

ILSI EUROPE CONCISE MONOGRAPH SERIES

ANTIOXIDANT AND ANTI-INFLAMMATORY COMPONENTS OF FOODS



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ANTIOXIDANT AND ANTI-INFLAMMATORY COMPONENTS OF FOODS

by
Professor Barry Halliwell



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FOREWORD

ILSI Europe's Functional Foods Task Force is exploring the scientific evidence supporting the concept that some diets/foods/ingredients are modulating health and/or some specific physiological functions beyond conventional nutrition. This concept has been agreed widely after the successful European Commission funded FUFOS and PASSCLAIM projects based on examples of various physiological functions sensitive to some foods/nutrients. A working group started from the diet perspective, and explored the scientific evidence regarding the beneficial effects of antioxidants, and/or foods rich in those compounds on health.

A rigorous scientific analysis is now more than needed in the complex area of oxidation and antioxidants where conflicting results have been published by mixing *in vitro*, *ex vivo* and *in vivo* experiments, ingredients and diets, and where many different markers have been used to monitor either specific mechanisms or a global effect on the host. Whereas a diet rich in foods providing a higher amount of antioxidants is considered to be beneficial for health, and antioxidants are able to protect *in vitro* against oxidative challenges, there is no convincing data on the beneficial effect *in vivo* of any specific antioxidant. One of the leading experts in the field, Prof. Barry Halliwell, delivered an impressive talk on antioxidants at the conference organised by ILSI Europe on 'Health benefits of foods – from emerging science to innovative products' in Prague in October 2011 and subsequently agreed to write an ILSI Europe Concise Monograph on the topic.

In this Concise Monograph, Prof. Halliwell has provided an unusual breeze full of stimulating oxygen in this complex area and summarised three main messages around the basic secret of a healthy life: balance!

1. Oxygen and its reactive oxygen species derivatives (ROS) are at the same time an indispensable fuel for life, an efficient weapon during acute inflammatory responses, and a dangerous poison involved in many diseases associated with chronic inflammation. Both too much and too little are associated with deleterious effects.
2. Antioxidants are very useful in buffering reactive oxygen metabolism, but some molecules, such as iron are "Janus" molecules. In fact there is a permanent adjustment between oxygen/ROS and antioxidants. The complex machinery of antioxidants with cofactors and endogenous antioxidants acts in many different ways in diverse tissues and needs different dietary constituents to work properly or to be regenerated.
3. Finally, it is a long way from the gut, where dietary antioxidants enter the circulation, to the mitochondria, where most oxidative reactions occur, and there is little evidence of a convincing increase in post-prandial blood concentrations of antioxidants after ingestion of either food or dietary supplements rich in those antioxidants.

The last part of this Concise Monograph deals with the conflicting epidemiological results and concludes that there is evidence on the benefit of some diets in reducing inflammation and/or oxidative damage, but no experimental data showing any benefit of high doses of antioxidant supplements.

There is still a nut to crack on the relationship between diet, dietary antioxidants, oxidation and inflammation.

To close this foreword, a big "thank you" and farewell to Dr Thomas Hatzold, recently retired from Mondelez, who was the driving force behind this Concise Monograph, which would not have been finalised without his dedicated effort.

Jean-Michel Antoine (Danone, France)
Sheila Wiseman (Unilever, The Netherlands)

INTRODUCTION

The good and the bad of oxygen

The air around us contains 21% by volume of oxygen (O_2), a gas essential to life. Eighty percent or more of the O_2 we consume is used for the process of aerobic respiration, which allows efficient energy production in our mitochondria in the form of adenosine 5'-triphosphate (ATP). Some O_2 is used by enzymes to catalyse essential reactions, which include the synthesis of the hormone adrenalin and the neurotransmitter dopamine, as well as the hydroxylation of amino acid residues during the formation of collagen, essential to making our connective tissues. Our cytochrome P450 enzymes use O_2 to metabolise xenobiotics ("foreign compounds"), including certain medicinal or recreational drugs, industrial chemicals, and toxins in the plants we eat, usually converting them into less harmful products that the body can easily dispose of.

Our need for O_2 obscures the fact that it is a toxic, mutagenic gas; we only survive its presence because we have evolved a wide range of antioxidant defences to help protect against its deleterious effects and allow us to use it safely. In addition to those antioxidants that we synthesise ourselves (often called the "endogenous antioxidants"), the human diet is rich in antioxidants, mostly from the plants we consume. Plants synthesise many antioxidants to protect themselves, because they produce O_2 during photosynthesis, and thus have to be able to tolerate high levels of it. Some of the antioxidants in plants in our diet are essential to us (e.g. vitamin E), whereas others are not essential but useful (e.g. carotenoids, flavonoids). Plants also contain many agents other than antioxidants, which may modulate different processes, including inflammation.

In recent years, the value of antioxidants contained in our diet, or consumed in more concentrated form as additives

and supplements, has been subject to intense scrutiny. Overall, the results of epidemiological studies, and more particularly intervention trials, have been confusing and contradictory, and have given no compelling evidence that high-dose supplements of "antioxidant" vitamins and minerals can benefit human health, at least in the populations studied. In particular, no convincing evidence has been obtained for protective effects of antioxidant supplements against the development of cancer and cardiovascular disease. Yet there is still a feeling embedded in the minds of the public, and many scientists, that antioxidants are good things (which is true, as we see below), and the more antioxidants the better (which is probably not true, as we also see below).

WHY IS OXYGEN TOXIC?

Reactive oxygen species

Over 80% of the O_2 we consume is used by mitochondria, and most of the rest helps to bring about useful metabolic transformations as described in the Introduction. However, a small percentage of the O_2 consumed (estimated as 1-2%, although these estimates are what they say ("estimates") and may be too high) is converted into reactive oxygen species (ROS). These are species more reactive than O_2 itself and capable of damaging biological molecules. Some ROS are free radicals, such as superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical (OH^{\bullet} , sometimes written as $\bullet OH$), and others are not, as Table 1 explains. Non-radical ROS include hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$).

The term "reactive" covers a broad range. Some ROS are highly reactive and others less so. The classic example of the former is hydroxyl radical (OH^{\bullet}), which reacts upon contact with all biological molecules and oxidises them instantly. For example, DNA exposed to OH^{\bullet} is rapidly damaged and the four DNA bases (adenine, thymine, cytosine, guanine) are converted into detrimental products. Thus, the purine base guanine is converted by OH^{\bullet} into a range of products, especially 8-hydroxyguanine (8OHG), which can mis-pair during DNA replication and generate mutations that contribute to cancer development. Exposure of lipids in membranes or lipoproteins to OH^{\bullet} starts a chain reaction ("lipid peroxidation") in which the lipids are oxidised to lipid peroxides via a series of intermediate lipid peroxy radicals (Table 1). Oxidation of lipids *in vivo* impairs cell membrane function, contributes to the development of atherosclerosis (especially oxidation of lipids in low density lipoproteins (LDL)) and is implicated in many other diseases. Lipid oxidation is also involved in the actions of several toxins, particularly those that affect the liver

(e.g. organic solvents such as carbon tetrachloride and excess ethanol). Oxidation of lipids in foodstuffs leads to rancidity and "off-flavours", so food chemists are just as interested in lipid peroxidation as biomedical scientists.

Hydroxyl radicals are produced *in vivo* when the body is exposed to ionising radiation, such as X-rays or gamma-rays. The intense energy splits water into hydroxyl and hydrogen radicals.



Excess exposure to ionising radiation damages DNA, largely via OH^{\bullet} , and hence raises the risk of cancer. It also oxidises lipids; OH^{\bullet} is again largely responsible. Hydroxyl radicals are also readily generated from hydrogen peroxide (H_2O_2). In contrast, superoxide radical ($O_2^{\bullet-}$) is much more selective in its actions. It does not attack DNA or lipids, but can inactivate a few enzymes critical to metabolism, including some in mitochondria, and this can lead to formation of the much more reactive OH^{\bullet} (Section 'The role of iron'). Hence, $O_2^{\bullet-}$ levels must be carefully controlled (Section 'Endogenous and exogenous antioxidants').

H_2O_2 , a non-radical ROS, is widely generated *in vivo* but is also rapidly broken down (Section 'Endogenous antioxidants') so that its steady-state levels are usually low (the micromolar range or less). It crosses membranes fairly easily, and so can diffuse between different subcellular compartments or even between cells. Like $O_2^{\bullet-}$, H_2O_2 is selectively reactive, being unable to attack most biomolecules. However, if H_2O_2 comes into contact with iron or copper ions, OH^{\bullet} can be generated. Indeed, a mixture of ferrous sulphate and H_2O_2 was described as long ago as 1894 as being able to oxidise every organic compound tested. This mixture is now called Fenton's reagent, after its discoverer. The Fenton reaction is



A similar reaction occurs with copper ions.



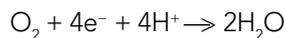
The damage caused to biomolecules (DNA, lipids, carbohydrates, proteins) by excessive ROS production (e.g. during exposure to ionising radiation, to toxins that generate ROS or in chronic inflammatory diseases (Section 'When inflammation goes awry') is often collectively called oxidative damage.

Why are ROS produced?

ROS are produced deliberately *in vivo*, e.g. during inflammation (Section 'Inflammation: The good and the bad of reactive oxygen species'). Some ROS, especially H_2O_2 , are generated to assist with signalling mechanisms that control the function, growth, division, and differentiation of cells. For example, H_2O_2 (at the correct levels) can stimulate the proliferation of several cell types, such as fibroblasts. Specific growth factor molecules bind to receptors on cell surfaces and trigger a cascade of biochemical reactions that influence cell behaviour and promote cell division. Prominent in this cascade is the phosphorylation of tyrosine or serine residues on proteins by kinase enzymes. However, cells also contain enzymes ("phosphatases") that remove these phosphate residues. When signalling starts, there is a transient rise in the cellular levels of H_2O_2 . This inactivates the phosphatases, increases net protein phosphorylation and enhances the signalling. There has to be careful coordination between H_2O_2 -generating and H_2O_2 -removing systems within the cell to allow this "burst" of H_2O_2 and make sure that H_2O_2 levels fall again when the signalling is complete. Careful regulation of signalling is essential – too much can lead to excessive cell proliferation and promote cancer development. Too little signalling and cells will not function properly, or organs

will not develop correctly. H_2O_2 levels also influence the behaviour of stem cells, helping to control their division, and to decide into which other cell types they differentiate.

In addition to this purposeful ROS production, some ROS appear to be produced "accidentally". We are continually exposed to background ionising radiation (e.g. cosmic rays, or radon) and so there is always a low level of OH^\bullet production *in vivo* by splitting of water. Many biomolecules are unstable in the presence of O_2 , undergoing chemical reaction with it to yield $\text{O}_2^{\bullet-}$. Examples are reduced folates, the hormones adrenalin and noradrenalin, and the neurotransmitter dopamine, but there are many others. Perhaps the most studied source of $\text{O}_2^{\bullet-}$ is the mitochondria. During normal mitochondrial functioning, electrons obtained by the oxidation of food-derived substrates are transferred to two cofactors, nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), reducing⁽¹⁾ them to give NADH and FADH_2 , respectively. These are re-oxidised back to NAD^+ or FAD by a chain of electron carriers (the "electron transport chain") in the inner mitochondrial membrane. A wide variety of electron carriers is present, including the iron-containing cytochrome proteins. After passing along this chain of carriers, the electrons are used to reduce O_2 to water by the enzyme cytochrome oxidase, a reaction that is the essence of respiration.



The energy released as electrons pass along the chain is used to make ATP.

Unfortunately, some of the electron carriers in the electron transport chain can themselves pass single electrons directly to O_2 , a process often referred to as "electron leakage", and $\text{O}_2^{\bullet-}$ is produced. Normally, over 98% of O_2 follows the correct path to produce water, but mitochondrial $\text{O}_2^{\bullet-}$ production occurs all the time and rises if more O_2 is

(1) Reduction is a gain of electrons. Food-derived substrates are oxidised, losing electrons to NAD^+ and FAD.

supplied (hence excess O_2 above the normal 21% is toxic to all aerobes, including humans) or if the mitochondria are damaged so that the electron carriers become disorganised and leak electrons to O_2 more readily. Excess mitochondrial $O_2^{\bullet-}$ formation occurs in diabetes (too much glucose leads to too much NADH and hence excess $O_2^{\bullet-}$) and contributes to the pathology of this disease. It also plays a role in cardiovascular and neurodegenerative diseases, among other conditions.

INFLAMMATION: THE GOOD AND THE BAD OF REACTIVE OXYGEN SPECIES

Acute inflammation

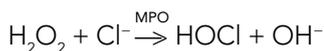
When body tissues are damaged by injury or infection, an acute inflammatory response occurs. Its typical characteristics have been known for centuries and include swelling, heat, pain and reddening. Blood flow to the inflamed area increases and the blood vessels increase their permeability. Some of the white blood cells normally present in the circulation begin to leave it and enter the inflamed tissue, attracted by a number of chemical signals ("chemotactic factors") generated in the damaged tissue. The body also makes more white cells, raising the numbers in the circulation and at the site of inflammation (Table 2, point 3). One chemotactic factor is H_2O_2 , generated in injured tissues in increased amounts, but there are many others. Lipids are broken down at the site of inflammation and the omega-6 polyunsaturated fatty acid (PUFA) arachidonic acid is released. It is converted by enzymes into prostaglandins and leukotrienes, families of compounds that contribute to the pain and swelling. Aspirin helps reduce the pain because it stops prostaglandin synthesis by inhibiting cyclooxygenase (COX), the first enzyme in the pathway. Lipoxygenases (LOX) are enzymes that initiate leukotriene synthesis.

An especially important type of white blood cell in early inflammation is the neutrophil, an amoeba-like cell with a multi-lobed nucleus. When a neutrophil that has entered the damaged tissue recognises injurious particles, such as bacteria or viruses, it engulfs them by a process termed phagocytosis, which takes them into the neutrophil cytoplasm in a vacuole. In the vacuole, they are exposed to a range of toxic agents that, hopefully,

kill these microorganisms. Among these toxins is $O_2^{\bullet-}$; when neutrophils respond to the presence of foreign material, they activate an enzyme system (NADPH oxidase) that oxidises NADPH (Fig. 1; mostly generated by a metabolic pathway called the pentose phosphate pathway) and uses the electrons to generate $O_2^{\bullet-}$.



The ingested organism is thus showered with excess $O_2^{\bullet-}$. Some of the $O_2^{\bullet-}$ is converted to H_2O_2 , which is also toxic to bacteria. In addition, the neutrophil enzyme myeloperoxidase (MPO) uses H_2O_2 to oxidise chloride (Cl^-) ion into the powerful antibacterial and antifungal agent hypochlorous acid (Table 1).



Many domestic bleaches are the sodium salt of hypochlorous acid. MPO has a green colour and its presence is thus easily recognised in infected tissues and body fluids.

Once the infecting organisms or other toxic agents have been destroyed, the neutrophils die, the inflammation resolves, tissue is remodelled and restored, and the body returns to normal. The acute inflammatory response is essential to human survival, otherwise we would die from overwhelming infections. In recent years, we have learned that NADPH oxidase enzymes are not unique to white blood cells; they are found (at much lower levels) in many other cell types. Some of them help to generate the H_2O_2 needed for signalling (Section 'Why are ROS produced?'), but their activity must be carefully controlled.

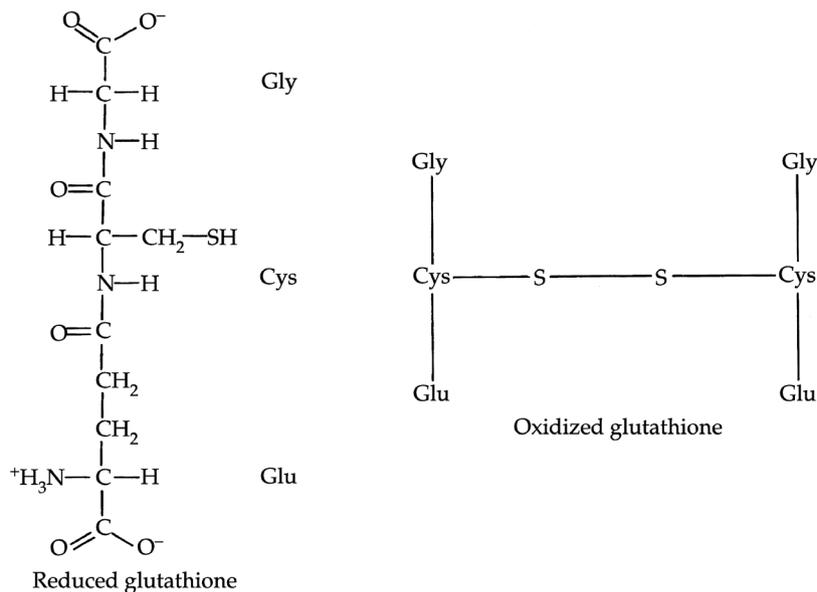
TABLE 1.
Nomenclature of reactive oxygen species (ROS);
a few examples

Free radical ROS	Non-radical ROS
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, OH^{\bullet}	Hypochlorous acid, HOCl
Lipid peroxy, lipid- OO^{\bullet}	Lipid hydroperoxide, lipid-OOH
Nitric oxide, NO^{\bullet}	Peroxynitrite, $ONOO^-$
Nitrogen dioxide, NO_2^{\bullet}	Nitrous acid, HNO_2

Atoms contain a nucleus with protons and neutrons, and electrons orbiting around the nucleus. In most atoms and molecules, all of the electrons are paired (i.e. in twos). A free radical is any atom or molecule with one or more unpaired electrons, orbiting by themselves. The presence of unpaired electrons increases the reactivity of free radicals, although different radicals show a range of reactivities. The superscript dot is the symbol used to denote a free radical.

Neutrophils have many mechanisms to kill infecting organisms, and the importance of ROS ($O_2^{\bullet-}$, H_2O_2 , HOCl) in killing varies with the infecting species. Nevertheless, unfortunate patients with inherited defects that render their neutrophils unable to generate $O_2^{\bullet-}$ (which also prevents H_2O_2 and HOCl generation) show a syndrome of persistent and multiple infections (chronic granulomatous disease) and need antibiotic prophylaxis. In addition, their inflammation often fails to subside as it normally should. Indeed, studies on animals show that 'normal' rates of ROS production by white blood cells are required not only for killing of certain infectious agents, but also for inflammation to resolve properly and for the tissue to return to normal. Products derived from the omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), such as the resolvins and protectins, also aid the resolution of inflammation.

FIGURE 1.
STRUCTURES OF REDUCED (GSH) AND OXIDISED (GSSG) GLUTATHIONE

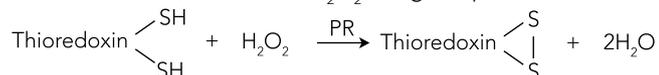


GSH is a tripeptide (glutamic acid-cysteine-glycine). In GSSG, two GSH molecules join together as the -SH groups of cysteine oxidise to form a disulphide bridge. The enzyme glutathione peroxidase removes H_2O_2 by the reaction:
 $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

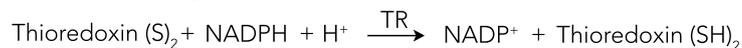
and GSH is then regenerated by glutathione reductase:
 $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

$NADP^+$ has a structure like NAD^+ and $NADPH$ like $NADH$, but $NADP^+$ and $NADPH$ have an extra phosphate group. In contrast, catalases degrade H_2O_2 directly to oxygen and water:
 $2H_2O_2 \rightarrow 2H_2O + O_2$

Peroxiredoxins (PR) remove H_2O_2 using the protein thioredoxin; two thiol groups on thioredoxin form a disulphide.



Thioredoxin (SH)₂ is then regenerated by thioredoxin reductase (TR).



When inflammation goes awry

The ROS and other toxins produced by neutrophils and other white cells during acute inflammation damage not only the invaders, but also the human tissues around them. This is why neutrophil NADPH oxidase is activated only when needed. Overall, however, the acute inflammatory response is beneficial – once the agents causing it have been dealt with, the inflammation subsides, biomolecules oxidised by the ROS in the tissues are repaired or replaced, and things return to normal.

However, anything causing excessive and/or prolonged activation of white blood cells has the potential to cause serious damage. In some diseases (autoimmune diseases), the body generates antibodies (“autoantibodies”) against constituents of its own tissues. Normally, antibodies are generated only in response to foreign organisms, to which they bind and facilitate attack by neutrophils and other cells of the immune system. This is the good side of antibodies. However, when autoantibodies bind to human tissues, they provoke persistent attack by white blood cells. This is bad, and can lead to serious tissue damage, to which ROS contribute. Chronic inflammation, and the accompanying severe tissue damage by ROS, are involved in many diseases. Let us look at some examples.

ROS and cancer: a complex relationship

Perhaps the most serious effect of chronic inflammation is an increased risk of cancer. For example, chronic inflammation of the liver, often caused by infection with viruses that resist attack by the immune system (e.g. hepatitis C), increases the risk of developing liver cancer. Chronic inflammatory diseases of the large intestine (e.g. ulcerative colitis) increase the risk of colorectal cancer. Cigarette smoke is a rich source of free radicals; this is one reason why smoking predisposes to cancer development.

ROS such as OH^{\bullet} damage DNA bases and create mutagenic lesions such as 8OHG. In the chronic inflammatory diseases, repair cannot keep up, 8OHG levels (and the levels of other mutagenic products resulting from DNA base damage by ROS) rise and mutation results, often activating oncogenes as well as inactivating tumour suppressor genes such as p53. ROS can also promote abnormal cell proliferation by causing excessive signalling (Section ‘Why are ROS produced?’). One important question is, given these roles of ROS, why have antioxidant supplements usually been ineffective in slowing cancer development in human intervention trials? This is explored in the Section ‘Biomarkers of oxidative damage’.

Atherosclerosis and dementia

Hepatitis and inflammatory bowel disease are examples of prolonged high-level inflammation. In recent years, we have realized that a lower level of chronic inflammation plays a key role in causing tissue damage in many diseases such as diabetes and atherosclerosis. Atherosclerosis can be described as a chronic low-level inflammation of blood vessel walls, leading to damage by ROS, especially to LDL in the vessel wall. In Alzheimer disease (AD), lumps of toxic proteins (plaques and tangles) accumulate in the brain and cause neuronal dysfunction. This leads to inappropriate activation of phagocytes found in the brain, the microglial cells. Normally microglia protect and assist neurons, but chronic and persistent microglial activation seems to aggravate brain damage in AD. Indeed, the affected brain regions of AD patients show markedly elevated levels of oxidative damage to proteins, lipids, DNA and RNA. Experiments on animals, and some human data, suggest that this damage precedes neuronal death, so preventing it by antioxidant agents that can cross the blood–brain barrier represents a therapeutic opportunity.

Obesity

Obesity is a risk factor for multiple conditions, most notably metabolic syndrome and type 2 diabetes. It is also associated with increased oxidative damage to biomolecules. Part of the reason seems to be that white adipose tissue, previously thought of as an inert storage depot for triglyceride, is in fact active in producing cytokines, chemical signals that can trigger inflammation and consequent ROS production (Table 2). If there is too much adipose tissue, too many cytokines can be produced. When they enter the circulation, they can promote inflammation and oxidative damage in other body tissues. In other words, obesity can result in a chronic low-grade inflammation. If metabolic syndrome and type 2 diabetes develop, provoked by obesity, the inflammation worsens.

Detecting inflammation

Inflammation is accompanied by a series of metabolic changes. First, there is increased production of cytokines, as mentioned in the Section 'Obesity'. Cytokine is a broad term that encompasses a wide range of molecules generated transiently and affecting several cell types. They play key roles in normal growth and development, inflammation, and tissue remodelling and repair after injury (e.g. wound healing). Some cytokines promote inflammation (e.g. Interleukin-1 (IL-1), Interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α); Table 2), whereas others seem to diminish it (e.g. Interleukin-10 (IL-10)). Cytokines are intimately related to ROS production: the production of pro-inflammatory cytokines by neutrophils and several other cell types is stimulated by ROS and the action of these cytokines on cells can in turn increase ROS production. The anti-inflammatory cytokine IL-10, in contrast, tends to decrease ROS levels.

TABLE 2.
Some biomarkers of inflammation

1. Plasma or serum levels of pro-inflammatory cytokines

Interleukin-6 (IL-6)

Tumour necrosis factor alpha (TNF- α)

Interleukin-1 β (IL-1 β)

Interferon- γ (IFN- γ)

2. Plasma or serum levels of acute-phase proteins

C-reactive protein (can rise 1000-fold or more during inflammation; levels low in healthy subjects)

Antiproteinases (proteins that slow down protein hydrolysis: usually only 2- to 4-fold changes)

Serum amyloid A protein (several hundred-fold rise)

Caeruloplasmin, haemopexin, haptoglobin (proteins with antioxidant properties; rises modest, ~50% to 400%)

3. Increased white cell numbers (referred to as "count") in the bloodstream

More neutrophils are made by the bone marrow and enter the circulation

Another consequence of inflammation is the acute phase response, a "whole body" response to inflammation that includes fatigue, breakdown of muscle proteins, and fever (caused by elevated levels of IL-6 and some other cytokines). In the acute phase response, the liver increases synthesis of several proteins, the acute-phase proteins. C-reactive protein (CRP) is the acute-phase protein showing the biggest change (Table 2).

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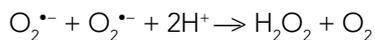
Measurement of pro-inflammatory cytokines and acute phase proteins is frequently carried out to detect inflammation. For the major chronic inflammatory diseases (hepatitis, ulcerative colitis, etc.), levels of all of them are increased. For chronic low-grade inflammation, as in obesity, it is hard to detect changes in many acute phase proteins, but CRP elevations are readily detectable since its fold change is so great (Table 2). Increases in IL-6, IL-1 β and TNF- α are also detectable, but often modest, in low-grade inflammation. The same is true of white blood cell count, clearly elevated in diseases such as hepatitis and inflammatory bowel disease, but sometimes the elevation is scarcely detectable in chronic low-grade inflammations such as atherosclerosis or obesity. Hence, to date, elevated CRP seems to be the "best" indicator of ongoing inflammation, although its high sensitivity can give rise to false positives (e.g. a minor infection on the day of measurement can lead to elevated values).

ENDOGENOUS AND EXOGENOUS ANTIOXIDANTS

Endogenous antioxidants

Humans have evolved to produce a wide range of antioxidants. They act in different ways and in different tissues, cells and subcellular compartments to minimise the damaging effects of ROS such as $O_2^{\bullet-}$ or OH^{\bullet} , whilst allowing some ROS to play useful roles. Since not all ROS are removed, some oxidative damage is inevitable, and the damaged molecules are repaired or replaced. DNA repair is particularly important, and multiple enzymes have evolved to remove lesions such as 8OHG from DNA, hopefully before the DNA replicates and the 8OHG causes mutations. Failures of repair, as a result of inherited defects in repair enzymes or the fact that repair becomes less efficient as we age, can contribute to the development of several diseases, especially cancer, because the mutagenic 8OHG is not removed as quickly as it should be. Free radicals may even play a role in the ageing process itself, although the role of ROS in ageing is a fiercely controversial area!

The first line of antioxidant defence in the human body, especially important in mitochondria, is the superoxide dismutase (SOD) enzymes, which convert $O_2^{\bullet-}$ into H_2O_2 .



Different types of SOD enzymes exist; one contains copper and zinc (CuZnSOD) and another manganese (MnSOD), all minerals essential in the human diet. MnSOD is located inside mitochondria and plays a key role in protecting them. Defects in mitochondrial MnSOD activity in animals cause severe mitochondrial damage and early death.

The H_2O_2 generated by SOD and by other enzymes (e.g. during signal transduction) is removed by a plethora of enzymes, including catalases and glutathione peroxidases. The most important peroxide removal mechanism *in vivo* is a complex group of enzymes called peroxiredoxins, whose activity is carefully controlled. Decreased activity of peroxiredoxins, achieved by a variety of mechanisms, allows H_2O_2 to accumulate during signal transduction, and restoration of their activity combined with the action of catalases and glutathione peroxidases rapidly decreases H_2O_2 levels to their normal low value when signalling is finished. Correct operation of the peroxiredoxin and glutathione peroxidase systems requires the element selenium from the diet (Table 3). It also requires a supply of the cofactor NADPH (Fig. 1), largely generated by the pentose phosphate pathway (Table 3).

Animal cells are rich in the tripeptide reduced glutathione (GSH) (Fig. 1). It acts as a cofactor for several enzymes, including the glutathione peroxidases that help remove H_2O_2 , as well as the glutathione transferases that work alongside cytochromes P450 to convert xenobiotics to safer products. GSH can directly scavenge several ROS (e.g. OH^{\bullet}) and helps to maintain the correct cellular redox state. Low levels of cysteine in the diet can impair the synthesis of GSH (Table 3).

The role of iron

Iron, like O_2 , is a Janus-faced molecule: essential (e.g. in catalase, in cytochromes P450, in haemoglobin to transport O_2 around the body, and in the cytochromes in the mitochondrial electron transport chains) but potentially damaging because it can catalyse free radical reactions such as Fenton chemistry. As a result, the human body handles it carefully, aiming to keep the amount of free Fe^{2+} as low as possible ("iron sequestration"). Thus, iron absorbed from the diet is carried from the

intestine to the organs that require it by the plasma protein transferrin. Iron-requiring cells unload iron from transferrin, and store any iron that is not immediately required in the protein ferritin. Neither ferritin nor transferrin is efficient in decomposing H_2O_2 to OH^\bullet . In iron overload diseases such as haemochromatosis and thalassaemia, iron ions are no longer fully sequestered, and iron-dependent free radical processes contribute to the resulting tissue injury. Copper is also essential in the

human body, and is similarly carefully handled to limit its pro-oxidant activity.

The severe consequences of losing MnSOD illustrate that too much $O_2^{\bullet-}$ can be toxic (Section 'Endogenous antioxidants'). Why is this? Many mitochondrial proteins contain iron, and $O_2^{\bullet-}$ can react with some of them to release iron from the proteins; this inactivates the proteins and, in addition, the released iron ions can react with H_2O_2 to form OH^\bullet .

TABLE 3.
How diet contributes to antioxidant defence

Type of link	Constituent	Role played
1) Direct antioxidant action <i>in vivo</i>	"Antioxidant nutrients"	Vitamin E, probably vitamin C. Vitamin C also has specific roles as a cofactor for enzymes. Possibly certain carotenoids, polyphenols (including flavonoids) and monophenols (e.g. caffeic or chlorogenic acids) under certain circumstances
2) Agents indirectly influencing antioxidant defences/oxidative damage	Fe	Constituent of catalase, cytochromes, haemoglobin. Both Fe deficiency (which can increase electron leakage from the mitochondrial electron transport chain) and Fe overload (e.g. by promoting Fenton chemistry) can promote oxidative damage. Careful control of "free" iron levels in the body is necessary
	Mn	Constituent of MnSOD
	Cu	Constituent of CuZnSOD. Cu is also needed for normal iron metabolism. Both Cu deficiency and overload cause oxidative damage, each involving increased mitochondrial ROS production
	Zn	Constituent of CuZnSOD. Can sometimes antagonise the pro-oxidant actions of iron by displacing it from sites where it is promoting oxidative damage (e.g. in atherosclerotic lesions)
	Magnesium	Cofactor for multiple enzymes (including some enzymes that supply cofactors for antioxidant defence, e.g. in the pentose phosphate pathway which supplies NADPH for the function of the glutathione and peroxiredoxin systems (Fig. 1)). Magnesium deficiency in animals causes inflammation and increased oxidative damage
	Amino acids/proteins	Adequate dietary intake needed for synthesis of GSH (glutamate, cysteine, glycine) and antioxidant defence enzymes, metal binding proteins, albumin (can scavenge ROS in blood). Human studies show that low protein intake decreases tissue GSH levels

TABLE 3.
How diet contributes to antioxidant defence (continued)

Type of link	Constituent	Role played
2) Agents indirectly influencing antioxidant defences/oxidative damage	Riboflavin (e.g. via flavin, adenine dinucleotide, FAD)	Glutathione reductase (has FAD cofactor) helps maintain GSH levels. FAD is essential for correct mitochondrial electron transport and fatty acid oxidation
	Thiamine	Used in several enzymes, including transketolase, an enzyme in the pentose phosphate pathway. Thiamine deficiency in animals causes neuronal and cardiac damage accompanied by oxidative damage
	Selenium	Cofactor for glutathione peroxidase and thioredoxin reductase enzymes, also needed for some of the methionine sulphoxide reductase enzymes that help repair oxidatively damaged proteins. Too much selenium is deleterious
	Nicotinamide	Used to make NAD ⁺ , NADP ⁺ , NADPH, NADH: energy metabolism, defence against infection (e.g. by NADPH oxidases), needed for repair of DNA, cofactor of glutathione and thioredoxin reductases
	Folic acid	Minimises levels of plasma homocysteine; high homocysteine is a risk factor for cardiovascular disease that may act by increased ROS generation
	Vitamin D	Deficiency seems associated with increased oxidative damage in animals: mechanism unclear
	Polyunsaturated fatty acids	Appear to minimise oxidative damage <i>in vivo</i> (see Table 7)
	Inducers of antioxidant defences	Agents that increase expression of genes encoding antioxidant and/or other defence systems. Examples are sulphoraphane, resveratrol, oltipraz, β -phenethyl isothiocyanate and curcumin
	Ethanol	Low levels may be cardioprotective, possibly not by antioxidant action. High intakes are deleterious to many body tissues, and oxidative damage is involved
	Total caloric intake	Eating only the recommended amount of calories can diminish the risk of obesity. Decreasing obesity decreases ROS production

CuZnSOD, copper, zinc-containing superoxide dismutase; FAD, flavin adenine dinucleotide; GSH, glutathione (reduced form); MnSOD, manganese-containing superoxide dismutase; NAD⁺, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP⁺, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

Regulation of the levels of endogenous antioxidants

When exposed to higher levels of ROS than normal, cells often respond by raising their antioxidant defence levels, especially that of MnSOD. For example, pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF- α) can increase ROS production in cells, but activities of MnSOD usually also rise to maintain a ROS/antioxidant balance. In another example, lipid peroxides can break down to aldehydes such as hydroxynonenal (HNE). HNE can damage DNA and proteins, but can also induce antioxidant defences. This is because HNE acts upon a protein in the cytoplasm known as Nrf2. The resulting activation of Nrf2 leads to increases in the cellular levels of certain antioxidants, especially glutathione (GSH), glutathione S-transferase enzymes, glutathione peroxidases, peroxiredoxins and other protective systems. GSH, among its other protective effects, can bind to HNE and safely remove it. Several dietary constituents, such as sulphoraphane, oltipraz, β -phenethyl isocyanate and curcumin can also lead to activation of Nrf2. Ironically therefore, pro-oxidants and dietary xenobiotics, can, by being perceived by the body via Nrf2 as mild toxins, raise defence levels that can protect against greater insults.

Another example of the same thing is some of the health beneficial effects of exercise. Modest exercise raises O₂ consumption, and ROS production, in skeletal muscle. Some of these ROS act on Nrf2 to increase antioxidant defences, which leads to an increased exercise capacity. However, if exercise is too strenuous (e.g. if a sedentary individual runs a marathon), the excessive ROS production can do more harm than good and contributes to muscle damage.

Diet-derived antioxidants

There has been much discussion over the role of antioxidants in the human diet in maintaining health, with special focus on vitamins C and E, carotenoids, as well as various phenols such as chlorogenic acids and flavonoids. One point worth making is that many dietary constituents contribute indirectly to antioxidant defence, and without them, the antioxidants cannot function optimally. Table 3 summarises several examples of this. Hence, one cannot overemphasise the importance of a balanced diet with all of the key nutrients in adequate, but not excessive, amounts. For example, high doses of antioxidant supplements are unlikely to negate the deleterious effects of malnutrition (e.g. if there is insufficient dietary protein; Table 3).

It is widely believed that diets rich in fruits and vegetables play a key role in the prevention of human disease, hence the "five a day"-type campaigns in many countries. Indeed, a recent article by Boeing *et al.* (see the Further Reading list) concluded that, for hypertension, coronary heart disease, and stroke, there is good evidence that more vegetables and fruits in the diet reduce the risk of developing these conditions. They further concluded that there is probable evidence that the risk of developing cancer in general (some specific cancers more than others) is inversely associated with the consumption of vegetables and fruits, and that there is reasonable evidence that increasing the consumption of vegetables and fruit lowers the risk of developing certain eye diseases, dementia and the risk of osteoporosis. Likewise, current data on asthma, chronic obstructive pulmonary disease, and rheumatoid arthritis indicate that an increase in vegetable and fruit consumption may help to decrease the risk of developing these diseases. In addition, diets rich in vegetables and fruit are often lower in calories and help to avoid obesity and its sequelae, including low-grade chronic inflammation, thus indirectly reducing the incidence of metabolic syndrome and

type 2 diabetes. Plants, of course, are rich in antioxidants and supply most of those present in the human diet. How much, then, of these beneficial effects of a plant-rich diet is due to the antioxidants present? Before answering this question, let us look at which antioxidants occur in plants.

Vitamin C (ascorbate) is essential *in vivo* as a cofactor for the action of several enzymes and lack of it causes the deficiency disease scurvy. It is also able to directly scavenge several ROS, and this role may be especially important in the respiratory tract, where it helps to absorb inhaled, powerfully oxidising ROS such as ozone and nitrogen dioxide that frequently pollute the air we breathe (Table 7). It also scavenges many of the free radicals in cigarette smoke, and so smokers use up ascorbate faster than non-smokers. Epidemiological studies show that low plasma levels of vitamin C are associated with increased risk of several diseases such as cancer and stroke, but there is little evidence from intervention studies that high doses of vitamin C are beneficial in preventing the onset of chronic diseases such as cancer or heart disease. Vitamin C *in vitro* easily oxidises to generate H_2O_2 and can reduce Fe^{3+} to Fe^{2+} and accelerate production by the Fenton reaction, by converting the Fe^{3+} product back to Fe^{2+} to react further with H_2O_2 . These “pro-oxidant” effects of vitamin C might be important in iron overload disease, but there is little evidence that they are important in the healthy human body.

RRR-α-Tocopherol, the most important (to humans) of the eight compounds that constitute vitamin E, is also essential *in vivo*; prolonged deprivation of it causes damage to several tissues, especially the brain and nervous system. Despite much debate as to possible alternative roles, the only established function of α -tocopherol *in vivo* seems to be as an inhibitor of lipid peroxidation: it is present in cell membranes and plasma lipoproteins, and intercepts some of the intermediate lipid peroxy radicals (Table 1), so slowing down the rate of lipid peroxidation. The optimal levels of vitamin E in the body are uncertain; fairly low intakes (the current

recommended daily allowance (RDA) or below) may be sufficient to give maximal antioxidant protection. Intervention trials with high doses of α -tocopherol have shown little or no protective effect against the development of cancer, diabetes, heart disease or mild cognitive impairment in humans, but there seems to be a modest protective effect against cognitive decline in patients with Alzheimer disease.

Carotenoids are widespread in diet and convey beautiful colours to our food, but their only established role in humans is that some of them, especially β -carotene, act as precursors of vitamin A. Nevertheless, other roles seem likely, as is suggested by the accumulation of the carotenoid lycopene in the prostate gland, and of lutein and zeaxanthin in the eye. However, there is no compelling evidence that carotenoids are essential to humans, and epidemiological studies of the effects of supplements containing them on macular degeneration or prostate cancer are inconclusive. Carotenoids can scavenge several ROS *in vitro*, but their antioxidant role *in vivo* (except perhaps in the eye and skin, where they can help to scavenge ROS produced by excessive light exposure) is probably minimal.

Foods and beverages (especially fruits, vegetables, grains, coffee, teas, and wine) have considerable antioxidant activity *in vitro*, as revealed by a series of “test tube” assays of total antioxidant activity such as oxygen radical absorbance capacity (ORAC), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS), etc. In these assays, an artificial radical is generated in the laboratory and the ability of the foodstuff to scavenge it is measured. However, the use of “biomarkers” (Section ‘The ROS-inflammation nexus: Biomarkers of oxidative damage’) to measure oxidative damage in humans has shown that most of these *in vitro* “antioxidant activities” do not translate into *in vivo* efficacy, i.e. the use of these test tube assays to “rank” foods according to their antioxidant levels may lead to conclusions that are biologically irrelevant.

In many plant-derived foods and beverages, the *in vitro* antioxidant activity is largely due to phenols, compounds containing an –OH group attached to a benzene ring. There are many types, including monophenols (one –OH group) such as caffeic acid (e.g. in coffee), and polyphenols (several –OH groups) such as the flavonoids (e.g. in wine and tea). The phenolic –OH group is a good scavenger of several ROS – indeed, that is how α -tocopherol (a monophenol) works. The tocopherol –OH group scavenges intermediate lipid peroxyl radicals (Table 1) and thus slows down lipid peroxidation. If there is more than one –OH group present on the aromatic rings, the compound can absorb more radicals. Hence, flavonoids (polyphenols) show up better than vitamin E or caffeic acid (both monophenols) in test tube assays of antioxidant activity. However, when humans consume polyphenols in the diet, they are not well absorbed, and those that are absorbed are rapidly metabolised, so that the levels present in the blood are low. In fact, the evidence that flavonoids or other phenols exert significant ROS-scavenging antioxidant effects *in vivo* is limited. Some evidence exists that high intakes of polyphenols (e.g. in cocoa, chocolate or tea) can produce improvements in vascular function in humans, but these may be exerted by mechanisms other than ROS scavenging. Some polyphenols or polyphenol-rich foods have been claimed to exert anti-inflammatory effects, but again, the evidence for this is limited.

Like ascorbate, polyphenols can readily oxidise to generate ROS. Indeed, some reports of the beneficial effects of phenols upon cells in culture have not been because of direct antioxidant effects, but rather the opposite. The phenols oxidise in the cell culture medium, producing oxidation products that activate the Nrf2 system and raise cellular GSH levels and other antioxidant defences.

The special case of the gastrointestinal tract

The stomach and intestinal contents contain a complex mixture of antioxidants derived from the diet, plus pro-oxidants, e.g. iron and copper ions, haem proteins (meat is an especially good source) and H_2O_2 . Indeed, some researchers have referred to the stomach as a “bioreactor”. It is possible that high levels of ascorbate, vitamin E, carotenoids and polyphenols can exert beneficial effects, antioxidant or otherwise, in the stomach, small intestine and colon, since absorption of vitamin E, carotenoids and flavonoids in the small intestine is rarely complete and thus some of them end up in the colon, and many are extensively metabolised by colonic bacteria. However, data on the effects of antioxidant supplements on the incidence of gastric or colorectal cancer in intervention trials are not supportive of benefit.

TABLE 4.
Criteria for an "ideal" biomarker

<p>A. Fundamental criterion (applicable both to biomarkers of inflammation and of oxidative damage)</p> <p>The biomarker predicts the later development of bodily dysfunction or overt disease</p>
<p>B. Technical criteria (applicable to both types of biomarker)</p> <ul style="list-style-type: none"> i. It must employ validated measurement technology, preferably simple to use (if achievable) ii. The result of the assay must be accurate, e.g. give the correct answer for the amount of biomarker present iii. The precision ("reproducibility") of the assay should be high iv. It must not be confounded by diet or other physiological factors v. It should be stable on storage, not being lost, or formed artefactually, in stored samples vi. For human use, it is preferable if it can be measured in easily obtainable samples, e.g. blood, urine, saliva, skin biopsy
<p>C. Technical criterion (applicable to biomarkers of oxidative damage)</p> <ul style="list-style-type: none"> i. The biomarker should measure a major part, or at least a fixed percentage, of total oxidative damage to the target molecule <i>in vivo</i>

THE ROS-INFLAMMATION NEXUS: BIOMARKERS OF OXIDATIVE DAMAGE

Inflammation is accompanied by increased ROS production from white cells and other sources. This is normally beneficial: ROS kill invading organisms and help to regulate the inflammatory process. In chronic inflammation, however, too many ROS are produced and cause extensive oxidative damage to tissues. Thus, inflammation is accompanied by increased levels of oxidative damage. Anti-inflammatory agents (e.g. steroids such as prednisolone) can act as "indirect antioxidants": if there is less inflammation, less ROS will be made. In the limited range of studies that have been done, correlations have been observed between levels of oxidative stress biomarkers and those of inflammation, as might be expected.

Biomarkers of oxidative damage

ROS are amazingly difficult to measure accurately *in vivo*, so scientists usually prefer to measure the damage that they cause to biomolecules. Many so-called "biomarkers of oxidative damage" have been described, but few can be trusted! Table 4 sets out the criteria they need to meet (which are also applicable to biomarkers of inflammation) and Table 5 gives some examples. The best oxidative damage biomarkers require sophisticated techniques for measurement, such as mass spectrometry. For lipid peroxidation, the best-validated biomarkers are the levels of F₂-isoprostanes in tissues, plasma and urine as measured by mass spectrometric methods (Table 5). For nucleic acid damage, the levels of 8-hydroxy-2'-deoxyguanosine (8OHdG) in urine seem to reflect whole-body oxidative damage to DNA and its precursors. For example, plasma or urinary F₂-isoprostane levels and excretion of 8OHdG are increased in subjects with iron overload, type 2 diabetes, obesity, other chronic inflammatory diseases, and in cigarette smokers.

TABLE 5.
Biomarkers of oxidative damage

Fairly robust (for human use)

- F₂-isoprostanes and other isoprostanes in tissues and body fluids (lipid peroxidation)*
- 8-Hydroxy-2'-deoxyguanosine (8OHdG) in urine (damage to DNA and its precursors)^Δ

Usable with cautious interpretation (e.g. controlling for possible effects of absorption of the products from the diet)

- Cholesterol oxidation products (COPs) / fatty acid hydroperoxides/hydroxides (lipid peroxidation)
- Lipid peroxide assays (lipid peroxidation)
- DNA base oxidation in DNA isolated from cells (with care taken to minimise artefactual oxidation of DNA during its isolation) or within whole cells, e.g. as measured by the comet assay with cleavage enzymes[◦]
- Direct chemical determination of malondialdehyde, other aldehydes (e.g. 4-hydroxynonenal) and their reaction products with proteins and DNA
- HPLC-based thiobarbituric acid (TBA) tests

To be avoided as results are often misleading

- Simple thiobarbituric acid (TBA) assays
- Total antioxidant capacity determination
- Many commercial kit methods for biomarkers

* Mass spectrometric methodology only.

Δ More research is needed on the origins of 8OHdG; how much from DNA and how much from the precursors of DNA and whether their relative contributions vary with age or disease.

◦ The comet assay measures strand breaks, which need not be due to oxidative DNA damage. Combining the assay with nuclease enzymes that act on oxidised DNA bases gives more useful results.

This table is adapted from Halliwell, 2011.

The Guidance on the scientific requirements for health claims related to antioxidants, oxidative damage and cardiovascular health (EFSA, 2011) contains a good discussion on the use of biomarkers in assessing health claims for antioxidants.

One obvious problem with plasma and urinary levels of oxidative damage products is that one cannot say which tissues they came from; they are an “average” of whole body oxidative damage. One technique widely used to measure DNA damage in isolated cells (e.g. human white blood cells) is the comet assay, which seems fairly reliable (Table 5).

The epidemiology of antioxidants

Many epidemiological studies have examined antioxidants. Let us begin by looking at some of the early ones that suggested the importance of antioxidants as disease-preventing agents. There are marked differences in cardiovascular disease incidence in different European countries: in general, the incidence is higher in the north (e.g. Finland and Scotland) and lower in the south (e.g. Southern Italy). If populations are standardised to correct for known risk factors (e.g. plasma cholesterol, smoking), there is a moderately strong inverse correlation between coronary events and lipid-standardised plasma α -tocopherol levels. Data from this and other studies allowed calculation of plasma antioxidant levels apparently associated with low risk of cardiovascular disease (Table 6). Similarly, the Zutphen elderly study examined risk factors for chronic disease in elderly men and found a decreased risk of mortality from heart disease in subjects with high intakes of certain flavonoids (Table 6). Later studies showed similar correlations. However, as explained earlier, intervention trials with high doses of ascorbate, α -tocopherol, β -carotene or other carotenoids, alone or in combination, have not been effective in preventing disease in humans, at least in the populations studied. There are several reasons for this.

One of the issues, which in fact led to the intervention trials, is that correlation does not prove a cause-consequence relationship. Diets rich in vegetables and fruits (such as the “Mediterranean diet”, also rich in fish and wines) are associated with lower incidences of cardiovascular diseases,

diabetes, stroke and certain types of cancer. β -Carotene, ascorbate, monophenols (including α -tocopherol) and polyphenols are common constituents of plants. Thus, eating more plants raises the antioxidant levels in the body and, at the same time, is associated with a lower risk of developing diseases. The two are not necessarily connected, i.e. higher antioxidant levels correlate with lower disease rates but should not be assumed to cause them.

Indeed, plants contain a huge range of agents that could potentially protect against disease. Some, such as sulphoraphane and curcumin (Section 'Regulation of the levels of endogenous antioxidants'), act as mild toxins that raise the level of antioxidant and other defences *in vivo*.

A fruit- and vegetable-rich diet is often low in saturated fat and *trans*-fat and richer in monounsaturated and polyunsaturated fats. PUFAs may have anti-inflammatory effects and can decrease plasma cholesterol levels in some subjects, ameliorating a risk factor (high plasma cholesterol) for cardiovascular and Alzheimer diseases. Fruits and vegetables are good sources of folic acid (Table 3), which helps to bring down levels of the amino acid homocysteine in the circulation. Homocysteine levels, if too high, damage the vascular system (probably by increasing ROS production) and are associated with increased risk of stroke and heart disease.

TABLE 6.
Relationship between risk of coronary heart disease (CHD) and plasma concentrations/intakes of certain antioxidants

Antioxidant	Concentration (μ M) associated with		
	Moderate risk of CHD ^a	Small risk of CHD ^a	
Carotene (mostly β -carotene)	<0.3	>0.4–0.6	
α -Tocopherol	<24	>27–28	
α -Tocopherol/cholesterol ratio (μ mol/mmol)	<4.1	>4.8–5.6	
Ascorbate	<24	>35–60	
	Daily flavonol intake (mg) ^b		
	0–19	19.1–29.9	>29.9
Mortality from CHD (per 1000 person years)			
Relative risk after correction for intake of total energy, saturated fats, blood pressure, physical activity, weight, smoking, total and HDL cholesterol	20.4 1.00	14.5 0.58	9.9 0.47

^aModerate risk of CHD, >250 deaths/10⁶; small risk, <130 deaths/10⁶. Data are taken from Gey, 1994 and Gey, 1995 and are based on a range of studies. Similarly, in elderly subjects (70–75 years) there was an inverse correlation between plasma α - plus β -carotene and mortality from cancer and cardiovascular disease (Buijsse et al. 2005).

^bFrom Hertog, 1997.

Antioxidant supplements: reasons for failure

As mentioned above, studies directly testing high-dose supplements of vitamin C, α -tocopherol, β -carotene or other carotenoids as agents to prevent human disease have, in general, yielded inconclusive or negative results. The logic behind such studies was that oxidative damage contributes significantly to disease development – the balance of the evidence suggests that this is probably true for neurodegenerative diseases (especially AD) and several cancers (especially those related to chronic inflammation), and may be true for atherosclerosis. Hence, if oxidative damage is lowered, less disease should occur. The assumption made in these studies was that the administered antioxidants would decrease oxidative damage levels. However, short-term studies administering high levels of antioxidants to humans reveal little or no effects on oxidative damage levels in people on adequate diets, as measured by reliable biomarkers (Table 5). Extrapolating this to the intervention studies, if the administered antioxidants did not alter oxidative damage levels, the prediction would be that disease risk would not change. As a specific example, if oxidative damage to DNA was not decreased by the “antioxidants” consumed, then no protective effect against cancer would be achieved, exactly as observed.

Some studies have suggested harm from high doses of vitamin E. If true, the harm does not appear to be great and it may involve other properties of α -tocopherol at high levels, e.g. its ability to impair blood coagulation by antagonising the action of vitamin K, perhaps raising the risk of haemorrhagic stroke. Another suggestion is that high-dose antioxidants interfere with the physiological functions of ROS, e.g. signalling and defence. Consistent with this, there is some evidence that antioxidant supplements can diminish the beneficial effects of exercise in humans.

Levels of biomarkers of oxidative damage vary widely among healthy individuals – perhaps it is only those with higher levels (genetics is one factor affecting this) who would benefit from extra antioxidants, just as only those with elevated blood pressure are likely to benefit from anti-hypertensive medication. Few studies have addressed this issue, and more work needs to be done to examine it.

Lifestyle aspects

If antioxidant supplements do not affect oxidative damage levels in humans, then what does? Many lifestyle aspects do, and Table 7 attempts to summarize our current knowledge. In several studies, obesity, hypercholesterolaemia, hyperglycaemia and hypertension have been found to be associated with increased risk of disease, and they also associate with increased oxidative damage and inflammation; indeed, inflammation may contribute a significant part of the oxidative stress measured in these diseases. Thus, if levels of F_2 -isoprostanes are high, losing weight and controlling plasma LDL cholesterol levels by exercising lifestyle choices may bring them down more readily than consuming α -tocopherol supplements (Table 7).

Do fruits and vegetables decrease the risk of disease development by lowering oxidative damage?

Both genetics and dietary patterns seem to influence F_2 -isoprostane levels in human plasma: high fruit/vegetable diets are associated with lower levels and meat-rich diets with higher ones. Does changing diet affect the levels? The evidence that it can, at least in the short term, is not compelling: while some studies have shown that antioxidant-rich foods can lower oxidative damage levels *in vivo* (e.g. tomato, soy sauce: Table 7), many have not or have given mixed messages.

For example, giving smokers 250 g of blueberries each day for 3 weeks decreased plasma lipid peroxides but not F₂-isoprostanes, and a “Mediterranean diet” did not decrease urinary F₂-isopropane excretion in healthy humans. Tomato paste decreased some biomarkers of oxidative damage but not others, in human volunteers.

Effects of long-term dietary changes have not been studied systematically, an area which needs further investigation.

TABLE 7.
If antioxidants cannot affect oxidative damage in humans then what does?

Parameter influencing level of oxidative damage	Suggested strategy
Obesity	Lose weight
Hyperglycaemia	Control blood glucose levels
Hypertension	Control blood pressure
High plasma LDL cholesterol	Control LDL cholesterol
Body iron levels	Avoid iron supplements, except when clinically necessary to treat anaemia. Donate blood regularly
Certain foods (e.g. dark soy sauce, various tomato products, e.g. tomato paste)*	Eat diet rich in fruits and vegetables
Intake of omega-3 polyunsaturated fatty acids (PUFAs) [^] (docosahexaenoic acid, possibly eicosapentaenoic acid)	Eat fish regularly
Excess alcohol intake	Drink less
Smoking	Don't smoke
Excessive exposure to sunlight	Avoid
Some environmental toxins (e.g. ozone, nitrogen dioxide, arsenic)	Attempt to avoid
Lack of exercise	Engage in regular exercise

* It is essential to include appropriate controls in testing the effects of foods, because the consumption of any food (antioxidant or not) can sometimes alter levels of certain biomarkers.

[^] Despite the propensity of omega-3 PUFAs to oxidise *in vitro*, growing evidence suggests that they minimise oxidative damage *in vivo*. This table is adapted from Halliwell, 2011, Halliwell, 2012 and Halliwell, 2013.

NUTRITION AND INFLAMMATION

There is some evidence for anti-inflammatory effects of specific nutrients in humans. For example, reasonable dietary intakes of omega-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in foods seem to have health benefits. Ironically, PUFAs *in vitro* are very susceptible to attack by ROS and oxidise readily (which generates a problem for the food industry in protecting them in cooking oils, baby foods, etc. to prevent their oxidation). *In vivo* however, they appear to decrease oxidative damage (Table 7). Exactly how is uncertain, but they may act to modestly decrease ROS and/or cytokine production by white blood cells, and they are precursors of the resolvins and defensins, which are anti-inflammatory agents; less inflammation usually means less ROS production. It is even possible that their oxidation products act as mild toxins and increase defences, by the Nrf2 system. The prostaglandins derived from EPA seem to be less pro-inflammatory than those from arachidonic acid. In contrast, consumption of high levels of *trans*-fats appears to raise levels of inflammatory markers such as CRP and IL-6.

Several other dietary constituents (e.g. some polyphenols) may be anti-inflammatory. Flavonoids are weak inhibitors of cyclooxygenase and lipoxygenase enzymes (Section 'Acute inflammation') and may be able to exert such effects in the gastrointestinal tract, although the levels present in the bloodstream are probably too low to be effective. It is possible that they could exert beneficial health effects (e.g. on the brain) by altering the composition of the gut flora, although more work is needed to validate this. Considerable attention has been paid to whole grains and to berries: in a few but not all studies, high intakes seem to lead to decreased biomarkers of low-grade inflammation, such as CRP levels.

Several studies have examined the association of the Mediterranean diet with markers of inflammation, and generally report inverse correlations, as do studies of subjects on vegetarian diets. Overall, it may be diets rich in fruits, vegetables, grains, etc. that are protective rather than any single constituent of them, combining the modest protective effects of several components in a synergistic way. Current evidence suggests, on balance, that the majority of these anti-inflammatory effects are not exerted by antioxidant mechanisms.

CONCLUSION

Reactive O₂ species and oxidative damage are features of aerobic life, useful in protecting us against infection but probably deleterious in predisposing to age-related diseases and possibly to ageing itself. A balanced diet, which provides multiple constituents directly or indirectly related to antioxidant defence (Table 3) plus moderate exercise are probably the best antioxidants and anti-inflammatory agents. Some alcohol consumption seems beneficial (exactly why is uncertain) but too much is bad (probably by making too many ROS). High-dose antioxidant supplements confer little, if any, added benefits, probably because their ability to influence oxidative damage in the human body is very limited. One area that needs more research is the development of antioxidants that really work as such in the human body, for the prevention and treatment of diseases. They should be especially useful in the dementias, where oxidative damage in the brain seems to be a prominent contributor to the pathology, and in chronic inflammation, to decrease the risk of cancer developing.

Another important point to consider is that many of the epidemiological studies carried out with antioxidants were on relatively young or middle aged people (indeed, people who volunteer for trials are also often healthier than average). Ageing can create a state of low-grade inflammation, sometimes called “inflammaging”, and oxidative damage levels tend to rise. It seems plausible that the elderly might benefit more from antioxidant supplementation, especially as their ability to absorb and process nutrients may be impaired. This is a key area for further study, given the increases in aged populations in many countries.

There is a complex and intimate relationship between free radicals and inflammation: diets rich in antioxidants tend to lower biomarkers of inflammation, although it may be a

range of constituents in addition to (or other than) the antioxidants that are achieving this. Chronic low-grade inflammation, resulting from obesity, metabolic syndrome, diabetes, atherosclerosis, and several other conditions, seems to be a major contributor to human morbidity. Whereas there has been some progress in developing biomarkers of oxidative damage (Table 6), there is less agreement on biomarkers of inflammation (Table 2). Plasma CRP level is widely used because of its high sensitivity; but should a rise in CRP in the absence of changes in other biomarkers (Table 2), as is sometimes observed, be taken as a real index of ongoing inflammation? More research is also needed in this area.

LIST OF ABBREVIATIONS

8OHdG: 8-Hydroxy-2'-deoxyguanosine

8OHG: 8-Hydroxyguanine

ABTS: 2,2'-Azinobis
(3-ethylbenzothiazoline-6-sulphonate)

AD: Alzheimer disease

ATP: Adenosine 5'-triphosphate

CHD: Coronary heart disease

COPs: Cholesterol oxidation products

COX: Cyclooxygenase

CRP: C-reactive protein

CuZnSOD: Copper, zinc-containing superoxide
dismutase

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FAD: Flavin adenine dinucleotide

FADH₂: Reduced flavin adenine dinucleotide

GSH: Glutathione (reduced form)

GSSG: Glutathione (oxidised form)

HDL: High density lipoprotein

HNE: Hydroxynonenal

IFN- γ : Interferon gamma

IL-1 β : Interleukin-1 beta

IL-6: Interleukin-6

IL-10: Interleukin-10

LDL: Low density lipoprotein

LOX: Lipoxygenase

MnSOD: Manganese-containing superoxide dismutase

MPO: Myeloperoxidase

NAD⁺: Nicotinamide adenine dinucleotide

NADH: Reduced nicotinamide adenine dinucleotide

NADP⁺: Nicotinamide adenine dinucleotide phosphate

NADPH: Reduced nicotinamide adenine dinucleotide
phosphate

ORAC: Oxygen radical absorbance capacity

PR: Peroxiredoxins

PUFA: Polyunsaturated fatty acid

RDA: Recommended daily allowance

ROS: Reactive oxygen species

SOD: Superoxide dismutase

TBA: Thiobarbituric acid

TNF- α : Tumour necrosis factor alpha

TR: Thioredoxin reductase

GLOSSARY

Antioxidant: Any substance that delays, prevents or removes oxidative damage to a target molecule.

Cytokines: Loosely-defined term that encompasses a wide range of polypeptides and glycoproteins. Their presence is usually transient and strictly controlled. Most cytokines are secreted, and act regionally by binding to receptors on their target cells, exerting effects at low concentrations.

Free Radical: Any atom or molecule with one or more unpaired electrons.

Oxidative Damage: The biomolecular damage caused by attack of reactive species upon the constituents of living organisms.

ROS: Reactive oxygen species. Species derived from O_2 that are more reactive than O_2 itself and capable of damaging biological molecules.

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