

MARKERS OF **OXIDATIVE DAMAGE AND**
ANTIOXIDANT PROTECTION
Current status and relevance to disease



SUMMARY REPORT OF A WORKSHOP HELD IN JUNE 1999

Organised by the ILSI Europe
Antioxidant Task Force

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Report on Markers of Oxidative Damage and Antioxidant Protection: Current status and relevance to disease
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***MARKERS OF OXIDATIVE DAMAGE
AND ANTIOXIDANT PROTECTION***
Current status and relevance to disease

REPORT OF A WORKSHOP HELD ON 28-30 JUNE 1999 IN PRAGUE, CZECH REPUBLIC
ORGANISED BY THE ILSI EUROPE ANTIOXIDANT TASK FORCE

AUGUST 2000

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GOAL AND PURPOSE OF THE WORKSHOP

Antioxidants are believed to be important in health maintenance through the modulation of oxidative processes in the body. One key approach to assess the importance of antioxidants in human health is the identification and validation of relevant markers which can detect potential benefits or risks relating to target functions of the body. Markers must be ethically acceptable and measurable in a non-invasive manner.

Validation of markers involves ratifying an analytical methodology and establishing a clear link between the marker and a particular biological function. There is a clear need at the present time to assess the relevance of currently proposed markers in terms of health benefits and reduction of disease risk. Eventually this scientific knowledge will provide evidence about the functionality of antioxidants in the diet and their contribution to long-term health benefits.

The goals of this workshop were to review:

- the markers of oxidative damage to DNA, lipids and proteins
- validation status
- link to function
- relevance to disease.

STRUCTURE OF THE WORKSHOP

The workshop was structured with plenary sessions and working groups focusing on antioxidants in relation to gene expression and DNA damage, lipid oxidation and vascular function, and protein oxidation. The workshop ended with a session summarising the conclusions, implications and recommendations for validation and use of markers in population studies related to health and disease.

SESSION I: GENERAL INTRODUCTION

1.1 Introduction to the workshop

Dr. B. Danse, ILSI Europe (B), gave a general introduction and welcome from ILSI Europe followed by a summary of the previous activities of the Antioxidant Task Force.

Prof. A. Diplock, International Antioxidant Research Centre (UK), introduced the workshop, pointing out the link between the successful European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) programme and one of the specific components within the functional foods area, antioxidants. The goal of FUFOSE was to critically assess the science base which provides evidence for a link between specific nutrients and function. The types of claims considered were: (i) Claims related to dietary guidelines of healthy diets; (ii) Nutrient content claims; (iii) Comparative claims; (iv) Nutrient function claims.

Two further claims would be relevant to functional foods:

Type A: enhanced function claims (e.g. caffeine improves cognitive function)

Type B: reduction of disease risk claims.

This workshop on antioxidants was designed to identify markers which could be used in population studies for proof of efficacy of antioxidants in support of claims types A or B.

1.2 Markers: definition and validation

Prof. Diplock introduced the terminology and criteria for markers. He proposed to replace the term biomarker by **marker** to avoid a false impression of biological relevance, which may lead to an over-reliance on the parameter if the validity and relevance has not been proven. If markers represent events directly involved in a process, they should be considered as **factors**, which are differentiated from **indicators** or **surrogate markers**, terms which represent correlated events. Criteria required for evaluation of markers include methodology, accessibility, reproducibility, relevance to the target tissue and safety of supplementation. A first example of how to validate the marker of DNA damage, 8-oxo-dG, was presented by Prof. J. Lunec, University of Leicester (UK), in the context of the ESCODD (European Standards Committee on Oxidative DNA Damage) initiative. Twenty-eight European laboratories used three different methods (GC-MS, HPLC, immunoassay) to measure this marker. A summary of the conclusions of this initiative is given under the report of working group A.

Discussion

The following discussion points were raised:

- The definition of markers as mentioned above was a proposal from Prof. Diplock; however, this was not a consensus view of the participants.
- Concerning validation of markers, the question was raised about whether there are standard operating procedures available? Apparently no yardsticks are available at the moment.
- It was stressed that a marker should be related to a **late** end-point, e.g. DNA damage as a marker for cancer. The question was raised about whether there is an intermediate end point for cancer, e.g. mutagenesis? However, only certain mutational events have the potential to participate in the process of carcinogenesis and the eventual appearance of cancer.
- A marker must be part of a **major** pathway in disease. It may be difficult to measure the major metabolite, but assurance should be made that what is being measured is related to the mainstream pathway. In addition, it is important to note that metabolism may vary from one individual to another.
- Comments were made that it would be useful to have estimates of turnover of antioxidant defences, both endogenous and ingested antioxidants (for example, using radiolabelled compounds). Initial pressure on antioxidant defences precedes damage. It would be useful to know how much antioxidant is consumed, and how much is regenerated. The importance of the dehydro-ascorbate/ascorbate balance and its measurement was emphasized. However, accurate plasma measurements are needed.
- The issue of compartmentalisation is critical, and the value of using circulating markers and the validity of lymphocytes as surrogates for other target tissues needs to be carefully considered. Unfortunately alternative tissues are often impracticable except in animal studies.

1.3 Introduction to oxidative damage

Three introductory talks were given on markers of oxidative damage to DNA, lipids and protein.

1.3.1 Oxidative DNA damage

Prof. H. Poulsen, Copenhagen University Hospita (DK), gave an overview of the antioxidant network function, the specific DNA bases susceptible to oxidation and the current methods used to measure these lesions. Oxidative modifications of DNA are abundant and potentially mutagenic lesions. It is important in the interpretation of the results to distinguish between concentration (steady-state pool of oxidized DNA bases and nucleosides) and rate (urinary excretion of DNA oxidation products). It is unlikely that oxidation of DNA bases will inflict severe disturbances on gene expression.

Discussion points

- Comment was made that 8-oxo-dG is more mutagenic than 8-oxo-dA, and very little is known about other oxidized bases. The sensitivity of methods for measuring mutations is limited.
- Oxidized bases cannot be linked to one single disease, namely cancer. For example, the relation with other diseases such as atherosclerosis is not known.
- Adaptive responses involving DNA repair should be considered. However, there are no methods to measure this at the present time.
- The origin of the enzymatic release of 8-oxo-dG/excretion was discussed: (i) the major repair pathway removes base, not nucleoside; (ii) not much is known about 8-oxo-dG coming from mitochondrial or genomic DNA.
- The source of material (urine, lymphocytes or target tissue) for measurement was discussed. The assumption is that lymphocytes behave the same way as tissue, but there is no evidence that they do. It may be necessary to distinguish between blood cells and solid organ cells.

1.3.2 Lipid peroxidation

Prof. J. Salonen, University of Kuopio (SF), spoke about the definition of markers and their use in population studies before he summarized the current status of markers for lipid peroxidation. Multiple markers affect disease involving multiple mediating processes (functions), therefore it is not possible to establish one marker:one function. The criterion of biological relevance for epidemiology is “predictive validity”.

Three levels of markers were mentioned:

- health outcome markers (e.g. arterial Intima-Media Thickness (IMT), other non-invasive markers)
- markers of function (endothelial, blood pressure, cognitive function)
- markers of damage (DNA, lipids, proteins).

Three types of studies were presented:

- Epidemiology studies: only prospective studies are relevant as disease processes affect markers. Markers need to be measured early on and followed through evolution of the disease. Prospective studies provide data on associations but do not necessarily establish causality
- Basic research studies to establish mechanism
- Intervention studies (supplementation) can test only one nutrient at a time. They are extremely time-consuming and expensive.

Lipid peroxidation products have several origins reflecting a balance of generation and elimination by absorption in the gut, generation in the circulation and generation in tissues. Examples of markers with their advantages and disadvantages were given, e.g. COPS (cholesterol oxidation products), hydroxy-fatty acids, F₂-isoprostanes, aldehydes, non-induced conjugated dienes, epitopes of oxidized LDL, electronegative LDL, redox ratios, etc. The discussion on these markers was continued and conclusions made in the working group B.

Discussion points

- It must be borne in mind that isoprostanes, apart from being useful markers of lipid peroxidation, also have very specific biological effects, such as vasoconstriction and effects on vascular smooth muscle.
- Doubts were expressed on the artefactual appearance of lipid peroxidation products in work-up as well as contamination from the diet. Comment was made that the creation of artefacts during work-up preparations applies to all markers. The advantages of COPS are that it is a stable marker and it is possible to separate endogenous and exogenous COPS.
- The role of case-control studies in “predictive validity” was questioned. In retrospective case-control studies, the disease may have influences, i.e. enhanced lipid peroxidation; thus increased lipid peroxidation may be a consequence and not the cause of the disease. If adipose tissue samples are used, the measurements reflect a situation from an earlier time, and this problem may not exist.

1.3.3 Protein oxidation

Prof. H. Griffiths, Aston University (UK), gave an overview of protein oxidation and its relevance to altered function. Many proteins may be modified by oxidation, including receptors, transcription factors, mitochondrial enzymes, extracellular matrix targets, etc. A number of amino acid residues are subject to oxidation including aromatic amino acids, sulphur containing, neutral aliphatic and basic aliphatic amino acids.

A spectrum of products is required to produce a biological fingerprint. Different proteins reflect a temporal index of oxidation. Oxidized proteins accumulate in certain tissues such as the lens. Stable species remaining a long time in the cell may confer memory in oxidatively stressed cells. The markers linked to disease include L-DOPA in atherosclerotic plaques and bityrosine in cataracts. L-DOPA is also a reducing species which confers memory.

The most commonly measured marker is carbonyls. To validate this marker the recommendation is to develop suitable material for interlaboratory validation and design of appropriate internal standards.

Discussion points

It was emphasized that the domain of markers of protein oxidation is in its infancy, therefore many questions remain to be answered, for example:

- Does oxidation of proteins result in regulation or damage?
- Are there differences in susceptibility to oxidation between nuclear and cytoplasmic proteins?
- How is the functionality of IgG affected in its ability to activate, complement or interact with Fc receptor?

SESSION 2: PARALLEL WORKING GROUPS

Reports from the working groups

Each working group session had 3 short 10-minute talks followed by 30 minutes of discussion and was concluded by a round-table discussion on the validation and relevance of the biomarkers presented. The report here will give a short summary of each speaker, discussion points, conclusions and recommendations.

2.1 Working group A: DNA oxidation measurement, DNA repair and gene expression

2.1.1 Measuring endogenous DNA oxidation

by Dr. A. Collins, Rowett Research Institute (UK)

A mutagenic lesion 8-oxoguanine is found in oxidized DNA and has been used as a measure of oxidative DNA damage in numerous studies. The most commonly used methods to detect 8-oxoguanine (or its deoxynucleoside, 8-oxo-dG), are HPLC with electrochemical detection (HPLC-ECD) and GC-MS (although HPLC-MS may be the method of choice in the near future). GC-MS is superior to HPLC-ECD in terms of unequivocal analyte identification and quantification. Both methods suffer from artefactual oxidation of guanine during the isolation and enzymatic digestion of DNA, and during the chemical derivatization procedure for the GC-MS method. The high levels of artefactual oxidation of guanine may have affected the conclusions drawn from many human studies where a wide range of estimates of endogenous damage has been reported (from about 100 down to 0.2 8-oxo-gua per 10^5 guanine) and raises concern about the validity of the analytical methods. An alternative procedure for measuring oxidation of DNA is through formamidopyrimidine glycosylase (FPG), a repair endonuclease that detects 8-oxoguanine in DNA and introduces a break, which may be measured by the comet assay (single cell gel electrophoresis). With this approach, artefactual oxidation of DNA is less likely to occur. However, this method may under-estimate damage, if some lesions in the DNA are inaccessible, or if lesions occur in close proximity (giving rise to a single measurable break).

Because of the large variation in estimates of 8-oxoguanine, ESCODD was established in 1997 to examine methodological approaches to measurement of 8-oxo-dG in various DNA samples. The aims of ESCODD are: (1) to validate analytical methods to measure 8-oxoguanine/8-oxo-dG with increased reproducibility and precision, (2) to compare analytical methods to the endonuclease-based comet assay, (3) to reach a consensus of the "average" level of oxidation in normal human DNA. The findings of this interlaboratory study revealed that further improvements in the analytical techniques are required to obtain a consensus of the average level of DNA damage in humans. The overall estimates of 8-oxoguanine formation in DNA continue to decrease as the analytical methods have been further refined.

The analysis of 8-oxo-dG is often conducted with lymphocytes and urine because these fluids are the most readily obtainable material from healthy subjects. It is uncertain whether measurement of oxidized DNA in lymphocytes reflects oxidative DNA damage in other tissues. Comparative studies of 8-oxo-dG formation in lymphocytes and other tissues of animals subjected to oxidative stress or antioxidant protection may answer this question. It is often assumed that urinary 8-oxo-dG is derived from cellular DNA repair processes. Estimates of the rate of input of oxidative

damage have been based on urinary 8-oxo-dG levels; however, it is 8-oxoguanine, and not 8-oxo-dG which is released by base excision repair. Urinary 8-oxo-dG instead may be derived from DNA breakdown products from dead cells that are oxidised during elimination. The degree of inter- and intra-individual variability in oxidized DNA base formation is unknown and the levels of oxidized DNA damage may be influenced by health status, diet, exercise and stress, all of which may vary. It may be possible to conduct population comparisons to measure oxidized DNA base formation, but it is currently impossible to assess individual disease risk with oxidized DNA levels. Based upon data from experimental animals and mutagenesis studies, it is believed that some oxidized lesions are mutagenic and may be involved in carcinogenesis, but the link between oxidized DNA damage and cancer in humans is tenuous.

More than 20 oxidized DNA bases are formed under various types of oxidative stress conditions. Some of these lesions are less prone to form through artefactual oxidation of DNA and may prove to be more reliable biomarkers than 8-oxo-dG. Several of these oxidized DNA bases should be considered as additional markers to assess oxidative DNA damage. DNA adducts derived from lipid oxidation also merit further applications in human biomonitoring studies.

2.1.2 *Repair of oxidative DNA damage and its relation to cancer*

by Dr. S. Boiteux, Commissariat de l'Energie Atomique – CEA (F)

The level of 8-oxo-dG in DNA represents a steady state and is distinct from the rate of input of oxidative damage. Its main determinant is presumably the rate of removal of damage by repair, more efficient repair resulting in a shorter half-life and a smaller pool of damage. In spite of the crucial role of DNA repair in maintaining a low steady state level of oxidized DNA, we know relatively little about the level of repair activity and interindividual variation, or the possible modulation of repair enzymes by genetic and dietary factors. The base excision repair pathway removes 8-oxoguanine lesions. In *E. coli* and yeast, lack of functional FPG (or yeast homologue OGG1) leads to a great elevation in the frequency of mutation (specifically, GC->TA transversions). The mammalian *ogg1* gene has also been identified. Knockout mice (*ogg1*^{-/-}) are now about one year old and thus far, there is no elevated incidence of cancer.

The 8-oxo-dG levels in lymphocytes in the knockout mice are only 50% higher than normal levels, which suggests that either the input of oxidative damage is much less than previously thought, or that a back-up repair process, perhaps nucleotide excision repair, takes over when base excision repair is impaired.

The human form of *ogg1* has been studied in various tissues. It occurs in two forms (splicing variants), one of which is located in mitochondria. Other repair functions have been found in mitochondria, dispelling an old myth that mitochondrial DNA damage is not repaired. Mutations have been identified in 1/25 and 6/40 human lung and kidney tumours, respectively, suggesting that mutations impairing *ogg1* activity may be associated with the carcinogenic process in these tissues.

There is currently no facile, reliable assay for DNA repair that is suitable for use in human biomonitoring studies. Repair assays utilizing cell free extracts *in vitro* may be used to assess endonuclease activity. These assays will most likely be limited to surrogate tissues such as lymphocytes, and it is uncertain whether the repair activities are representative of other tissues. There is little known about inter- and intraindividual variability in repair.

2.1.3 Markers of gene expression

by Prof. R. Tyrrell, University of Bath (UK)

Changes in gene expression can provide an extremely sensitive marker of oxidative stress (or changes in oxidative status) of tissue, but the development of markers based on such changes is at an early stage. Expression of the genes selected should be specifically induced by oxidative stress, if possible, and the functional significance of the gene product and its induction should be known. In general, markers based on events in signal transduction or transcription factors (e.g. NFkB) activation are not considered suitable because their induction tends to be transient, non-specific, and hard to quantify.

Haem oxygenase 1 (HO-1) is, at present, the most appropriate gene for study as a marker in relation to oxidative stress. Its level of induction under physiological conditions is very high in several cell types (e.g. skin fibroblasts) although not in epidermal keratinocytes. Techniques and reagents are available to measure expression of this gene at three levels (mRNA accumulation by Northern blotting or competitive RT-PCR, protein expression by flow cytometry using specific antibodies, or enzymatic activity by HPLC). However, levels of intraindividual and interindividual variability are expected to be large especially for the mRNA measurements and the parameters require validation before exploitation as a marker will be feasible.

It should be emphasised that to select markers of gene expression that will respond to oxidative stress, genes whose expression is modulated by cellular reducing equivalents or exogenous stress are required. It should be demonstrated that the up-regulation attributable to oxidative stress is reduced by antioxidants in appropriate target cells (in *in vitro* test systems prior to application in *in vivo* testing). This is quite distinct from the response to antioxidants that involves the up-regulation of certain specific genes (e.g. glutathione-S-transferase) as a consequence, for example, of the antioxidant response element (ARE) present in the promoter. Such genes respond to a diverse selection of antioxidants and do not reflect oxidative status. They are not suitable as markers in the context discussed here.

Discussion points

- An important, unanswered question is whether lymphocytes represent an adequate surrogate tissue for the target tissues.
- An additional question was whether 8-oxo-dG is really the best marker of oxidative damage. Although it is relatively abundant, the proneness of guanine to artefactual oxidation is a disadvantage. It was suggested that 8-oxo-dG should not be the only oxidation product measured.
- It was pointed out that the background level of endogenous oxidative DNA damage is uncertain.
- The aspect of storage of tissue and DNA sample was discussed. The method of storage, i.e. at -20°C and -80°C or in liquid nitrogen will have potential effects on the level of oxidation markers of DNA.
- It is unknown how much DNA oxidation occurs – and is repaired – per cell, per day. It was commented that the measurement of urinary 8-oxo-dG represents both DNA repair and oxidation of dead cell DNA and that it would be useful to be able to evaluate DNA repair capability in individuals. However, no such assay is available at the present time.
- The extent and cause of intraindividual variability in oxidative DNA damage was discussed. Factors such as health status, dietary factors, exercise and general stress may significantly affect intraindividual variability.
- Concerning the extent and origin of interindividual variations, it seems unlikely that we will ever link individual levels of oxidative damage in DNA with individual disease risk, but comparisons of mean values for groups of individuals will continue to be informative.

- The question arose about which population group would be the most suitable for studies to establish the validity of biomarkers of DNA damage in terms of human health outcomes. In particular, should the study groups consist of people who are at elevated risk, such as patients on chemo/radiotherapy, old people, or smokers, to maximise the chance of seeing effects on the biomarker as well as on clinical outcome? This is an ongoing debate.

2.1.4 Conclusions: Current status of the markers for DNA and gene expression

- *Markers for DNA*

Marker	Method of measurement	Comment
8-oxo-dG (3 years standardization between about 30 laboratories in Europe is ongoing as a Framework Five project, ESCODD. First two reports published).	HPLC-EC	EC is the most commonly used method, it is sensitive and the easiest method to use. Useful for tissue measurement. Used for urine measurement by very few groups
	GC-MS	Specific with potential for measuring other oxidative modifications. Resource demanding and used in few laboratories. Measures the base and not the nucleoside. Not used for urine measurements.
	LC-MSMS	Under development. Theoretically the most appealing method, but also resource demanding. Useful for both urine and tissue measurement.
	Immunological methods	Commercially available. Not validated and problems with specificity. Not recommended before validated.
Oxidized pyrimidines, 8-oxoguanine	Enzymic method (comet assay)	Very sensitive, quantitative, rapid and inexpensive. Uses specific repair enzymes to detect oxidized bases.

- *Markers for gene expression*

Markers for gene expression are still in their infancy.

2.2 Working group B: Antioxidants and vascular function

2.2.1 Antioxidants and LDL oxidation

by Prof. M. Aviram, Rambam Medical Center (IL)

The multistage process of atherogenesis was described. No one single marker is sufficient to follow this process. Oxidation of LDL is a major risk factor involved in the atherogenic process. Within the LDL particle, antioxidants are first used up, then lipid peroxidation and protein oxidation products are formed. 27-OH cholesterol is highly abundant in coronary lesions, but not in plasma. 7 β -OH cholesterol is highly abundant in carotid lesions. Oxidized LDL can be removed by paraoxonase.

2.2.2 Antioxidant and measures of lipid oxidation

Prof. B. Halliwell, National University of Singapore (SG)

Criteria for an “ideal” marker of lipid peroxidation were listed:

- measured by accurate, chemically robust, MS-based, validated method
- a major product, or minor product indicative of an important pathway
- not confounded by the diet (short-term effects e.g. oxidation products from food)
- applicable to both total and steady state levels
- stable upon storage.

Isoprostanes fulfil most of these criteria. It is an MS method, is not confounded by the diet (short-term effects) and is stable upon storage. It is, however, a minor product and therefore it still has to be established if it is predictive of a major pathway in disease. Further methodological improvement and harmonization is needed. It may also be appropriate to develop methods for measuring “total peroxides” but this would be complicated by confounding factors in the diet.

2.2.3 Antioxidants, nitric oxide and smooth muscle activity

by Prof. E. Anggard, William Harvey Research Institute (UK)

The importance of the signalling pathways in the endothelium in relation to cardiovascular disease were emphasised. Physiological stimuli such as NO and O₂⁻ are necessary for cellular signalling pathways. However, excess formation of reactive species (superoxide, peroxynitrite) leads to cell proliferation and vasoconstriction. Disease progresses from oxidative stress to endothelial dysfunction to insulin resistance to diabetes type II and complications.

Pulse-flow radiology was proposed as a non-invasive method to monitor endothelial function through measurement of blood flow in the finger. This measurement can reflect NO dysfunction. However, the basic work is not yet done; we need to find the right patients, antioxidant, dose, and test in a large-scale intervention study.

Discussion points

- The choice of measuring early versus late peroxidation products (such as oxysterols) was discussed. It was pointed out that early products are unstable with a short half-life, whereas late products are stable with a long half-life. It was generally agreed that more than one marker should be measured.
- The specificity of the markers was discussed. It was considered preferable to use specific markers when the appropriate methodology was developed and validated (e.g. methodology for oxysterols developed by R. Riemersma). However, non-specific methods, such as MDA, should not be discarded as they are practical to handle and give a lot of information (M. Aviram). It was commented that when measuring MDA, it must be done with the GC-MS method to be sure of what is being measured. Further validation is required on markers such as 27-OH-cholesterol, especially if it is considered as a major risk factor.
- The practicability of every laboratory having MS equipment was questioned. It may be necessary to accept some alternative methods.
- Storage conditions were seen as an important factor, in particular whether the addition of antioxidants for stability would influence the result.
- Concern was expressed on how well plasma measurements reflect tissue events.
- Selection of the population was once again debated. Should it be healthy vs. high-risk individuals or non-responders? Lipid peroxidation in healthy people has a large range, but the people at the top of the range may be more sensitive to risk. Diseased tissues oxidize more rapidly than normal tissues.
- Concerning isoprostanes, the major question is whether as minor lipid peroxidation products, they are indicative of a major pathway involved in the disease process. There is a large range even in healthy people. It would be interesting to know if people with high levels of isoprostanes have a higher risk of CVD and diabetes type II. The most significant elevation of isoprostanes is found in diabetes (greater than in smokers). Another important point is whether isoprostanes are influenced by the diet. It has been shown that there is no influence of diet in the short-term. However, clearly diet in the longer term affects progression of the disease and the level of isoprostanes measured.
- What happens in the *ex-vivo* LDL model was considered as much more destructive than what happens *in vivo*.
- Endothelial cell function was of great interest but it was concluded that much more work is needed to validate markers and correlate them to function.

2.2.4 Recommendations

- An ESCODD-type of initiative for standardization and validation of the isoprostane method was welcomed by the group.
- It was strongly recommended to include markers in **all** intervention trials.

2.2.5 Conclusions: markers of lipid peroxidation

I. Short list of recommended markers of lipid peroxidation

Marker	Measurement method	Comments
Isoprostanes	MS	Meets all criteria for a useful marker except that it is a minor pathway – is it indicative?
COPS	MS	Expect coronary events in people with high COPS levels. The marker is confounded by the diet but is this not more physiological? Prospective studies ongoing.
OH-fatty acids	MS	Specific fatty acids need to be further investigated as possible markers linked to disease
Ox-LDL Ab	ELISA	Clear relation to disease. Problem of availability and standardization of antibodies

II. Interesting markers needing further development

Marker	Measurement method	Comments
Aldehydes	HPLC, MS	Urinary, not plasma malondialdehyde MDA-lysine adducts are worth investigating further
Oxidized phospholipids	HPLC, ELISA	Interesting marker, but further studies are required
Electronegative LDL	HPLC, FPLC	Difficult to measure reproducibly

III: Markers not retained

Marker	Comments
LOOH	Samples are not stable to storage
Exhaled pentane	Non-specific
Total antioxidant status	Non-specific
<i>In vitro</i> LDL ox	Non-physiological

Complementary markers of oxidant/antioxidant status would be redox ratios, antioxidant and fatty acid levels, glutathione status, paraoxonase activity

2.3 Working group C: Protein oxidation

It quickly became clear that, as compared with DNA and lipid oxidation, the field of protein oxidation is very much in its infancy. On the other hand, it was clearly felt that, since proteins do play a crucial role in many (patho)physiological processes, the area of markers for protein oxidation should and will grow in years to come.

2.3.1 Oxidation of immunoglobulins

by Prof. H. Griffiths, Aston University (UK)

In vitro, protein oxidation can be specifically measured in immunoglobulin and these oxidations influence functional characteristics of immunoglobulins, possibly inducing a “pro-inflammatory state”. *In vivo* evidence was presented showing a reversible reduction in IgG carbonyl content in subjects receiving vitamin C supplementation.

2.3.2 Direct protein modification in LDL

by Prof. C. Rice-Evans, International Antioxidant Research Center (UK)

The oxidation of low-density lipoproteins (LDL) is well accepted to be a risk factor for development of atherosclerosis. It is not yet known what events *in vivo* promote the oxidation process, but such agents as those that catalyze the oxidative/reductive decomposition of pre-formed lipid peroxides, lipoxygenases, peroxynitrite, hypochlorite have all been invoked. In this presentation, Prof Rice-Evans presented data showing that apolipoprotein B in LDL can be modified directly by oxidants such as hypochlorous acid, independently of lipid peroxidation. Direct oxidation or modification of amino acids on the surface of the apolipoprotein B may have important consequences on physiological function.

2.3.3 Protein oxidation in the eye lens and its relation to cataract

by Prof. A. Taylor, University of Tel Aviv (IL)

A pathophysiological situation where protein oxidation seems to be a key step in the causal pathway was highlighted. Lens opacification leading to cataract formation involves protein oxidation in the lens of the eye as a consequence of insufficient proteolytic “repair” activity leading to accumulation of oxidized proteins. The ubiquitin-conjugation proteolytic system that removes oxidized proteins is influenced by the redox status in eye lens and tissues. Moreover, antioxidants, especially the water solubles, e.g. vitamin C, seem to play a role in preventing protein oxidation in the lens. This is also indicated by epidemiological data.

Validation

Biological validation, preferably in prospective epidemiological studies, is an important issue. Before this, method validation and standardization are areas that do need further work, given the relative infancy of the research field. Issues in method validation should include sample collection and storage, extraction, hydrolysis and standardization. Criteria as used in procedures for GLP (Good Laboratory Practice) should be applied here.

Link with disease

Several markers of protein oxidation have been linked to a specific disease, although mainly in cross-sectional studies. These diseases included Alzheimer, diabetes, Parkinson, cardiovascular disease and cataract. With a possible exception for cataract, the biological validity of the protein markers is not yet clear.

Discussion points

- There is a generally recognized problem of broad interindividual variation in basal levels of markers.
- Concerns were raised on the influence of sampling and storage conditions and of possible artefacts created during sample preparation, extraction and protein digestion.
- An important question is whether plasma values reflect protein oxidation in the target tissue. However, a major problem is the accessibility of samples from the target tissue.

2.3.4 Recommendations

- Validation of the carbonyl assay
- Investigation of function of oxidized proteins and their relation to disease

2.3.5 Conclusions: markers of protein oxidation

Marker	Measurement method	Comments
Carbonyls	ELISA in plasma or specific proteins	Most widely used non-specific
SH groups	Derivatisation + spectrophotometry	Widely used – low specificity
Nitro-tyrosine; Chlorotyrosine	HPLC + ECD or immunodetection	Nitro-tyrosine is reportedly degraded by HOCl, questioning its validity as an oxidative biomarker. Chlorotyrosine appears to be highly sensitive and specific <i>in vitro</i> . Little application <i>ex vivo</i> yet.
Tyrosine dimers	HPLC + fluorescence	Specific
L-DOPA	HPLC + fluorescence	Successfully used in two laboratories – no interlaboratory comparison
Kynurenines	HPLC + fluorescence	Successfully used in two laboratories – no interlaboratory comparison
Hydroxylated valine and leucine; oxidation products of lysine.	Derivatisation + HPLC + fluorescence	Stable during extraction. Used in one laboratory for determining oxidative stress in excised atherosclerotic plaques and excised cataractous lenses.
Lens opacity	Physicochemical methods	<i>In situ</i> analysis with functional relevance.

SESSION 3: VALIDATION OF METHODOLOGY AND RELEVANCE

3.1 Total antioxidant status

Prof. C. Rice-Evans, International Antioxidant Research Centre (UK), reviewed the current methodology for measuring total antioxidant status (FRAP, ORAC, TEAC). These assays measure the combined reducing capacity. Although these assays are good for *in vitro* testing of antioxidants as single components or as part of complex food matrices, the use of them in plasma is limited for the following reasons:

- (i) The chemistry of the assay system must be right. The contribution of individual serum antioxidant components to the total antioxidant status is different for each assay.
- (ii) The sensitivity is too low to detect the very small changes resulting after antioxidant supplementation (above the background of albumin and uric acid reacting in the assay).

Discussion points

- Cyclic voltammetry was proposed as an alternative method which is based on the redox potential.
- Total antioxidant status assays are measuring the static situation, they are not measuring rates or transient changes, fast- and slow-reacting compounds. For example, a low concentration of a compound which is regenerated may have a more significant contribution than a compound in high concentration which is rapidly used up.

3.2 Review of human studies on DNA damage and repair, LDL and protein oxidation

3.2.1 Oxidative damage and antioxidant protection related to DNA damage

Prof. S. Loft, University of Copenhagen (DK), gave an overview of studies involving antioxidants interventions and measurement of oxidative DNA damage. The results are inconclusive with insignificant changes in 8-oxo-dG urinary levels after intervention with antioxidants. Interestingly, phytochemicals coming from Brussels sprouts were more effective than antioxidants. In addition, there was a large variation in measurement of 8-oxo-dG levels following antioxidant intervention, possibly reflecting differences in populations, regimens, functional correlates of biomarkers and between laboratories and assays. Depletion or oxidative stress prior to antioxidant intervention might facilitate demonstration of protective effects. Prof. Loft pointed out that in molecular epidemiology studies, DNA adducts and mutations would be considered as **exposure** markers, and not as markers of biological effect or altered structure-function. The concept of steady-state balance between damage and repair must also be borne in mind.

Discussion points

- Comment was made that one of the difficulties in interpreting the data is because of the different ways of reporting the levels of 8-oxo-dG. It was recommended to introduce a standard way of reporting 8-oxo-dG values (per 10⁵ dG) for publication.
- The recent trend in attempts to validate 8-oxo-dG is towards precision in measurement rather than absolute accuracy.
- The “comet” assay may overestimate DNA damage.
- As previously mentioned under working group A, the unanswered questions remain:
 - Is 8-oxo-dG the ideal marker?
 - Measurement in surrogate versus the target tissue?
 - What does urinary 8-oxo-dG represent – repair products or oxidation of dead cell DNA?
 - The degree of intra- and interindividual variability?
- There is no good assay for DNA repair that would be suitable for biomonitoring studies.
- The link between oxidative DNA damage and cancer is tenuous.
- It is important to distinguish between oxidant (e.g. haem oxygenase) and antioxidant regulated genes (via the antioxidant responsive element).

3.2.2 Oxidative damage and antioxidant protection related to cardiovascular diseases.

Prof. M. Aviram, Rambam Medical Centre (IL), summarized the risk factors for atherosclerosis and the human studies carried out with antioxidants. LDL oxidation is a major risk factor for atherosclerosis.

Oxidized LDL-induced macrophage foam cell formation and atherosclerosis are inhibited by dietary antioxidants such as phenolics and flavonoids found in red wine, olive oil, licorice and pomegranate. The mechanisms for these phenomena involve protection against cell-mediated oxidation of LDL via effects on the lipoprotein, the arterial cells (oxygenases such as NADPH oxidase vs. antioxidants such as glutathione) and HDL-associated paraoxonase (which hydrolyzes oxidized lipids).

Discussion points

- Testing of antioxidants for their ability to inhibit LDL oxidation (*in vitro*) is acceptable for experimental studies but not recommended for population studies (*ex vivo*).
- Criticism of the mouse apoE model: contrary to humans, mice synthesize their own vitamin C and therefore do not need it from the diet. It is therefore important to know the vitamin C status of the apoE mice and how that might influence the results found with antioxidant intervention with, for example, flavonoids.
- Flavonoids are poorly absorbed in humans. Tea flavonoids are strongly bound to proteins. How does this compare to the mouse model?

3.2.3 *Oxidative damage and antioxidant protection related to protein oxidation*

Prof. M. Chevion, The Hebrew University of Jerusalem (IL), summarized the human studies related to protein oxidation and protection by antioxidants. The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content (PCC). It can be monitored by several modifications of the reaction of PCC with dinitrophenylhydrazine. Human studies have focused on diabetes, neurodegenerative disease and aging. In most studies an increase, typically by 40%-100%, was observed and correlated with the severity of the pathology.

A concerted international effort is needed for validation of the PCC and the newly developing methodologies.

Discussion points

- Which of the available assays for protein carbonyls should preferably be used?
- What is the explanation for the variable baseline levels of plasma carbonyls in the different studies?
- Could carbonyls also be derived from glucose? How are we sure that this does not introduce noise in the measurement?
- All available studies on plasma carbonyls so far are cross-sectional: there is a need for intervention and follow-up studies.

SESSION 4: IMPLICATIONS AND CONCLUSIONS – FUTURE DIRECTIONS

4.1 Implications for the design of future studies

Prof. F. Kok, Wageningen University (NL), discussed the implications of marker development on the design of future human intervention and epidemiology studies. Human studies generally aim at establishing either enhanced function (type A claim) or reduced disease risk (type B claim). Regarding study design, type A claims would at least require human intervention studies. Type B claims would require prospective data, if possible experimental. The type of study design depends not only on the markers available but also on the time frame of the causal pathway to the eventual occurrence of chronic diseases. Application of markers in human studies raises questions related to study size and choice of the study population, validity of markers, determinants of variability in marker levels, use of proxy versus target-tissue markers and time-integration of enhanced function and disease risk markers. An important practical point is the feasibility of the methodology to be applied to a large sample size. Collection, storage and transport of samples as well as time-consuming laboratory procedures have to be taken into account. Human studies do not generally need perfect validity of the marker; but an adequate ranking of subjects within the population usually is sufficient. Internal validity of the study must be ensured by proper comparability of intervention and control groups, or of cases and controls. Precision is more important than accuracy.

In conclusion, to establish causality for antioxidant involvement in either enhanced function or reduced disease risk, development of laboratory methodology with validation of appropriate markers and designing of human intervention and epidemiology studies should go hand in hand.

Discussion points

- Subclassification of effect or response is enhanced function and modulation of the disease process.
- Concerning functional claims, one can speak of increased or decreased function. This may be beneficial or adverse. Perhaps it would be better to say “altered” rather than “enhanced” function.
- It is important to distinguish between markers of exposure, effect and susceptibility although, it is agreed that marker classification is somewhat arbitrary.

4.2 Validation of markers for their relevance to health and disease

A summary of the requirements for methodological validation was given by Dr. J-M. Antoine, Groupe Danone (F). Briefly, two keystones were identified for selection of markers of oxidative damage and antioxidant protection:

- (i) Analytical validation to assure that the marker can be measured accurately and reproducibly.
- (ii) Pathophysiological validation to assure relevance of the marker to biological function.

To achieve validation of a standardized analytical method, the following points will need to be considered: choice of the analytical apparatus (e.g. GCMS), a standardized procedure for extraction and analysis including the use of a standard reference, assessment of intralaboratory variance (reproducibility), assessment of interlaboratory variance (round-test), production and measurement of a standard reference material (quality control), measurement of critical points (quality assurance), assessment of sensitivity, measurement of intersubject variance.

Such an approach is well underway for measurement of DNA oxidation products via the European Standards Committee on Oxidative DNA Damage (ESCODD). Similar approaches are recommended for measurement of markers of lipid and protein oxidation products.

Prof. Diplock continued the discussion on the relevance of markers to physiology and pathology. A distinction is required between cause and secondary consequences of disease. A marker may be involved in several metabolic or disease pathways, therefore understanding of the role of the marker is crucial to assess its validity and specificity. Markers must be minimally invasive and ethical if they are to be used in experimental studies. Clearly the validation of the relevance of markers to health and disease represents a major challenge for future research in the field of antioxidants and health.

Discussion points

- Lipid peroxidation markers, particularly isoprostanes, need clarification on the relation to a major disease pathway. Some argue that the case for isoprostanes as the ideal marker has been overstated. Cholesterol oxidation products (COPS) have been linked to disease, whereas there is no evidence for the health relevance of isoprostanes.
- DNA repair assays are in their infancy. It is possible to show persistence of DNA damage.
- Although changes in markers may be indicative of disease development, the appearance of these markers could also be part of the disease process.
- Claims: It was strongly pointed out that despite the proposed type A and type B classification for the strength of the links between antioxidants and health, it is currently not legally possible to make health-related claims for antioxidant ingredients of food. In the USA, nutrients are considered only as food, and therefore they have to be safe. In Europe an antioxidant has to be **safe** and to have **proven efficacy** before going forward with health claims. There is no procedure in the EU for evaluating health-related claims.
- Management of results: Managing the communication involving interpretation of the scientific results and explanation of a position on functional foods to the public, as well as advising the legislative organizations in Europe are important.

4.3 Recommendations

- There is a need for a coordinated biomarker approach in Europe.
- An ESCODD-like approach to protein and lipid oxidation markers is recommended. However, there would need to be a consensus on which was the most relevant marker(s) to be validated.

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