorbic acid and other antioxidants of the 2.2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) oxidation catalyzed by peroxidase: a new approach for determining total antioxidant status of foods. Anal. Biochem. 236 (2), 255–261.

Arts, M.J., Haenen, G.R., Voss, H.P., Bast, A., 2001. Masking of antioxidant capacity by the interaction of flavonoids with protein. Food Chem. Toxicol. 39 (8), 787–791.

Asensi, M., Sastre, J., Pallardo, F.V., Garcia, d.l.A., Estrela, J.M., Vina, J., 1994. High-performance liquid chromatography method for measurement of oxidized glutathione in biological samples. Anal. Biochem. 217 (2), 323–328.

Asmis, R., Jelk, J., 2000. Vitamin E supplementation of human macrophages prevents neither foam cell formation nor increased susceptibility of foam cells to lysis by oxidized LDL. Arterioscler. Thromb. Vasc. Biol. 20 (9), 2078–2086.

Astley, S., Langrish-Smith, A., Southon, S., Sampson, M., 1999. Vitamin E supplementation and oxidative damage to DNA and plasma LDL in type 1 diabetes. Diabetes Care 22 (10), 1626–1631.

Atsumi, T., Iwakura, I., Kashiwagi, Y., Fujisawa, S., Ueha, T., 1999. Free radical scavenging activity in the nonenzymatic fraction of human saliva: a simple DPPH assay showing the effect of physical exercise. Antioxid. Redox. Signal 1 (4), 537–546.

Ayaori, M., Hisada, T., Suzukawa, M., Yoshida, H., Nishiwaki, M., Ito, T., Nakajima, K., Higashi, K., Yonemura, A., Ohsuzu, F., Ishikawa, T., Nakamura, H., 2000. Plasma levels and redox status of ascorbic acid and levels of lipid peroxidation products in active and passive smokers. Environ. Health Perspect. 108 (2), 105–108.

Babbs, C.F., Cregor, M.D., Turek, J.J., Badylak, S.F., 1991. Endothelial superoxide production in the isolated rat heart during early reperfusion after ischemia. A histochemical study. Am. J. Pathol. 139 (5), 1069–1080.

Bartosz, G., Bartosz, M., 1999. Antioxidant activity: What do we measure? Acta Biochim. Pol. 46 (1), 23–29.

Bartosz, G., Janaszewska, A., Ertel, D., 1998a. Spectrophotometric determination of peroxyl-radical trapping capacity. Curr. Top. Biophys. 22 (Suppl. B), 11–13.

Bartosz, G., Janaszewska, A., Ertel, D., Bartosz, M., 1998b. Simple determination of peroxyl radical-trapping capacity. Biochem Mol. Biol. Int. 46 (3), 519–528.

Bartsch, H., 1999. In: Singer, B., Bartsch, H. (Eds.), Exocyclic DNA adducts in Mutagenesis and Carcinogenesis, vol. 150. IARC Scientific Publications, Lyon, pp. 1–16.

Bartsch, H., Barbin, B., Marion, M.-J., Nair, J., Guichard, Y., 1994. In: Di Carlo, F.J. (Ed.), Drug Metabolism Reviews, vol. 26 (1 & 2). Marcel Dekker Inc., New York, pp. 349–371.

Beatty, E.R., O'Reilly, J.D., England, T.G., McAnlis, G.T., Young, I.S., Geissler, C.A., Sanders, T.A., Wiseman, H., 2000. Effect of dietary quercetin on oxidative DNA damage in healthy human subjects. Br. J. Nutr. 84 (6), 919–925.

Beckman, J.S., Ye, Y.Z., Anderson, P.G., Chen, J., Accavitti, M.A., Tarpey, M.M., White, C.R., 1994. Excessive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol. Chem. Hoppe-Seyler 375, 81–88.

Bedossa, P., Houglum, K., Trautwein, C., Holstege, A., Chojkier, M., 1994. Stimulation of collagen alpha 1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: A link to tissue fibrosis? Hepatology 19 (5), 1262–1271.

Benedetti, A., Malvaldi, G., Fulceri, R., Comporti, M., 1984. Loss of lipid peroxidation as a histochemical marker for preneoplastic hepatocellular foci of rats. Cancer Res. 44 (12 Pt 1), 5712–5717.

Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239 (1), 70–76.

Benzie, I.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15–27.

Berger, M.M., 2001. Influence of early trace element and vitamin E supplement on antioxidant status after major trauma – a controlled trial. Nutr. Res. 21, 41–54.

Berlett, B.S., Stadtman, E.R., 1997. Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. 272 (33), 20313–20316.

Bianchini, F., Elmstahl, S., Martinez-Garcia, C., Kappel, A.L., Douki, T., Cadet, J., Ohshima, H., Riboli, E., Kaaks, R., 2000. Oxidative DNA damage in human lymphocytes: correlations with plasma levels of alpha-tocopherol and carotenoids. Carcinogenesis 21 (2), 321–324.

Biewenga, G.P., Veening-Griffioenen, D.H., Nicastia, A.J., Haenen, G.R.M.M., 1998. Effects of dihydrolipoic acid on peptide methionine sulfoxide reductase – implications for antioxidant drugs. Arneimittel-Forschung-drug Res. 48, 144–148.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 181, 1199–1200.

Bonassi, S., Fontana, V., Ceppi, M., Barale, R., Biggeri, A., 1999. Analysis of correlated data in human biomonitoring studies. The case of high sister chromatid exchange frequency cells. Mutat. Res. 438 (1), 13–21.

Bors, W., Bell, G.W., Korroll, 1984. Radiolysis and photolysis of aqueous aerated tryptophan solutions. In: Saran, M., Tait, D. (Eds.), Oxygen Radicals in Chemistry and Biology. Walter de Gruyter Co., Berlin, pp. 491–500.

Boyle, S.P., Dobson, V.L., Duthie, S.J., Hinselwood, D.C., Kyle, J.A., Collins, A.R., 2000a. Bioavailability and efficiency of rutin as an antioxidant: a human supplementation study. Eur. J. Clin. Nutr. 54 (10), 774–782.

Boyle, S.P., Dobson, V.L., Duthie, S.J., Kyle, J.A., Collins, A.R., 2000b. Absorption and DNA protective effects of flavonoid glycosides from an onion meal. Eur. J. Nutr. 39 (5), 213–223.

Brame, C.J., Salomon, R.G., Morrow, J.D., Roberts, L.J., 1999. Identification of extremely reactive gamma-ketoaldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. J. Biol. Chem. 274 (19), 13139–13146.

Branca, F., Hanley, A.B., Pool-Zobel, B., Verhagen, H., 2001. Biomarkers in disease and health. Br. J. Nutr. 86 (Suppl. 1), S55–S92.

Brennan, L.A., Morris, G.M., Wasson, G.R., Hannigan, B.M., Barnett, Y.A., 2000. The effect of vitamin C or vitamin E supplementation on basal and H_2O_2-induced DNA damage in human lymphocytes. Br. J. Nutr. 84 (2), 195–202.

Bunout, D., Garrido, A., Suazo, M., Kauffman, R., Venegas, P., la, M.P., Petermann, M., Hirsch, S., 2000. Effects of supplementation with folic acid and antioxidant vitamins on homocysteine levels and LDL oxidation in coronary patients. Nutrition 16 (2), 107–110.

Burcham, P.C., Kuhan, Y.T., 1996. Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. Biochem Biophys. Res. Commun. 220 (3), 996–1001.

Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M., Winterbourn, C.C., 1997. Protein carbonyl measurement by a sensitive ELISA method. Free Radic. Biol. Med. 23 (3), 361–366.

Cambiaggi, C., Dominici, S., Comporti, M., Pompella, A., 1997. Modulation of human T lymphocyte proliferation by 4-hydroxynonenal, the bioactive product of neutrophil-dependent lipid peroxidation. Life Sci. 61 (8), 777–785.

Cao, G., Cutler, R.G., 1995. Protein oxidation and aging. I. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. Arch. Biochem. Biophys. 320 (1), 106–114.

Cao, G., Prior, R.L., 1999. Measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 299, 50–62.

Cao, G., Alessio, H.M., Cutler, R.G., 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol. Med. 14 (3), 303–311.

Cao, G., Verdon, C.P., Wu, A.H., Wang, H., Prior, R.L., 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clin. Chem. 41 (12 Pt 1), 1738–1744.

Cao, G., Prior, R.L., Cutler, R.G., Yu, B.P., 1997. Effect of dietary restriction on serum antioxidant capacity in rats. Arch. Gerontol. Geriatr. 25, 245–253.

Carlberg, C., Möller, L., Paaki, P., Kantola, M., Stockmann, H., Purkunen, R., Wagner, P., Lauper, U., Kaha, M., Elovaara, E., Kirkinen, P., Pasanen, M., 2000. DNA adducts in human placenta as biomarkers for environmental pollution, analyzed by the ^{32}P-HPLC method. Biomarkers 5, 182–191.

Carp, H., Janoff, A., 1979. In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J. Clin. Invest. 63 (4), 793–797.

Carr, A.C., van den Berg, J.J., Winterbourn, C.C., 1998. Differential reactivities of hypochlorous and hypobromous acids with purified *Escherichia coli* phospholipid: formation of haloamines and halohydrins. Biochim. Biophys. Acta 1392 (2–3), 254–264.

Carr, A.C., Winterbourn, C.C., van den Berg, J.J., 1996. Peroxidase-mediated bromination of unsaturated fatty acids to form bromohydrins. Arch. Biochem. Biophys. 327 (2), 227–233.

Carty, J.L., Bevan, R., Waller, H., Mistry, N., Cooke, M., Lunec, J., Griffiths, H.R., 2000. The effects of vitamin C supplementation on protein oxidation in healthy volunteers. Biochem. Biophys. Res. Commun. 273 (2), 729–735.

Carvalho, V.M., Asahara, F., DiMascio, P., de I, A.C., Cadet, J., Medeiros, M.H., 2000. Novel 1, N(6)-etheno-2′-deoxyadenosine adducts from lipid peroxidation products. Chem. Res. Toxicol. 13 (5), 397–405.

Castenmiller, J.J., Lauridsen, S.T., Dragsted, L.O., Hof, K.H., Linssen, J.P., West, C.E., 1999. beta-carotene does not change markers of enzymatic and nonenzymatic antioxidant activity in human blood. J. Nutr. 129 (12), 2162–2169.

Chakrapani, B., Yedavally, S., Leverenz, V., Giblin, F.J., Reddy, V.N., 1995. Simultaneous measurement of reduced and oxidized glutathione in human aqueous humor and cataracts by electrochemical detection. Ophthalmic Res. 27 (Suppl. 1), 69–77.

Chaudhary, A.K., Nokubo, M., Reddy, G.R., Yeola, S.N., Morrow, J.D., Blair, I.A., Marnett, L.J., 1994. Detection of endogenous malondialdehyde–deoxyguanosine adducts in human liver. Science 265 (5178), 1580–1582.

Chaudhary, A.K., Nokubo, M., Oglesby, T.D., Marnett, L.J., Blair, I.A., 1995. Characterization of endogenous DNA adducts by liquid chromatography/electrospray ionization tandem mass spectrometry. J. Mass Stectrom. 30, 1157–1166.

Chen, H.J., Zhang, L., Cox, J., Cunningham, J.A., Chung, F.L., 1998. DNA adducts of 2,3-epoxy-4-hydroxynonanal: detection of 7-(1′,2′-dihydroxyheptyl)-3H-imidazo[2,1-i]purine and 1,N6-ethenoadenine by gas chromatography/negative ion chemical ionization/mass spectrometry. Chem. Res. Toxicol. 11 (12), 1474–1480.

Chen, H.J., Chiang, L.C., Tseng, M.C., Zhang, L.L., Ni, J., Chung, F.L., 1999. Detection and quantification of 1, N(6)-ethenoadenine in human placental DNA by mass spectrometry. Chem. Res. Toxicol. 12 (12), 1119–1126.

Chen, S., Chang, L.S., Wei, Y., 2001. Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. Free Radic. Biol. Med. 30 (11), 1328–1334.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., Loeb, L.A., 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions. J. Biol. Chem. 267 (1), 166–172.

Chevion, M., Berenshtein, E., Stadtman, E.R., 2000. Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radic. Res. 33 (Suppl.), S99–S108.

Chojkier, M., Houglum, K., Solis-Herruzo, J., Brenner, D.A., 1989. Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. A role for lipid peroxidation? J. Biol. Chem. 264 (28), 16957–16962.

Chopra, M., Fitzsimons, P.E., Strain, J.J., Thurnham, D.I., Howard, A.N., 2000. Nonalcoholic red wine extract and quercetin inhibit LDL oxidation without affecting plasma antioxidant vitamin and carotenoid concentrations. Clin. Chem. 46 (8 Pt 1), 1162–1170.

Chou, S.-T., Ko, L.-E., Yang, C.-S., 2001. High performance liquid chromatography with fluorimetric detection for the determination of total homocysteine in human plasma: method and clinical applications. Anal. Chim. Acta 429, 331–336.

Choy, C.K., Benzie, I.F., Cho, P., 2000. Ascorbic acid concentration and total antioxidant activity of human tear fluid measured using the FRASC assay. Invest. Ophthalmol. Vis. Sci. 41 (11), 3293–3298.

Chung, F.L., Chen, H.J., Nath, R.G., 1996. Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts. Carcinogenesis 17 (10), 2105–2111.

Chung, F.-L., Zhang, L., Ocando, J.E., Nath, R.G., 1999a. In: Singer, B., Bartsch, H. (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, vol. 150. IARC Scientific Publications, Lyon, pp. 45–54.

Chung, F.L., Nath, R.G., Nagao, M., Nishikawa, A., Zhou, G.D., Randerath, K., 1999b. Endogenous formation and significance of 1,N2-propanodeoxyguanosine adducts. Mutat. Res. 424 (1–2), 71–81.

Ciabattoni, G., Davi, G., Collura, M., Iapichino, L., Pardo, F., Ganci, A., Romagnoli, R., Maclouf, J., Patrono, C., 2000. In vivo lipid peroxidation and platelet activation in cystic fibrosis. Am. J. Respir. Crit. Care Med. 162 (4 Pt 1), 1195–1201.

Clermont, G., Lecour, S., Lahet, J., Siohan, P., Vergely, C., Chevet, D., Rifle, G., Rochette, L., 2000. Alteration in plasma antioxidant capacities in chronic renal failure and hemodialysis patients: a possible explanation for the increased cardiovascular risk in these patients. Cardiovasc. Res. 47 (3), 618–623.

Collins, A.R., 2000. Measurement of oxidative DNA damage using the comet assay. In: Griffiths, H.R., Lunec, J. (Eds.), Measuring in Vivo Oxidative Damage: A Practical Appraoch. Wiley, New York, pp. 83–94.

Collins, A.R., Dusinska, M., Gedik, C.M., Stetina, R., 1996. Oxidative damage to DNA: Do we have a reliable biomarker? Environ. Health Perspect. 104 (Suppl. 3), 465–469.

Collins, A.R., Duthie, S.J., Fillion, L., Gedik, C.M., Vaughan, N., Wood, S.G., 1997. Oxidative DNA damage in human cells: the influence of antioxidants and DNA repair. Biochem. Soc. Trans. 25 (1), 326–331.

Collins, A.R., Gedik, C.M., Olmedilla, B., Southon, S., Bellizzi, M., 1998a. Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates. FASEB J. 12 (13), 1397–1400.

Collins, A.R., Olmedilla, B., Southon, S., Granado, F., Duthie, S.J., 1998b. Serum carotenoids and oxidative DNA damage in human lymphocytes. Carcinogenesis 19 (12), 2159–2162.

Collins, B.H., Horska, A., Hotten, P.M., Riddoch, C., Collins, A.R., 2001. Kiwifruit protects against oxidative DNA damage in human cells and in vitro. Nutr. Cancer 39 (1), 148–153.

Cooke, M.S., Evans, M.D., Podmore, I.D., Herbert, K.E., Mistry, N., Mistry, P., Hickenbotham, P.T., Hussieni, A., Griffiths, H.R., Lunec, J., 1998. Novel repair action of vitamin C upon in vivo oxidative DNA damage. FEBS Lett. 439 (3), 363–367.

Cooke, M.S., Evans, M.D., Herbert, K.E., Lunec, J., 2000. Urinary 8-oxo-2′-deoxyguanosine—source, significance and supplements. Free Radic. Res. 32 (5), 381–397.

Cooper, A.J., Pulsinelli, W.A., Duffy, T.E., 1980. Glutathione and ascorbate during ischemia and postischemic reperfusion in rat brain. J. Neurochem. 35 (5), 1242–1245.

Corongiu, F.P., Poli, G., Dianzani, M.U., Cheeseman, K.H., Slater, T.F., 1986. Lipid peroxidation and molecular damage to polyunsaturated fatty acids in rat liver. Recognition of two classes of hydroperoxides formed under conditions in vivo. Chem. Biol. Interact. 59 (2), 147–155.

Courderot-Masuyer, C., Dalloz, F., Maupoil, V., Rochette, L., 1999. Antioxidant properties of aminoguanidine. Fundam. Clin. Pharmacol. 13 (5), 535–540.

Creed, D., 1984. The photochemistry and photophysics of the near UV absorbing amino acids. III Cystine and its simple derivatives. Photochem. Photobiol. 39, 577–584.

Crews, H., Alink, G., Andersen, R., Braesco, V., Holst, B., Maiani, G., Ovesen, L., Scotter, M., Solfrizzo, M., van den, B.R., Verhagen, H., Williamson, G., 2001a. A critical assessment of some biomarker approaches linked with dietary intake. Br. J. Nutr. 86 (Suppl. 1), S5–35.

Crews, H.M., Hanley, A.B., Verhagen, H., Wild, C. (Eds.) 2001b. Biomarkers of exposure and effect in relation to quality of life and human risk assessment. Br. J. Nutr. 86 (Suppl. 1).

Crow, J.P., 1999. Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxyphenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection. Methods Enzymol. 301, 151–160.

Cui, X.S., Torndal, U.B., Eriksson, L.C., Moller, L., 1995. Early formation of DNA adducts compared with tumor formation in a long-term tumor study in rats after administration of 2-nitrofluorene. Carcinogenesis 16 (9), 2135–2141.

Cyrus, T., Pratico, D., Zhao, L., Witztum, J.L., Rader, D.J., Rokach, J., FitzGerald, G.A., Funk, C.D., 2001. Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein E-deficient mice. Circulation 103 (18), 2277–2282.

Daneshvar, B., Frandsen, H., Dragsted, L.O., Knudsen, L.E., Autrup, H., 1997. Analysis of native human plasma proteins and haemoglobin for the presence of bityrosine by high-performance liquid chromatography. Pharmacol. Toxicol. 81 (5), 205–208.

Davi, G., Alessandrini, P., Mezzetti, A., Minotti, G., Bucciarelli, T., Costantini, F., Cipollone, F., Bon, G.B., Ciabattoni, G., Patrono, C., 1997. In vivo formation of 8-Epi-prostaglandin F2 alpha is increased in hypercholesterolemia. Arterioscler. Thromb. Vasc. Biol 17 (11), 3230–3235.

Davi, G., Ciabattoni, G., Consoli, A., Mezzetti, A., Falco, A., Santarone, S., Pennese, E., Vitacolonna, E., Bucciarelli, T., Costantini, F., Capani, F., Patrono, C., 1999. In vivo formation of 8-iso-prostaglandin F2 alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation. Circulation 99 (2), 224–229.

Davies, M.J., Fu, S., Wang, H., Dean, R.T., 1999. Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic. Biol. Med. 27 (11–12), 1151–1163.

de la Asuncion, J.G., Olmo, M.L., Sastre, J., Millan, A., Pellin, A., Pallardo, F.V., Vina, J., 1998. AZT treatment induces molecular and ultrastructural oxidative damage to muscle mitochondria. Prevention by antioxidant vitamins. J. Clin. Invest. 102 (1), 4–9.

Dean, R.T., Fu, S., Stocker, R., Davies, M.J., 1997. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 324 (Pt 1), 1–18.

Degan, P., Shigenaga, M.K., Park, E.M., Alperin, P.E., Ames, B.N., 1991. Immunoaffinity isolation of urinary 8-hydroxy-2′-deoxyguanosine and 8-hydroxyguanine and quantitation of 8-hydroxy-2′-deoxyguanosine in DNA by polyclonal antibodies. Carcinogenesis 12 (5), 865–871.

Degan, P., Bonassi, S., Caterina, M., Korkina, L.G., Pinto, L., Scopacasa, F., Zatterale, A., Calzone, R., Pagano, G., 1995. In vivo accumulation of 8-hydroxy-2′-deoxyguanosine in DNA correlates with release of reactive oxygen species in Fanconi's anaemia families. Carcinogenesis 16 (4), 735–741.

Dekhuijzen, P.N., Aben, K.K., Dekker, I., Aarts, L.P., Wielders, P.L., Herwaarden, C.L., Bast, A., 1996. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 154 (3 Pt 1), 813–816.

Dianov, G., Bischoff, C., Piotrowski, J., Bohr, V.A., 1998. Repair pathways for processing of 8-oxoguanine in DNA by mammalian cell extracts. J. Biol. Chem. 273 (50), 33811–33816.

Dizdaroglu, M., 1991. Chemical determination of free radical-induced damage to DNA. Free Radic. Biol. Med. 10 (3–4), 225–242.

Dizdaroglu, M., 1994. Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry. Methods Enzymol. 234, 3–16.

Doerge, D.R., Churchwell, M.I., Fang, J.L., Beland, F.A., 2000. Quantification of etheno-DNA adducts using liquid chromatography, on-line sample processing, and electrospray tandem mass spectrometry. Chem. Res. Toxicol. 13 (12), 1259–1264.

Duthie, S.J., Ross, M., Collins, A.R., 1995. The influence of smoking and diet on the hypoxanthine phosphoribosyltransferase (hprt) mutant frequency in circulating T lymphocytes from a normal human population. Mutat. Res. 331 (1), 55–64.

Duthie, S.J., Ma, A., Ross, M.A., Collins, A.R., 1996. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Res. 56 (6), 1291–1295.

Ellman, G.L., 1959. Tissue sulphydryl groups. Arch. Biochem. Biophys. 82, 70–77.

England, T.G., Jenner, A., Aruoma, O.I., Halliwell, B., 1998. Determination of oxidative DNA base damage by gas chromatography-mass spectrometry. Effect of derivatization conditions on artifactual formation of certain base oxidation products. Free Radic. Res. 29 (4), 321–330.

Erhola, M., Nieminen, M.M., Kellokumpu-Lehtinen, P., Metsa-Ketela, T., Poussa, T., Alho, H., 1997. Plasma peroxyl radical trapping capacity in lung cancer patients: a case-control study. Free Radic. Res. 26 (5), 439–447.

ESCODD (European Standards Committee on Oxidative DNA Damage) 2000. Comparison of different methods of measuring 8 oxoguanine as a marker of oxidative DNA damage. Free Radic. Res. 32 (4), 333–341.

Esterbauer, H., Schaur, R.J., Zollner, H., 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11 (1), 81–128.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E.A., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388 (2), 261–266.

Feig, D.I., Reid, T.M., Loeb, L.A., 1994. Reactive oxygen species in tumorigenesis. Cancer Res. 54 (Suppl. 7), 1890–1894.

Fenech, M., Perepetskaya, G., Mikhalevich, L., 1997. A more comprehensive application of the micronucleus technique for biomonitoring of genetic damage rates in human populations – experiences from the Chernobyl catastrophe. Environ. Mol. Mutagen. 30 (2), 112–118.

Ferro, D., Basili, S., Pratico, D., Iuliano, L., FitzGerald, G.A., Violi, F., 1999. Vitamin E reduces monocyte tissue factor expression in cirrhotic patients. Blood 93 (9), 2945–2950.

Floyd, R.A., 1990. The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis 11 (9), 1447–1450.

Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H., Rickard, R.C., 1986. Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. Free Radic. Res.Commun. 1 (3), 163–172.

Foksinski, M., Kotzbach, R., Szymanski, W., Olinski, R., 2000. The level of typical biomarker of oxidative stress 8-hydroxy-2′-deoxyguanosine is higher in uterine myomas than in control tissues and correlates with the size of the tumor. Free Radic. Biol. Med. 29 (7), 597–601.

Frank, J., Kelleher, D.K., Pompella, A., Thews, O., Biesalski, H.K., Vaupel, P., 1998. Enhancement of oxidative cell injury and antitumor effects of localized 44 °C hyperthermia upon combination with respiratory hyperoxia and xanthine oxidase. Cancer Res. 58 (13), 2693–2698.

Frei, B., Yamamoto, Y., Niclas, D., Ames, B.N., 1988. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. Anal. Biochem. 175 (1), 120–130.

Frelon, S., Douki, T., Ravanat, J.L., Pouget, J.P., Tornabene, C., Cadet, J., 2000. High-performance liquid chromatography–tandem mass spectrometry measurement of radiation-induced base damage to isolated and cellular DNA. Chem. Res. Toxicol. 13 (10), 1002–1010.

Fridovich, I., 1997. Superoxide anion radical ($O_2^{\bullet -}$), superoxide dismutases, and related matters. J. Biol. Chem. 272 (30), 18515–18517.

Fu, S., Gebicki, S., Jessup, W., Gebicki, J.M., Dean, R.T., 1995a. Biological fate of amino acid, peptide and protein hydroperoxides. Biochem. J. 311 (Pt 3), 821–827.

Fu, S., Hick, L.A., Sheil, M.M., Dean, R.T., 1995b. Structural identification of valine hydroperoxides and hydroxides on radical-damaged amino acid, peptide, and protein molecules. Free Radic. Biol. Med. 19 (3), 281–292.

Fu, S., Davies, M.J., Stocker, R., Dean, R.T., 1998a. Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem. J. 333 (Pt 3), 519–525.

Fu, S., Dean, R., Southan, M., Truscott, R., 1998b. The hydroxyl radical in lens nuclear cataractogenesis. J. Biol. Chem. 273 (44), 28603–28609.

Fu, S.L., Dean, R.T., 1997. Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins. Biochem J. 324 (Pt 1), 41–48.

Funabiki, R., Takeshita, K., Miura, Y., Shibasato, M., Nagasawa, T., 1999. Dietary supplement of G-rutin reduces oxidative damage in the rodent model. J. Agric. Food Chem. 47 (3), 1078–1082.

Funk, M.O., Baker, W.J., 1985. Determination of organic peroxides by high performance liquid chrmoatography with electrochemical detection. J. Liq. Chromatogr. 8 (4), 663–675.

Gackowski, D., Rozalski, R., Roszkowski, K., Jawien, A., Foksinski, M., Olinski, R., 2001. 8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine levels in human urine do not depend on diet. Free Radic. Res. 35 (6), 825–832.

Garner, B., Witting, P.K., Waldeck, A.R., Christison, J.K., Raftery, M., Stocker, R., 1998. Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. J. Biol. Chem. 273 (11), 6080–6087.

Garsetti, M., Pellegrini, N., Baggio, C., Brighenti, F., 2000. Antioxidant activity in human faeces. Br. J. Nutr. 84 (5), 705–710.

Gasbarrini, A., Addolorato, G., Simoncini, M., Gasbarrini, G., Fantozzi, P., Mancini, F., Montanari, L., Nardini, M., Ghiselli, A., Scaccini, C., 1998. Beer affects oxidative stress due to ethanol in rats. Dig. Dis. Sci 43 (6), 1332–1338.

Gavella, M., Lipovac, V., Vucic, M., Rocic, B., 1997. Evaluation of ascorbate and urate antioxidant capacity in human semen. Andrologia 29 (1), 29–35.

Gee, C.A., Kittridge, K.J., Willson, R.L., 1985. Peroxy free radicals, enzymes and radiation damage: sensitisation by oxygen and protection by superoxide dismutase and antioxidants. Br. J. Radiol. 58 (687), 251–256.

Ghiselli, A., Serafini, M., Natella, F., Scaccini, C., 2000. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. Free Radic. Biol. Med. 29 (11), 1106–1114.

Giessauf, A., vanWickern, B., Simat, T., Steinhart, H., Esterbauer, H., 1996. Formation of N-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. FEBS Lett. 389 (2), 136–140.

Girard, P.M., Boiteux, S., 1997. Repair of oxidized DNA bases in the yeast *Saccharomyces cerevisiae*. Biochimie 79 (9–10), 559–566.

Good, P.F., Werner, P., Hsu, A., Olanow, C.W., Perl, D.P., 1996. Evidence of neuronal oxidative damage in Alzheimer's disease. Am. J. Pathol. 149 (1), 21–28.

Goode, H.F., Richardson, N., Myers, D.S., Howdle, P.D., Walker, B.E., Webster, N.R., 1995. The effect of anticoagulant choice on apparent total antioxidant capacity using three different methods. Ann. Clin. Biochem. 32 (Pt 4), 413–416.

Gopaul, N.K., Halliwell, B., Anggard, E.E., 2000. Measurement of plasma F2-isoprostanes as an index of lipid peroxidation does not appear to be confounded by diet. Free Radic. Res. 33 (2), 115–127.

Green, M.H., Lowe, J.E., Waugh, A.P., Aldridge, K.E., Cole, J., Arlett, C.F., 1994. Effect of diet and vitamin C on DNA strand breakage in freshly-isolated human white blood cells. Mutat. Res. 316 (2), 91–102.

Griffiths, H.R., 2000. Antioxidants and protein oxidation. Free Radic. Res. 33 (Suppl.), S47–S58.

Griffiths, H.R., Lunec, J., Gee, C.A., Willson, R.L., 1988. Oxygen radical induced alterations in polyclonal IgG. FEBS Lett. 230 (1–2), 155–158.

Griffiths, H.R., Lunec, J., Blake, D.R., metabolite, N-formyl, 1992. Oxygen radical induced fluorescence in proteins; identification of the fluorescent tryptophan kynurenine, as a biological index of radical damage. Amino Acids 3, 183–194.

Griffiths, H.R., Bevan, R., Lunec, J., 1999a. Measurement of oxidised amino acids by HPLC. In: Lunec, J., Griffiths, H.R. (Eds.), Handbook of Oxidative Methods. Wiley, London.

Griffiths, H.R., Wright, J., Bevan, R., Lunec, J., 1999b. Oxidative modification of a specific apolipoprotein B lysine residue confers altered receptor specificity on LDL. Redox Rep. 4 (6), 337–339.

Griffiths, H.R., Aldred, S., Bevan, R., Waller, H., Mistry, N., Cooke, M., Lunec, J., 2002 Vitamin C supplementation causes a decrease in protein bound N-formlykynuernine in healthy subjects, submitted.

Guengerich, F.P., 1992. Roles of the vinyl chloride oxidation products 1-chlorooxirane and 2-chloroacetaldehyde in the in vitro formation of etheno adducts of nucleic acid bases [corrected]. Chem. Res. Toxicol. 5 (1), 2–5.

Gupta, R.C., Lutz, W.K., 1999. Background DNA damage for endogenous and unavoidable exogenous carcinogens: a basis for spontaneous cancer incidence? Mutat. Res. 424 (1–2), 1–8.

Gurr, M., 1999. In: Lipids in Nutrition and Health: A Reappraisal. Oily Press, Bridgewater, p. 97.

Gutteridge, J.M., 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. FEBS Lett. 128 (2), 343–346.

Hagmar, L., Brogger, A., Hansteen, I.L., Heim, S., Hogstedt, B., Knudsen, L., Lambert, B., Linnainmaa, K., Mitelman, F., Nordenson, I., 1994. Cancer risk in humans predicted by increased levels of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage. Cancer Res. 54 (11), 2919–2922.

Halliwell, B., 1999. Oxygen and nitrogen are pro-carcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen species: measurement, mechanism and the effects of nutrition. Mutat. Res. 443 (1–2), 37–52.

Halliwell, B., Gutteridge, J.M., 1981. Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. FEBS Lett. 128 (2), 347–352.

Halliwell, B., Gutteridge, J.M., 1989. Free Radicals in Biology and Medicine, second ed. Oxford University Press, New York.

Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57 (Suppl. 5), 715S–724.

Halliwell, B., Clement, M.V., Long, L.H., 2000. Hydrogen peroxide in the human body. FEBS Lett. 486 (1), 10–13.

Hamilton, I.M., Gilmore, W.S., Benzie, I.F., Mulholland, C.W., Strain, J.J., 2000. Interactions between vitamins C and E in human subjects. Br. J. Nutr. 84 (3), 261–267.

Harris, M.E., Carney, J.M., Hua, D.H., Leedle, R.A., 1994. Detection of oxidation products in individual neurons by fluorescence microscopy. Exp. Neurol. 129 (1), 95–102.

Hazen, S.L., Hsu, F.F., Duffin, K., Heinecke, J.W., 1996. Molecular chlorine generated by the myeloperoxidase-hydrogen peroxide-chloride system of phagocytes converts low density lipoprotein cholesterol into a family of chlorinated sterols. J. Biol. Chem. 271 (38), 23080–23088.

Hazra, T.K., Izumi, T., Maidt, L., Floyd, R.A., Mitra, S., 1998. The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. Nucleic Acids Res. 26 (22), 5116–5122.

Hedley, D., Chow, S., 1992. Flow cytometric measurement of lipid peroxidation in vital cells using parinaric acid. Cytometry 13 (7), 686–692.

Helbock, H.J., Beckman, K.B., Shigenaga, M.K., Walter, P.B., Woodall, A.A., Yeo, H.C., Ames, B.N., 1998. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc. Natl. Acad. Sci. USA 95 (1), 288–293.

Hendin, B.N., Kolettis, P.N., Sharma, R.K., Thomas Jr., A.J., Agarwal, A., 1999. Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. J. Urol. 161 (6), 1831–1834.

Hertog, M.G.L., deVries, A., Ocké, M.C., Schouten, A., Bueno-de-Mesquita, B., Verhagen, H., 1997. Oxidative DNA-damage in humans: comparison between high and low habitual fruits and vegetables consumption. Biomarkers 2, 259–262.

Hicks, M., Gebicki, J.M., 1979. A spectrophotometric method for the determination of lipid hydroperoxides. Anal. Biochem. 99 (2), 249–253.

Hirai, K.-I., Moriguchi, K., Wang, G.-Y., 1992. Real time histochemistry of oxygen free radical production in human leukocytes. Acta Histochem. Cytochem. 25, 319–323.

Holvoet, P., Perez, G., Zhao, Z., Brouwers, E., Bernar, H., Collen, D., 1995. Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. J. Clin. Invest. 95 (6), 2611–2619.

Howard, D.J., Ota, R.B., Briggs, L.A., Hampton, M., Pritsos, C.A., 1998. Oxidative stress induced by environmental tobacco smoke in the workplace is mitigated by antioxidant supplementation. Cancer Epidemiol. Biomarkers Prev. 7 (11), 981–988.

Huang, H.Y., Helzlsouer, K.J., Appel, L.J., 2000. The effects of vitamin C and vitamin E on oxidative DNA damage: results from a randomized controlled trial. Cancer Epidemiol. Biomarkers Prev. 9 (7), 647–652.

Hussain, S.P., Aguilar, F., Amstad, P., Cerutti, P., 1994. Oxy-radical induced mutagenesis of hotspot codons 248 and 249 of the human p53 gene. Oncogene 9 (8), 2277–2281.

Ikeda, K., Nagai, R., Sakamoto, T., Sano, H., Araki, T., Sakata, N., Nakayama, H., Yoshida, M., Ueda, S., Horiuchi, S., 1998. Immunochemical approaches to AGE-structures: characterization of anti-AGE antibodies. J. Immunol. Methods 215 (1–2), 95–104.

Ingrosso, D., D'angelo, S., Carlo, E., Perna, A.F., Zappia, V., Galletti, P., 2000. Increased methyl esterification of altered aspartyl residues in erythrocyte membrane proteins in response to oxidative stress. Eur. J. Biochem. 267 (14), 4397–4405.

Iversen, S.A., Cawood, P., Madigan, M.J., Lawson, A.M., Dormandy, T.L., 1984. Identification of a diene conjugated component of human lipid as octadeca-9,11-dienoic acid. FEBS Lett. 171 (2), 320–324.

Iversen, S.A., Cawood, P., Dormandy, T.L., 1985. A method for the measurement of a diene-conjugated derivative of linoleic acid, 18:2(9,11), in serum phospholipid, and possible origins. Ann. Clin. Biochem. 22 (Pt 2), 137–140.

Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C.H., Hall, D.O., 1978. Subcellular localisation and identification of superoxide dismutase in the leaves of higher plants. Eur. J. Biochem. 91 (2), 339–344.

Jacob, R.A., Kelley, D.S., Pianalto, F.S., Swendseid, M.E., Henning, S.M., Zhang, J.Z., Ames, B.N., Fraga, C.G., Peters, J.H., 1991. Immunocompetence and oxidant defense during ascorbate depletion of healthy men. Am. J. Clin. Nutr. 54 (Suppl. 6), 1302S–1309.

Jacobson, J.S., Begg, M.D., Wang, L.W., Wang, Q., Agarwal, M., Norkus, E., Singh, V.N., Young, T.L., Yang, D., Santella, R.M., 2000. Effects of a 6-month vitamin intervention on DNA damage in heavy smokers. Cancer Epidemiol. Biomarkers Prev. 9 (12), 1303–1311.

Jaruga, E., Lapshina, E.A., Bilinski, T., Plonka, A., Bartosz, G., 1995. Resistance to ionizing radiation and antioxidative defence in yeasts. Are antioxidant-deficient cells permanently stressed? Biochem. Mol. Biol. Int. 37 (3), 467–473.

Jaruga, P., Zastawny, T.H., Skokowski, J., Dizdaroglu, M., Olinski, R., 1994. Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer. FEBS Lett. 341 (1), 59–64.

Jaruga, P., Speina, E., Gackowski, D., Tudek, B., Olinski, R., 2000. Endogenous oxidative DNA base modifications analysed with repair enzymes and GC/MS technique. Nucleic Acids Res. 28 (6), E16.

Jenkins, G.J., Morgan, C., Baxter, J.N., Parry, E.M., Parry, J.M., 2001. The detection of mutations induced in vitro in the human p53 gene by hydrogen peroxide with the restriction site mutation (RSM) assay. Mutat. Res. 498 (1–2), 135–144.

Jenkinson, A.M., Collins, A.R., Duthie, S.J., Wahle, K.W., Duthie, G.G., 1999. The effect of increased intakes of polyunsaturated fatty acids and vitamin E on DNA damage in human lymphocytes. FASEB J. 13 (15), 2138–2142.

Jozwik, M., Jozwik, M., Jozwik, M., Szczypka, M., Gajewska, J., Laskowska-Klita, T., 1997. Antioxidant defence of red blood cells and plasma in stored human blood. Clin. Chim. Acta 267 (2), 129–142.

Kamiya, H., Kasai, H., 1995. Formation of 2-hydroxydeoxyadenosine triphosphate, an oxidatively damaged nucleotide, and its incorporation by DNA polymerases. Steady-state kinetics of the incorporation. J. Biol. Chem. 270 (33), 19446–19450.

Kamiya, H., Miura, H., Murata-Kamiya, N., Ishikawa, H., Sakaguchi, T., Inoue, H., Sasaki, T., Masutani, C., Hanaoka, F., Nishimura, S., 1995. 8-Hydroxyadenine (7,8-dihydro-8-oxoadenine) induces misincorporation in in vitro DNA synthesis and mutations in NIH 3T3 cells. Nucleic Acids Res. 23 (15), 2893–2899.

Karnovsky, M.J., 1994. Robert Feulgen Lecture 1994. Cytochemistry and reactive oxygen species: a retrospective. Histochemistry 102 (1), 15–27.

Kasai, H., Nishimura, S., 1984. Hydroxylation of deoxy guanosine at the C-8 position by polyphenols and aminophenols in the presence of hydrogen peroxide and ferric ion. Gann 75 (7), 565–566.

Kasai, H., Iwamoto-Tanaka, N., Fukada, S., 1998. DNA modifications by the mutagen glyoxal: adduction to G and C, deamination of C and GC and GA cross-linking. Carcinogenesis 19 (8), 1459–1465.

Kato, Y., Maruyama, W., Naoi, M., Hashizume, Y., Osawa, T., 1998. Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain. FEBS Lett. 439 (3), 231–234.

Kato, Y., Miyake, Y., Yamamoto, K., Shimomura, Y., Ochi, H., Mori, Y., Osawa, T., 2000. Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem. Biophys. Res. Commun. 274 (2), 389–393.

Komosinska-Vassev, K., Olczyk, K., Kucharz, E.J., Marcisz, C., Winsz-Szczotka, K., Kotulska, A., 2000. Free radical activity and antioxidant defense mechanisms in patients with hyperthyroidism due to Graves' disease during therapy. Clin. Chim. Acta 300 (1–2), 107–117.

Kondo, Y., Ohnishi, M., Kawaguchi, M., 1999. Detection of lipid peroxidation catalyzed by chelated iron and measurement of antioxidant activity in wine by a chemiluminescence analyzer. J. Agric. Food Chem. 47 (5), 1781–1785.

Koshiishi, I., Imanari, T., 1997. Measurement of ascorbate and dehydroascorbate contents in biological fluids. Anal. Chem. 69 (2), 216–220.

Koshiishi, I., Mamura, Y., Liu, J., Imanari, T., 1998. Evaluation of an acidic deproteinization for the measurement of ascorbate and dehydroascorbate in plasma samples. Clin. Chem. 44 (4), 863–868.

Kostka, T., Drai, J., Berthouze, S.E., Lacour, J.R., Bonnefoy, M., 2000. Physical activity, aerobic capacity and selected markers of oxidative stress and the anti-oxidant defence system in healthy active elderly men. Clin. Physiol. 20 (3), 185–190.

Langley, S.C., Brown, R.K., Kelly, F.J., 1993. Reduced free-radical-trapping capacity and altered plasma antioxidant status in cystic fibrosis. Pediatr. Res. 33 (3), 247–250.

LePage, F., Margot, A., Grollman, A.P., Sarasin, A., Gentil, A., 1995. Mutagenicity of a unique 8-oxoguanine in a human Ha-ras sequence in mammalian cells. Carcinogenesis 16 (11), 2779–2784.

Lean, M.E., Noroozi, M., Kelly, I., Burns, J., Talwar, D., Sattar, N., Crozier, A., 1999. Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA. Diabetes 48 (1), 176–181.

Lee, A., Thurnham, D.I., Chopra, M., 2000. Consumption of tomato products with olive oil but not sunflower oil increases the antioxidant activity of plasma. Free Radic. Biol. Med. 29 (10), 1051–1055.

Lee, A.T., Plump, A., DeSimone, C., Cerami, A., Bucala, R., 1995. A role for DNA mutations in diabetes-associated teratogenesis in transgenic embryos. Diabetes 44 (1), 20–24.

Lee, B.M., Lee, S.K., Kim, H.S., 1998. Inhibition of oxidative DNA 8-OHdG, and carbonyl contents in smokers treated with antioxidants (vitamin E, vitamin C, beta-carotene and red ginseng). Cancer Lett. 132 (1–2), 219–227.

Leighton, F., Cuevas, A., Guasch, V., Perez, D.D., Strobel, P., Martin, A., Urzua, U., Diez, M.S., Foncea, R., Castillo, O., Mizon, C., Espinoza, M.A., Urquiaga, I., Rozowski, J., Maiz, A., Germain, A., 1999. Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans. Drugs Exp. Clin. Res. 25 (2–3), 133–141.

Lenton, K.J., Therriault, H., Fulop, T., Payette, H., Wagner, J.R., 1999. Glutathione and ascorbate are negatively correlated with oxidative DNA damage in human lymphocytes. Carcinogenesis 20 (4), 607–613.

Letters, J.M., Witting, P.K., Christison, J.K., Eriksson, A.W., Pettersson, K., Stocker, R., 1999. Time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E knockout mice. J. Lipid Res. 40 (6), 1104–1112.

Leuratti, C., Singh, R., Deag, E.J., Griech, E., Hughes, R., Bingham, S.A., Plastaras, J.P., Marnett, L.J., Shuker, D.E.G., 1999. In: Singer, B., Bartsch, H. (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, vol. 150. IARC Scientific Publications, Lyon, pp. 197–203.

Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357.

Li, H., Lawson, J.A., Reilly, M., Adiyaman, M., Hwang, S.W., Rokach, J., FitzGerald, G.A., 1999. Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F(2)-isoprostanes in human urine. Proc. Natl. Acad. Sci. USA 96 (23), 13381–13386.

Lindahl, T., 2001. Keynote: past, present, and future aspects of base excision repair. Prog. Nucleic Acid Res. Mol. Biol. 68, xvii–xxx.

Loft, S., Poulsen, H.E., 1998. Estimation of oxidative DNA damage in man from urinary excretion of repair products. Acta Biochim. Pol. 45 (1), 133–144.

Loft, S., Poulsen, H.E., 1999. Markers of oxidative damage to DNA: antioxidants and molecular damage. Methods Enzymol. 300, 166–184.

Loft, S., Astrup, A., Buemann, B., Poulsen, H.E., 1994. Oxidative DNA damage correlates with oxygen consumption in humans. FASEB J. 8 (8), 534–537.

Lonnrot, K., Metsa-Ketela, T., Molnar, G., Ahonen, J.P., Latvala, M., Peltola, J., Pietila, T., Alho, H., 1996. The effect of ascorbate and ubiquinone supplementation on plasma and CSF total antioxidant capacity. Free Radic. Biol. Med. 21 (2), 211–217.

Loureiro, A.P., Mascio, P., Gomes, O.F., Medeiros, M.H., 2000. trans,trans-2,4-decadienal-induced 1,N(2)-etheno-2′-deoxyguanosine adduct formation. Chem. Res. Toxicol. 13 (7), 601–609.

Lovell, D.P., Thomas, G., Dubow, R., 1999. Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog. Carcinog. Mutagen. 19 (2), 109–119.

Lunec, J., 1998. ESCODD: European Standards Committee on Oxidative DNA Damage. Free Radic. Res. 29 (6), 601–608.

MacKinnon, K.L., Molnar, Z., Lowe, D., Watson, I.D., Shearer, E., 1999. Measures of total free radical activity in critically ill patients. Clin. Biochem. 32 (4), 263–268.

Marangon, K., Devaraj, S., Tirosh, O., Packer, L., Jialal, I., 1999. Comparison of the effect of alpha-lipoic acid and alpha-tocopherol supplementation on measures of oxidative stress. Free Radic. Biol. Med. 27 (9–10), 1114–1121.

Marnett, L.J., 1999. Lipid peroxidation-DNA damage by malondialdehyde. Mutat. Res. 424 (1–2), 83–95.

Masuda, Y., Yamamori, Y., 1991a. Histological detection of lipid peroxidation following infusion of tert-butyl hydroperoxide and ADP-iron complex in perfused rat livers. Jpn. J. Pharmacol. 56 (2), 133–142.

Masuda, Y., Yamamori, Y., 1991b. Histological evidence for dissociation of lipid peroxidation and cell necrosis in bromotrichloromethane hepatotoxicity in the perfused rat liver. Jpn. J. Pharmacol. 56 (2), 143–150.

Matsuse, T., Ohga, E., Teramoto, S., Fukayama, M., Nagai, R., Horiuchi, S., Ouchi, Y., 1998. Immunohistochemical localisation of advanced glycation end products in pulmonary fibrosis. J. Clin. Pathol. 51 (7), 515–519.

McKelvey-Martin, V.J., Green, M.H., Schmezer, P., Pool-Zobel, B.L., Meo, M.P., Collins, A., 1993. The single cell gel electrophoresis assay (comet assay): a European review. Mutat. Res. 288 (1), 47–63.

Meagher, E.A., Barry, O.P., Lawson, J.A., Rokach, J., FitzGerald, G.A., 2001. Effects of vitamin E on lipid peroxidation in healthy persons. J. Am. Med. Assoc. 285 (9), 1178–1182.

Metsa-Ketela, T., 1991. Luminescent assay for total peroxyl radical-trapping capability of plasma. In: Stanley, P., Kricka, L. (Eds.), Bioluminescence and Chemiluminescence: Current Status. Wiley, Chichester, pp. 389–392.

Miller III, E.R., Appel, L.J., Risby, T.H., 1998. Effect of dietary patterns on measures of lipid peroxidation: results from a randomized clinical trial. Circulation 98 (22), 2390–2395.

Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A., 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin. Sci. (Colch.) 84 (4), 407–412.

Minuz, P., Andrioli, G., Degan, M., Gaino, S., Ortolani, R., Tommasoli, R., Zuliani, V., Lechi, A., Lechi, C., 1998. The F2-isoprostane 8-epiprostaglandin F2alpha increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of NO. Arterioscler. Thromb. Vasc. Biol. 18 (8), 1248–1256.

Mistry, N., Evans, M.D., Griffiths, H.R., Kasai, H., Herbert, K.E., Lunec, J., 1999. Immunochemical detection of glyoxal DNA damage. Free Radic. Biol. Med. 26 (9–10), 1267–1273.

Mitchell, J.H., Collins, A.R., 1999. Effects of a soy milk supplement on plasma cholesterol levels and oxidative DNA damage in men – a pilot study. Eur. J. Nutr. 38 (3), 143–148.

Moller, L., Hofer, T., 1997. [^{32}P]ATP mediates formation of 8-hydroxy-2′-deoxyguanosine from 2′-deoxyguanosine, a possible problem in the ^{32}P-postlabeling assay. Carcinogenesis 18 (12), 2415–2419.

Moller, L., Grzybowska, E., Zeisig, M., Cimander, B., Hemminki, K., Chorazy, M., 1996. Seasonal variation of DNA adduct pattern in human lymphocytes analyzed by ^{32}P-HPLC. Carcinogenesis 17 (1), 61–66.

Morin, B., Bubb, W.A., Davies, M.J., Dean, R.T., Fu, S., 1998. 3-Hydroxylysine, a potential marker for studying radical-induced protein oxidation. Chem. Res. Toxicol. 11 (11), 1265–1273.

Morrow, J.D., Hill, K.E., Burk, R.F., Nammour, T.M., Badr, K.F., Roberts, L.J., 1990. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc. Natl. Acad. Sci. USA 87 (23), 9383–9387.

Morrow, J.D., Zackert, W.E., Yang, J.P., Kurhts, E.H., Callewaert, D., Dworski, R., Kanai, K., Taber, D., Moore, K., Oates, J.A., Roberts, L.J., 1999. Quantification of the major urinary metabolite of 15-F2-isoprostane (8-iso-PGF2alpha) by a stable isotope dilution mass spectrometric assay. Anal. Biochem. 269 (2), 326–331.

Naguib, Y.M., 2000. A fluorometric method for measurement of oxygen radical-scavenging activity of water-soluble antioxidants. Anal. Biochem. 284 (1), 93–98.

Nair, J., 1999. In: Singer, B., Bartsch, H., Lyon, (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, vol. 150. IARC Scientific Publications, pp. 55–61.

Nair, J., Barbin, A., Velic, I., Bartsch, H., 1999. Etheno DNA-base adducts from endogenous reactive species. Mutat. Res. 424 (1–2), 59–69.

Nakajima, M., Takeuchi, T., Takeshita, T., Morimoto, K., 1996. 8-Hydroxydeoxyguanosine in human leukocyte DNA and daily health practice factors: effects of individual alcohol sensitivity. Environ. Health Perspect. 104 (12), 1336–1338.

Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S., Tannenbaum, S.R., 1992. DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc. Natl. Acad. Sci. USA 89 (7), 3030–3034.

Nielsen, S.E., Young, J.F., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., andstrom, B., Dragsted, L.O., 1999. Effect of parsley (Petroselinum crispum) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. Br. J. Nutr. 81 (6), 447–455.

Niemela, O., Parkkila, S., Yla-Herttuala, S., Halsted, C., Witztum, J.L., Lanca, A., Israel, Y., 1994. Covalent protein adducts in the liver as a result of ethanol metabolism and lipid peroxidation. Lab. Invest. 70 (4), 537–546.

Nohammer, G., 1982. Quantitative microspectrophotometrical determination of protein thiols and disulfides with 2,2′-dihydroxy-6,6′-dinaphthyldisulfide (DDD). The variety of DDD-staining methods demonstrated on Ehrlich ascites tumor cells. Histochemistry 75 (2), 219–250.

Nohammer, G., Desoye, G., Khoschsorur, G., 1981. Quantitative cytospectrophotometrical determination of the total protein thiols with "mercurochrom": optimization and calibration of the histochemical reaction. Histochemistry 71 (2), 291–300.

Nohammer, G., Bajardi, F., Benedetto, C., Schauenstein, E., Slater, T.F., 1986. Quantitative cytospectrophotometric studies on protein thiols and reactive protein disulphides in samples of normal human uterine cervix and on samples obtained from patients with dysplasia or carcinoma-in-situ. Br. J. Cancer 53 (2), 217–222.

Nourooz-Zadeh, J., 1999. Ferrous ion oxidation in presence of xylenol orange for detection of lipid hydroperoxides in plasma. Methods Enzymol. 300, 58–62.

Nyyssonen, K., Porkkala-Sarataho, E., Kaikkonen, J., Salonen, J.T., 1997. Ascorbate and urate are the strongest determinants of plasma antioxidative capacity and serum lipid resistance to oxidation in Finnish men. Atherosclerosis 130 (1–2), 223–233.

O'Donnell, V.B., Eiserich, J.P., Bloodsworth, A., Chumley, P.H., Kirk, M., Barnes, S., Darley-Usmar, V.M., Freeman, B.A., 1999. Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. Methods Enzymol. 301, 454–470.

Offord, E., Poppel, G., Tyrrell, R., 2000. Markers of oxidative damage and antioxidant protection: current status and relevance to disease. Free Radic. Res. 33 (Suppl.), S5–S19.

Ohmori, S., Kawase, T., Higashiura, M., Chisaka, Y., Nakata, K., Yamasaki, Y., 2001. High-performance liquid chromatographic method to analyze picomole levels of glutathione, cysteine and cysteinylglycine and its application to pre-cancerous rat livers. J. Chromatogr. B Biomed. Sci. Appl. 762 (1), 25–32.

Okamoto, K., Toyokuni, S., Uchida, K., Ogawa, O., Takenewa, J., Kakehi, Y., Kinoshita, H., Hattori-Nakakuki, Y., Hiai, H., Yoshida, O., 1994. Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in human renal-cell carcinoma. Int. J. Cancer 58 (6), 825–829.

Olinski, R., Zastawny, T., Budzbon, J., Skokowski, J., Zegarski, W., Dizdaroglu, M., 1992. DNA base modifications in chromatin of human cancerous tissues. FEBS Lett. 309 (2), 193–198.

Olinski, R., Zastawny, T.H., Foksinski, M., Barecki, A., Dizdaroglu, M., 1995. DNA base modifications and antioxidant enzyme activities in human benign prostatic hyperplasia. Free Radic. Biol. Med. 18 (4), 807–813.

Olinski, R., Jaruga, P., Foksinski, M., Bialkowski, K., Tujakowski, J., 1997. Epirubicin-induced oxidative DNA damage and evidence for its repair in lymphocytes of cancer patients who are undergoing chemotherapy. Mol. Pharmacol. 52 (5), 882–885.

Ono, T., Negishi, K., Hayatsu, H., 1995. Spectra of superoxide-induced mutations in the lacI gene of a wild-type and a mutM strain of *Escherichia coli* K-12. Mutat. Res. 326 (2), 175–183.

Opara, E.C., Abdel-Rahman, E., Soliman, S., Kamel, W.A., Souka, S., Lowe, J.E., Abdel-Aleem, S., 1999. Depletion of total antioxidant capacity in type 2 diabetes. Metabolism 48 (11), 1414–1417.

Palinski, W., Rosenfeld, M.E., Yla-Herttuala, S., Gurtner, G.C., Socher, S.S., Butler, S.W., Parthasarathy, S., Carew, T.E., Steinberg, D., Witztum, J.L., 1989. Low density lipoprotein undergoes oxidative modification in vivo. Proc. Natl. Acad. Sci. USA 86 (4), 1372–1376.

Palmer, A.M., Thomas, C.R., Gopaul, N., Dhir, S., Anggard, E.E., Poston, L., Tribe, R.M., 1998. Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesenteric arteries of the streptozotocin-diabetic rat. Diabetologia 41 (2), 148–156.

Panayiotidis, M., Collins, A.R., 1997. Ex vivo assessment of lymphocyte antioxidant status using the comet assay. Free Radic. Res. 27 (5), 533–537.

Paolicchi, A., Minotti, G., Tonarelli, P., Tongiani, R., Cesare, D., Mezzetti, A., Dominici, S., Comporti, M., Pompella, A., 1999. Gamma-glutamyl transpeptidase-dependent iron reduction and LDL oxidation–a potential mechanism in atherosclerosis. J. Investig. Med. 47 (3), 151–160.

Paradis, V., Kollinger, M., Fabre, M., Holstege, A., Poynard, T., Bedossa, P., 1997a. In situ detection of lipid peroxidation by-products in chronic liver diseases. Hepatology 26 (1), 135–142.

Paradis, V., Mathurin, P., Kollinger, M., Imbert-Bismut, F., Charlotte, F., Piton, A., Opolon, P., Holstege, A., Poynard, T., Bedossa, P., 1997b. In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. J. Clin. Pathol. 50 (5), 401–406.

Park, E.M., Shigenaga, M.K., Degan, P., Korn, T.S., Kitzler, J.W., Wehr, C.M., Kolachana, P., Ames, B.N., 1992. Assay of excised oxidative DNA lesions: isolation of 8-oxoguanine and its nucleoside derivatives from biological fluids with a monoclonal antibody column. Proc. Natl. Acad. Sci. USA 89 (8), 3375–3379.

Pasqualotto, F.F., Sharma, R.K., Nelson, D.R., Thomas, A.J., Agarwal, A., 2000. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertil. Steril. 73 (3), 459–464.

Patrignani, P., Panara, M.R., Tacconelli, S., Seta, F., Bucciarelli, T., Ciabattoni, G., Alessandrini, P., Mezzetti, A., Santini, G., Sciulli, M.G., Cipollone, F., Davi, G., Gallina, P., Bon, G.B., Patrono, C., 2000. Effects of vitamin E supplementation on F(2)-isoprostane and thromboxane biosynthesis in healthy cigarette smokers. Circulation 102 (5), 539–545.

Pellegrini, N., Re, R., Yang, M., Rice-Evans, C., 1999. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2′-azinobis-3-ethylenebenzothiazoline-6-sulfonic acid radical cation decolorization assay. Methods Enzymol. 299, 379–389.

Pellegrini, N., Riso, P., Porrini, M., 2000. Tomato consumption does not affect the total antioxidant capacity of plasma. Nutrition 16 (4), 268–271.

Peng, J., Jones, G.L., Watson, K., 2000. Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. Free Radic. Biol. Med. 28 (11), 1598–1606.

Petropoulos, I., Mary, J., Perichon, M., Friguet, B., 2001. Rat peptide methionine sulphoxide reductase: cloning of the cDNA, and down-regulation of gene expression and enzyme activity during aging. Biochem. J. 355 (Pt 3), 819–825.

Pfeiffer, J.M., Askew, E.W., Roberts, D.E., Wood, S.M., Benson, J.E., Johnson, S.C., Freedman, M.S., 1999. Effect of antioxidant supplementation on urine and blood markers of oxidative stress during extended moderate-altitude training. Wilderness. Environ. Med. 10 (2), 66–74.

Pflaum, M., Will, O., Epe, B., 1997. Determination of steady-state levels of oxidative DNA base modifications in mammalian cells by means of repair endonucleases. Carcinogenesis 18 (11), 2225–2231.

Piretti, M.V., Pagliuca, G., 1989. Systematic isolation and identification of membrane lipid oxidation products. Free Radic. Biol. Med. 7 (2), 219–221.

Pirie, A., 1971. Formation of N'-formylkynurenine in proteins from lens and other sources by exposure to sunlight. Biochem. J. 125 (1), 203–208.

Podmore, I.D., Griffiths, H.R., Herbert, K.E., Mistry, N., Mistry, P., Lunec, J., 1998. Vitamin C exhibits pro-oxidant properties. Nature 392 (6676), 559.

Pompella, A., Comporti, M., 1991. The use of 3-hydroxy-2-naphthoic acid hydrazide and Fast Blue B for the histochemical detection of lipid peroxidation in animal tissues – a microphotometric study. Histochemistry 95 (3), 255–262.

Pompella, A., Comporti, M., 1993. Imaging of oxidative stress at subcellular level by confocal laser scanning microscopy after fluorescent derivatization of cellular carbonyls. Am. J. Pathol. 142 (5), 1353–1357.

Pompella, A., Maellaro, E., Casini, A.F., Comporti, M., 1987. Histochemical detection of lipid peroxidation in the liver of bromobenzene-poisoned mice. Am. J. Pathol. 129 (2), 295–301.

Pompella, A., Cambiaggi, C., Dominici, S., Paolicchi, A., Tongiani, R., Comporti, M., 1996a. Single-cell investigation by laser scanning confocal microscopy of cytochemical alterations resulting from extracellular oxidant challenge. Histochem. Cell Biol. 105 (3), 173–178.

Pompella, A., Paolicchi, A., Dominici, S., Comporti, M., Tongiani, R., 1996b. Selective colocalization of lipid peroxidation and protein thiol loss in chemically induced hepatic preneoplastic lesions: the role of gamma-glutamyltranspeptidase activity. Histochem. Cell Biol. 106 (3), 275–282.

Pool-Zobel, B.L., Bub, A., Muller, H., Wollowski, I., Rechkemmer, G., 1997. Consumption of vegetables reduces genetic damage in humans: first results of a human intervention trial with carotenoid-rich foods. Carcinogenesis 18 (9), 1847–1850.

Pool-Zobel, B.L., Bub, A., Liegibel, U.M., Lishaut, S., Rechkemmer, G., 1998. Mechanisms by which vegetable consumption reduces genetic damage in humans. Cancer Epidemiol. Biomarkers Prev. 7 (10), 891–899.

Pool-Zobel, B.L., Adlercreutz, H., Glei, M., Liegibel, U.M., Sittlingon, J., Rowland, I., Wahala, K., Rechkemmer, G., 2000. Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells. Carcinogenesis 21 (6), 1247–1252.

Popov, I., Lewin, G., 1999. Antioxidative homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol. 300, 437–456.

Popov, I.N., Lewin, G., 1994. Photochemiluminescent detection of antiradical activity: II. Testing of nonenzymic water-soluble antioxidants. Free Radic. Biol. Med. 17 (3), 267–271.

Porkkala-Sarataho, E., Salonen, J.T., Nyyssonen, K., Kaikkonen, J., Salonen, R., Ristonmaa, U., Diczfalusy, U., Brigelius-Flohe, R., Loft, S., Poulsen, H.E., 2000. Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men. Arterioscler. Thromb. Vasc. Biol. 20 (9), 2087–2093.

Porrini, M., Riso, P., 2000. Lymphocyte lycopene concentration and DNA protection from oxidative damage is increased in women after a short period of tomato consumption. J. Nutr. 130 (2), 189–192.

Pratico, D., 1999. F(2)-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. Atherosclerosis 147 (1), 1–10.

Prieme, H., Loft, S., Nyyssonen, K., Salonen, J.T., Poulsen, H.E., 1997. No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion in smokers. Am. J. Clin. Nutr. 65 (2), 503–507.

Proteggente, A.R., Rehman, A., Halliwell, B., Rice-Evans, C.A., 2000. Potential problems of ascorbate and iron supplementation: Pro-oxidant effect in vivo? Biochem. Biophys. Res. Commun. 277 (3), 535–540.

Pryor, W.A., Castle, L., 1984. Chemical methods for the detection of lipid hydroperoxides. Methods Enzymol. 105, 293–299.

Quinn, M.T., Linner, J.G., Siemsen, D., Dratz, E.A., Buescher, E.S., Jesaitis, A.J., 1995. Immunocytochemical detection of lipid peroxidation in phagosomes of human neutrophils: correlation with expression of flavocytochrome b. J. Leukoc. Biol. 57 (3), 415–421.

Radicella, J.P., Dherin, C., Desmaze, C., Fox, M.S., Boiteux, S., 1997. Cloning and characterization of hOGG1, a human homolog of the OGG1 gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 94 (15), 8010–8015.

Rao, A.V., Agarwal, S., 1998. Bioavailability and in vivo antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. Nutr. Cancer 31 (3), 199–203.

Ravanat, J.L., Guicherd, P., Tuce, Z., Cadet, J., 1999. Simultaneous determination of five oxidative DNA lesions in human urine. Chem. Res. Toxicol. 12 (9), 802–808.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26 (9–10), 1231–1237.

Reddy, S., Halliwell, B., Jones, A.D., Longhurst, J.C., 1999. The use of phenylalanine to detect hydroxyl radical production in vivo: a cautionary note. Free Radic. Biol. Med. 27 (11–12), 1465.

Regoli, F., Winston, G.W., 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicol. Appl. Pharmacol. 156 (2), 96–105.

Rehman, A., Collis, C.S., Yang, M., Kelly, M., Diplock, A.T., Halliwell, B., Rice-Evans, C., 1998. The effects of iron and vitamin C co-supplementation on oxidative damage to DNA in healthy volunteers. Biochem. Biophys. Res. Commun. 246 (1), 293–298.

Reilly, M., Delanty, N., Lawson, J.A., FitzGerald, G.A., 1996. Modulation of oxidant stress in vivo in chronic cigarette smokers. Circulation 94 (1), 19–25.

Renner, S., Rath, R., Rust, P., Lehr, S., Frischer, T., Elmadfa, I., Eichler, I., 2001. Effects of beta-carotene supplementation for six months on clinical and laboratory parameters in patients with cystic fibrosis. Thorax 56 (1), 48–52.

Requena, J.R., Fu, M.X., Ahmed, M.U., Jenkins, A.J., Lyons, T.J., Baynes, J.W., Thorpe, S.R., 1997. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. Biochem. J. 322 (Pt 1), 317–325.

Retsky, K.L., Chen, K., Zeind, J., Frei, B., 1999. Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation. Free Radic. Biol. Med. 26 (1–2), 90–98.

Riely, C.A., Cohen, G., Lieberman, M., 1974. Ethane evolution: a new index of lipid peroxidation. Science 183 (121), 208–210.

Roberts, L.J., Davies, S., Roden, D., Yang, T., Morrow, J.D., Brame, C., 1999a. Formation of unique isolevuglandin protein adducts esterified to phospholipids in vivo. Free Radic. Biol. Med. 27 (Suppl. 1), 47.

Roberts, L.J., Salomon, R.G., Morrow, J.D., Brame, C.J., 1999b. New developments in the isoprostane pathway: identification of novel highly reactive gamma-ketoaldehydes (isolevuglandins) and characterization of their protein adducts. FASEB J. 13 (10), 1157–1168.

Robinson, J.M., Batten, B.E., 1990. Localization of cerium-based reaction products by scanning laser reflectance confocal microscopy. J. Histochem. Cytochem. 38 (3), 315–318.

Robinson, S., Bevan, R., Lunec, J., Griffiths, H., 1998. Chemiluminescence determination of hydroperoxides following radiolysis and photolysis of free amino acids. FEBS Lett. 430 (3), 297–300.

Rodriguez, H., Akman, S.A., 1998. Mapping oxidative DNA damage at nucleotide level. Free Radic. Res. 29 (6), 499–510.

Rodriguez, H., Holmquist, G.P., D'Agostino Jr., R., Keller, J., Akman, S.A., 1997. Metal ion-dependent hydrogen peroxide-induced DNA damage is more sequence specific than metal specific. Cancer Res. 57 (12), 2394–2403.

Rodriguez, H., Valentine, M.R., Holmquist, G.P., Akman, S.A., Termini, J., 1999. Mapping of peroxyl radical induced damage on genomic DNA. Biochemistry 38 (50), 16578–16588.

Rodriguez, H., Jurado, J., Laval, J., Dizdaroglu, M., 2000. Comparison of the levels of 8-hydroxyguanine in DNA as measured by gas chromatography mass spectrometry following hydrolysis of DNA by *Escherichia coli* Fpg protein or formic acid. Nucleic Acids Res. (online) 28 (15), E75.

Rojas, C., Cadenas, S., Lopez-Torres, M., Perez-Campo, R., Barja, G., 1996. Increase in heart glutathione redox ratio and total antioxidant capacity and decrease in lipid peroxidation after vitamin E dietary supplementation in guinea pigs. Free Radic. Biol. Med. 21 (7), 907–915.

Rota, C., Chignell, C.F., Mason, R.P., 1999. Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. Free Radic. Biol. Med. 27 (7–8), 873–881.

Rouzer, C.A., Chaudhary, A.K., Nokubo, M., Ferguson, D.M., Reddy, G.R., Blair, I.A., Marnett, L.J., 1997. Analysis of the malondialdehyde-2′-deoxyguanosine adduct pyrimidopurinone in human leukocyte DNA by gas chromatography/electron capture/negative chemical ionization/mass spectrometry. Chem. Res. Toxicol. 10 (2), 181–188.

Royall, J.A., Ischiropoulos, H., 1993. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch. Biochem. Biophys. 302 (2), 348–355.

Rust, P., Eichler, I., Renner, S., Elmadfa, I., 1998. Effects of long-term oral beta-carotene supplementation on lipid peroxidation in patients with cystic fibrosis. Int. J. Vitam. Nutr. Res. 68 (2), 83–87.

Ryan, M., Grayson, L., Clarke, D.J., 1997. The total antioxidant capacity of human serum measured using enhanced chemiluminescence is almost completely accounted for by urate. Ann. Clin. Biochem. 34 (Pt 6), 688–689.

Salomon, R.G., Batyreva, E., Kaur, K., Sprecher, D.L., Schreiber, M.J., Crabb, J.W., Penn, M.S., DiCorletoe, A.M., Hazen, S.L., Podrez, E.A., 2000a. Isolevuglandin-protein adducts in humans: products of free radical-induced lipid oxidation through the isoprostane pathway. Biochim. Biophys. Acta 1485 (2–3), 225–235.

Salomon, R.G., Kaur, K., Batyreva, E., 2000b. Isolevuglandin-protein adducts in oxidized low density lipoprotein and human plasma: a strong connection with cardiovascular disease. Trends Cardiovasc. Med. 10 (2), 53–59.

Salonen, J.T., Nyyssonen, K., Salonen, R., Porkkala-Sarataho, E., Tuomainen, T.P., Diczfalusy, U., Bjorkhem, I., 1997. Lipoprotein oxidation and progression of carotid atherosclerosis. Circulation 95 (4), 840–845.

Sasaki, N., Fukatsu, R., Tsuzuki, K., Hayashi, Y., Yoshida, T., Fujii, N., Koike, T., Wakayama, I., Yanagihara, R., Garruto, R., Amano, N., Makita, Z., 1998. Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. Am. J. Pathol. 153 (4), 1149–1155.

Schacter, E., Williams, J.A., Stadtman, E.R., Levine, R.L., 1996. Determination of carbonyl groups in oxidised proteins. In: Punchard, A., Kelly, F.J. (Eds.), Free Radicals. A practical Approach. IRL Press, Oxford, pp. 159–170.

Schaeffer, H.J., 1989. Gas chromatographic analysis of traces of light-hydrocarbons – a review of different systems in practise. J. High Resolut. Chromatogr. 12 (2), 69–81.

Sheehy, M., 2002. A flow-cytometric method for quantification of neurolipofuscin and comparison with existing histological and biochemical approaches. Arch. Gerontol. Geriat.

Shigenaga, M.K., 1999. Quantitation of protein-bound 3-nitrotyrosine by high-performance liquid chromatography with electrochemical detection. Methods Enzymol. 301, 27–40.

Shigenaga, M.K., Gimeno, C.J., Ames, B.N., 1989. Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. Proc. Natl. Acad. Sci. USA 86 (24), 9697–9701.

Shlafer, M., Brosamer, K., Forder, J.R., Simon, R.H., Ward, P.A., Grum, C.M., 1990. Cerium chloride as a histochemical marker of hydrogen peroxide in reperfused ischemic hearts. J. Mol. Cell Cardiol. 22 (1), 83–97.

Sian, J., Dexter, D.T., Cohen, G., Jenner, P.G., Marsden, C.D., 1997. Comparison of HPLC and enzymatic recycling assays for the measurement of oxidized glutathione in rat brain. J. Pharm. Pharmacol. 49 (3), 332–335.

Silvester, J.A., Timmins, G.S., Davies, M.J., 1998. Photodynamically generated bovine serum albumin radicals: evidence for damage transfer and oxidation at cysteine and tryptophan residues. Free Radic. Biol. Med. 24 (5), 754–766.

Sinclair, A.J., Girling, A.J., Gray, L., Le Guen, C., Lunec, J., Barnett, A.H., 1991. Disturbed handling of ascorbic acid in diabetic patients with and without microangiopathy during high dose ascorbate supplementation. Diabetologia 34 (3), 171–175.

Singh, R., Blattler, W.A., Collinson, A.R., 1993. An amplified assay for thiols based on reactivation of papain. Anal. Biochem. 213 (1), 49–56.

Smith, M.A., Perry, G., Richey, P.L., Sayre, L.M., Anderson, V.E., Beal, M.F., Kowall, N., 1996. Oxidative damage in Alzheimer's. Nature 382 (6587), 120–121.

Srigiridhar, K., Nair, K.M., 2000. Supplementation with alpha-tocopherol or a combination of alpha-tocopherol and ascorbic acid protects the gastrointestinal tract of iron-deficient rats against iron-induced oxidative damage during iron repletion. Br. J. Nutr. 84 (2), 165–173.

Stadtman, E.R., 1993. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. 62, 797–821.

Stadtman, E.R., Berlett, B.S., 1991. Fenton chemistry. Amino acid oxidation. J. Biol. Chem. 266 (26), 17201–17211.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L., 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. 320 (14), 915–924.

Sumida, S., Doi, T., Sakurai, M., Yoshioka, Y., Okamura, K., 1997. Effect of a single bout of exercise and beta-carotene supplementation on the urinary excretion of 8-hydroxy-deoxyguanosine in humans. Free Radic. Res. 27 (6), 607–618.

Sun, M., Yokoyama, M., Ishiwata, T., Asano, G., 1998. Deposition of advanced glycation end products (AGE) and expression of the receptor for AGE in cardiovascular tissue of the diabetic rat. Int. J. Exp. Pathol. 79 (4), 207–222.

Sundelin, S.P., Nilsson, S.E., 2001. Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants. Free Radic. Biol. Med. 31 (2), 217–225.

Suzuki, J., Inoue, Y., Suzuki, S., 1995. Changes in the urinary excretion level of 8-hydroxyguanine by exposure to reactive oxygen-generating substances. Free Radic. Biol. Med. 18 (3), 431–436.

Szabo, C., Ohshima, H., 1997. DNA damage induced by peroxynitrite: subsequent biological effects. Nitric Oxide 1 (5), 373–385.

Takayama, F., Aoyama, I., Tsukushi, S., Miyazaki, T., Miyazaki, S., Morita, T., Hirasawa, Y., Shimokata, K., Niwa, T., 1998. Immunohistochemical detection of imidazolone and N(epsilon)-(carboxymethyl)lysine in aortas of hemodialysis patients. Cell Mol. Biol. (Noisy.-le-grand) 44 (7), 1101–1109.

Tanaka, M., Sotomatsu, A., Kanai, H., Hirai, S., 1992. Combined histochemical and biochemical demonstration of nigral vulnerability to lipid peroxidation induced by DOPA and iron. Neurosci. Lett. 140 (1), 42–46.

Terao, J., Matsushita, S., 1987. Application of HPLC for the determination of phospholipid hydroperoxides in food and biological systems. Free Radic. Biol. Med. 3 (5), 345–347.

ter Steege, J.C., Koster-Kamphuis, L., Straaten, E.A., Forget, P.P., Buurman, W.A., 1998. Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. Free Radic. Biol. Med. 25 (8), 953–963.

Thompson, H.J., Heimendinger, J., Haegele, A., Sedlacek, S.M., Gillette, C., O'Neill, C., Wolfe, P., Conry, C., 1999. Effect of increased vegetable and fruit consumption on markers of oxidative cellular damage. Carcinogenesis 20 (12), 2261–2266.

Thompson, S., Smith, M.T., 1985. Measurement of the diene conjugated form of linoleic acid in plasma by high performance liquid chromatography: A questionable non-invasive assay of free radical activity? Chem. Biol. Interact. 55 (3), 357–366.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35 (3), 206–221.

Torbergsen, A.C., Collins, A.R., 2000. Recovery of human lymphocytes from oxidative DNA damage; the apparent enhancement of DNA repair by carotenoids is probably simply an antioxidant effect. Eur. J. Nutr. 39 (2), 80–85.

Toyokuni, S., Okada, S., Hamazaki, S., Minamiyama, Y., Yamada, Y., Liang, P., Fukunaga, Y., Midorikawa, O., 1990. Combined histochemical and biochemical analysis of sex hormone dependence of ferric nitrilotriacetate-induced renal lipid peroxidation in ddY mice. Cancer Res. 50 (17), 5574–5580.

Toyokuni, S., Miyake, N., Hiai, H., Hagiwara, M., Kawakishi, S., Osawa, T., Uchida, K., 1995. The monoclonal antibody specific for the 4-hydroxy-2-nonenal histidine adduct. FEBS Lett. 359 (2–3), 189–191.

Toyokuni, S., Tanaka, T., Hattori, Y., Nishiyama, Y., Yoshida, A., Uchida, K., Hiai, H., Ochi, H., Osawa, T., 1997. Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. Lab. Invest. 76 (3), 365–374.

Tretyakova, N.Y., Burney, S., Pamir, B., Wishnok, J.S., Dedon, P.C., Wogan, G.N., Tannenbaum, S.R., 2000. Peroxynitrite-induced DNA damage in the supF gene: correlation with the mutational spectrum. Mutat. Res. 447 (2), 287–303.

Tsimikas, S., Shortal, B.P., Witztum, J.L., Palinski, W., 2000. In vivo uptake of radiolabeled MDA2, an oxidation-specific monoclonal antibody, provides an accurate measure of atherosclerotic lesions rich in oxidized LDL and is highly sensitive to their regression. Arterioscler. Thromb. Vasc. Biol. 20 (3), 689–697.

Tsuchiya, M., Suematsu, M., Suzuki, H., 1994. In vivo visualization of oxygen radical-dependent photoemission. Methods Enzymol. 233, 128–140.

Tubaro, F., Ghiselli, A., Rapuzzi, P., Maiorino, M., Ursini, F., 1998. Analysis of plasma antioxidant capacity by competition kinetics. Free Radic. Biol. Med. 24 (7–8), 1228–1234.

Tuo, J., Liu, L., Poulsen, H.E., Weimann, A., Svendsen, O., Loft, S., 2000. Importance of guanine nitration and hydroxylation in DNA in vitro and in vivo. Free Radic. Biol. Med. 29 (2), 147–155.

Uchida, K., Fukuda, A., Kawakishi, S., Hiai, H., Toyokuni, S., 1995. A renal carcinogen ferric nitrilotriacetate mediates a temporary accumulation of aldehyde-modified proteins within cytosolic compartment of rat kidney. Arch. Biochem. Biophys. 317 (2), 405–411.

Upritchard, J.E., Sutherland, W.H., Mann, J.I., 2000. Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. Diabetes Care 23 (6), 733–738.

Vaca, C.E., Fang, J.L., Mutanen, M., Valsta, L., 1995. 32P-Postlabelling determination of DNA adducts of malonaldehyde in humans: total white blood cells and breast tissue. Carcinogenesis 16 (8), 1847–1851.

Valkonen, M., Kuusi, T., 1997. Spectrophotometric assay for total peroxyl radical-trapping antioxidant potential in human serum. J. Lipid Res. 38 (4), 823–833.

Van Bakel, M.M., Printzen, G., Wermuth, B., Wiesmann, U.N., 2000. Antioxidant and thyroid hormone status in selenium-deficient phenylketonuric and hyperphenylalaninemic patients. Am. J. Clin. Nutr. 72 (4), 976–981.

Van den Berg, J.J., Winterbourn, C.C., Kuypers, F.A., 1993. Hypochlorous acid-mediated modification of cholesterol and phospholipid: analysis of reaction products by gas chromatography-mass spectrometry. J. Lipid Res. 34 (11), 2005–2012.

Van den Berg, R., Haenen, G.R.M.M, van Den, B.H., Bast, A., 1999. Applicabillity of an improved Trolox equivalent antioxidant assay for evaluation of antioxidant capacity measurements of mixtures. Food Chemistry 66, 511–517.

Van den Berg, R., van Vliet, T., Broekmans, W.M., Cnubben, N.H., Vaes, W.H., Roza, L., Haenen, G.R., Bast, A., Berg, H., 2001. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. J. Nutr. 131 (6), 1714–1722.

van Overveld, F.W., Haenen, G.R., Rhemrev, J., Vermeiden, J.P., Bast, A., 2000. Tyrosine as important contributor to the antioxidant capacity of seminal plasma. Chem. Biol. Interact. 127 (2), 151–161.

van Poppel, G., Kok, F.J., Duijzings, P., de Vogel, N., 1992. No influence of beta-carotene on smoking-induced DNA damage as reflected by sister chromatid exchanges. Int. J. Cancer 51 (3), 355–358.

van Poppel, G., Poulsen, H., Loft, S., Verhagen, H., 1995. No influence of beta carotene on oxidative DNA damage in male smokers. J. Natl. Cancer Inst. 87 (4), 310–311.

Varma, S.D., 1989. Radio-isotopic determination of subnanomolar amounts of peroxide. Free Radic. Res. Commun. 5 (6), 359–368.

Venditti, P., DiMeo, S., de Martino, R.P., Leo, T., 1995. Determination by enhanced luminescence technique of liver antioxidant capacity. Arch. Physiol. Biochem. 103 (4), 484–491.

Verhagen, H., Poulsen, H.E., Loft, S., van Poppel, G., Willems, M.I., van Bladeren, P.J., 1995. Reduction of oxidative DNA-damage in humans by brussels sprouts. Carcinogenesis 16 (4), 969–970.

Verhagen, H., Rauma, A.L., Torronen, R., de Vogel, N., Bruijntjes-Rozier, G.C., Drevo, M.A., Bogaards, J.J., Mykkanen, H., 1996. Effect of a vegan diet on biomarkers of chemoprevention in females. Hum. Exp. Toxicol. 15 (10), 821–825.

Verhagen, H., de Vries, A., Nijhoff, W.A., Schouten, A., van Poppel, G., Peters, W.H., van Den, B.H., 1997. Effect of Brussels sprouts on oxidative DNA-damage in man. Cancer Lett. 114 (1–2), 127–130.

Vina, J., Vento, M., Garcia-Sala, F., Puertes, I.R., Gasco, E., Sastre, J., Asensi, M., Pallardo, F.V., 1995. L-cysteine and glutathione metabolism are impaired in premature infants due to cystathionase deficiency. Am. J. Clin. Nutr. 61 (5), 1067–1069.

Vina, J., Servera, E., Asensi, M., Sastre, J., Pallardo, F.V., Ferrero, J.A., Garcia-De-La-Asuncion, J., Anton, V., Marin, J., 1996. Exercise causes blood glutathione oxidation in chronic obstructive pulmonary disease: prevention by O_2 therapy. J. Appl. Physiol. 81 (5), 2198–2202.

Virag, L., Scott, G.S., Cuzzocrea, S., Marmer, D., Salzman, A.L., Szabo, C., 1998. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly (ADP-ribose) synthetase (PARS) activation. Immunology 94 (3), 345–355.

Virella, G., Virella, I., Leman, R.B., Pryor, M.B., Lopes-Virella, M.F., 1993. Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers. Int. J. Clin. Lab. Res. 23 (2), 95–101.

Visioli, F., Galli, C., 1997. Evaluation of antioxidant capacity by chemiluminescence. Anal. Biochem. 249 (2), 244–246.

Wade, C.R., van Rij, A.M., 1985. In vivo lipid peroxidation in man as measured by the respiratory excretion of ethane, pentane, and other low-molecular-weight hydrocarbons. Anal. Biochem. 150 (1), 1–7.

Waeg, G., Dimsity, G., Esterbauer, H., 1996. Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins. Free Radic. Res. 25 (2), 149–159.

Wander, R.C., Du, S.H., 2000. Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. Am. J. Clin. Nutr. 72 (3), 731–737.

Wardman, P., von Sonntag, C., 1995. Kinetic factors that control the fate of thiyl radicals in cells. Methods Enzymol. 251, 31–45.

Watanabe, K., Yamamoto, S., Hayaishi, O., 1979. Reactions of prostaglandin endoperoxides with prostaglandin I synthetase solubilized from rabbit aorta microsomes. Biochem. Biophys. Res. Commun. 87 (1), 192–199.

Watson, W.P., Aston, J.P., Barlow, T., Crane, A.E., Potter, D., Brown, T., 1999. In: Singer, B., Bartsch, H. (Eds.), Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis, vol. 150. IARC Scientific Publications, Lyon, pp. 63–73.

Wayner, D.D., Burton, G.W., Ingold, K.U., Locke, S., 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. FEBS Lett. 187 (1), 33–37.

Wayner, D.D., Burton, G.W., Ingold, K.U., 1986. The antioxidant efficiency of vitamin C is concentration-dependent. Biochim. Biophys. Acta 884 (1), 119–123.

Wayner, D.D., Burton, G.W., Ingold, K.U., Barclay, L.R., Locke, S.J., 1987. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim. Biophys. Acta 924 (3), 408–419.

Weimann, A., Belling, D., Poulsen, H.E., 2001. Measurement of 8-oxo-2′-deoxyguanosine and 8-oxo-2′-deoxyadenosine in DNA and human urine by high performance liquid chromatography-electrospray tandem mass spectrometry. Free. Radic. Biol. Med. 30 (7), 757–764.

Weimann, A., Belling, D., Poulsen, H.E., 2002. Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry. Nucleic Acids Res. 30 (2), E7.

Welch, R.W., Turley, E., Sweetman, S.F., Kennedy, G., Collins, A.R., Dunne, A., Livingstone, M.B., McKenna, P.G., McKelvey-Martin, V.J., Strain, J.J., 1999. Dietary antioxidant supplementation and DNA damage in smokers and nonsmokers. Nutr. Cancer 34 (2), 167–172.

Whitehead, T.P., Thorpe, G.H.G., Maxwell, S.R.J., 1992. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. Anal. Chim. Acta 266, 264–265.

Whiteman, M., Halliwell, B., 1999. Loss of 3-nitrotyrosine on exposure to hypochlorous acid: implications for the use of 3-nitrotyrosine as a bio-marker in vivo. Biochem. Biophys. Res. Commun. 258 (1), 168–172.

Wild, C.P., Andersson, C., O'Brien, N.M., Wilson, L., Woods, J.A., 2001. A critical evaluation of the application of biomarkers in epidemiological studies on diet and health. Br. J. Nutr. 86 (Suppl. 1), S37–S53.

Wilks, A., Ortiz de Montellano, P.R., 1992. Intramolecular translocation of the protein radical formed in the reaction of recombinant sperm whale myoglobin with H_2O_2. J. Biol. Chem. 267 (13), 8827–8833.

Williams, M.D., Remmen, H., Conrad, C.C., Huang, T.T., Epstein, C.J., Richardson, A., 1998. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. J. Biol. Chem. 273 (43), 28510–28515.

Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S., 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254 (5034), 1001–1003.

Wink, D.A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M.W., Mitchell, J.B., 1998. The multifaceted roles of nitric oxide in cancer. Carcinogenesis 19 (5), 711–721.

Winterbourn, C.C., Chan, T., Buss, I.H., Inder, T.E., Mogridge, N., Darlow, B.A., 2000. Protein carbonyls and lipid peroxidation products as oxidation markers in preterm infant plasma: associations with chronic lung disease and retinopathy and effects of selenium supplementation. Pediatr. Res. 48 (1), 84–90.

Wood, L.G., Fitzgerald, D.A., Gibson, P.G., Cooper, D.M., Garg, M.L., 2000a. Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma. Lipids 35 (9), 967–974.

Wood, S.G., Gedik, C.M., Collins, A.R., 2000. Controlled oxidation of calf thymus DNA to produce standard samples for 8-oxodeoxyguanosine analysis; effects of freeze-drying, storage and hydrolysis conditions. Free Radic. Res. 32 (4), 327–332.

Wood, S.G., Gedik, C.M., Vaughan, N.J., Collins, A.R., 2000b. Measurement of 8-oxo-deoxyguanosine in lymphocytes, cultured cells and tissue samples by HPLC with electrochemical detection. In: Barnett, Y.A., Barnett, C.R. (Eds.), Methods in Molecular Medicine, vol. 38.

Yagi, N., Yoshikawa, T., Naito, Y., Matsuyama, K., Tanaka, Y., Ochiai, J., Yoshida, N., Kondo, M., 1999. Protective effect of green tea extract against reperfusion injury in rats: antioxidant and anti-inflammatory properties. J. Clin. Biochem. Nutr. 27, 89–101.

Yamamoto, Y., Brodsky, M.H., Baker, J.C., Ames, B.N., 1987. Detection and characterization of lipid hydroperoxides at picomole levels by high-performance liquid chromatography. Anal. Biochem. 160 (1), 7–13.

Yaqoob, P., Pala, H.S., Cortina-Borja, M., Newsholme, E.A., Calder, P.C., 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur. J. Clin. Invest. 30 (3), 260–274.

Yasuda, M., Narita, S., 1997. Simultaneous determination of phospholipid hydroperoxides in human plasma by high performance liquid chromatography with chemiluminescence detection. J. Chromatog. 693, 211–217.

Yen, T.Y., Christova-Gueoguieva, N.I., Scheller, N., Holt, S., Swenberg, J.A., Charles, M.J., 1996. Quantitative analysis of the DNA adduct N2,3-ethenoguanine using liquid chromatography/electrospray ionization mass spectrometry. J. Mass Spectrom. 31 (11), 1271–1276.

Yermilov, V., Rubio, J., Becchi, M., Friesen, M.D., Pignatelli, B., Ohshima, H., 1995a. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. Carcinogenesis 16 (9), 2045–2050.

Yermilov, V., Rubio, J., Ohshima, H., 1995b. Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. FEBS Lett. 376 (3), 207–210.

Yermilov, V., Yoshie, Y., Rubio, J., Ohshima, H., 1996. Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoguanine and base-propenal mediated by peroxynitrite. FEBS Lett. 399 (1–2), 67–70.

Yi, P., Zhan, D., Samokyszyn, V.M., Doerge, D.R., Fu, P.P., 1997. Synthesis and 32P-postlabeling/high-performance liquid chromatography separation of diastereomeric 1,N2-(1,3-propano)-2′-deoxyguanosine 3′-phosphate adducts formed from 4-hydroxy-2-nonenal. Chem. Res. Toxicol. 10 (11), 1259–1265.

Yi, P., Sun, X., Doerge, D.R., Fu, P.P., 1998. An improved 32P-postlabeling/high-performance liquid chromatography method for the analysis of the malondialdehye-derived 1, N2-propanodeoxyguanosine DNA adduct in animal and human tissues. Chem. Res. Toxicol. 11 (9), 1032–1041.

Yla-Herttuala, S., Rosenfeld, M.E., Parthasarathy, S., Glass, C.K., Sigal, E., Witztum, J.L., Steinberg, D., 1990. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. Proc. Natl. Acad. Sci. USA 87 (18), 6959–6963.

Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E.R., Mizuno, Y., 1996. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. Proc. Natl. Acad. Sci. USA 93 (7), 2696–2701.

Young, J.F., Nielsen, S.E., Haraldsdottir, J., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., Crozier, A., Sandstrom, B., Dragsted, L.O., 1999. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am. J. Clin. Nutr. 69 (1), 87–94.

Young, J.F., Dragsted, L.O., Daneshvar, B., Lauridsen, S.T., Hansen, M., Sandstrom, B., 2000. The effect of grape-skin extract on oxidative status. Br. J. Nutr. 84 (4), 505–513.

Yu, T.C., Sinnhuber, R.O., 1957. 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. Food Technology 11, 104–108.

Yu, T.W., Ong, C.N., 1999. Lag-time measurement of antioxidant capacity using myoglobin and 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid): rationale, application, and limitation. Anal. Biochem. 275 (2), 217–223.

Zastawny, T.H., Dabrowska, M., Jaskolski, T., Klimarczyk, M., Kulinski, L., Koszela, A., Szczesniewicz, M., Sliwinska, M., Witkowski, P., Olinski, R., 1998. Comparison of oxidative base damage in mitochondrial and nuclear DNA. Free Radic. Biol. Med. 24 (5), 722–725.

Zeisig, M., Moller, L., 1995. ^{32}P-HPLC suitable for characterization of DNA adducts formed in vitro by polycyclic aromatic hydrocarbons and derivatives. Carcinogenesis 16 (1), 1–9.

Zeisig, M., Moller, L., 1997. ^{32}P-HPLC improvements to characterize DNA adduct stereoisomers from benzo(*a*)pyrene and benzo(*c*)phenanthrene and to separate DNA adducts from 7,12-dimethylbenzanthracene. J. Chrom. 691, 341–350.

Zeisig, M., Hofer, T., Cadet, J., Moller, L., 1999. ^{32}P-postlabeling high-performance liquid chromatography (^{32}P-HPLC) adapted for analysis of 8-hydroxy-2′-deoxyguanosine. Carcinogenesis 20 (7), 1241–1245.

Zhu, H., Bannenberg, G.L., Moldeus, P., Shertzer, H.G., 1994. Oxidation pathways for the intracellular probe 2′,7′-dichlorofluorescein. Arch. Toxicol. 68 (9), 582–587.