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Review

Approaching reactive species in the frame of their clinical significance: A toxicological appraisal

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ABSTRACT

Redox biology and toxicology are interrelated fields that have produced valuable evidence regarding the role and clinical significance of reactive species. These issues are analyzed herein by presenting 6 arguments, as follows: *Argument 1*: There is no direct connection of redox-related pathologies with specific reactive species; *Argument 2:* The measurement of reactive species concentration is a major challenge due to their very short half lives; *Argument 3:* There is an interplay between reactive species generation and fundamental biological processes, such as energy metabolism; *Argument 4:* Reactive species exert beneficial biological action; *Argument* 5: *Reactive species follow the hormesis phenomenon*; *Argument 6:* Oxidative modifications of redox-related molecules are not necessarily interpreted as oxidative damage. We conclude that reactive species do not seem to exert clinical significance, which means that they lack a measurable cause-effect relation with chronic diseases. Unpredictable results could, nevertheless, arise through novel experimental setups applied in the field of toxicology. These are related to the real-life exposure scenario via the regimen of long-term low-dose (far below NOAEL) exposure to mixtures of xenobiotics and can potentially offer perspectives in order to investigate in depth whether or not reactive species can be introduced as clinically significant redox biomarkers.

1. Introduction

Redox biology is a wide research field that is rapidly expanding and is interrelated with molecular biology and toxicology. Its main objective is to investigate the biological roles of reductions and oxidations and, thus, molecules such as reactive species (and other redox biomarkers) are of main concern due to their ability to alter redox status of cells, tissues, organs and whole organisms (Halliwell and Gutteridge, 2015). The modification of redox homeostasis is a distinct property of reactive species indicating their fundamental role not only in health but also in disease (Quijano et al., 2016; Ghezzi et al., 2017). Free radicals, molecules that belong to the class of reactive species, were associated with pathology for the first time in 1956 when Denham Harman formulated the "*free radical theory of aging*" (Harman, 1956). During the following decades free radicals, and reactive species in general, were inextricably linked to oxidative stress and numerous pathological conditions (Valko et al., 2007). As a result, several chronic pathologies are often known as redox-related as is the case for type 2 diabetes (Watson, 2014) and neurodegenerative diseases (Sbodio et al., 2019). Nevertheless, the clinical significance of reactive species and other redox biomarkers is still questioned and this is one of the main research challenges in the topic of redox biology for the upcoming years. To this end, the main objective of this opinion article is to examine the existing evidence with respect to whether reactive species exert clinical significance on the basis of the currently available knowledge under the real life risk simulation (RLRS) concept (Tsatsakis et al., 2019a). This is a very recently adopted idea that approaches the real-life exposure scenario concerning the putative harmful impact of exposure of humans to mixtures of xenobiotics normally encountered during their everyday routine (Tsatsakis et al., 2016; Fountoucidou et al., 2019). In fact, we describe with vivid examples how reactive species participate in disease onset and in toxicity induced by xenobiotics such as pesticides and heavy metals and we try to analyze the future perspectives based on novel experimental approaches in the field of toxicology (Tsatsakis et al., 2016, 2017, 2019b).

2. Oxidative stress: defined and re-defined

The oxidative stress theory of disease has been quite popular in the field of redox biology during the last decades. The term *oxidative stress*,

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although implied in older studies, did not appear in the literature before 1970 when Paniker and colleagues linked it to glutathione metabolism in oxidatively challenged erythrocytes (Paniker et al., 1970). The first and most influential definition of this biological phenomenon was given by Helmut Sies in the middle 80's as "a *disturbance in the prooxidant-antioxidant balance in favor of the former*" (Sies, 1985). Due to the progress of modern research and the accumulation of knowledge, oxidative stress was re-defined by Dean Jones 21 years later as "*a disturbance in the prooxidant/antioxidant balance in favor of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage*" (Jones, 2006). The latter definition associates oxidative stress with redox signalling, an essential biological process for normal cell function. Helmut Sies in his definition linked oxidative stress with prooxidants and antioxidants in the first place. Noteworthy, this definition satisfied the needs of the field during that time and although it still remains popular, it is considered today as vague and obsolete. The most important reason is that it refers to an equilibrium, which obviously can literally be built only if its two legs are numerically expressed in a given studied tissue. However, this seems to be a very hard task since the estimation of the levels of all prooxidants and antioxidants is impaired. Regarding prooxidants, this definition comprises reactive species also. We should note here that, according to the current knowledge, specific reactive species are oxidants because they directly oxidize specific substrates, whereas other reactive species [e.g., hydrogen peroxide (H_2O_2)] are pro-oxidants because they lead to the formation of oxidants, thus, acting indirectly as oxidizing agents.

3. The role of reactive species in disease according to the theory of causes introduced by Kenneth Rothman and the theory of signs and semiotics proposed by Charles Sanders Peirce

Redox biomarkers, as defined recently by our group, include antioxidant molecules that their concentration or activity is affected by reactive species, biological entities that are generated by the interaction of reactive species with biomolecules and the reactive species *per se* (Veskoukis et al., 2019). Such biomarkers are used to monitor the potential alterations in tissue redox profile induced by stimuli the most common of them being exercise, nutrition, aging, disease and toxicological interventions (Veskoukis et al., 2012a,b; Ghezzi et al., 2017; Docea et al., 2018). With respect to disease, there is increasing experimental evidence correlating oxidative stress (thus, reactive species and other redox biomarkers as its endpoints/indices) with numerous chronic pathologies (Halliwell and Gutteridge, 2015; Poprac et al., 2017). Indeed, it is established that reactive species are involved in several chronic pathologies (e.g., ocular disease, tissue injury in general and cardiovascular diseases) (Kehrer and Klotz, 2015; Griendling et al., 2016; Ung et al., 2017). Nevertheless, although there is almost no disease negatively correlated with oxidative stress, at least according to our knowledge to date several redox biomarkers and reactive species in particular do not seem to have a cause-effect relation with any pathological condition, that is there is not even one reactive species that has been described as the main cause of a chronic disease. What we want to retain here, hence, is that reactive species, on the basis of the currently available research findings, do not exert clinical significance regarding chronic diseases. In other words, there is no experimental evidence indicating that specific redox biomarkers (i.e., reactive species) can be used as indices for the diagnosis of redox-related chronic pathologies and, as a result, no antioxidants are known to have the ability to act as remedies or therapeutic agents against chronic diseases. However, they have been correlated to the effects of acute conditions that lead to disruption of tissue redox status.

Here, we should stress two important issues regarding firstly the oxidative stress-based mechanisms of metal toxicity and, secondly, the role of antioxidants on facing the detrimental impact of several toxicological treatments in order to approach recent evidence regarding the role of reactive species in chronic diseases under a toxicological appraisal. Oxidative stress is the basic mechanism of toxicity of several metals, such as lead and cadmium (Flora et al., 2015; Andjelkovic et al., 2019). Indeed, it has been demonstrated that lead and cadmium induce lipid peroxidation as manifested by enhancement of malondialdehyde concentration in plasma and kidney and decrease total antioxidant capacity of liver in rats (Andjelkovic et al., 2019). This study is in line with previous experimental evidence supporting the idea that metal toxicity is redox-related via inducing oxidative stress and causing the depletion of the antioxidant mechanisms in a tissue-specific manner (Matović et al., 2015). It has also been pointed out that the toxic action of chromium, whose sources are food, water and commercial products depends on its oxidation state since compounds that contain hexavalent chromium are approximately 100 times more toxic than their trivalent counterpart (Soares et al., 2010). To this end, an interesting study has observed that chromium induces anomalies in the immune system by decreasing the number of myeloid cells and neutrophils (Karaulov et al., 2019). Interestingly, these detrimental effects are oxidative stress-based as the authors have shown that the levels of conjugated dienes and malondialdehyde are increased in spleen and liver and the activities of catalase and superoxide dismutase are impaired in erythrocytes in rats (Karaulov et al., 2019). Similarly, oxidative stress is the basic mechanism for the toxic action of fully brominated diphenyl ether (BDE-209) that is a flame retardant present in textiles and plastic and, therefore, humans can be exposed to it during their everyday routine (Milovanovic et al., 2018). Specifically, BDE-209 leads to elevated thiobarbituric acid reactive substances concentration and reduced levels of total –SH groups in rat kidney, findings that are backed up by previous relevant literature results (Buha et al., 2015). Concerning the second issue, it has been reported that vitamin C, a strong antioxidant molecule, is effective on ameliorating the toxic effects of paraquat, a widely used herbicide (Hu et al., 2018). Indeed, two clinical studies have indicated that therapy with antioxidants can alleviate the harmful effects of paraquat (Hong et al., 2002; Moon et al., 2011). In addition, reduced glutathione (GSH) is also a decisive factor for paraquat toxicity since tissues and cells that suffer from GSH depletion are more vulnerable to paraquat (Nakagawa et al., 1995). The beneficial role of numerous antioxidants, namely GSH, quercetin, gallic acid, N-acetyl cysteine and α-lipoic acid against heavy metal toxicity cannot be also overlooked (Flora et al., 2013).

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor and the master signalling regulator of antioxidant and oxidant responses following redox altering stimuli, such as exposure to toxic agents and pathological conditions (i.e., cancer and diabetes) (Tebay et al., 2015; Fu et al., 2016). Under physiological conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap-1) in the cytoplasm, which is a cysteine-rich negative regulator and leads to the ubiquitination and, finally, to the degradation of Nrf2 (Vargas-Mendoza et al., 2019). In oxidative stress context, however, the formed reactive species oxidize Keap-1 and Nrf2 is released. Subsequently, Nrf2 enters the nucleus and binds to the antioxidant response elements promoting the expression of genes coding for enzymes belonging to the antioxidant mechanism (e.g., catalase, superoxide dismutase) and for detoxifying enzymes (e.g., glutathione-S transferase) (Raghunath et al., 2018). After Nrf2 has acted towards the reinforcement of the antioxidant defence and following the elimination of the oxidative stress-induced stimulus, it is transported outside the nucleus via the nucleus export signal (Sun et al., 2007). It appears that the activated Nrf2 pathway is a major regulator of the antioxidant defence of tissues against oxidative insults. Concomitantly, it has been proposed that Nrf2 activation may be detrimental since it promotes oxidative situations in chronic diseases and mainly cancer (DeNicola et al., 2011). The idea is that the antioxidant defence of cancer cells is stronger through constitutive Nrf2 activation, which can be the result of the induction of the transcription Kruppel-like factor 9 (Zucker et al., 2014). Several studies have reported such findings suggesting that Nrf2 activation may lead to cancer promotion and/or progression and metastasis, hence Nrf2 inhibitors could be used

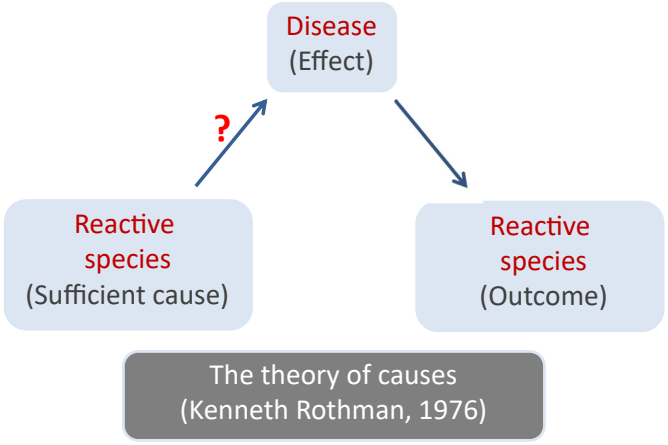

Fig. 1. The absence of causality between reactive species and disease. A cause-effect relation between reactive species and chronic disease presupposes that reactive species are the sufficient cause of the effect (i.e., disease), as defined by Kenneth Rothman in 1976. However, this is not established yet. Reactive species are the outcome of numerous pathologies, though.

as anticancer drugs (Panieri et al., 2020; Wang et al., 2016; Tao et al., 2017).

Based on the above and taking into account that several antioxidants are beneficial against several cancer types due to Nrf2 inhibition and against the toxicity of a wide range of xenobiotics, it is implied that reactive species are crucial factors for the manifestation of these toxic effects. However, such toxic effects are not chronic pathologies, and do not constitute the main consideration of the present article. We examine herein the role of reactive species as a cause for the onset of chronic diseases that are routinely encountered in the general population. On that basis, we cannot refuse the inextricable relation of reactive species with several diseases. However, this relation cannot be characterised as bidirectional for the moment. Indeed, as highlighted above, the generation of reactive species and their harmful action on biomolecules is a symptom but we are not sure whether they are also a *sufficient cause* of disease (Fig. 1). The term *sufficient cause* has been defined by Kenneth Rothman more than 40 years ago as "*a cause that inevitably, alone or in conjunction with other causes initiates or permits a sequence of events resulting in an effect*" (Rothman, 1976).

This is a vivid semiotic approach that reminds us the theory of signs as postulated by the father of semiotics Charles Sanders Peirce, (1932). Peirce defined the idea of *semiosis* as "*an action, or influence, which is, or involves, a cooperation of three subjects, such as a sign, its object, and its interpretant, this trirelative influence not being in any way resolvable into actions between pairs*" and the term *semiotic* as the "*quasi-necessary, or formal doctrine of signs*" (Peirce, 1932). The theory of signs is easily described in the form of examples pumped out of everyday life. As defined by Peirce, this theory involves a triangle of subjects namely the sign, its object and the interpretant. Let us give an example (Fig. 2). A person walks across a road and observes a lightning behind the hill far away from the small town he resides. The *observation of the lightning is the interpretant* according to Peirce and *the lightning itself is the sign* of rain at a village behind the hill. In this example, *rain is the object*. Although this sign is linked to its object, there is also the case that there are further signs with one or two degrees of separation from the aforementioned object (i.e., rain behind the hill). Interestingly, the black clouds seen from far away constitute a sign with one degree of separation from rain since their existence does not necessarily mean that it is raining in the village in question. Furthermore, a clap heard by the person of our story during his presence inside his house is a sign with two degrees of separation from the object. It is possible that the person misunderstood the origin of this noise, which could be derived from fireworks, for example, and not from a lightning (i.e., the sign of rain). Therefore, the impression that it is raining somewhere close is totally mistaken. Apart from the signs there is also the notion of *icon*. In this example, an icon could be a TV weather forecast that predicts a rainy day in the village behind the hill. However, there is also the possibility of failure because it is largely based on probabilities (Peirce, 1955).

The experimental evidence available to date with respect to the clinical significance of reactive species could be aptly parallelized with this quite insightful theory (Fig. 2). A *redox-related disease (e.g., cancer) or condition (e.g., aging) is the object* according to Peirce. A *sign of the object (i.e., cancer) is a tumour metastasis* and *a general weakness or chronic low-grade inflammation in several tissues are considered as signs of aging*. As stated in the previous paragraphs, the bidirectional relation of redox biomarkers with redox-related diseases is not yet established (Ghezzi et al., 2018). As a corollary, *reactive species are considered as icons of disease and not as signs*. To further get into it, both cancer and aging process induce tissue damage associated with oxidative modifications of biomolecules (Sosa et al., 2013; Liguori et al., 2018). Apart from reactive species, biomarkers of protein and lipid oxidation, such as protein carbonyls and malonyl dialdehyde (MDA), respectively are icons with one degree of separation from the object since they are referred to a molecular symptom without being connected to diagnosis or therapy. The activity of antioxidant enzymes (e.g., catalase) on the other hand is an icon with two degrees of separation from the object because it is not clear what exactly they stand for. To sum up, it appears that reactive species and other redox biomarkers are not part of the semiotic triangle (i.e., object, sign, interpretant) proposed by Peirce meaning that, as far as we know today, they lack clinical relevance regarding chronic pathologies and not acute stimuli able to alter redox status.

4. Six arguments to support why reactive species lack clinical significance, on the basis of the currently available knowledge

In the previous sections we have focused on the role of redox biomarkers and mostly reactive species in disease and degenerative conditions (e.g., aging). Furthermore, using the vivid example illustrated in Fig. 2 we have tried to extrapolate this role on the grounds of the theory of signs and semiosis. We assert that, based on the available scientific evidence, reactive species seem to lack clinical relevance. According to our opinion there are 6 arguments for that. These arguments are depicted in Fig. 3 and analyzed hereby.

Argument 1. *There is no direct connection of redox-related pathologies with specific reactive species*

As mentioned above, it is indisputable that there are diseases whose pathology is redox-related. A few of the most common examples are cancer (Bekeschus et al., 2017), neurodegenerative diseases (Lepka et al., 2016) and diabetes (Watson, 2014). It is well established that the generation of reactive species in blood and tissues is an outcome of such pathologies (Halliwell and Gutteridge, 2015). Nevertheless, reactive species are not yet considered as real causes of diseases, although this could hold true in the future (Fig. 3). The issue is much more complicated because there is an obvious question emerging: "which reactive species are correlated to the development of cancer, neurological pathologies or diabetes?" The answer is easy to be given: "We still do not know". Consequently, it is unknown whether reactive species cause disease onset or which of them could be related to specific pathologies. This is why antioxidants, the compounds that fight or inhibit the generation of reactive species have not been validated for therapeutic reasons. This could mean that reactive species are not causes of disease but more possibly that, given the unknown role of each reactive species in specific diseases, the antioxidants that do not also exert specificity for reactive species are ineffective. Additionally, it is usually a hard task to translate the results obtained by the measurement of specific redox biomarkers, other than reactive species, in clinical context. For

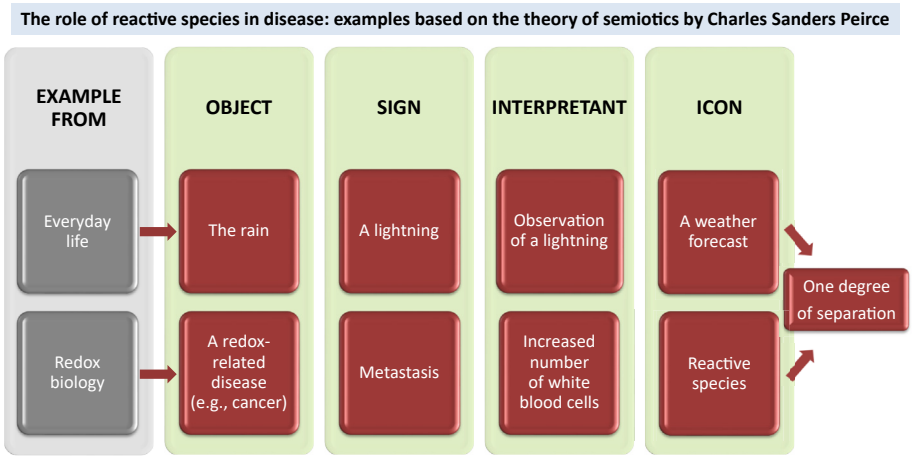

Fig. 2. A vivid illustration of the current knowledge regarding the clinical significance of reactive species. This figure depicts a parallelization of the main question of this opinion article with the theory of semiotics as formulated by Charles Sanders Peirce approximately 9 decades ago. We report that to date there is no sufficient evidence supporting the notion that reactive species are clinically significant. They could rather be characterized as an icon of the object (i.e., disease) with one degree of separation, according to Peirce.

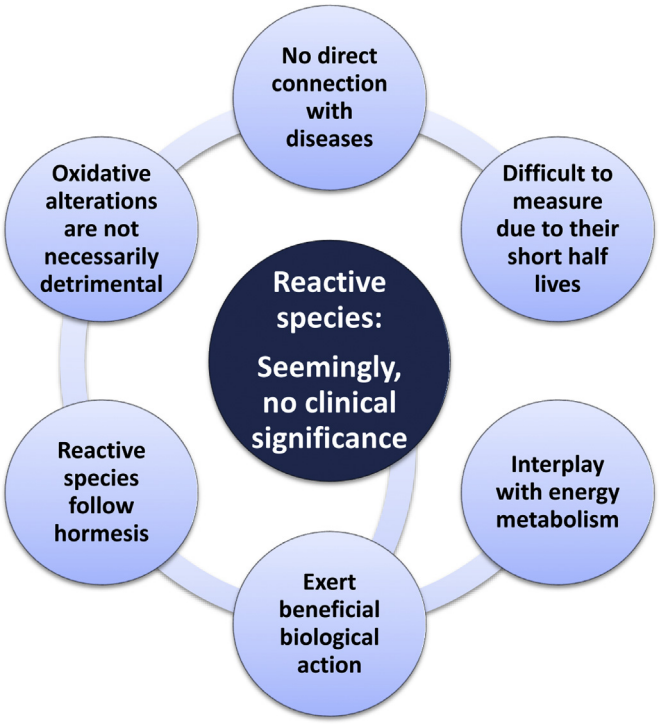

Fig. 3. Six arguments, as analyzed in the present study, why reactive species (defined as redox biomarkers) do not seem to exert clinical significance on the basis of the currently available knowledge.

example, the increase in the activity of catalase (a crucial antioxidant enzyme) in erythrocytes collected by a patient suffering from a redox-related disease after antioxidant administration as a putative therapeutic strategy can be interpreted twofold. The administered compound could have reinforced the tissue redox profile potentially protecting the patient. Nevertheless, it is possible that the patient will not be benefited since catalase scavenges H_2O_2 that in the specific pathology may not play a pivotal role. On the other hand, H_2O_2 could possibly be a determinant factor for the outcome of this particular redox-related disease, therefore induction of catalase activity is a defensive mechanism for a systemic protection. Today, there is not enough evidence, however, thus we cannot reach any of the two aforementioned conclusions.

Argument 2. *The measurement of the concentration of reactive species is a major challenge due to their very short half lives*

Two decades ago the measurement of the concentration of reactive species directly even *in vitro* was a really difficult drill due to their extremely low half lives estimated between 10^{-9} s and 10^{-3} s for hydroxyl radical ($HO^{\bullet}$) and H_2O_2, respectively (Dickinson and Chang, 2011). This was mainly due to the experimental tools, which did not offer the ability to conduct such advanced research. During the last years, though, several assays have been developed in order to assess their levels both *in vitro*, *in vivo* and *ex vivo* (Pavelescu, 2015). Among them, the technologies of reactive oxygen species (ROS) measurement with fluorescent dyes, genetically encoded reporters, nanoparticle systems and nanotube probes have been developed and the researchers have achieved to conduct this kind of experiments with worth mentioning accuracy (Woolley et al., 2013). Nevertheless, the problem remains. Regarding the fluorescent probes, the results gleaned by their usage can be easily compromised. Indeed, the irreversible reaction of a probe with a reactive species will give higher fluorescence values if it is found in compartments or organelles with high oxidative potential. Furthermore, due to the high rate of reaction of reactive species with antioxidant compounds the fluorescence dyes cannot compete them, thus the obtained concentration of reactive species measured will not be true again (Wooley et al., 2013). Therefore, it becomes evident that the spatiotemporal characteristics of the measured reactive species are an essential factor for the quantification of their amounts, a fact that confines the translational value of the fluorescence dyes. Similar issues emerge when using genetically encoded reporters since they are dependent from the pH of the subcellular compartment that the reactive species in question is present. This means that the factor of the spatial presence of reactive species is also confounding (Roma et al., 2012). From the above it can be concluded that, although useful tools have been developed and can offer important insight for the *in vitro* measurement of reactive species, they have specific drawbacks, hence the interpretation of the findings they generate needs extra caution (Fig. 3). In order to partially overcome this issue, a common practice is to use tests that measure the concentration of a wide range of reactive species indirectly. By doing this, interesting experimental evidence can be acquired, with questionable accuracy though. Therefore, the measurement of a battery of specific biomarkers functionally clustered that holistically assess oxidative stress as a result of the action of reactive species is recommended (Veskoukis et al., 2016a, 2018a, 2019).

Argument 3. *There is an interplay between reactive species generation and fundamental biological processes, such as energy metabolism*

A rule that cannot be violated regarding the adoption of a chemical molecule as a clinical significant biomarker, that is an index of a disease, is that it is not affected by fundamental biological processes. This is because such biological procedures are able to seriously alter its levels independently on the presence of a pathological condition or not. Reactive species are not characterised as clinically relevant indices, at

least given the currently available experimental data, partly due to the interplay between them and energy metabolism (Fig. 3). This matter has been touched upon by excellent review articles that gather the most effective studies in this topic (Quijano et al., 2016; Zhang et al., 2019). It is indisputable that oxidative phosphorylation in the inner membrane of mitochondrion is a major generator pathway for reactive species. Specifically, one of the less reactive ROS, namely superoxide anion ($O_2^{\bullet-}$) is a by-product of metabolism through the action of complex I (i.e., NADH:ubiquinone oxidoreductase) (Tahara et al., 2009). Apart from oxidative phosphorylation, the enzyme α-ketoglutarate dehydrogenase of the tricarboxylic acid cycle is responsible for H_2O_2 production (Kowaltowski et al., 2009). These ROS may not constitute a serious threat for oxidatively modifying macromolecules directly, however they react with other reactive species and form less stable and much more detrimental oxidizing agents. A relevant example is the formation of peroxynitrite ($ONOO^-$) following the reaction between nitric oxide radical ($NO^{\bullet}$) and $O_2^{\bullet-}$ (Zhang et al., 2019). Such reactive species are readily implicated in the development and progression of several redox-related diseases. However, given that an unknown, for the moment, amount of the specific reactive species is generated through energy metabolism, it is not possible to differentiate the concentration that is effective to the disease development, thus making the effort to offer to them a clinically relevant meaning a real challenge.

Argument 4. *Reactive species exert beneficial biological actions*

Free radicals and reactive species in general were inextricably linked to harmful biological outcomes for many decades (Halliwell and Gutteridge, 1984). Specifically, they have been associated with oxidative stress that is considered, in turn, a synonymous term with pathology and detrimental oxidative modifications of biomolecules (Fridovich, 1978). The domination of this idea, however, has been threatened recently as it is now a fact that the role of reactive species is not necessarily nocuous. In specific cases they are not only harmless but also essential for the progression of critical biological procedures (Fig. 3). Towards this direction there is evidence indicating that, depending on their concentration, they exert beneficial biological roles, such as signal transduction (Carroll and Conte, 2013; Winterbourn, 2015), gene expression (Ji et al., 2006) and useful adaptations after repeated bouts of exercise (Radak et al., 2001; Margaritelis et al., 2018). This was a revolutionary finding that was based on scarce evidence from the 70's (Mittal and Murad, 1977) and 90's (Sundaresan et al., 1995; Bae et al., 1997) and shifted the field of redox biology to a new era. This kind of knowledge that emerged recently made the connection of reactive species with the development and progression of clinical situations even more difficult (Murphy et al., 2011). Taking into account that the intrinsically generated reactive species are physiologically present in the cellular environment in specific amounts because they are necessary for its survival, whereas they can be detrimental below or above this optimal concentration, they cannot be linked to pathological conditions. On the basis of the knowledge available to date there is not the ability to discriminate their harmful from their harmless levels. Thus, there is not yet the necessary experimental evidence in order to plainly conclude that reactive species exert clinical significance.

Argument 5. *Reactive species follow the hormesis phenomenon*

The term hormesis was firstly introduced in the literature by Southam and Ehrlich in the field of plant physiology and it is still relevant to this research area providing important evidence (Southam and Ehrlich, 1943; Agathokleous et al., 2019). Hormesis applies perfectly to the fields of pharmacology and toxicology as it describes the dose-response relationship (Tsatsakis et al., 2018). In particular, it expresses the stimulatory effect of drugs when administered in low doses and their detrimental impact when their dose exceeds a given upper threshold (i.e., the so-called bell-shaped curve) (Calabrese, 2005). Recent experimental results suggest that hormesis is a biological phenomenon regulating the role of reactive species in the living organisms. Two physiological stimuli, namely exercise and aging are classical modalities that induce reactive species and, interestingly, the impact of their effects is dominated by the amounts of reactive species (Radak et al., 2005, 2017). Exercise in particular is an excellent model for the investigation of both the physiological and pathological roles of reactive species (Veskoukis et al., 2012b, 2016b; 2018b). However, it has been demonstrated that both excessive and diminished production of reactive species are harmful because oxidative and reductive stress emerge, respectively (Spanidis et al., 2018). In other words, someone could hypothesize that the presence of reactive species below a concentration threshold in a living organism is beneficial. This, however, seems to be challenged (Spanidis et al., 2018). Therefore, the idea of hormesis seems to shift according to the notion that there is an optimal concentration of reactive species that is favouring and in concentration both below and above this amount, detrimental effects may be observed. It appears that this concept might apply also to chronic diseases implying that it is not easy to delineate which reactive species are sufficient causes for specific pathologies.

Argument 6. *Oxidative modifications of redox-related molecules are not necessarily interpreted as oxidative damage*

Several molecules that are formed following the oxidative alteration of biomolecules by reactive species, such as MDA and protein carbonyls, are considered as redox biomarkers, since they depict the disruption of redox equilibrium in blood or other tissues (Veskoukis et al., 2008, 2009, 2010, 2019). Alternatively, MDA and protein carbonyls are the outcome of the detrimental, oxidizing action of reactive species, thus they are interpreted as the outcome of oxidative damage of lipids and proteins, respectively. On the contrary, there are other situations where one molecule is oxidized but the generated oxidized moiety is not considered as the product of the oxidatively damaged parental molecule (Fig. 3). Herein, we will refer to two appropriate examples. The reduced form of glutathione (i.e., GSH) is normally in equilibrium with its oxidized form (i.e., GSSG) in cells and tissues. One of the manifold biological functions of GSH is to get oxidized to GSSG in order an antioxidant enzyme, such as glutathione peroxidase, to act protecting the cell compartments from the harmful peroxides by reducing them. Therefore, GSH is in a way sacrificed through its oxidation without meaning that the presence of GSSG is necessarily a product of oxidative damage of GSH. In that case GSSG is a molecule that its formation is imposed in order the cell to overcome a putative oxidative attack. Another example indicating that the oxidative modification of a redox-related molecule is not always interpreted as oxidative damage is that of peroxiredoxin. In particular, it has been reported that hyperoxidation (i.e., irreversible oxidation) of a cysteine moiety in the molecule of human (Moon et al., 2005) and yeast (Lim et al., 2008) peroxiredoxin switches it from a peroxidase to a chaperone protein. Therefore, peroxiredoxin is over-oxidized, however it does not undergo alterations that cause its malfunction but it remains a useful molecule with another role. Conclusively, the two aforementioned examples show that the harmful action of reactive species may result in positive effects making the effort to connect them with a clinical outcome very ambitious.

5. We are still far from crediting reactive species with clinical significance: a proof

It has been perceptively proposed that one of the myths that will not die is that "antioxidants are good and free radicals are bad" (Scudellari, 2015). This phrase could drive anyone to think that since free radicals, and reactive species in general, are bad they must be directly associated with diseases. Therefore, the antioxidants that by definition inhibit reactive species generation or scavenge them after they have been formed due to numerous redox status-altering treatments could be beneficial against redox-related pathologies. This is far from reality,

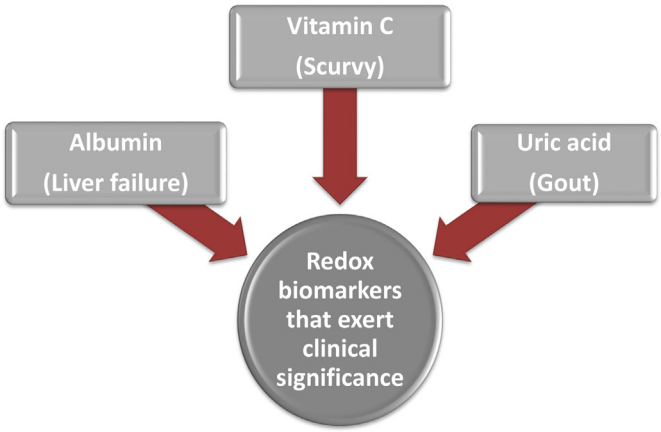

Fig. 4. Three examples of redox biomarkers (i.e., albumin, vitamin C and uric acid) that exert clinical significance and the pathologies mainly caused by their deficiency.

though. It is astonishing that no antioxidants have been approved for therapeutic indications by the Food and Drug Administration or other similar organizations (Ghezzi et al., 2017). The only exception is edaravone, a free radical scavenger that has been approved against stroke and amyotrophic lateral sclerosis in Japan (Wada et al., 2014; Abe et al., 2014). In addition, it has been reported that antioxidants may be either ineffective or even detrimental when administered against several diseases (Bjelakovic et al., 2012). Many relevant findings have been obtained from experiments in animals and not humans. Here the problem is more serious, though, because the issues of validity (Johnson, 1997) and extrapolation (Veskoukis et al., 2012b) of these findings to clinic remain. Furthermore, it appears that they make even less hopeful the possibility to state that reactive species exert clinical significance, that is they have a relation of causation with specific pathological conditions, as analyzed above based on the 6 arguments. It has to be mentioned that, unlike reactive species, other redox biomarkers exert clinical significance. Specifically, as illustrated in Fig. 4, oxidation of albumin is correlated to liver failure (Oettl et al., 2013), deficiency of vitamin C is the basic cause for the development of scurvy (Delanghe et al., 2011), whereas increased accumulation of uric acid in blood plasma causes gout (Sautin and Johnson, 2008).

6. Future perspectives on the basis of toxicology

Toxicology is a research field inextricably related to redox biology. It investigates the toxic effects of xenobiotics on diverse organisms and the basis of such effects is often redox-related, and on the same time they are associated with the occurrence of pathologies whose molecular background is linked to reactive species. Consequently, toxicology touches upon two basic pillars of redox biology, which are the biological role of reactive species and the pathological background of redox-related diseases. Given that toxicology is a rapidly expanding research area during the last decades, due to accumulated knowledge, one could probably hypothesize that it can be the appropriate field to check the putative clinical significance of reactive species. Recent experimental evidence has emerged forming a strong bond between redox biology and toxicology and can potentially provide an important clue regarding the clinical relevance of reactive species (Fountoucidou et al., 2019). A series of papers published the last four years have challenged an idea that the scientific community has not previously touched at all. On that basis, we have firstly to keep in mind that humans come to everyday contact with differential xenobiotics. Two characteristics of this fact are of great interest. The first is that the concentration of these compounds is very low (usually below the NOAEL, no-observed-adverse-effect level) and, secondly, they are in the form of mixtures and not as individual chemicals. Intriguingly, there is the possibility that, although this motif of exposure is not seemingly detrimental, it is proven to be harmful for human health and may be followed by severe pathologies (Tsatsakis et al., 2016; Fountoucidou et al., 2019). Describing a recent experiment with astonishing findings that introduces for the first time the notion that the long-term low-dose regimen of exposure to chemicals is associated with detrimental effects, a mixture of xenobiotics (pesticides, food preservatives and food packaging materials) were administered to rats for 18 months in three doses well below NOAEL and a variety of biomarkers with or without clinical significance were evaluated. According to some groundbreaking results, the administered chemical mixture induced monotonic and non-monotonic effects on several biochemical and redox-related parameters after 6 and 12 months of exposure (Docea et al., 2018, 2019). Additionally, 18 months of exposure to the higher dose of the mixture induced toxicity and oxidative stress indicated by the increased oxidation burden that was evaluated by several blood and tissue redox biomarkers (Fountoucidou et al., 2019). Therefore, the cumulative risk assessment needs to be redefined since this idea gains adherents during the last years (Tsatsakis et al., 2016, 2017, 2019c). Using this evidence as our base, we believe that the monotonic alterations in biochemical parameters along with the changes induced in redox biomarkers by the administered mixture may keep the keys for unraveling the mystery for the clinical relevance of specific biomarkers and reactive species among them. Indeed, monotonic changes were observed in parameters of liver function (i.e., alanine transaminase and alkaline phosphatase) and immune system (i.e., lymphocyte number). These biomarkers have indisputable clinical relevance. Therefore, we propose that the positive correlation of redox biomarkers that have been monotonically altered with the aforementioned indices could offer a first clue in order to acquire evidence implying that redox biomarkers, and reactive species in particular, exert clinical significance.

A third pillar, which is also of great importance regarding the association of the excessive reactive species generation with chronic diseases is the theory of "metaflammation" through the influence of long-term low-dose exposure to anthropogens. Under this frame, infectious diseases are pathologies with rather obvious causality (i.e., the presence of a pathogenic microorganism in the host organism). However, the causality of chronic and especially redox-related diseases is difficult to be defined, but a description has been pertinently referred to by Egger and Dixon (2014). The above mentioned authors have analyzed the terms of proximal or downstream causes of disease that are more immediate to disease and the distal or upstream causes that, as Peirce would probably say, they have one degree of separation than the aforementioned ones (Egger and Dixon, 2014). To this end, cardiovascular diseases have an unhealthy way of life (e.g., absence of exercise, smoking and poor nutrition) as determinant and several social and economical factors as upstream causes. The majority of the redox-related pathologies are associated with excessive reactive species production and, thus, oxidative stress and disrupted redox-signalling and on the same time they are linked to enhanced inflammatory response (Libby, 2007; Rius-Pérez et al., 2020). In particular a form of chronic, low-grade inflammation reported as "metaflammation" that was originally associated with obesity (Hotamisligil et al., 1993), was later linked to other oxidative stress-related diseases (Hotamisligil, 2006; Khansari et al., 2009). Since metaflammation seems to be a determinant in the appearance of chronic diseases, reactive species are putatively serious risk factors especially when the role of anthropogens is studied (Egger and Dixon, 2014). Anthropogens have been defined as "*man-made environments, their byproducts and/or lifestyles encouraged by these, some of which may be detrimental to human health*" that induce inflammation (Egger, 2012; Egger and Dixon, 2011). Therefore, due to their concomitant ability to induce reactive species generation, the study of anthropogen-induced metaflammation could provide useful insight in the investigation in the topic of the potential clinical significance of reactive species.

7. Concluding remarks

The issue concerning the putative clinical significance of redox biomarkers and particularly reactive species is present in the scientific queries of the researchers in the fields of redox biology and toxicology. Surprisingly, though, it has not yet been touched upon meticulously probably due to the difficulty in organizing the appropriate experimental set ups and to the absence of advanced research tools. In the present article we have tried to approach this essential question in our modern world and the real-life risk simulation concept under the theoretical and even philosophical frame by parallelizing the currently available knowledge with the ideas of the theory of causes introduced by Kenneth Rothman and the theory of semiotics proposed by Charles Sanders Peirce. We present a vivid illustration of the topic by analyzing 6 arguments that, according to our point of view, are crucial and justify why reactive species do not seem to have a cause-effect relation with disease. Toxicology offers future perspectives, though, through the novel experimental setups discussed here and the notion of the simulation of real-life exposure via the long-term low-dose exposure regimen. Our conclusions are based on the experimental findings that are available to date and the accumulated knowledge they have generated. On this basis, it appears that today we cannot say positively that a specific reactive species is the sufficient cause of a chronic pathological condition unlike acute conditions that induce oxidative stress. Nevertheless, this could possibly be a reality which may be proven in the future, taking into account that research in the relevant fields is expanding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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