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Ageing, Age-related Diseases and Oxidative Stress: What to Do Next?

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Highlights

- Damage caused by oxidative stress is an important hallmark of ageing
- Whereas experimental work shows clear antioxidant effects, clinical trials failed
- There is a lack of reliable markers of oxidative stress in large epidemiological studies
- Functional level, but not circulation level, of antioxidants could provide more insights

Abstract

Among other mechanisms, oxidative stress has been postulated to play an important role in the rate of ageing. Oxidative damage contributes to the hallmarks of ageing and essential components in pathological pathways which are thought to drive multiple age-related diseases. Nonetheless, results from studies testing the hypothesis of oxidative stress in ageing and diseases showed controversial results. While observational studies mainly found detrimental effects of high oxidative stress levels on disease status, randomized clinical trials examining the effect of antioxidant supplementation on disease status generally showed null effects. However, re-evaluations of these counterinitiative observations are required considering the lack of reliability and specificity of traditionally used biomarkers for measuring oxidative stress. To facilitate these re-evaluations, this review summarizes the basic knowledge of oxidative stress and the present findings regarding oxidative damage and ageing and age-related diseases. Meanwhile, two approaches are highlighted, namely proper participants selection, together with the development of reliable biomarkers. We propose that oxidized vitamin E metabolites may be used to accurately monitor individual functional antioxidant level, which might serve as promising key solutions for future elucidating the impact of oxidative stress on ageing and age-related diseases.

Key words

Ageing, Age-related diseases, Oxidative stress, Vitamin E metabolites

1. Introduction

It has been widely acknowledged that life expectancy has increased over the past centuries as a specific result of improved medical care, vaccination and hygiene (Eggleston and Fuchs, 2012; Rappuoli et al., 2014). The process of ageing is a dynamic, chronological process characterized by the gradual accumulation of damage to cells, progressive functional decline and increased susceptibility and vulnerability to diseases. In addition, ageing is closely connected to the onset and progression of multiple age-related diseases, such as cancer, type 2 diabetes mellitus, and cardiovascular and neurodegenerative diseases (Finkel et al., 2007; Samani and van der Harst, 2008; Wyss-Coray, 2016). The ageing process is postulated to originate from several basic molecular changes, better known as the hallmarks of ageing, which include four primary hallmarks, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis, three antagonistic hallmarks, deregulated nutrient sensing, mitochondrial dysfunction, and cellular senescence, and two integrative hallmarks stem cell exhaustion, and altered intercellular communication (López-Otín et al., 2013).

These hallmarks contributing to the ageing process could be caused by oxidative damage. For example, telomeres are highly sensitive to oxidative damage and their repair capacity is less well than other parts of the chromosome (von Zglinicki, 2000; von Zglinicki, 2002). Hence, oxidative damage may result in telomere attrition that accelerates ageing and increases the risk of age-related diseases (Blackburn et al., 2015). The concept of oxidative stress was introduced in 1985 and updated later (Sies, 1985; Sies, 2015; Sies et al., 2017). Oxidative stress refers to "an imbalance between the generation of oxidants and their elimination systems, i.e. antioxidants, in favor of oxidants, leading to disruption of redox signaling and control and/or molecular damage" (Sies et al., 2017). Conceptually, the level of oxidative stress ranges from physiological level for redox signaling to toxic level of molecular or organelle damage (**Figure 1**). Redox signaling is essential for host defense as well as in a diverse array of signaling pathways (Holmström and Finkel, 2014; Schieber and Chandel, 2014). Other damages caused by non-physiological high oxidative stress leads to a wide range of phenotypic changes, including altered gene expression, arrested cell proliferation and cell growth, and cellular senescence (Burdon, 1995; Hensley et al., 2000; Kreuz and Fischle, 2016).

Antioxidants may act as scavengers of oxidants to maintain the biological redox steady states. Therefore, since the oxidative stress theory was proposed (Harman, 1972), antioxidants were postulated to potentially play a protective role in ageing and age-related diseases. Considering the premise that adverse health consequences caused by oxidative stress can be counteracted by antioxidants, a comprehensive body of studies aiming to examine the beneficial effects of antioxidants on diseases have been carried out in the past three decades. However, results were often disappointing and counterintuitive. The most appealing and well-known example is vitamin E, a well elucidated chain-breaking antioxidant. Although lower disease risks in individuals with higher vitamin E concentration have been found in many observational studies (Bostick et al., 1993; Li et al., 2012; Mayer-Davis et al., 2002; Rimm et al., 1993; Stampfer et al., 1993; Wright et al., 2006), as well as protective properties of vitamin E in animal experiments (Banks et al., 2010; Muller et al., 2007), most clinical trials examining vitamin E supplementation failed to demonstrate any advantageous effects on the prevention or treatment of various age-related diseases (Bjelakovic et al., 2007; Farina et al., 2017; Klein et al., 2011; Miller et al., 2005; Myung et al., 2013; Wang et al., 2014).

Along with these conflicting evidences, it seems like the controversy about the oxidative stress theory in ageing and age-related diseases has never stopped (**Figure 2**). In addition, over the past 30 years, fluctuations in the use of antioxidant supplements were also observed, for example in the US (**Figure 3**) (Kantor et al., 2016; Kim et al., 2014). The percentage of individuals using antioxidant supplements gradually increased from 1980s and peaked in 1990s. Of note, specifically the use of vitamin E supplements steeply dropped in the early 21st century, where after decline turned to be stabilized. However, these observations neither imply that any consensus about the effect of antioxidants on diseases has been reached, nor that the oxidative stress theory has been refuted. Conversely, the annual publication count of antioxidant articles steadily increased since the 1990s, and more than 30,000 papers have been published in 2018 alone about this research topic (Yeung et al., 2019).

So far, oxidative damages are thought to play a pivotal role in the pathological processes implicated in ageing and age-related diseases and the underlying biochemical mechanisms have been clarified in detail (Cui et al., 2012; López-Otín et al., 2013). However, there are still several questions unsettled such as the existing paradox regarding the preventive and therapeutic role of antioxidants (such as vitamin E), the lack of stable and representative biomarkers of oxidative stress, and whether oxidative stress is causally associated with ageing and age-related disease in the general population setting. Therefore, this review is organized as such to provide an overview of the chemical processes involved in oxidative stress and an update on the available evidences about associations with ageing and age-related diseases. In the last part of the review, antioxidants, especially the controversial role of vitamin E will be addressed, together with novel insights and directions for future research.

2. Generation of Reactive Oxygen Species (ROS) and health roles

2.1 Endogenous generation of ROS

According to the “free radical theory” that was proposed in the 1950s and revised in the 1970s, damages induced by free radicals are the main cause of ageing and a shorter lifespan (Cadenas and Davies, 2000; Harman, 1956, 1972). Reactive oxygen species (ROS) are highly reactive molecules, primarily including typical free radicals that contain at least one unpaired electron (superoxide $O_2^-\bullet$, hydroxyl radical $\bullet OH$) and hydrogen peroxides (H_2O_2), and have been considered the main source of endogenous oxidative stress damage (Liochev, 2013).

It is widely accepted that the bulk of ROS are generated by the mitochondrial electron transport chain during normal oxidative respiration in addition to numerous intracellular pathways (**Figure 4**). It is estimated that about 1-2% of the daily overall oxygen molecules consumed are reduced into $O_2^-\bullet$

with the leak of electrons (Turrens, 2003). This process occurs mainly in two discrete complexes of mitochondrial electron transport chain in the matrix side of inner mitochondrial membrane, notably complex I (NADH-ubiquinone oxidoreductase) and complex III (ubiquinone-cytochrome c reductase) (Finkel and Holbrook, 2000). Iron-sulphur centers are thought as the most likely site of ROS production in complex I (Andreyev et al., 2005). Complex III, also known as the Q cycle, which refers to a set of ubiquinone oxidation reactions, is responsible for the robust production of superoxide, the precursor of other ROS, by non-enzymatic transfer of electrons to molecular oxygen. Once generated, superoxide could be catalyzed by superoxide dismutase (SOD) into H_2O_2, which is unstable and membrane-diffusible peroxide. Subsequently, in the presence of transition cation with reduced form (Fe^{2+} or Cu^{+}, referred to as the Fenton reaction) or myeloid peroxide (MPO) (Castagna et al., 2008), H_2O_2 further dismutates to $\bullet OH$, the extremely unstable and most reactive ion among all ROS. In summary, the main process of ROS generation in mitochondria could be schematically presented as $O_2 \rightarrow O_2^{-}\bullet \rightarrow H_2O_2 \rightarrow \bullet OH$.

Hydroxyl radicals may lead to detrimental damages to macromolecules owing to its chemical properties (Halliwell, 1989). Moreover, its formation relies on the presence of a reduced form transition cation, mainly iron generated from the hemoglobin heme group during hemolysis. Hence any component which can stabilize heme iron within hemoglobin could prevent oxidative damage caused by hydroxyl radicals. In recent years, haptoglobin (Hp), an abundant, acute-phase inflammatory glycoprotein, which is predominantly synthesized in the liver and is regulated by cytokines, has been indicated to have an important role in the prevention of the generation of hydroxyl radicals by virtue of its ability to bind hemoglobin with high affinity and stability, thus preventing the release of heme iron from hemolysis into the circulation (Andersen et al., 2017), as shown in **Figure 4**. The Hp gene basically contains two common alleles, namely Hp1 and Hp2, with homozygous (1-1 or 2-2) and heterozygous (2-1) genotypes. In parallel with the theoretical evidence, both haptoglobin concentration and genotype, specifically Hp2-2, are associated with various age-related diseases, such as cancer, cardiovascular disease, etc. (Levy et al., 2010). However, the underlying mechanisms related to the pathophysiology of these diseases still remain to be demonstrated.

2.2 Complex role of ROS in health maintenance and diseases

The role of ROS in the body is rather complex, and the influences on health vary largely along with changing ROS levels. ROS levels, as a reflection of oxidative stress, are modulated by oxidant generation and their elimination, and are linked to many pathophysiological processes. Within physiological levels, ROS are in a biological redox steady state (Sies et al., 2017) and facilitate the maintenance of cellular homeostasis and function. However, ROS levels would go toward either side beyond dynamic balance (pathological states). Thus ROS levels have both beneficial and damaging aspects, as put forward in the concept of mitohormesis (Ristow and Schmeisser, 2014), Consequently, both (too) low and (too) high levels of ROS will have adverse health effects, as illustrated in **Figure 1**.

2.2.1 Physiological levels: beneficial health effects

ROS may act as second messengers owing to the characteristics of having an intricate system for synthesis and removal as well as reversible signaling effects. Both superoxide and hydrogen peroxide could be potential messengers to regulate reduction-oxidation-dependent signaling mechanisms, while hydrogen peroxide has higher advantage in signaling capacity given its stability and membrane permeability. The major mechanism underlying most redox-dependent signaling has been considered as the reversible modulation of cysteine thiol groups (thiolate anion to sulfenic form Cys-SOH) regulated by hydrogen peroxide (Holmström and Finkel, 2014; Reczek and Chandel, 2015).

Within physiological levels (Range II in **Figure 1**), ROS can promote host defense mechanisms such as for the optimal activity for macrophages against bacteria, as well as in signaling pathways, such as toll-like receptors initiated pathways, Mitogen-Activated Protein Kinase (MAPK) signaling pathways, NF-??B signaling pathway and Keap1-Nrf2-ARE signaling pathway (Hekimi et al., 2011; Holmström and Finkel, 2014; Schieber and Chandel, 2014; Zhang et al., 2016). Therefore, ROS levels within the physiological range are critical signaling molecules for many redox-dependent signaling processes including gene expression, metabolic regulation, inflammatory response, stem cell proliferation and differentiation, cancer pathogenesis as well as ageing. Intensive discussion regarding ROS signaling physiological consequences have been provided in previous reviews (Holmström and Finkel, 2014; Schieber and Chandel, 2014).

Given the signaling effect of ROS, we can speculate that a certain increase of ROS in the physiological range would lead to better health effects, from health maintenance to health promotion, such as decreased risk of diseases, or even prolonged lifespan (Ristow and Schmeisser, 2014), as illustrated in IIA, **Figure 1**. However, with further increase of ROS levels and more damage events, this beneficial effect may decrease or disappear, but with no manifestation of pathological symptoms (IIB, **Figure 1**).

2.2.2 Elevated or Decreased Physiological levels (Pathological state): adverse health effects

When ROS levels are beyond the range of physiological levels, either (too) low or (too) high, adverse health effects can happen. For example, upon inflammatory stimulation, neutrophils are activated and generate large amounts of ROS for host defense. However, Ncf1 (neutrophil cytosolic factor 1) mutated mice, with low production of ROS, have higher type I interferon and develop an accelerated lupus-like disease (Urbonaviciute et al., 2019). Similarly, the Ncf1-339 T allele, related to reduced extracellular ROS production in neutrophils and increased type 1 interferon-regulated genes expression, was found enriched in systemic lupus erythematosus patients compared to healthy controls (Olsson et al., 2017). Moreover, patients with NADPH oxidase 2 (Nox2) mutations that are associated with lower ROS level, were more susceptible to develop chronic granulomatous disease (Kelkka et al., 2014). These findings indicated an association between lower ROS generation that is not sufficient to maintain physiological processes which could lead to inferior health outcomes, as shown in Range I, **Figure 1**.

On the other hand, in the situation of high ROS levels (toxicity), excessive ROS might irreversibly react with intracellular macromolecules, including lipids, proteins and DNA, both in the mitochondria and nucleus. Hazardous products are formed and accumulated, and the functions of macromolecules and organelles are altered. Once these exceed the repair capacity of the body, devastating damage will occur and accelerated ageing or multiple diseases might manifest or progress (discussion in part 2.3 and part 3), as shown in Range III, **Figure 1**.

2.2.3 What is the physiological level?

Though **Figure 1** shows the possible ranges of ROS levels, several caveats should be noted. Firstly, how to define "physiological levels". ROS generation happens all the time and signaling merits and damaging events occurs simultaneously. For example, protein oxidation will produce many harmful byproducts, meanwhile it is also involved in redox signaling and control, especially cysteine side chain peroxidation. When we are talking about the levels of either ROS or oxidative stress, we should be more cautious of using "high" and "low", given that "normal" is undefined. Possibly, the so-called "high" or "low" ROS levels close to the physiological range should also be regarded as tolerable levels. For example, transgenic animal model with lower SOD production did not show a shortened lifespan in mice (Jang et al., 2009; Zhang et al., 2009), and even an extended lifespan in C.elegans (Van Raamsdonk and Hekimi, 2009). This "lower SOD production" may lead to a relatively "higher ROS level", but notably, this level could be tolerated by the experimented animals and they also function well for maintaining or promoting health, thus being potential physiological. In addition, antioxidants would have this similar dose-response manner since the change of antioxidant levels are mostly inversely associated with oxidants, for example, in C elegans, an inverted U-shaped dose-response relationship between antioxidants and lifespan was observed (Desjardins et al., 2017). Consequently, the same situation might also be there for oxidative stress.

2.3 Damages caused by oxidative stress

2.3.1 Lipid peroxidation

Polyunsaturated fatty acid (PUFA), particularly with a high number of double bonds, such as arachidonic acid and linoleate, are highly susceptible to ROS and free radicals, known as autocatalytic chain reaction (Yin et al., 2011). Lipid peroxidation includes three steps: initiation, propagation and termination, as shown in **Figure 5**. Phospholipids peroxidation in lipid membranes may lead to a decrease in membrane fluidity and permeability, and inactivation of receptors, resulting in cell apoptosis. Furthermore, lipid radicals generated during oxidation processes can form a myriad of deleterious end products, including reactive aldehydes, alkanes and alkenes (Sousa et al., 2017). Among those products, malondialdehyde and 4-hydroxy-2-nonenal (4-HNE) are two of the most widely studied aldehydes. However, due to the high reactivity of these aldehydes, for example they can react with proteins through the Michael addition reaction or with DNA to form adducts (Il'yasova et al., 2012; Spickett, 2013), it is difficult to accurately measure their free concentrations as valid oxidative damage levels. Moreover, urinary malondialdehyde levels are affected by dietary malondialdehyde content (Brown et al., 1995), and glutathione S-transferases genetic polymorphisms could confound the metabolism of 4-HNE (Il'yasova et al., 2012). Therefore, both the properties of the products themselves, xenobiotic sources, and genetic predispositions present obstacles for their development and further use as reliable biomarkers.

F_2-Isoprostanes (F_2-IsoPs), another important class of lipid peroxidation end products, can be formed through non-enzymatic oxidation of arachidonic acid. These are prostaglandin-like compounds initially formed in-situ esterified to phospholipids and subsequently released in free form by phospholipases (Milne et al., 2005). Isoprostanes have widely been considered as reliable biomarkers quantitating oxidative stress with their important merits of being (i) stable, both chemically and unaffected by diet or health status; (ii) sensitive to changes in oxidative stress; (iii) present in detectable quantities in many biological fluids, such as urine and plasma; and (iv) accessible to define a normal range in the population level which means the change of their formation can serve as the reflection of different oxidative stress levels and further relate to pathological status or diseases (Gopaul et al., 2000; Montuschi et al., 2004; Roberts and Morrow, 2000). A meta-analysis identified different quantitative levels of 8-isoprostane-$F_{2\alpha}$ in patients across different pathological conditions, with relatively small increased levels observed in patients with, for example hypertension and metabolic syndrome, while large increased levels were observed with patients for example kidney related pathologies and respiratory tract disorders (van't Erve et al., 2017).

2.3.2 Protein oxidation

Proteins are also important targets for ROS. Protein oxidation involves (i) oxidative modification of site-specific amino acid residues, including oxidation of sulphur-containing amino acid residues, particularly cysteine and methionine, as well as reactions with aromatic amino acid residues and peroxynitrite; (ii) protein fragmentation resulting from oxidative cleavage of the peptide backbone; (iii) generation of protein carbonyl derivatives; and (iv) generation of protein-protein cross-linkages (Berlett and Stadtman, 1997; Davies, 2016; Stadtman, 2006). Protein oxidation may lead to a change in its three-dimensional structures, modification of physiological properties such as enzyme activities and signal transduction networks which are related to cellular regulation and function, and further protein proteolytic degradation on one hand, or protein aggregation, partial unfolding and altered conformation on the other hand (Davies, 1990; Davies, 2016; Hohn et al., 2014). Carbonyl derivatives are a large group of end-products of protein oxidation which have been considered as the most widely used biomarkers of oxidative damage to protein. They can be formed through oxidative cleavage of backbones, direct oxidation of amino acid residues including lysine, arginine, proline and threonine, as well as reaction of amino acid residues with aldehyde resulting from lipid peroxidation or carbohydrate (Stadtman and Levine, 2003). Among the protein carbonyls, advanced glycation end products (AGEs) are a heterogenous group of additive derivatives produced by reactions between proteins and oxidation end products from PUFAs or carbohydrates, such as hazardous aldehyde (Singh et al., 2001). Other protein oxidation end-products include advanced oxidation protein products (AOPP) formed mainly by myeloperoxidase-derived chlorinated oxidants (Witko-Sarsat et al., 1998), oxidized low-density lipoprotein (oxLDL) derived from oxidative modification (Trpkovic et al., 2015), and nitrotyrosine resulting from tyrosine oxidation (Bartesaghi et al., 2007). However, because of the structural heterogeneity caused by different biochemical pathways as well as the low specificity and sensitivity of antibodies in available detection assays, it is difficult to use them as stable and efficient biomarkers in large epidemiological studies.

2.3.3 Nuclear DNA oxidation

ROS, in particular the hydroxyl radicals, can cause oxidative damage to the DNA, which includes: (i) base mutation; (ii) strand breaking, both single and double; (iii) DNA-protein cross-links; and (iv) formation of DNA-adducts. In general, hydroxyl radicals could react with DNA bases and sugar-phosphate backbone, leading to erroneous base pairing and further common mutation of G to T and C to A bases (Grollman and Moriya, 1993). Meanwhile, hydroxyl radicals could also react with the deoxyribose moiety leading to the loss of DNA bases, generating base-free sites, thus single or double DNA strand(s) could break. DNA strand breaks are well-established risk factors of genome instability, cell cycle disruption, as well as cell death (Dehennaut et al., 2013; Johnson et al., 2000; Yan et al., 2003). DNA-protein cross-links involving thymine and tyrosine in the nucleoprotein complex of histones and DNA (nucleo-histone) can also be induced by the hydroxyl radicals (Dizdaroglu et al., 1989). In addition, adducts to DNA can be formed by reaction of deoxyguanosine (M(1)G) and deoxyadenosine with other macromolecular modifications trigged by ROS, such as reactive aldehyde products generated during the lipid peroxidation (Marnett, 2002).

Specific to the most common base mutations, there are mainly three ways: (i) adding double bonds to heterocyclic DNA bases; or (ii) abstracting hydrogen atom from the methyl group of thymine; or (iii) forming allyl radical of thymine and carbon-centered sugar radicals (Dizdaroglu et al., 2002; Winterbourn, 2008). For pyrimidines, hydroxyl radicals react with the particularly sensitive C5- and C6- double bond of thymine and cytosine, generating a spectrum of adducts, including thymine glycol, cytosine glycol, 5-hydroxymethyl uracil and 5-formyluracil, 5-hydroxycytosine, uracil glycol and 5-hydroxyuracil (Breen and Murphy, 1995; Dizdaroglu et al., 2002). For purines, similarly, C4-, C5, and C8- sites are more sensitive to radicals (Breen and Murphy, 1995; Dizdaroglu et al., 2002). Among all these DNA oxidative modifications, the mutagenic lesions of saturated imidazole ring 7,8-dihydroxy-8-oxo-7,8-dihydroguanine (8-oxodG), formed from hydroxylation of the C8 residue of guanine, has been the most abundant, best characterized, and widely considered as a potential biomarker of DNA oxidation (Cadet et al., 2003; Dizdaroglu et al., 2002). Theoretically, the concentration of 8-oxodG measured in urine, with its merits of long-term stability in urine and multiple methods for measurement, reflects the accumulative DNA oxidation in the whole body and is thus considered being predictive for ageing and multiple diseases. However, unlike other molecules, oxidative damage to DNA might be repaired by the DNA repair systems (Chatterjee and Walker, 2017). So the ROS-induced 8-oxodG concentration is not only related to oxidative damage, but also depends on the DNA repair capacity. Yet, due to the unknown inter-individual difference in DNA repair capacity, it is difficult to quantify the real 8-oxodG concentration induced by ROS accurately in individuals, therefore, the further use of this biomarker has been hampered.

2.3.4 Mitochondrial dysfunction

The mitochondrial respiratory chain is the main intracellular place for endogenous ROS generation, with steady higher concentrations of free radicals in the mitochondrial matrix, and this leads to close proximity of mitochondria to ROS. Evidence showed that an about 5- to 10-fold higher superoxide anion level is present in the mitochondrial matrix than in the cytosolic and nuclear spaces (Cadenas and Davies, 2000), making the mitochondria primary targets for ROS-induced damages.

Mitochondrial DNA (mtDNA) casts a critical role in energy generation during oxidative phosphorylation by the function of encoding important bioenergetic genes including 13 polypeptides, 22 transfer RNA, 2 ribosomal RNA, which are essential for the synthesis of multi-subunit enzymatic components of the electron transport chain (Anderson et al., 1981). However, there are several factors ascribed that contributed to the higher vulnerability of mtDNA to oxidative damage: (i) close proximity to the oxidants forming site; (ii) the absence of histones in mtDNA; and (iii) limited mtDNA repair activity. This less active repair capacity is partly attributed to the multiple copies of mtDNA and a circular and compact coding arrangements in mtDNA (Druzhyna et al., 2008). Therefore, damaged mtDNA would be degraded and replaced by newly produced ones copied from undamaged genomes, which gives a higher ability to mitochondria against detrimental mutation effects. Meanwhile, there are also fewer mtDNA repair mechanisms than that in nuclear DNA and mostly are poorly understood. Though base excision repair, mismatch repair as well as recombination or nonhomologous end joining repair mechanisms do exist in mtDNA, nucleotide excision repair is still absent, and the specific roles of each pathway, as well as proteins involved in repair processes remain to be fully elucidated (Croteau et al., 1999; Druzhyna et al., 2008). It is estimated that oxidized bases are as 10 times frequent in mtDNA than nuclear DNA, for instance, the concentration of the common DNA lesion 8-oxodG is much higher in mtDNA than in nuclear DNA (Ames et al., 1995; Cui et al., 2012). When a mutation occurs, mutated and normal mtDNA co-exist in the same mitochondria, which is known as heteroplasmy (Ozawa et al., 1990; Stewart and Chinnery, 2015). The percentage of erroneous sequence of mtDNA changes during cell replication and division, and mitochondria could aggregate different mutations over time, reducing their bioenergetic capacity (Wallace and Chalkia, 2013). When the increasing proportion of mutated mtDNA exceeds the critical threshold level of normal mitochondrial energy generation, bioenergetic defects in cells may occur. Besides, the sustaining existence and accumulation of damaged mtDNA in the mitochondria, will inevitably lead to more ROS generation, which in turn causes further damage, making a vicious cycle (Ames et al., 1995).

The mtDNA damages would lead to loss of redox homeostasis, perturbed Ca^{2+} homeostasis, damage to membrane proteins and lipids, as well as to abnormal mitochondrial energy transduction (Wallace and Chalkia, 2013). All these modifications are the driving force for further mitochondrial dysfunction and loss of integrity, which will affect cell viability and cellular function (Lin and Beal, 2006; Wallace, 2013). In addition, organs, especially the brain, heart and muscles, being high-energy consuming and sensitive to bioenergetic defects, are strongly affected by mitochondrial dysfunctions, resulting in organ-specific pathologies. Moreover, variation in mtDNA have also been shown to associate with several clinical phenotypes, including cardiovascular diseases, anthropometric and metabolic measures (Kraja et al., 2019).

In addition to mtDNA, mitochondrial sirtuins have found to be involved in regulation of redox homeostasis. Sirtuins are a group of proteins (from SIRT1 to SIRT7), acting predominantly as nicotinamide adenine dinucleotide (NAD^+) dependent deacetylases and ADP-ribosyltransferases. SIRT3, 4 and 5 are exclusively localized within mitochondrial, altering the NAD/NADPH ratio in mitochondria to respond to metabolic changes. SIRT3 is supposed as the major mitochondrial deacetylase and is the most thoroughly studied mitochondrial sirtuin. It plays an important role in mitochondrial bioenergetics (Guarente, 2011a; Imai and Guarente, 2016; Sack and Finkel, 2012; Westphal et al., 2007), as well as promote resistance to oxidative stress by reducing ROS via magnesium SOD deacetylation and activation (Guarente, 2011a; Sack and Finkel, 2012). Though SIRT4 and SIRT5 are less well understood, SIRT5 have also been shown to be strongly associated with oxidative stress signaling and protect cells from ROS, suppressing oxidative stress levels (Singh et al., 2018). In contrast, SIRT4 might have dual roles, either to induce ROS production or to have antioxidative function (Singh et al., 2018). In addition, these mitochondrial sirtuins work in concert and present complicated interaction profiles (Yang et al., 2016), for example, SIRT3 and SIRT4 may function together to protect cells from stress and DNA damage (Jeong et al., 2013). A series of reviews regarding sirtuins on mitochondrial and oxidative regulation have been published before (Grabowska et al., 2017; Guarente, 2008, 2011a; Guarente, 2011b; Imai and Guarente, 2016; Sack and Finkel, 2012; Westphal et al., 2007). Nevertheless, additional studies are needed to in-depth ascertain and illustrate the mechanism of mitochondrial sirtuins in maintaining mitochondrial biological functions and redox homeostasis.

3. Causes and consequences of oxidative damage on ageing and age-related diseases

The progressive accumulation of oxidative damage to macromolecules and mitochondria will finally lead to pathophysiological alterations, functional decline and accelerated ageing. Here we will discuss the harmful consequences of oxidative stress, mainly oxidative damage which are more closely related to accelerated ageing and multiple age-related diseases, with a special focus on ageing and lifespan, cardiovascular diseases (CVD) and neurodegenerative diseases (NNDs).

3.1 Ageing and lifespan

The best way to determine the effect of oxidative stress on ageing is to test whether changes in lifespan are dependent on alterations in oxidative stress levels. Numerous studies aimed to investigate this effect have been conducted in animals. Based on the free radical theory of ageing, attempts to extend lifespan have been carried out with a focus on two different targets: alterations of oxidant load (i.e.

ROS generation) or alternations of mitochondrial antioxidant capacity. Alternations of oxidant load are usually investigated through calorie restriction, while alternation of mitochondrial antioxidant capacity is often examined through inducing genetic alterations, or through dietary supplementations.

Calorie reduction (CR) is defined as a 10-50% reduction of total energy intake without inducing malnutrition. A large body of evidence suggested that CR might modulate the mitochondrial activity and lower ROS production resulting in reduction of oxidative damage through sirtuins regulation, for example through primary activation of sirtuin 1 and further activation of PPARγ coactivator-1α (PGC-1α), leading to a slower rate of aging-related decline and extended lifespan (Guarente, 2013; López-Lluch and Navas, 2016; Zullo et al., 2018). Several reviews indicated that, in lower model organisms ranging from yeast to mammals, CR is capable to extend both average and maximum lifespan and health span (Fontana et al., 2010; Heilbronn and Ravussin, 2003; Ingram and de Cabo, 2017; Most et al., 2017; Testa et al., 2014). Interestingly, studies in humans also detected some favorable biomarkers of longevity induced by CR, mainly hormonal adaptions, such as lower insulin, and lower IGF-1 level, though the latter was mainly in response to protein restriction (Heilbronn et al., 2006; Lettieri-Barbato et al., 2016).

With respect to induced genetic alternations, antioxidants-related transgenic lower models have been frequently used, which have altered antioxidant capacity or a disrupted oxidative-related signaling pathway, leading to either a reduced or extended lifespan (Chen et al., 2010; Liang et al., 2003; Liao and Kennedy, 2014; Salmon et al., 2010; Selman and Withers, 2011). Mice with antioxidant overexpression, for example, catalase (Dai et al., 2017), or with modifications in other signaling pathway components, such as $p66^{sch-/-}$ (Migliaccio et al., 1999), $Igf1r^{+/-}$ (Junnila et al., 2013) are generally considered to have increased longevity. However, other results are inconclusive about altered expression levels of, especially SOD (Jang et al., 2009; Perez et al., 2009; Van Remmen et al., 2003; Zhang et al., 2009), thioredoxin (Cunningham et al., 2018; Perez et al., 2011), or methionine sulfoxide reductase A ($MsrA^{-/-}$) that function to protect against protein oxidation (Moskovitz et al., 2001; Salmon et al., 2009). Moreover, $Gpx1^{-/-}$ knocked-out mice with reduced glutathione peroxidase-1 surprisingly did not show accelerated ageing (Van Remmen et al., 2003; Zhang et al., 2009). Similarly, results on longevity of transgenic invertebrate models, such as C. elegans or drosophila, with alternations of antioxidants capacity, are also quite divergent, and some studies even found a large extension of lifespan with elevated oxidative stress level (Doonan et al., 2008; Hwang et al., 2014; Oberacker et al., 2018; Reveillaud et al., 1994; Uno and Nishida, 2016; Van Raamsdonk and Hekimi, 2009; Yang and Hekimi, 2010; Yang et al., 2007; Yee et al., 2014). The inconsistence was also found in experiments with dietary antioxidants supplementation (Sadowska-Bartosz and Bartosz, 2014).

However, oxidative stress may act as a double-edged sword to human health, as discussed in part 2.2. It is difficult to determine a lifespan-affecting ROS level and further lead to the ambiguity on the relation of oxidative stress and ageing. Nevertheless, a recent review still conclude that mitochondria play a critical role in the ageing process, but it remains unclear whether it is a cause or consequence (Sanz, 2016).

3.2 Cardiovascular diseases (CVDs)

Cardiac myocytes have a high number of mitochondria which makes them more susceptible to oxidative damage. In addition to the mitochondrial pathway of ROS generation, crosstalk exists between mitochondria and NADPH oxidase, the major sources of ROS in blood vessels, affecting vascular function by dysregulation or uncoupling eNOS, leading to endothelium dysfunction (Daiber et al., 2017). Together with other ROS-induced damages involved in mitochondrial dysfunction, and ROS-induced macromolecule damages (e.g. ox-LDL), they all contribute to the pathogenesis and progress of CVD (Daiber, 2010; Daiber et al., 2017). To date, several lines of evidences have found an association between onset and progression of cardiovascular diseases (CVD), which include atherosclerosis (Kattoor et al., 2017), hypertension (Chrissobolis et al., 2017), heart failure (Munzel et al., 2017), and peripheral artery disease (Steven et al., 2017).

In rodents, deletion of the NADPH oxidases gene (Nox1 and Nox2) or p47phx deficiency resulted in markedly lower ROS generation, and yielded a lower risk of atherosclerosis, while Nox4 deletion gave an increased risk (Forstermann et al., 2017). Similarly, over/under-expression of different antioxidant enzymes modulated atherogenesis in different genetically-altered mouse models (Forstermann et al., 2017; Munzel et al., 2017). In human studies, increased expression and activity of NADPH oxidase and xanthine oxidase subunits were associated with higher ROS production, and an increased risk of coronary artery disease (Guzik et al., 2006). Moreover, mtDNA copy number, the increase of which is indicative of early molecular changes compensating for oxidative stress-induced mitochondrial defects, has been shown to be associated with CVD. A study composed of 21870 participants from three large cohorts found that the CVD incidence increased 23% with one-standard deviation decrease in mtDNA copy number after adjustment for traditional CVD risk factors (Ashar et al., 2017). In addition, epidemiological cohort studies consistently showed that higher dietary intake of antioxidants, either as diet components or supplements, including flavonoids, vitamin E and C, are associated with lower CVD risk, such as coronary heart disease, ischemic stroke as well as CVD mortality (Siti et al., 2015; Ye and Song, 2008). However, large randomized controlled clinical trials, generally failed to show any beneficial effect of antioxidants supplementation, including vitamin E, A, C, B12, B6 and folic acid, on CVD risk, especially in atherosclerosis (Gori and Munzel, 2011; Siti et al., 2015). Meta analyses also concluded that there is insufficient evidence to support vitamins or other antioxidants supplementation to decrease CVD incidence (Boeing et al., 2017; Myung et al., 2013). Interestingly, a very recent Mendelian Randomization study found there is causal association between higher circulating vitamin E and the risk of coronary artery disease or myocardial infarction (Wang and Xu, 2019). However, the three vitamin E associated instrumental SNPs used in this study are all associated with blood lipid levels or coronary artery disease itself. This basically violates the important condition for conducting Mendelian Randomization that no horizontal pleiotropy for instruments.

Though sensitivity analysis was performed, this is only for one SNP but not for others, thus the results are of limit value. Therefore, despite the biologically evidence from consistent results, the premise of interventions with the intention to decrease oxidative levels and consequently CVD risk is still limited.

3.3 Neurodegenerative diseases (NDDs)

NDDs are characterized by dynamic and progressive neuronal cell damage and loss of neurons, typically including Alzheimer’s disease (AD) and Parkinson’s disease (PD). Ageing is considered to be the predominant risk factor for NDDs, and accumulative oxidative damages during ageing are the main culprits of neurological deterioration (Islam, 2017; Lin and Beal, 2006). Neurological systems are extremely sensitive and vulnerable to ROS-induced damage due to their higher energy demand, lower rate of cellular renewal, membrane PUFA levels, as well as less active oxidative defense mechanisms (Rego and Oliveira, 2003). Notably, studies suggested that mitochondrial dysfunction plays a causal role in the pathogenesis of NDDs (Cheignon et al., 2018; Islam, 2017). Sirtuins, which provide a key role in mitochondrial function and oxidative stress regulation (part 2.3) are also associated with neuron fates, being either neuroprotective or neurotoxic (Sansone et al., 2013; Satoh et al., 2017; Westphal et al., 2007). A large number of proteins that are implicated in NDDs are found to have mitochondrial involvement. AD is pathologically characterized by extracellular deposition of amyloid- β peptide (Aβ) and intracellular neurofibrillary tangles composed of hyper-phosphorylated tau. Oxidative stress may active signaling pathways, such as Jun amino-terminal kinase and p38 mitogen-activated protein kinase, and give rise to amyloid precursor protein and β-secretases to form Aβ, as well as increase tau phosphorylation (Lin and Beal, 2006; Zhao and Zhao, 2013). In turn, Aβ proteins would directly induce ROS generation through activating NADPH oxidase (as shown in **Figure 2**), further exacerbating neuronal damage, amplifying neurotoxicity (Liu et al., 2017). Besides, mtDNA control-region adopted more mutations in 23 pathologically confirmed AD brain samples compared with 40 controls, and heteroplasmic mtDNA control-region mutations increased 63% on average in all AD brains, while markedly increased 130% in patients older than 80 years (Coskun et al., 2004). 1-methyl-4-phenylpyridinium (MPP+), oxidized product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone, both of which are mitochondrial complex I inhibitors, have been demonstrated to result in clinical PD phenotype by producing pathological degeneration of dopaminergic neurons of the substantia nigra (Betarbet et al., 2000; Fornai et al., 2005). Other PD-related proteins including α-Synuclein, Parkin, DJ-1, PINK1, LRRK2 and HTRA2 were associated with mitochondrial dysfunction as well (Jiang et al., 2016; Lin and Beal, 2006). In addition, ROS-induced macromolecule damage, for example MDA and HNE, lipid peroxidation end products, may also play an important role in NDDs (Butterfield et al., 2010; Coppede and Migliore, 2015; Niedzielska et al., 2016). This can be evidenced by the fact that increased levels of oxidative stress biomarkers and decreased levels of antioxidative biomarkers were observed in pharmacologically induced or transgenic animal models with AD or PD (Niedzielska et al., 2016). Several papers have speculated oxidative stress as the key component in the etiology of NNDs (Blesa et al., 2015; Islam, 2017; Jiang et al., 2016; Khusnutdinova et al., 2008; Kim et al., 2015; Lin and Beal, 2006; Mariani et al., 2005; Niedzielska et al., 2016; Rego and Oliveira, 2003).

Many, but not all, observational studies indicated that higher intake of antioxidants or supplements such as vitamin E could reduce the risk of NDDs (Engelhart et al., 2002; Luchsinger et al., 2003; Morris et al., 1998; Morris et al., 2002; Yaffe et al., 2004; Zandi et al., 2004; Zhang et al., 2002). Plasma vitamin E levels are associated with better cognitive performance (Boccardi et al., 2016), and patients with AD were found to have a lower nutrient status of vitamin E, C, B12 in blood and brain or cerebrospinal fluid (de Wilde et al., 2017; Lopes da Silva et al., 2014). Animal experimental studies also showed preventive effect of vitamin E supplementation on AD risk (Gugliandolo et al., 2017). However, most clinical trials have shown disappointing results in terms of the benefits on cognitive decline or dementia of antioxidants supplementation for NDDs, including vitamin E, C, B12, B6 and coenzyme Q10. Meta-analyses also suggested that there is no evidence of vitamin supplementation for prevention of cognitive decline or dementia in cognitively healthy adults (Rutjes et al., 2018), or for prevention of progression from mild cognitive impairment (MCI) to dementia, or for treatment of MCI, or for improvement of cognitive function in people with MCI or dementia due to AD (Farina et al., 2017; McCleery et al., 2018). Similar results were partly observed for PD (Negida et al., 2016; Zhu et al., 2017). A series of seminal papers on antioxidant therapy as well as the challenges in AD have been reviewed thoroughly (Mecocci et al., 2018; Mecocci and Polidori, 2012; Polidori and Nelles, 2014).

In order to disentangle the causality of antioxidants and AD, two Mendelian Randomization studies were conducted recently. In European-ancestry individuals, there is no causal association between circulating vitamin E levels (Liu et al., 2018), as well as ascorbate, β-carotene, retinol (Williams et al., 2018) and AD risk. However, the SNPs selected as vitamin E genetic instrument are also associated with lipid metabolism, and only each single SNP for ascorbate and retinol was selected, limiting the power of the analyses. Meanwhile, these instruments only explained a small portion of the total variance thus may not fully represent the circulating vitamin levels. It remains unclear to what extend the vitamin levels are associated with oxidative stress. Therefore, nevertheless the compelling biological plausibility and observational evidences, there is also lack of convincing data for the antioxidant supplementation aiming to prevent or treat NDDs.

4. Future Perspectives and Concluding Remarks

4.1 Reasons for the failure of antioxidant supplements in clinical trials

Based on the free radical theory and positive results from both experimental and observational studies, numerous clinical trials examining antioxidants, particularly vitamin E, have been conducted for the prevention and/or treatment of age-related morbidity and mortality. However, results are apparently disappointing as partly discussed earlier in part 3. Here, we will have an in-depth summary of the critical points in clinical trials with a focus on the widely-studied vitamin E supplementation.

Vitamin E is a well-known chain breaking antioxidant, as shown in **Figure 5**. Alpha-tocopherol competitively reacts with lipid peroxyl radical with higher reaction rate to inhibit chain propagation. Theoretically, abundant vitamin E will ameliorate oxidative damage by preventing lipid peroxidation. Not surprisingly, most observational studies specifically found detrimental/protective effects of high oxidative stress levels/antioxidants levels on disease status. However, oral vitamin E supplementation in middle-aged participants failed to demonstrate protective effects on neither primary and secondary prevention, nor on treatment of various age-related diseases. Several potential reasons for this failure have been discussed extensively (Brewer, 2010; Murphy, 2014; Robinson et al., 2006; Steinhubl, 2008). Data reported that the plasma vitamin E levels of participants with normal baseline blood tocopherol levels could only increase less than 2-3 times no matter the amount or duration of supplementation (Munteanu et al., 2004). Therefore, when individuals have a relatively low oxidative level and/or sufficient or high antioxidative level at enrollment in a study, it is highly unlikely to generate much benefit via extra supplementation, which might be explained as potential "functional concentration limit". This hypothesis is supported by studies in vitamin E supplementation stratified by haptoglobin genotype. Vitamin E supplementation provided cardiovascular-protective effects only in discriminatingly selected subgroup of individuals with both diabetes and Hp2-2 genotype (Hochberg et al., 2017; Seferovic et al., 2018; Vardi et al., 2013), who had high levels of oxidative stress and inferior antioxidant protection, in whom supplementation decreased cardiovascular events by 34% (Asleh et al., 2018). Besides, larger improvement was observed in nonalcoholic steatohepatitis patients carrying the Hp2 allele after vitamin E treatment compared with Hp1 allele carriers (Banini et al., 2018). Still, biological mechanisms underlying these observations should be explored in greater detail in future studies.

Another potential reason for the failure of vitamin E supplementation in clinical trials might lie in the difference of forms of tocopherols. In many studies, for example, in observational studies, vitamin E has been used, but this "vitamin E" was not specific to α-tocopherol and it is more about the combination of complicated tocopherol or tocotrienol isomers, while only α-tocopherol can be called "vitamin E" given its proved ability to avoid vitamin E deficiency (Azzi, 2018). Natural vitamin E has the highest bioactivity in the human body compared to other tocopherols or tocotrienols. However, most of the oral supplementation of vitamin E is synthetic, with lower bioactivity than the natural one, characterizing by preferentially non-oxidation metabolites in urine (Traber et al., 1998).

Besides, administration heterogeneity could result from various reasons and become critical in clinical trials, such as timing, monotherapy or not, dose and duration. Normally, it is hard to define a standardized strategy for supplement administration since chronic diseases often have an ambiguous onset time and the progression can take several years. Administration of the antioxidant supplements after irreversible ROS damage could have a negligible beneficial effect at that moment in time. Therefore, there might be a certain "critical window", and antioxidants administrated at an optimal timing before disease onset or during the early stages of disease could be able to ameliorate ROS-induced damage. Similarly, duration of intervention needs to be sufficiently long to observe these effects.

Moreover, there is still a lack of consensus about the proper dose regimen for antioxidant supplements. The doses used in clinic trials are derived from observational studies and are relatively safe and low. However, the dose for vitamin E to suppress plasma F_2-isoprostane concentration is 1600 IU or 3200 IU without observing any adverse effect after 16 weeks of supplementation, while the suppression of isoprostane were 35% and 49% respectively (Roberts et al., 2007). Importantly, the authors suggested that 49% reduction with the largest dose of 3200 IU/day was not so profound. Davies et al also concluded from two modeling techniques, that only a sufficiently high concentration of non-enzymatic antioxidants would have chance for collision and interaction with free radicals (Davies and Holt, 2018). However, oral intake of vitamin E only modestly increases the plasma and tissue vitamin E levels, and this decreases the concentration of vitamin E at target cells or organelles and slows down the reaction rate with ROS, which was quite unlikely to affect health outcomes (Stephens et al., 1996). And also, endogenously present defense systems will weaken the effects of non-enzymatic antioxidants when the reaction rate with free radicals is below a necessary threshold (Davies and Holt, 2018). Besides, some studies suggested vitamin E could also act as pro-oxidant after denoting a hydrogen atom to lipid radical (Kontush et al., 1996), so regenerating the reduced form from the oxidized form guarantees the lower depletion rate as well as the antioxidant function of vitamin E. Theoretically, combined administration of vitamin E and other antioxidants, such as ascorbic acid, coenzyme Q10 which can reduce oxidized vitamin E, might be protective. However, trials regarding this point are also inconsistent (Bjelakovic et al., 2007; Zandi et al., 2004).

Ageing and age-related diseases are multifactorial, heterogeneous and multidimensional. They do not respond to the organ-centered paradigm of “one cause - one mechanism - one disease/condition - one therapy”. Therefore, single therapeutic component that targets to only one aspect of the several factors contributing to aging and age-related diseases, even if highly weighted like oxidative stress, might be less effective as the solution by itself. Similarly, it is highly possible that only one component (vitamin E) might not be enough to show physiological significant effects on the complex, heterogeneous pathways of organ dysfunction with increasing age.

In addition to these potential explanations for the lack of significant findings of vitamin E supplementation, some opinions argued that oxidative damage could merely play a role in the pathophysiology instead of being a direct cause of diseases (Kim et al., 2015; Murphy, 2014; Niki, 2015). However, emerging evidence points to the possible etiological role of oxidative stress, especially ROS-induced mitochondrial dysfunction in NDDs (Cheignon et al., 2018; Islam, 2017; Zhao and Zhao, 2013). To demonstrate the causality, the best way is to directly measure the association of ROS, or ROS-induced damage change with pathological disorders or diseases, or the improvement of clinical phenotypes leaded by prevention of oxidative damage (Murphy, 2014; Murphy et al., 2011; Winterbourn, 2008). Yet, it is almost impossible to measure total ROS levels in the human body because of their chemical properties of short-existence and high-reactivity. Similarly, it remains a challenge to accurately measure oxidative damage because of the lack of reliable biomarkers as well as technical difficulties, further leading to the uncertainty of causality.

4.2 Potential oxidative stress biomarkers in clinical trials

After oral supplementation, vitamin E is digested and absorbed into the circulation by the body. However, plasma vitamin E concentrations after supplementation in healthy population with normal range of baseline vitamin E could largely vary from one individual to another which may arise from variations in the regulation of vitamin E uptake and metabolism between subjects, such as genetic factors, particularly apolipoprotein polymorphism (Roxborough et al., 2000). Besides, though no causal association was shown in the previous Mendelian Randomization of circulating vitamin E and Alzheimer's disease (Liu et al., 2018), this may raise another argument that even circulating levels, especially induced by synthetic vitamin E, should not be considered the same as functional levels. First, vitamin E has two metabolic ways, to go either through non-enzymatic, namely free radical-dependent metabolism, opening the hydroxy-chromanol ring, or through enzymatic (mainly CYP4F2 of cytochrome P-450 family) metabolism with only successive shortening of phytyl side chain (Torquato et al., 2016) (**Figure 6**). As synthetic vitamin E preferentially goes through non-oxidation pathway, it is essential to determine whether or not, it acts as antioxidant in the body. Second, oxidative damage is often located and gathered in certain tissues, specific cell types, or organelles. Although plasma circulating vitamin E are incorporated into lipoproteins and non-specifically transported to tissues, the acquisition of vitamin E seems to be largely mediated via the delivery and selective uptake of vitamin E after lipoprotein particles binding to receptor (Hacquebard and Carpentier, 2005). However, the mechanisms of the delivery and uptake of tissue vitamin E has been relatively poorly-characterized in other tissues except only for the hepatic tissue, and it appears to be essentially related to the expression and function of lipoprotein receptor. It is still hard to define the uptake level by many tissues precisely. Thus the accumulation of vitamin E could be different in various tissues, for instance, only limited amount of vitamin E in brain was observed after supplementation, or could be different for regions within the same organ, for example striatum contains the lowest amount of vitamin E compared with other areas in the brain (Shukitt-Hale et al., 2000). In addition, once taken up by cells, intracellular distribution of vitamin E to organelles is regulated by different transport proteins, but these proteins and their activity could differ from one tissue to another (Hacquebard and Carpentier, 2005). Hence the vitamin E concentration at different target tissues or organelles are largely differed, and there might be insufficient concentration in certain target locations to counteract with overwhelmed oxidative stress damage, particularly in the high energy-consuming organs such as brain. Obviously, the real functional vitamin E might be totally different with, to be more precisely, far less than the circulating vitamin E. Therefore, due to the differences in susceptibility to oxidative damage and response to vitamin E supplementation among individuals, even identical supplementation strategies (e.g. vitamin E type, administration protocol) may possibly not lead to same health outcomes. What matters most should be the ability to take advantage of circulating vitamin E by the body, namely to what extent, it acts as antioxidants. In brief, these issues can be simplified as the detection and monitoring of the antioxidative function of vitamin E.

Ideally, there are two aspects to monitor the effect of vitamin E, one is to measure robust oxidative stress levels in a range of pathologies of specific targets, as well as its alterations corresponding to vitamin E supplementation, and the other is to measure the direct level change of vitamin E acting as antioxidants. However, the major limitation of the first approach is the identification of reliable biomarkers. An oxidative damage biomarker which relates to the pathology could be promising, such as MDA, HNE, carbonyls, and particularly isoprostanes. These biomarkers have been identified in experimental and population-based studies (Bartoli et al., 2011; Dalleau et al., 2013; Strobel et al., 2011; van't Erve et al., 2017), together with the potential associations with ageing and age-related diseases. Nevertheless, the measured oxidative damages are often the result of the complex network of both endogenous and exogenous antioxidants systems. Moreover, these biomarkers are exclusive to certain macromolecule damage which cannot reflect the whole oxidative damage of the body. Although isoprostanes have been regarded as the most reliable biomarker with many merits mentioned in part 2.2.1 so far, notwithstanding, several possible drawbacks impede its further use. In the pathway of isoprostane formation, direct oxygen addition would compete with the second 5-exo cyclization involving a carbon-centered reaction, so the formation relies on low oxygen tension which gives priority to the cyclization reaction (Fessel et al., 2002; Milne et al., 2005). Besides, due to the ubiquitous existence of F_2-isoprostane in normal tissues, the production derived from kidney may confound the urine isoprostane concentration (Milne et al., 2005; Roberts and Morrow, 2000). This could also be an explanation why a larger increase of isoprostane was found in the kidney pathologies than other disease status (van't Erve et al., 2017). Apart from the generation pathway, quantitation of isoprostanes remains a challenge due to multiple steps in the detection method, which is labor intensive, as well as due to the impossibility to specify each isomer (Il'yasova et al., 2012). All these drawbacks hindered the possibility as a biomarker in large epidemiological studies or clinical trials to assess the specific oxidative damage change caused by vitamin E accurately.

In recent years, vitamin E metabolites have been proposed to help better elucidate vitamin E roles (Galli et al., 2017). Those metabolites reveal the biological process and reflect the authentic utility of vitamin E. Notably, identification of metabolites, especially free radical-dependent vitamin E metabolites, along with the determination of their levels may provide insights into oxidative stress-related physiological functions, as well as disease mechanisms and therapeutic strategies. When vitamin E acts as peroxyl lipid radical scavenger, it forms vitamin E radical, which further reacts with lipid peroxides into α-tocopherol quinone (α-TQ) (**Figure 6**) (Herrera and Barbas, 2001). In presence of NAD(P)H, α-TQ captures hydrogens converting into α-tocopherol hydroquinone (α-THQ), following the β-oxidation and cyclisation of the phytyl side chain, α-tocopheronic acid and α-tocopherol lactone (α-TL) are generated (Brigelius-Flohe and Traber, 1999). Finally, α-TL are excreted as polar glucuronidated and sulfated conjugates of α-TL hydroquinone (α-TLHQ) glucuronide and α-TLHQ sulphate (Sharma et al., 2013). Theoretically, disease states that are associated with increased oxidative stress are likely to have higher antioxidants requirements, which would result in depletion of circulating vitamin E, and consequently, higher concentration of oxidized vitamin E metabolites in urine, as a reflection of real antioxidant capacity.

Few studies have shown that oxidized vitamin E has been associated with diseases. A level of about 3% to 11% of α-tocopherol as oxidized form of α-TQ was found in all lipoprotein density fractions prepared from advanced human atherosclerotic plaque (Niu et al., 1999). Older individuals without baseline cognitive impairment and with the highest tertile of α-TQ/cholesterol had higher risk of prevalent dementia (Ravaglia et al., 2008). A recent study also indicated that both plasma α-TQ and 4-HNE were higher in participants with non-alcoholic fatty liver diseases, with lipid peroxidation as one of the earliest pathogenic events, compared with healthy participants (Torquato et al., 2019). Though it has been claimed that formation of α-tocopheronic acid and α-TL could be artificial products from oxidation of α carboxyethyl hydroxy chromans during the analytical process (Pope et al., 2000), Sharma and colleagues developed a feasible way to measure these conjugates with avoiding the artefacts (Gayatri et al., 2010), and found that the mean concentrations of α-TL conjugates were significant higher in children with type 1 diabetes compared with age-matched controls (Sharma et al., 2013). Notably, all these studies were carried out in a small sample size, and it remains unclear whether the metabolites levels are consistent with the oxidative damage. Efforts are still required to validate the potential biomarkers in large population-based studies, and further use as clinical monitoring at the individual level after supplementation of vitamin E.

4.3 Remarks and Conclusions

There is ongoing controversy in terms of antioxidant supplementation for prevention and/or therapy of ageing and age-related diseases. However, large amounts of money are still spent, and tens of trials with different population set and various administration strategies are in plan, regarding single antioxidants clinical trials. Of note, "multifactor - multi treatments" might be more effective since there might be an additional or a synergistic benefit from two agents with acceptable safety and efficacy, for example the combination of treatments (traditional treatment plus antioxidants treatment) to achieve better effects than antioxidants only. Interestingly, a recent trial showed that middle-aged type 2 diabetic patients who received metformin treatment plus vitamin E and/or vitamin C had significantly improvement of glucose measures as well as lipid profiles compared to patients with metformin treatment alone, indicating that antioxidants use might be an adjuvant therapy in the management in type 2 diabetic patients (El-Aal et al., 2018). But the combination use still requires additional efforts.

In summary, there are two critical points in clinical trials related to oxidative stress that need to be further discussed: (i) Target population. Participants should be selected by the stratification of certain features (e.g., genotype) which could induce significantly heterogeneous responses. So the selected participants subgroup are most likely to benefit from vitamin E supplementation. For example, vitamin E supplementation has been shown to be associated with an approximately 35% reduction in CVD specifically in individuals with both diabetes and Hp 2-2 (Hochberg et al., 2017). Besides, a recent study also suggested that alpha-tocopherol supplementation was beneficial for cancer prevention only among carriers of homozygous low-activity alleles in the catechol-O-methyltransferase gene (Hall et al., 2019). Therefore, there is still need for further studies aiming to identify potential geno-/pheno- types which could be a determinant of the possible benefits of supplementations, as well as the elucidation of underlying mechanisms. (ii) Reliable biomarkers. To date, there is still little consensus about the best systemic biomolecular measurements of oxidative stress. An optimal biomarker should be an authentic reflection of overall redox status, easily accessible, reasonably stable, easy to be detected accurately. Moreover, since ROS has properties of second messenger which is necessary for maintenance of cellular homeostasis, as well as normal physiological function, it remains obscure to define an optimal range of an oxidative damage-induced biomarker from physiological to pathological disorders, i.e. which level is detrimental for health, or promotes longevity and metabolic health outcomes.

There are rather convincing evidence that oxidative damage has an important role in ageing and the pathogenesis multiple age-related diseases. Yet, clinical trials did not demonstrate the preventive or therapeutic role of antioxidants supplementation. However, this does not mean the failure of the oxidative stress theory and use of antioxidants. On the contrary, it compels us to rethink this issue with several open-ended questions: (i) Are these associations reliable in observational studies which could be biased by confounders and reverse causality. (ii) How do we infer the causality of oxidative stress and diseases on population level. (iii) What should be the best biomarker of oxidative stress in clinical trials. (iv) How to develop proper preventive or therapeutic strategies taking all the critical points into account. Therefore, researches in the coming years should be devoted to more in-depth studies examining the role of more direct measure of oxidative stress in biofluids in the pathogenesis of age-related diseases and the ageing process, and to clarify the causality lying between oxidative stress and age-related diseases and the ageing process.

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Declarations of interest

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Figure captions

Figure 1 ROS levels and health effects: a dose-response model

Detailed discussion in the text part 2.2.

Figure 2 Schematic review of oxidative stress theory in ageing and age-related diseases

Figure 3 Trends in Antioxidants supplementation in the US

NHS: Nurses' Health Study, HPFS: Health Professionals Follow-up Study, NHANES: National Health and Nutrition Examination Survey. Data presented in the graph of NHS and HPFS are from 1986 to 2006, and data of NHANES are from 1999 to 2012.

Figure 4 Reactive oxygen species generation

O_2-•:Super oxide anion, SOD: super oxidize dismutase, H_2O_2: hydrogen peroxide, •OH hydroxyl radical, MPO: myeloid peroxide, HClO: hypochlorous acid, $^1O_2$: singlet oxygen, NOS: nitric oxide synthase, NO•: nitric oxide radical, ONOO-: peroxynitrite, ONOOH: peroxynitrous acid, ONO•: nitrogen dioxide, GSH: glutathione, GSSG: glutathione disulfide, GPx: glutathione peroxidase, CAT: catalase, NADP+: Nicotinamide adenine dinucleotide phosphate, NADPH: Nicotinamide adenine dinucleotide phosphate reduced form, GR: glutathione reductase.

The bulk of ROS are mainly generated by the mitochondrial electron transport chain during normal aerobic metabolism in addition to multiple ways encompassing cytosolic enzyme systems (NADPH oxidase), monoamine oxidase on the outer membrane of mitochondrial, xanthine oxidase and uncoupled NOS. In the electron transport chain, oxygen molecules are univalent reduced into O_2-• with the leak of electrons. The formation of superoxide is the initial step and the start of a cascade reaction of other ROS generation. Once generated, it could be catalyzed spontaneously by SOD into H_2O_2. Subsequently in the presence of transition cation with reduced form (Fe^{2+} or Cu^+, referred to as the Fenton reaction) or myeloid peroxide (MPO), H_2O_2 further dismutates to •OH. Meanwhile, H_2O_2 can also be reduced into water by the enzymatic antioxidants such as CAT and GPx. Haptoglobin binds hemoglobin with high affinity and stability, preventing the release of heme iron from hemolysis into circulation, consequently terminating Fenton reaction and preventing the production of •OH.

Figure 5 Lipid peroxidation chain reaction induced by free radicals

L: lipid radical, LOO•: lipid peroxyl radical, LOOH: lipid hydriperoxides, LOOL: peroxide bridged dimer, L-L: fatty acid dimer, LOH: aldehydes (e.g., MDA, HNE).

Three steps of lipid peroxide chain reaction:

Initiation: ROS (especially hydroxy radicals) initially trigger a reactive hydrogen atom extraction from the methylene group of polyunsaturated fatty acid forming carbon-centered radicals at the allylic position, and then react with oxygen molecule thus forming a peroxyl radical. Propagation: Peroxyl radical, as an intermittent radical in the reaction chain, may further react with nearby lipids, yielding lipid hydroperoxides (LOOH), as well as new carbon-centered radicals, resulting in the chain propagation and amplification. With the presence of transition metals, lipid peroxide can generate lipid alkoxyl (LO•) which further extracts a hydrogen atom from nearby lipids forming another new carbon-centered radical, thereby repeating the whole process as a vicious circle since the initial proton extraction, and sustain the chain oxidation. Termination: Through cyclization or degradation, lipid peroxyl radical and lipid alkoxyl can form a myriad of reactive aldehydes, alkanes and alkenes.

Figure 6 Alpha-tocopherol metabolism

The left panel shows the non-enzymatic metabolic pathway of α-tocopherol. Alpha-tocopherol reacts with lipid peroxyl radicals, forming α-tocopherol radical, which further reacts with lipid peroxides, following hydrolyzation, generating α-tocopherol quinone (α-TQ). In presence of NAD(P)H, α-TQ captures hydrogens converting into α-tocopherol hydroquinone (α-THQ), following the β-oxidation and cyclisation of the phytyl side chain, α-tocopheronic acid and α-tocopherol lactone (α-TL) are generated. Finally, α-TL are excreted as polar glucuronidate and sulfate conjugates of α-TL hydroquinone (α-TLHQ). The right panel presents the enzymatic metabolic pathway of α-tocopherol. This process is initiated in the liver, with the hydroxylation of the methyl group by hepatic CYP enzyme. Subsequently, the phytyl side chain shortens successively with the removal of carbon units in β-oxidation. Finally, α-carboxyethyl-hydroxychroman (α-CEHC) are generated and excreted as their polar glucuronide or sulfate conjugates.

Figure 1

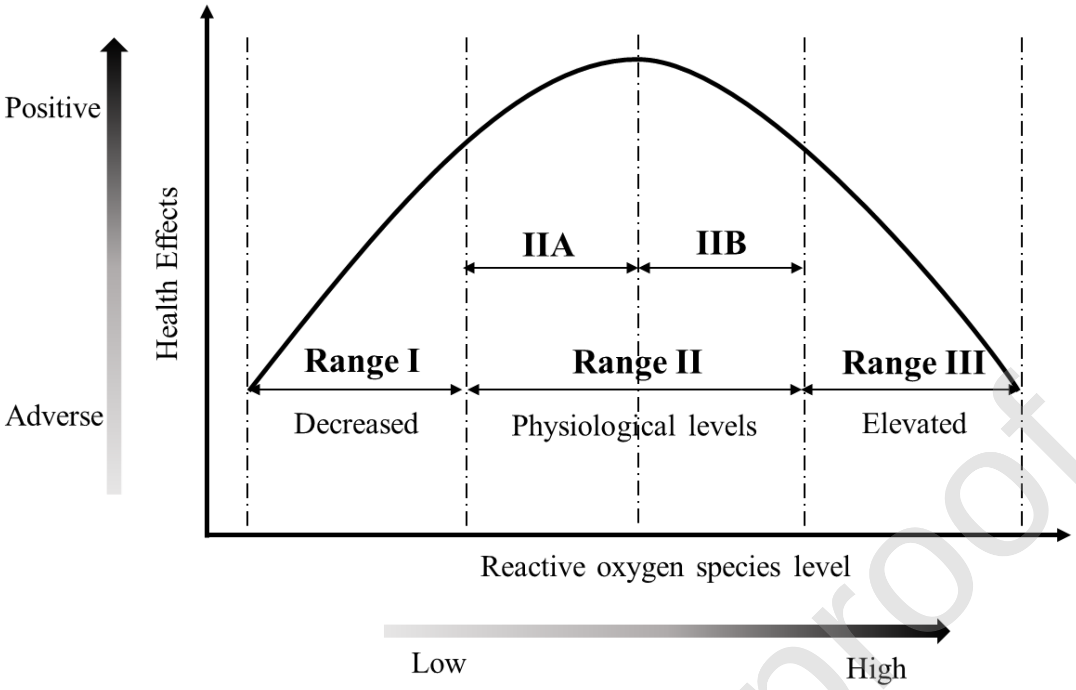

Positive
Adverse
Health Effects
IIA
IIB
Range I
Range II
Range III
Decreased
Physiological levels
Elevated
Reactive oxygen species level
Low
High

Figure 2

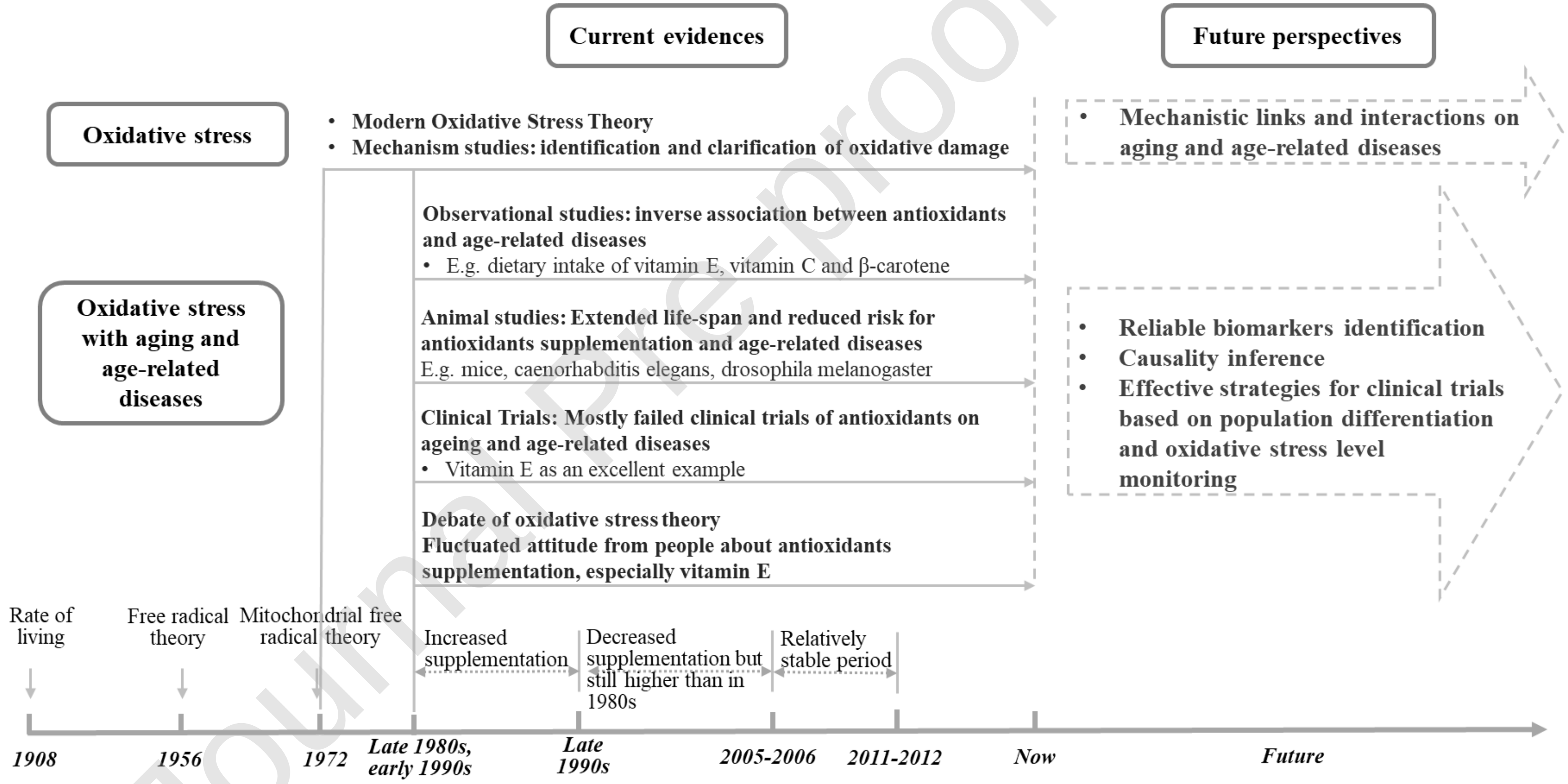

Current evidences
Future perspectives
Oxidative stress
• Modern Oxidative Stress Theory
• Mechanism studies: identification and clarification of oxidative damage
• Mechanistic links and interactions on aging and age-related diseases
Oxidative stress with aging and age-related diseases
Observational studies: inverse association between antioxidants and age-related diseases
• E.g. dietary intake of vitamin E, vitamin C and β-carotene
Animal studies: Extended life-span and reduced risk for antioxidants supplementation and age-related diseases
E.g. mice, caenorhabditis elegans, drosophila melanogaster
Clinical Trials: Mostly failed clinical trials of antioxidants on ageing and age-related diseases
• Vitamin E as an excellent example
Debate of oxidative stress theory
Fluctuated attitude from people about antioxidants supplementation, especially vitamin E
• Reliable biomarkers identification
• Causality inference
• Effective strategies for clinical trials based on population differentiation and oxidative stress level monitoring
Rate of living
Free radical theory
Mitochondrial free radical theory
Increased supplementation
Decreased supplementation but still higher than in 1980s
Relatively stable period
1908
1956
1972
Late 1980s, early 1990s
Late 1990s
2005-2006
2011-2012
Now
Future

Figure 3


Proportion of population (%)
55
50
45
40
35
30
25
20
15
10
5
0
1986
1990
1994
1998
2000
2002
2004
2006
2008
2010
2012
Year
β-carotene (HPFS)
β-carotene (NHS)
Vitamin B6 (HPFS)
Vitamin B6 (NHANES
Vitamin B6 (NHS)
Vitamin C (HPFS)
Vitamin C (NHANES)
Vitamin C (NHS)
Vitamin E (HPFS)
Vitamin E (NHANES)
Vitamin E (NHS)

Figure 4

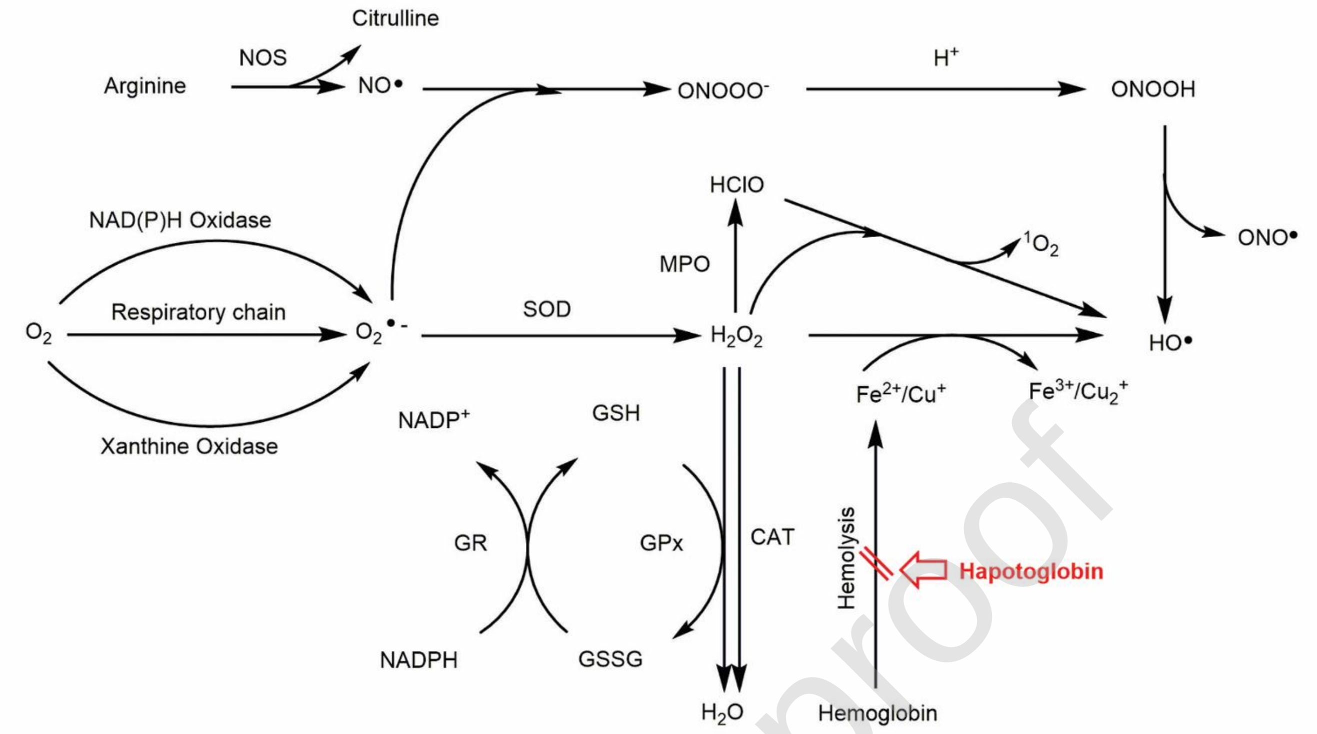

Citrulline
NOS
Arginine
NO•
ONOOO-
H+
ONOOH
HClO
NAD(P)H Oxidase
MPO
1O2
ONO•
Respiratory chain
O2
O2•-
SOD
H2O2
HO•
Fe2+/Cu+
Fe3+/Cu2+
Xanthine Oxidase
NADP+
GSH
GR
GPx
CAT
Hemolysis
Hapotoglobin
NADPH
GSSG
H2O
Hemoglobin

Figure 5

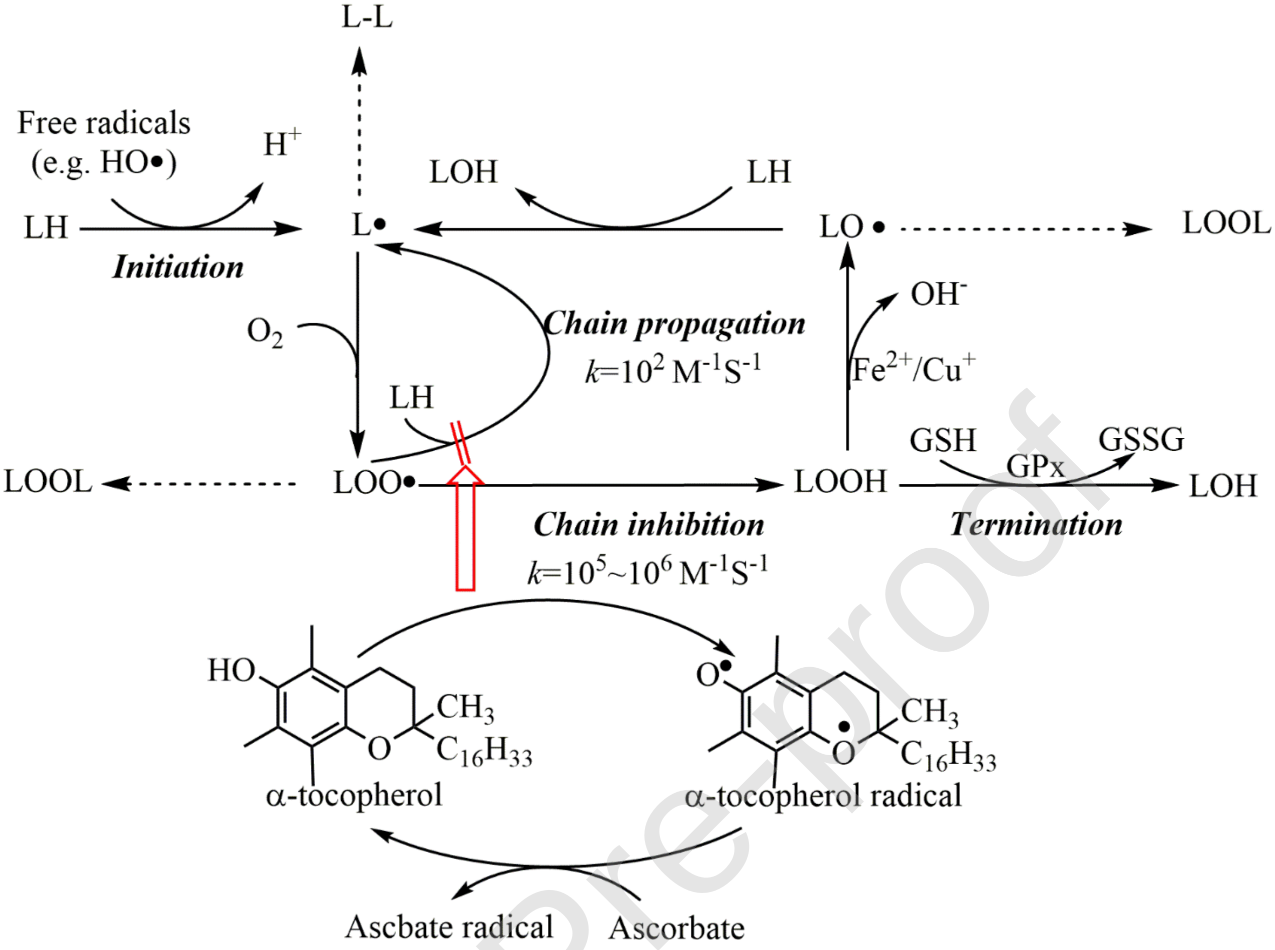
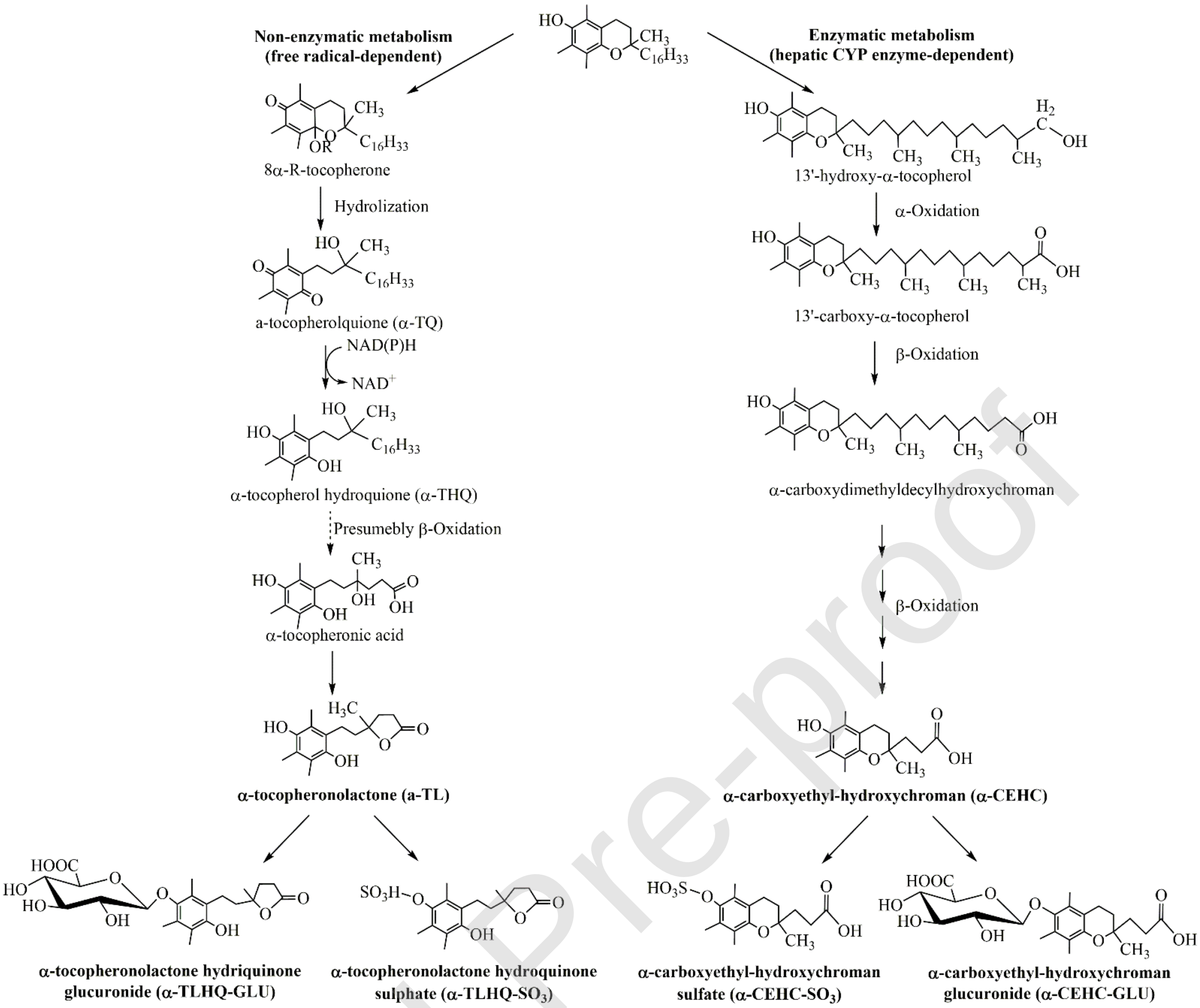

L-L
Free radicals
(e.g. HO•)
H+
LOH
LH
LH
L•
LO •
LOOL
Initiation
OH-
O2
Chain propagation
k=10^2 M^-1S^-1
Fe2+/Cu+
LH
GSH
GSSG
GPx
LOOL
LOO•
LOOH
LOH
Chain inhibition
k=10^5~10^6 M^-1S^-1
Termination
HO
CH3
C16H33
O
O•
CH3
C16H33
O
α-tocopherol
α-tocopherol radical
Ascbate radical
Ascorbate

Figure 6


HO
CH_3
$C_{16}H_{33}$
Non-enzymatic metabolism
(free radical-dependent)
Enzymatic metabolism
(hepatic CYP enzyme-dependent)
OR
8α-R-tocopherone
Hydrolization
a-tocopherolquione (α-TQ)
NAD(P)H
NAD^+
α-tocopherol hydroquione (α-THQ)
Presumebly β-Oxidation
α-tocopheronic acid
H_3C
α-tocopheronolactone (a-TL)
13'-hydroxy-α-tocopherol
α-Oxidation
13'-carboxy-α-tocopherol
β-Oxidation
α-carboxydimethyldecylhydroxychroman
β-Oxidation
α-carboxyethyl-hydroxychroman (α-CEHC)
HOOC
SO_3H
HO_3S
α-tocopheronolactone hydriquinone glucuronide (α-TLHQ-GLU)
α-tocopheronolactone hydroquinone sulphate (α-TLHQ-SO_3)
α-carboxyethyl-hydroxychroman sulfate (α-CEHC-SO_3)
α-carboxyethyl-hydroxychroman glucuronide (α-CEHC-GLU)