Lipid peroxidation and ferroptosis: The role of GSH and GPx4

Free Radical Biology and Medicine 152 (2020) 175–185

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Lipid peroxidation and ferroptosis: The role of GSH and GPx4

Fulvio Ursini*, Matilde Maiorino

Department of Molecular Medicine, University of Padova, Viale G. Colombo, 3, I-35131, Padova, Italy

ARTICLE INFO

Keywords:
cell death
ferroptosis
GSH
GPx4
Lipid hydroperoxide
Lipid peroxidation
Oxygen toxicity
Selenium

ABSTRACT

Ferroptosis (FPT) is a form of cell death due to missed control of membrane lipid peroxidation (LPO). According to the axiomatic definition of non-accidental cell death, LPO takes place in a scenario of altered homeostasis. FPT, differently from apoptosis, occurs in the absence of any known specific genetically encoded death pathway or specific agonist, and thus must be rated as a regulated, although not "programmed", death pathway. It follows that LPO is under a homeostatic metabolic control and is only permitted when indispensable constraints are satisfied and the antiperoxidant machinery collapses.

The activity of the selenoperoxidase Glutathione Peroxidase 4 (GPx4) is the cornerstone of the antiperoxidant defence. Converging evidence on both mechanism of LPO and GPx4 enzymology indicates that LPO is initiated by alkoxyl radicals produced by ferrous iron from the hydroperoxide derivatives of lipids (LOOH), traces of which are the unavoidable drawback of aerobic metabolism. FPT takes place when a threshold has been exceeded. This occurs when the major conditions are satisfied: i) oxygen metabolism leading to the continuous formation of traces of LOOH from phospholipid-containing polyunsaturated fatty acids; ii) missed enzymatic reduction of LOOH; iii) availability of ferrous iron from the labile iron pool.

Although the effectors impacting on homeostasis and leading to FPT in physiological conditions are not known, from the available knowledge on LPO and GPx4 enzymology we propose that it is aerobic life itself that, while supporting bioenergetics, is also a critical requisite of FPT. Yet, when the homeostatic control of the steady state between LOOH formation and reduction is lost, LPO is activated and FPT is executed.

1. Introduction

Following the historical distinction between "accidental" and "regulated" cell death, a continuously growing list of regulated cell death (RCD) routines had been compiled. Agonists, inhibitors, metabolic pathways and genetic regulation of different forms of non-accidental cell death have been identified and classified by the Nomenclature Committee of Cell Death. Ferroptosis (FPT) is a newly described form of regulated cell death, defined as the result of missing or insufficient activity of the selenoperoxidase Glutathione Peroxidase 4 (GPx4), which causes a specific form of cell death operated by membrane lipid peroxidation (LPO) [1].

Introducing the concept of FPT, we recall the fundamental axiom of oxygen metabolism and toxicity stating that the high thermodynamic oxidative potential of molecular oxygen is mitigated by the kinetic sluggishness, due to incompatibility between the ground spin status of molecular oxygen and the reduced carbon of organic compounds [2]. To get fire, a spark is used to provide the energy exceeding the high energetic barrier of the transition state of the reaction between oxygen and fuel. In the case of the biological reactions of oxygen, the spin restriction can be bypassed at low temperature, when molecular oxygen interacts –accepting a single electron-with free radicals or transition metals [3]. Thus, the membrane damaging LPO, although thermodynamically favorable, is kinetically controlled and can only initiate when the spin barrier of oxygen is circumvented.

Oxidative damage has been for decades the leitmotif of studies on accidental free radical damage associated to spontaneous or experimental diseases. The remarkable insight, recently brought by the discovery of FPT, is framed in the notion that LPO is proposed today as a "regulated" event.

Abbreviations: ACSLs, Acyl-CoA synthetases; Aox-OH, Phenolic antioxidant; CoQH, reduced Coenzyme Q; Cys_2, Cys disulfide; DTD, DT-Diaphorase; EAA, Glutamate transporter; FPT, Ferroptosis; FST, Ferrostatin-1; GCL, γ-Glu-Cys Ligase; GGT, γ-glutamyl Transpeptidase; GPx4, Glutathione Peroxidase 4; $L^{\bullet}$, lipid carbon-centered radical; LIP, labile iron pool; $LO^{\bullet}$, lipid alkoxy radical; $LOO^{\bullet}$, lipid hydroperoxyl radical; LOOH, lipid hydroperoxide; LOX, lipoxygenase; LPO, lipid peroxidation; NOX, NADPH oxidase; PHGPx, Phospholipid Hydroperoxide Glutathione Peroxidase; RCD, regulated cell death; Mrp/Abcc, Multidrug Resistance-Associated Proteins; SOD, Superoxide Dismutase; Tf, Transferrin; TfR, Transferrin receptor

* Corresponding author.
E-mail addresses: fulvio.ursini@unipd.it (F. Ursini), matilde.maiorino@unipd.it (M. Maiorino).

https://doi.org/10.1016/j.freeradbiomed.2020.02.027
Received 2 December 2019; Received in revised form 3 February 2020; Accepted 26 February 2020
Available online 09 March 2020
0891-5849/

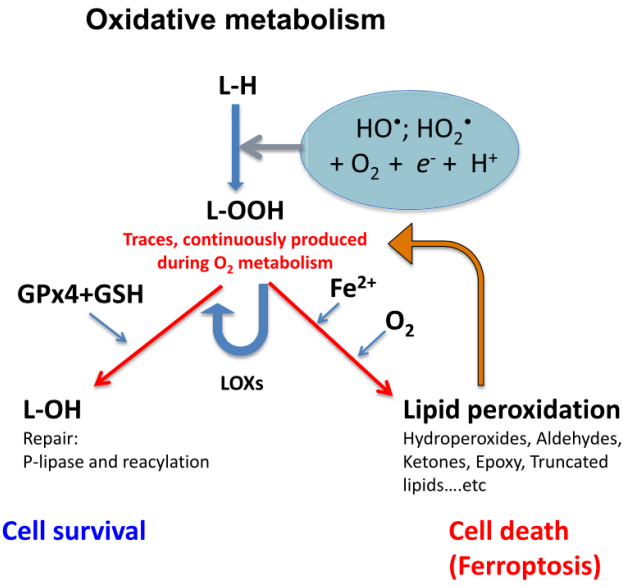

Fig. 1. Connection between oxygen metabolism, lipid hydroperoxides and ferroptosis.
The scheme summarizes the description on constraints and events linking oxygen metabolism to cell death. During aerobic metabolism traces of lipid hydroperoxides (LOOH) are continuously produced in membranes. The chemical steps are: i) formation of free radicals from oxygen metabolism ($HO^{\bullet}$; $HO_2^{\bullet}$) operating an H abstraction from a polyunsaturated lipid; ii) O_2 addition to the carbon-centered radical of the lipid; iii) H addition to the lipid hydroperoxyl radical, yielding LOOH. LOOH in the membranes, possibly multiplied by lipoxygenases (LOXs), are continuously reduced to the corresponding hydroxy derivatives (LOH) by GPx4. This reaction competes with initiation of membrane LPO by ferrous iron. Membranes containing LOH are repaired, while lipid peroxidation executes FPT.

LPO has been identified as the executor of FPT from the results obtained by pharmacologic manipulations leading to either GSH depletion or GPx4 inhibition in cells [4,5]. These results agree with the earlier observations that whole gene deletion of GPx4 is embryonically lethal [6], and, in the adult or in a specific tissue, its depletion results in specific cell death phenotypes [7–13].

This set of evidence supports the notion that FPT is not the outcome of a death pathway sparked by a specific agonist, but, rather, a kind of distortion of the integrated mechanisms controlling oxygen metabolism and toxicity. The implicit fall-out is that these mechanisms must be continuously operating for keeping low the steady state between reactions permitting and inhibiting LPO.

In this review, we will critically discuss the specific aspects and the constraints of the relationship between GPx4 activity, LPO and FPT as schematically outlined in Fig. 1.

Our proposal is that the traces of hydroperoxide derivatives of lipids (LOOH), unavoidably produced as a consequence of oxygen activation during aerobic metabolism, are continuously reduced by GPx4 in the presence of GSH. Not until the GPx4 reaction becomes limiting, ferrous iron initiates LPO by LOOH decomposition, leading to cell death by FPT. This features the Janus face of the oxygen molecule and highlights the proposed notion of a threshold that is exceeded when not only the indispensable constraints of LPO are fulfilled, and GPx4 activity is insufficient.

2. Lipid autoxidation and peroxidation

Both terms, “autoxidation” and “peroxidation”, indicate the formation of LOOH and their decomposition leading to a series of products, including reactive electrophiles. Although these terms are indifferently used in the scientific literature on oxidative degradation of lipids, here we will refer to this semantic distinction solely to point out the peculiar analogy and difference between the slow oxidative degradation of organic matter, typically occurring in polymers, foods or oils (autoxidation), and the rapidly progressing event, taking place in biological membranes (lipid peroxidation). In both cases, indeed, the sparking event is formation of free radicals from LOOH, while the most remarkable difference between the two processes - autoxidation taking place taking in days and peroxidation in minutes - is the kinetics of the reactions producing radicals from LOOH, which paradoxically are both, the initiators and the products of oxidative chain reactions. Consistently, a major antioxidant mechanism, in both cases, is the reduction of LOOH, which integrates the well-known antioxidant effect of free radical trapping.

The oxidative degradation of lipids is a free radical process encompassing three phases: i) formation of the first lipid radical initiating the peroxidative chain reaction (*initiation*); ii) a peroxidative chain reaction where remarkably the number of radicals remains constant (*propagation*); and iii) arrest of the peroxidative chain reaction by radical-radical interaction (*termination*) [14].

LOOH is the indispensable precursor of free radicals competent for the initiation of peroxidative chain reaction [15]. From LOOH, initiating radicals are formed from the O–O bond either by the slow molecule-assisted homolysis reaction, (fat autoxidation) [16], or by the fast one-electron transfer reaction from transition metals (membrane LPO) [15]. It is the enormous difference in the kinetics of the formation of initiating radicals that mainly accounts for the difference between rancidity of fats in foods and LPO in biological membranes.

In LOOH, the energy of the O–O bond, although insufficient to support an uni-molecular decomposition, is compatible with a molecule-assisted homolysis, favored by hydrogen bonding. A peculiar case is that of homolysis of a LOOH operated by another LOOH (Eq (1)) [16]:

$$2\ LOOH \rightarrow LO^{\bullet} + LOO^{\bullet} + H_2O \quad (1)$$

This reaction is critical for initiating autoxidation of fats in bulk phase, but definitely much less important, if any, for initiating membrane LPO. In membranes, initiation is operated by the electron transfer from a reduced transition metal to a LOOH in a Fenton-like reaction, producing the initiating alkoxy radical ($LO^{\bullet}$) [15] (Eq (2)):

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+} + H_2O \quad (2)$$

These notions highlight the relevance, discussed in paragraph 3, of the “first” LOOH in both autoxidation and LPO.

$LO^{\bullet}$ either extracts a H from a neighbor lipid (L’H), forming a carbon-centered radical (L$^{\bullet}$), (Eq (3)):

$$LO^{\bullet} + L'H \rightarrow LOH + L^{\bullet} \quad (3)$$

or, more likely, rearranges forming an epoxy group and a new carbon-centered radical in the same fatty acid chain (Epoxy-L$^{\bullet}$) [14].

The carbon-centered radicals (L$^{\bullet}$ or Epoxy-L$^{\bullet}$) reversibly add oxygen forming a lipid hydroperoxyl radical (L’OO$^{\bullet}$), (Eq (4)):

$$L^{\bullet}\ (\text{or Epoxy-L}^{\bullet}) + O_2 \longleftrightarrow L'OO^{\bullet}\ (\text{or Epoxy-LOO}^{\bullet}) \quad (4)$$

For simplicity, the evolution of the epoxy group will not be further discussed.

From $LOO^{\bullet}$, LOOH is generated by an H transfer that stabilizes the reversible oxygen addition. When H is donated by a methylenic carbon of a divinyl methane moiety of an unsaturated fatty acid esterified in a complex lipid, the newly formed L$^{\bullet}$ adds oxygen, thus propagating lipid peroxidation (the number of radicals does not increase) (Eq (5)):

$$LOO^{\bullet} + L'H \rightarrow LOOH + L^{\bullet} \quad (5)$$

Alternatively, the oxygen addition leading to $LOO^{\bullet}$ can be stabilized with a much higher rate constant by H donors such as tocopherols or coenzymes Q that for this reaction are regarded as antioxidants (Aox-

OH). Since the radical of the antioxidant (Aox-O$^{\bullet}$) is not kinetically competent for efficiently propagating peroxidative chain reaction [17], the reaction preventing propagation is defined as “chain breaking” (Eq (6)):

$$LOO^{\bullet} + Aox\text{-}OH \rightarrow LOOH + Aox\text{-}O^{\bullet} \quad (6)$$

The length of the peroxidative chain relies on membrane structure and composition. In general, it is acknowledged being very short, propagation being terminated by radical-radical interaction.

A typical arrest reaction, which results in a decreased number of free radicals, is the disproportion of LOO$^{\bullet}$ through the Russell mechanism (Eq (7)):

$$2\ LOO^{\bullet} \rightarrow LOH + L{=}O + O_2 \quad (7)$$

A hydroxyl and a keto derivative of fatty acid chain (LOH and L=O) are produced, while molecular oxygen is released in an electronically excited state that decays emitting a photon [18].

It is worth noting that the chain breaking reaction (Eq. (6)), although limiting propagation, kinetically favors the formation of LOOH from which, in the presence of Fe^{2+}, the peroxidation-initiating species LO$^{\bullet}$ is produced.

The description of the complex series of reactions producing a long series of minor species is besides the scope of present discussion and has been extensively reported [14].

About the role of endogenous free radical scavengers two recent back to back papers reported the identification of a gene able to complement GPx4 loss [19,20]. The product of this gene is the "Ferroptosis Suppressor Protein 1" (FSP1), previously known as "Apoptosis Inducing Factor Mitocondria Associated 2" (AiFM2). This protein, indeed, coincides with the DT-Diaphorase (DTD) discovered in 1958 by Lars Ernster [21], the oxidoreductase that reduces quinones to corresponding hydroquinones using either NADH or NADPH. Although the mechanism of protection has not been conclusively unraveled, it has been proposed to rely on the increased steady-state of the reduced Coenzyme Q (CoQH) that scavenges LOO$^{\bullet}$ yielding LOOH (Eq (6)). However, this partially conflicts with the above discussion about the role of LOOH as the species producing LO$^{\bullet}$. As a matter of fact, reduction of LOO$^{\bullet}$ becomes fully efficient in inhibiting LPO only when associated to reduction of the LOOH produced [22]. To give an account for the effect of the increased concentration of reduced CoQH by DTD, it seems more chemically sound the option that the anti-ferroptotic mechanism of CoQH is scavenging of LO$^{\bullet}$, as recently proposed for Ferrostatin-1 (FST), the paradigmatic synthetic antioxidant that inhibits FPT [23]. It is possible, indeed, that, *in vivo,* the local concentration of CoQH in the membrane could be sufficient to compete with a polyunsaturated lipid for the reaction with LO$^{\bullet}$, despite the limited kinetic advantage of phenolic antioxidants in this reaction [24]. A similar mechanism can be evoked in explaining, at least in part, the protection against FPT afforded by vitamin E in the absence of GPx4, either in isolated cells or brain [25].

Interestingly, in the case of FST, the radical of the antioxidant, generated upon the reaction with LO$^{\bullet}$, is regenerated back by Fe^{2+}. It follows that FST in the presence of ferrous iron operates a kind of peroxidatic cycle where LOOH is reduced to LOH by two single electron transitions (Fig. 2). The final antioxidant effect of FST is, therefore, similar to that of GPx4, although, notably, the molecular mechanism is distinct: two single electron transfer reactions *vs* one nucleophilic substitution. What is relevant to point out here is that LPO, as well as autoxidation, cannot proceed unless continuously re-initiated from LOOH. Consistently, a major antioxidant mechanism in both cases is the reduction of LOOH to LOH. The description and the major relevance of the antioxidant “peroxidolytic mechanism” has been masterfully discussed by G. Scott [26]. He also had to acknowledge that “to many biochemists and material technologists, ‘antioxidant’ is synonymous of spin - trap”.

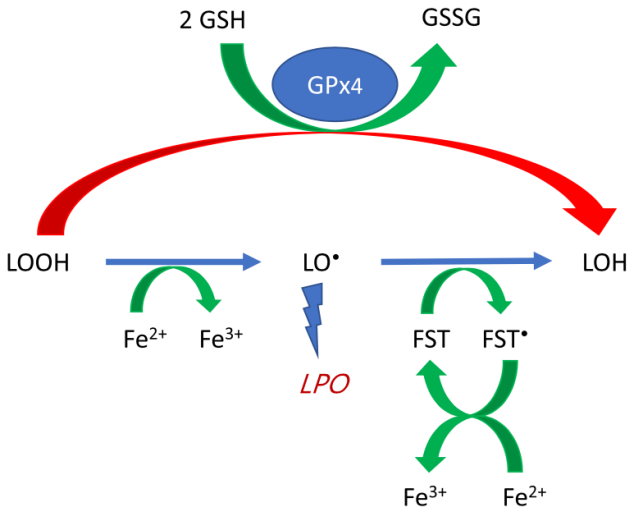

Fig. 2. Antioxidant mechanism of GPx4 and Ferrostatin.
Both, GPx4 activity and Ferrostatin (FST), in the presence of ferrous iron reduce LOOH to LOH and this prevents LPO initiation. The chemical mechanism is a nucleophilic substitution in LOOH for GPx4, and one-electron transition to LO$^{\bullet}$ for FST. The proposed recycling of ferrostatin radical (FST$^{\bullet}$) by Fe^{2+} is indicated [23].

3. How is the “first” LOOH required to initiate oxidative chain reactions produced?

The energetic barrier of the reaction between molecular oxygen and carbon, due to the ground electron status of carbon (singlet) and dioxygen (triplet), is escaped when either oxygen reacts with a free radical or it is reduced by an uneven number of electrons unveiling the reactivity as free radical itself. The species produced by monovalent reduction of molecular oxygen is the superoxide anion ($O_2^{\bullet -}$).

The major biological sources of $O_2^{\bullet -}$, are NADPH oxidases (NOXs) [27], and mitochondria, which leak electrons from the transport chain [28–30].

Superoxide anion is the precursor of the so called “reactive oxygen species”, (ROS) as cumulatively indicated in cell biology studies, which are endowed of both, toxic and physiological effects. The identification of the radical species competent for abstracting H from a polyunsaturated fatty acid chain and thus producing the “first” LOOH, after oxygen addition plus H transfer from a new lipid or an antioxidant, is here synthetically worked-out, taking into account the chemical and biochemical constraints of the reaction.

Superoxide anion is not a good oxidant [31], and thus indirect pathways, producing the lipid carbon centered radical (L$^{\bullet}$) that adds molecular oxygen yielding LOO$^{\bullet}$, eventually stabilized as LOOH, must be considered. What is known is that $O_2^{\bullet -}$ interacts with iron complexes forming perferryl species, which have been proposed as competent for generating the L$^{\bullet}$, escaping the spin restriction [32]. Also, $O_2^{\bullet -}$ is reduced either spontaneously or enzymatically to hydrogen peroxide and then, by Fenton chemistry, to hydroxyl radical (HO$^{\bullet}$). This species is, indeed, by far the most potent oxidant produced by incomplete reduction of oxygen, the role of which was defined in the earliest studies on free radical in biology. HO$^{\bullet}$ is a strong electrophile and reacts at diffusion-limited rate with practically any biological molecule. However, it is just this extremely elevated reaction rate that makes unlikely the specific oxidation of a methylene carbon of a polyunsaturated fatty acid of a phospholipid inserted in a membrane. Another, often neglected, oxidant suitable for H abstraction, is the protonated form of $O_2^{\bullet -}$ (i.e. $HO_2^{\bullet}$) also named per-hydroxyl or hydroperoxyl radical [30,33]. Against the relevance of this radical, there are both, the extremely low concentration and presence of Superoxide

Dismutases activities (SODs). Conversely, in favor, are the low pK_a value of $O_2^{\bullet-}$ (4.7 in aqueous solution [34]) that is compatible with the protonation in the acidic environment of the cytosolic side of internal mitochondrial membrane, and the fact that the non-charged $HO_2^{\bullet}$ is expected to be a poor substrate of SODs, missing the electrostatic enzyme-substrate interaction [35].

In summary, although the nature of the species involved in the formation of $L^{\bullet}$ in cell membrane evolving to the 'first' LOOH inside the membrane is not rigorously defined, available data allow to safely assume that the first critical event is $O_2^{\bullet-}$ production.

FPT descends from LPO and mitochondria have been proposed to have a role in FPT [36], although this view has been also criticized [37,38]. What we know is that a respiratory chain fully reduced by respiratory control is a condition favoring electron leakage [29], that α-keto-acid dehydrogenases are an even better source of $O_2^{\bullet-}$ in mitochondria [39], that oxidation of α-ketoglutarate, produced by glutaminolysis, supports erastin-inducing FPT [40] and that the cytosolic vital form of GPx4 is located in the somatic tissue mitochondria [41] and/or contact sites between the inner and the outer membrane [42]. In addition, while this article was under review, a paper appeared showing that silencing of the dihydrolipoate dehydrogenase component of the alpha ketoacid dehydrogenases protects FPT induced by cystine deprivation [43]. This evidence is strongly in favor with the role of these mitochondrial enzymes in producing the species leading to LOOH production and eventually FPT when GSH is limiting. The seemingly solid conclusion is, therefore, that mitochondrial activity is competent for the formation of the traces of LOOH from which LPO is initiated when reduction by GPx4 is limiting.

4. Role of lipoxygenases

Besides the above mechanisms, LOOH can be produced also by some dioxygenase homologs, namely lipoxygenases (LOXs) [44]. Evidence has been produced for their role in FPT [45,46], and thus, since antioxidants suppress LOXs activity [47], a relevant question emerges whether FPT inhibition by antioxidants could be due to LOXs inhibition [48]. Against there is the observation that ferrostatin and liprostatin 1, the two paradigmatic FPT inhibitors, are poor inhibitors of 12/15 LOX but excellent inhibitors of LPO, instead [49]. Nevertheless, the issue of a possible control of LOXs by Vitamin E could be of physiological relevance. Antioxidant analogs of Vitamin E inhibit LOXs activity and the acknowledged mechanism is scavenging of a free radical intermediate of the catalytic cycle [47]. However, it has been reported that the most specific inhibitor of 12/15 LOX is tocotrienol-containing the unsaturated phytic chain [50], and that esterified derivatives of tocopherol inhibit 12/15 LOX in spite the absence of the redox moiety [46]. This addresses a possible role of the side chain of vitamin E, besides the redox properties of the chromanol ring. In this view, LOXs inhibition should be due, at least in part, to the competition for the active site between the tocopherol side chain and the fatty acid chain substrate. Unfortunately, this non-canonic, innovative evidence is mainly supported by molecular modeling and requires a further in deep validation.

Thus, at the present level of knowledge, it can be safely concluded that tocopherol dampens LOXs activity just by operating a radical trapping reaction on the active site.

A possibly relevant aspect of LOXs impact on LPO and FPT is the activation of these enzymes by LOOH and thus the control by GPx4. This interesting observation emerged from studies on the function of 12/15 LOX. This enzyme has a role in the final maturation of reticulocytes [51]. It forms LOOH from intact phospholipids and this is the critical event priming the destruction of mitochondria in the last phase of differentiation of reticulocytes into red blood cells [52], indicating that mitochondria LPO is a physiological event in red blood cell maturation. The detailed mechanism of the controlled activation of 12/15 LOX in reticulocytes is still unclear, besides the fact that the catalytic iron in the active site must be kept in the ferric form. What it has been demonstrated is that, in sub-mitochondrial particles, GPx4 inhibits 12/15 LOX activity by reducing LOOH that, by oxidizing the catalytic iron, act as indispensable activators [53]. Consistent with this function, is the evidence of oxidative damage and severe anemia caused by GPx4 silencing in erythropoietic cells [54] when peroxidation, not kept under the necessary control, widens. Hence, it appears conceivable that LOOH also have a role in activating LOXs, and that by this mechanism LPO is enhanced. This further confirms the notion that the whole series of oxidative events on membrane phospholipids is controlled by GPx4.

In summary, the emerging view is that LOXs are better enhancers than indispensable agonists of FPT. Consistently, in animals lacking *alox15*, the gene expressing the isoform active on intact phospholipids [55], fail to complement the embryo lethality due to inactive GPx4 expression [56].

5. From microsomal lipid peroxidation to GPx4

The rancidity of fats, a typical consequence of autoxidation, was known from antiquity, and carpenters and painters used oxidation-polymerized linseed oil for centuries. The first scientific description dates back to early XIX century when Théodore de Saussure provided manometric and gravimetric evidence for the spontaneous addition of oxygen to walnut oil [57]. Since then, the process of oxidative degradation, and the procedures to prevent it, attracted the attention primarily of rubber chemists and food technologists. The observation in 1963 by P. Hochstein and L. Ernster of a very fast oxygen consumption rate in a suspension of rat liver microsomes exposed to NADPH and ADP while seeking for an oxidative phosphorylation pathway, was attributed to an unforeseen extremely fast and massive oxidative degradation of membrane lipids [58]. The discovery of microsomal LPO had, in our opinion, the main merit of bridging the gap between fat rancidity and a biological event, *i.e.* between autoxidation and peroxidation according to the semantic distinction presented in paragraph 2.

The features of the microsomal lipid peroxidation were soon identified. Traces of iron complexed by the phosphates of ADP, and kept reduced by electrons flowing through the microsomal electron transport chain from the reduced pyridine nucleotide, were indispensable activators. Soon it was observed that NADPH and the microsomal electron transport chain could be replaced by other sources of electrons, such as ascorbate [59].

The wishful thinking that LPO could be relevant in human diseases inspired a long series of studies addressed to understand the basic mechanism of LPO and the biological or pharmacological mechanisms to prevent it. In the late seventies we also stepped, by a coincidence, on LPO and this prompted studies that led to the discovery of GPx4. While addressing the microsomal HMG-CoA reductase activity, we noticed a rotten-like smell emanated by the reaction mixture: a clear evidence of peroxidation. The mixture indeed contained, among other reagents, microsomes, adenine nucleotides and NADPH. The hit that mostly drove us toward the discovery of GPx4, was the report by P. McCay that liver cytosol, in the presence of GSH, inhibits LPO [60]. Adopting a typical biochemical approach, we purified the protein accounting for this GSH-dependent anti-peroxidant effect. This "Peroxidation Inhibiting Protein" [61], was named "Phospholipid Hydroperoxide Glutathione Peroxidase" (PHGPx) when the enzymatic activity on oxidized membrane phospholipid was characterized [62]. Notably, the PHGPx activity was not shared with the previously known glutathione peroxidase, known today as GPx1. PHGPx was later systematically renamed GPx4, after the description of the corresponding gene [63] and the identification of other GPx homologs. However, how the suffix 4 originated, is unclear.

In our early experiments, we already observed that neither SODs, nor H_2O_2 removing enzymes inhibit membrane LPO. This draw our attention to traces of membrane LOOH as the indispensable species for initiating LPO.

Combining this set of evidence with the known chemistry of lipid peroxidation, it clearly emerges that a unique and specific "activator" of FPT does not exist, and that the initiation of LPO relies on insufficient GPx4 activity only. In this perspective, formation of LOOH in membranes emerges as a continuous unavoidable constraint related to oxidative metabolism operating in aerobic life. The lesson from these *in vitro* studies is that, in microsomes, membrane LPO, instead of being "activated", is rather "not sufficiently inhibited". This concept indeed, could be extended today to the most popular FPT.

The series of studies on GPx4 and LPO also disclosed a critical reason why selenium is indispensable to life. GPx4 indeed, is a vital enzyme and even the substitution of sulfur for selenium in mice GPx4, is incompatible with life, despite the fact that the recombinant sulfur-containing mutant catalyzes the same reaction and is expressed at much higher level [64]. This is due, besides the kinetic advantage of the wild type enzyme [65] to a much higher oxidative stability of the latter *vs.* the cysteine-containing variant [66].

6. The products of lipid peroxidation and ferroptosis

The occurrence of LPO leading to FPT, although inferred *a priori* from the missed anti-peroxidant activity of GPx4, is still far from being evaluated with rigorous quantitative precision.

In the early studies on LPO, which were carried out on lipid dispersions or subcellular fractions, the analytical procedures were borrowed from oil and food analysis. Diene conjugation, colorimetric determination of malondialdehyde or titration of hydroperoxides have been measured for decades. In our experience, none of these procedures is sensitive and precise enough for studies at cellular level, such as FPT. Here, only tiny amount of malondialdehyde in fact can be measured [67], and oxygenated species derived from phospholipids are hardly detectable only by sophisticated MS procedure [68].

In the majority of cell biology studies, the evidence of ongoing membrane LPO was inferred, instead, by measuring the oxidation of the probe C11-BodiPy 581/591 [69]. This probe monitors the formation of oxygen-centered lipid radicals in cells, taking advantage of insertion in the membrane due to the lipophilic tail, and excitation and emission in the non-toxic visible range. The signal produced by fluorescence emission of oxidized C11-Bodipy is therefore *bona fide* acknowledged today as the evidence of ongoing membrane LPO in cells.

An elegant, although not quantitative, proof of the link between LPO and FPT has been produced by the observation that cells loaded with deuterated fatty acids become more resistant to FPT, due to the isotopic effect [45]. Additionally, a chemoproteomic study showed that during FPT several proteins are carbonylated by hydroxynonenal, the product of LPO [70] a specific unsaturated hydroxyaldehyde first described in detail in 1984 [71,72].

In conclusion, although a precise quantitative evidence of LPO in FPT is still missing, the link between the two phenomena is convincing. Yet, whether either a specific oxidized lipids is involved or, instead, it is a mixture of LPO products that impairs specific vital functions remains undefined. Future oxy-lipidomic procedures are expected to bring a major contribution in this respect, when the sensitivity of the MS analysis and computational data processing will be further implemented.

7. Role of glutathione

GSH, the reducing substrate of GPx4 activity, is indispensable for preventing FPT. Consistently, GSH is indispensable for life as the silencing of γ-Glu-Cys ligase (GCL), the rate-limiting enzyme of GSH synthesis, is lethal [73,74]. Cell death is seemingly due to LPO, although other mechanisms limiting the maintenance of vital homeostasis are also possible.

More generally, the relationship between GSH redox status and regulated cell death –originally cumulatively referred to as apoptosis- attracted a huge number of studies focusing the alleged, although never fully revealed, relationship between "oxidative stress" and cell death [75,76].

The straightforward impact of GSH levels on FPT emerged from the use of erastin, a compound that, by lowering the intracellular level of GSH activates, in sensitive cells, a form of death morphologically identical to that induced by missing GPx4 [37].

Cellular GSH concentration is maintained by a complex homeostatic mechanism where the steady-state concentration is under kinetic control of specific enzymatic reactions [77].

GSH concentration is controlled by the rate of oxidation, conjugation, extrusion, uptake of thiol containing precursors and re-synthesis. Intracellular reduction rate of GSSG, instead, seems of relatively minor relevance as suggested by the observation that absence of glutathione reductase produces a mild phenotype in animals, revealed only as increased sensitivity to oxidative challenges [78].

7.1. Role of Nrf2

GSH biosynthesis is under the control of Nrf2, the key regulator of the cellular response against "stress", which is switched on by electrophiles [79]. These, by interacting with the nucleophilic Cys residues of the adaptor protein Keap1, which facilitates Nrf2 ubiquitination, allow the newly synthesized transcription factor to escape ubiquitination and thus to transit to the nucleus. Here, after combining with other proteins, Nrf2 promotes transcription of target antioxidant genes [80], among which there are the catalytic and the regulatory subunits of GCL. This mechanism emerges as a feed-back control of the nucleophilic tone activated by electrophiles. However, Nrf2 not only activates GSH biosynthesis, but also primes the expression of the multidrug resistance-associated proteins (Mrp/Abcc), competent for exporting of GSH-conjugates, GSSG and GSH itself [75,81,82]. In this respect, Nrf2 activation, by decreasing intracellular GSH, at least under specific experimental conditions, could also sensitize cells to FPT. In support to this, it has been reported that silencing of Mrp/Abcc increases GSH concentration while overexpression, increasing extrusion, is associated with cell death [75].

Cumulatively, while it is largely accepted that Nrf2 activation, by priming GSH synthesis, increases the nucleophilic tone in the frame of a protective, anti-stress function, it is not clear whether also the GSSG/GSH extrusion, promoted by Nrf2 activation, could also modulate sensitivity to FPT. In this context, it is relevant to mention that the seldom reported observations describing GPx4 as a Nrf2 target, although theoretically conceivable, should be considered with caution. The GPx4 promoter(s) indeed do not contain Nrf2 sites and systematic studies addressing the issue are missing.

It is reasonable, therefore, to conclude that the final effect of Nrf2 on FPT is still incompletely defined if not still frankly ambiguous.

7.2. Uptake of Cys and role of γ-glutamyl transpeptidase

The Cys required for GSH biosynthesis comes from diet. Besides Cys and its disulfide (Cys_2) the extracellular *milieu* also contains GSH and GSSG extruded from the cells via the Mrp/Abcc [75]. Both, Cys and GSH are rapidly oxidized in the extracellular environment, yielding cystine (Cys_2) and GSSG. The canonical pathway of amino acid uptake from glutathione, starts with the reaction of γ-glutamyl transpeptidase (GGT), an enzyme facing the outer layer of cell membrane [77]. By hydrolyzing glutamate from GSH or GSSG, di- or tetra-peptides containing Gly and Cys or 2Gly and Cys_2 are formed, respectively. The peptide bonds of these peptides are immediately hydrolyzed by a peptidase releasing Gly, Cys and Cys_2. Uptake of Cys and Cys_2 takes place via the specific AA transporter and the X_c^- antiport, respectively (see paragraph 7.3).

In the cycle described several years ago by Meister, the glutamate of extracellular GSH is transferred by GGT to amino acids and the γ-

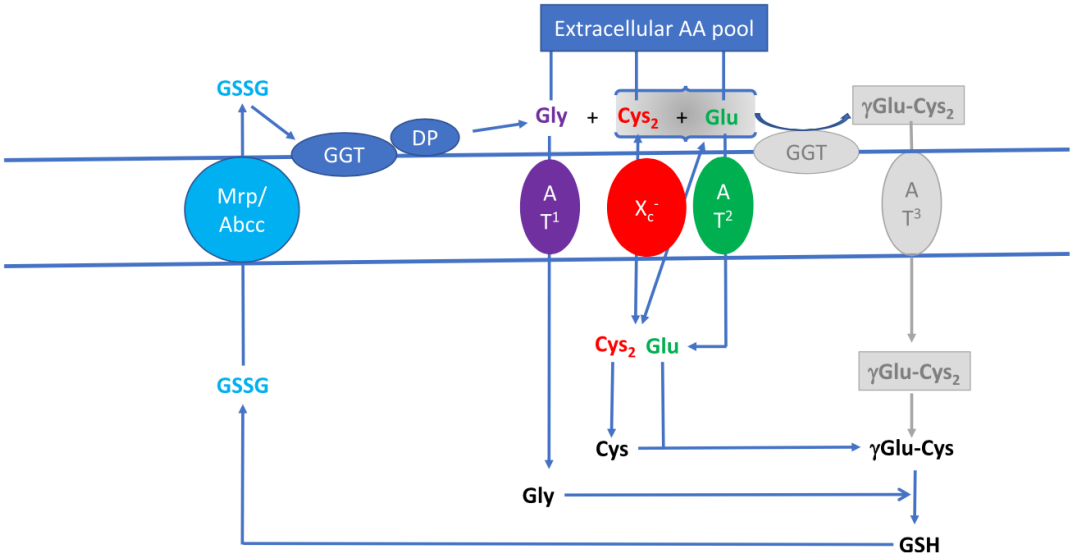

Fig. 3. Recycling of GSSG.
GSSG is extruded via the Mrp/Abcc transporter and hydrolyzed by GGT and a dipeptidase (DP) to Gly, Glu and Cys_2 contributing to the extracellular amino acid pool. Gly and Glu are taken up by the specific amino acid transporters (AT^1 and AT^2) and Cys_2 is reversibly exchanged with Glu by the X_c^- transporter. In the classical Meister cycle (grey, on the right) GGT transpeptidase activity catalyzes the binding of Cys_2 to Glu forming γGlu-Cys_2 that is imported by the γ-glutamyl-amino acid transporter (AT^3). This transferase component of GGT, which is not indispensable to the process, is debated and reported here for historical completeness only. For simplicity, oxidations and reductions of different thiols and disulfides are not specifically indicated.

glutamyl amino acids are eventually imported [83].

The relevance of the transpeptidase activity of GGT and the γ-glutamyl amino acid transporter has been recently questioned and evidence is available that the enzyme, despite the name, solely complies with the hydrolase activity [84,85]. Although the question remains open, we can safely conclude that GGT, irrespective of the issue of transpeptidase activity relevance, as hydrolase makes available Cys and Cys_2 derived from GSH or GSSG for uptake, for which the above transport systems are indispensable. A synthetic outline of the recycling of GSSG is summarized in Fig. 3.

7.3. Role of cystine and the X_c^- transporter

The relative relevance of distinct pathways providing Cys for GSH synthesis from extra cellular precursors has never been comparatively analyzed in detail, thus supporting the assumption that large differences might exist in different cells and tissues.

The Cys_2/Glu antiporter system X_c^- has been by far the most exhaustively investigated [86]. This descends from a practical issue too, since cultured cells practically exclusively rely on high levels of Cys_2 in culture media. The other main reason of the focus on X_c^- is its irreversible inhibition by erastin, a compound first used to disclose the features of FPT. Consequently, inhibiting X_c^- by erastin became a paradigmatic experimental tool for inducing FPT.

Yet, being a bidirectional transporter, flow direction is controlled by substrate concentration, where the differential intra- and extra-cellular concentration of Glu and Cys_2 drives the fluxes. Since Glu can enter by its own transporter (EAA), a cycle can be envisaged, where a continuous uptake via EAA provides Glu that is exported via the X_c^- antiporter system, thus supporting cellular Cys_2 uptake (Fig. 3).

Yet, although the activity of the system X_c^- is usually acknowledged promoting GSH biosynthesis, it can also impair it, an effect particularly relevant in neurons. The most relevant pathophysiological condition where X_c^- proved relevant in favoring cell death in fact, is Glu neurotoxicity [87]. This neuronal form of death, named “oxytosis” several decades ago, is primed by extracellular Glu and it is, indeed, largely overlapping FPT in encompassing GSH depletion, oxidative stress, LOXs activation, and calcium influx [88]. The high concentration of extracellular Glu, in fact inverts the flow direction so that Cys_2 is extruded.

It has been recently reported that the gene encoding for the tumor suppressor protein p53, (TP53) sensitizes cells to FPT by down-regulating SLC7A11, the light chain subunit of the X_c^- transporter [89]. Consistently, SLC7A11 overexpression decreases the sensitivity of cells to FPT [90]. On the other hand, it has been also reported that stabilization of p53 by upregulating p21 has a pro-survival effect, instead [91]. This preserves GSH concentration and delays the induction of FPT, indicating that the p53-p21 may also help cancer cells to cope with metabolic stress induced by Cys_2 depletion [89]. This set of evidence discloses an unexpected mechanism descending from the activation of the gene TP53, although further studies are required for discriminating among seemingly contradictory results.

7.4. Role of protein turnover

Cys available for GSH synthesis can also derive from intra-lysosomal protein degradation. Following proteolysis, the specific Cys_2 transporter cystinosin releases Cys_2 in the cytosol. Interestingly, in humans, a defect of this transporter yields a specific pathology known as cystionosis, where Cys_2 crystallize within lysosomes [92]. A relevance of this pathway on FPT regulation has never been specifically addressed.

7.5. Role of the transulfuration pathway

By the transulfuration pathway methionine is converted to homocysteine which is transformed to cystathionine and finally Cys [93]. Regulation by S-adenosylmethionine suggests that the major role of the transulfuration pathway is balancing methionine or homocysteine degradation with methionine availability, rather than Cys biosynthesis. Apparently, synthesis of Cys by the transulfuration pathway can be viewed as the vehicle for sulfur conversion to sulfate and taurine end products [94]. Yet, in experiments seeking for genetic suppressors of FPT it was shown that when cysteinyl-tRNA synthetase was knocked down, the transulfuration pathway is activated, thus protecting against

FPT induced by system X_C^- inhibitors [95,96]. This complies with a possible role of the transulfuration pathway in coping with GSH synthesis needed to decrease sensitivity to FPT, at least in some cell types.

In conclusion, although GSH depletion is the widely used experimental procedure for inducing FPT, the sensitivity is largely different in distinct cell lines and distinct metabolic conditions. The basic question, therefore, about the role of GSH depletion as positive agonist of FPT under physiological conditions must be rated as highly likely, although conclusive evidence about mechanisms is far from being positively achieved.

8. Role of iron

Iron has an indispensable function in LPO, and it is the cellular pool of redox-active free Fe^{2+} – i.e. the so called "labile iron pool" (LIP) [97] - having the key role of initiating LPO from LOOH.

Iron is ingested with food and is continuously recycled among tissues, leaving the organism only by bleeding, or by cell loss from intestine or skin. Intracellular concentration and biological availability of iron is carefully controlled, resulting from the rates of uptake, utilization, protein turnover, storage and export. Cellular uptake occurs via the transferrin receptor (TfR) at least in the most studied hematopoietic cells, while in the non-hematopoietic cells the routes of uptake are much less clearly defined [98]. TfR takes up transferrin (Tf) carrying Fe^{3+} from plasma by clathrin-mediated endocytosis. In the endosome, a proton pump allows releasing Fe^{3+}, while iron-free Tf, losing affinity for the TfR, is freed and recycled together with the TfR. Inside the endosome, Fe^{3+} is reduced to Fe^{2+}, transferred to cytosol by the divalent metal transporter-1 (DMT1), and associated to LIP. Heme oxygenase-1 (HO1), by releasing Fe^{2+} from heme catabolism, also contributes to LIP iron content. Here Fe^{2+}, besides operating a translational homeostatic control of the proteins of iron metabolism via the assembly of Fe–S clusters in iron regulatory proteins (IRP) [99], is either utilized in other biosynthetic pathways, which may require mitochondria import by mitoferrin, or sequestered in the cytoplasm by ferritin in the Fe^{3+} form. Also ferritinophagy, therefore, releases iron [100]. Consistently, recent reports indicate that increased iron by either HO1 activity, or ferritinophagy, or increased expression of the TfR, sensitizes cells to death by FPT [40,101,102]. Interestingly, a report indicating that in the cardiomyopathy induced by doxorubicin and ischemia-reperfusion, Nrf2 activation primes the expression of HO1, which, by releasing iron, primes FPT [103], further highlights the ambiguous role of this transcription factor in the context of ferroptosis.

In conclusion, in the frame of the complex metabolic regulation, the dimension of the cytosolic LIP emerges as a possible regulator of FPT. However, while it is unquestionable that traces of iron are indispensable to FPT, whether a sudden increase of the amount of available reduced iron could be a physiological agonist priming FPO is hard to establish and seems rather unlikely.

9. Role of PUFAs in membrane phospholipids

As outlined above, LPO requires polyunsaturated phospholipids (see paragraph 2). It can be anticipated, therefore, that phospholipid PUFA content is a major indispensable constraint of FPT.

In cells, long chain free fatty acids (FFA) are trapped and channeled to the different metabolic pathways upon activation by acyl-CoA synthetases (ACSLs). Mammalian cells are endowed of five homologs of these enzymes, which select FFA, although specificity is not strict [104]. Specifically, ACSL4 exhibits a well-documented preference for arachidonic acid and other ω-6 fatty acids, thus controlling membrane phospholipid fatty acid composition [68,105]. Based on this, it is not surprising that ACSL4 impacts on cell sensitivity to FPT [68,106]. Interestingly, mouse embryonal fibroblasts devoid of wild type GPx4, but expressing the homozygous Cys mutant, display a reduced ACSL4 expression. This is suggestive of a compensatory mechanism activated to decrease cell sensitivity to LPO of in the absence of an efficient LOOH reduction [64].

10. Transcriptional and translational regulation of GPx4 expression

GPx4 activity is obviously regulated by selenium availability. Besides its intrinsic role in enzyme catalysis, Se affects the level of the different isoforms of the $tRNA^{[Ser]Sec}$ [107] and, for some glutathione peroxidases, which in this respect are the most studied among selenoproteins, also mRNA stability [108]. In addition, the 3' UTR of the mRNA encoding for selenoproteins, which is indispensable in recoding the UGA termination codon for selenocysteine insertion, also modulates Sec incorporation efficiency at the translational level [109]. These mechanisms account for the non-uniform supply of Sec to selenoproteins (also called hierarchy). Solid evidence converges to the notion that GPx4 ranks among the highest in the hierarchy, which means that, following selenium deprivation, GPx4 mRNA generally remains rather stable [110]. Yet, cultured cells usually lack of a useful source of selenium, and the supply of nanomolar amount of $NaSeO_3$ to the culture medium, increases, depending on the cell type, up to 50% GPx4 protein expression and activity [111], [unpublished]. This probably mostly occurs by a translational effect, and accounts for the identification of Se availability as a constraint of sensitivity to FPT [112].

Besides Se availability, other mechanisms regulating GPx4 expression are ill defined. At the level of gene transcription, the fact that three different proteins can be synthesized from *gpx4*, due to the presence of multiple transcriptional starts, yielding the mitochondrial (m-), nuclear (n-) and cytosolic (c-) GPx4, renders the issue of gene regulation extremely complex [113]. Yet, reverse genetics indicates that only the c-GPx4 isoform is indispensable for embryo survival [41] and seemingly for preventing FPT. The m- and n-forms, instead, are apparently just connected to male fertility [114] and sperm chromatin condensation [115] respectively. According to Imai et al. [116], the specific promoter of the vital c-GPx4 is located after the transcriptional start for the m-form and is apparently further regulated by a stretch of sequences upstream. Interestingly, it is devoid of a classical TATA box, suggesting that c-GPx4 is a housekeeping enzyme, the expression of which is seemingly under the control of general transcription factors such as SP1, NF–Y and AP2 [117], this latter, notably, stressing the role of this isoform in embryonic development. In addition, in CaCo2 cells, a combined CREB and C/EBP (CRE) element located in the upstream region seems necessary for full promoter activation during differentiation [118]. Available information on the issue of *gpx4* expression regulation has been exquisitely summarized in a recent review [119].

Yet, it can be concluded that present knowledge fails to provide hits on the option that FPT is regulated by a sudden shift in GPx4 expression. Just the simplistic view stands, indicating that higher GPx4 expression leads to higher resistance to FPT inducing stimuli.

11. Inactivation of GPx4

FPT was identified as a novel form of RCD by screening cancer-specific drug candidates whose suggested molecular mechanism was inhibition of cellular GPx4 activity.

The observation that, *in vitro, 1S, 3R*-RSL3 does not inhibit purified GPx4 was an intriguing, unexpected evidence [120]. Since inhibition required cytosol, presence of an "inhibition permitting activity" was postulated and the protein accounting for it purified. This protein, 14.3.3ε, interacts with GPx4 and permits the alkylation of the Sec moiety at the active site, thus reasonably playing a scaffold function. Following GPx4 alkylation, the protein-protein interaction is lost. A notable corollary of this mechanism is the presence of a functional thiol-disulfide switch in 14.3.3ε which allows the "inhibition permitting activity" only when reduced. The existence of such a sophisticated

mechanism would be suggestive of a physiological role of 14.3.3ε in FPT beyond just permitting GPx4 inhibition by a synthetic drug. What emerges is a possible feed-back pathway where the more oxidizing environment decreases the sensitivity of GPx4 to inactivation.

All together, these observations further contribute to frame GPx4 activity and FPT in a scenario of a regulated cell death and will encourage the search for the physiological species of which *1S, 3R*-RSL3 could be a pharmacologic mimetic.

12. Conclusions and perspectives

Congruence between enzymology and cell biology is a desirable achievement for the accurate understanding of a (patho)physiological event. This is the case of GPx4, first described *in vitro* as a unique anti-peroxidant, fully characterized enzyme, and re-discovered decades later as the controller of survival or death operated by FPT. It was known from decades that missing GPx4 activity leads to LPO, and what additionally we learned today is that this has a major impact in neurodegenerative diseases, kidney failure, metabolic diseases, ischemia-reperfusion, sepsis and immunological cell killing. Furthermore, the function of GPx4 as critical controller of LPO did also open an interesting window for drug targeting aimed to innovative cancer therapies.

Although descriptions of the specific cellular phenotypes produced by missing GPx4 activity contributed to defining a novel form of regulated cell death, the precise, final mechanism linking LPO to cell death is still obscure. It is not known, indeed, whether it is a specific peroxidation product or the perturbation of membrane order and structure that generates the irreversible loss of homeostatic control executing the death sentence.

From the enzymology of GPx4, the biochemistry of LPO and the features of FPT, we can safely conclude that cells are susceptible to FPT when a convergence is fulfilled between necessary, although not sufficient, constraints:

1) presence of polyunsaturated fatty acids in membrane phospholipids;
2) continuous formation of traces of LOOH, produced as byproduct of oxygen activation associated to oxidative metabolism, and possibly multiplied by LOXs activity;
3) availability of traces of ferrous iron in a complexed form compatible with the reaction with LOOH.

Critical is the notion that the above necessary constraints can lead to FPT only when GPx4 activity is missing or insufficient. This corroborates the proposal that FPT is indispensably activated when the threshold of anti-peroxidant capacity, facing the continuous oxidative challenge produced by ongoing metabolism, is overcame.

Some considerations about the alleged inactivation of GPx4 are suggested by basic enzymology and protein chemistry. What we know is that:

i) GPx4 content and activity do not correlate in different tissues, in agreement with the presence of inactive or less active protein [121];
ii) the Sec moiety of GPx1, and by analogy of GPx4, is prone to beta cleavage forming dehydroalanine (DHA) under conditions of an endogenous oxidative stress [122] or when linked to bulky residues and oxidized to selenoxide [123];
iii) in the absence of GSH, the catalytic Sec of GPx4 forms selenenylamide that evolves to DHA during proteolysis, when tertiary structure is lost [66];
iv) GPx4 inhibition by the electrophile *1S, 3R*-RSL3 indispensably requires the scaffold protein 14.3.3ε seemingly operating a conformational switch on GPx4 [120].

This set of heterogeneous information converge to the challenging hypothesis that GPx4 could be functionally inactivated by beta-cleavage of its selenium moiety under oxidizing conditions and in the absence of GSH, a reaction possibly assisted by a conformational shift.

A condition of extremely low GSH concentration is the final phase of spermatogenesis when *m*-GPx4, by oxidizing as alternative substrate protein thiols, builds up the mitochondrial capsule where it remains co-polymerized [124]. We could wonder, therefore, why spermatozoa do not massively die of FPT while building up mitochondrial capsule. An intriguing explanation is suggested by the fact that the major cardiolipin species in spermatozoa contains four residues of palmitic acid [125], fully resistant to LPO. A case, to our knowledge, unique in biology, consistent with the role of mitochondria and cardiolipin in FPT [126,127].

In conclusion, at the present level of knowledge, FPT emerges as a form of cell death due to missed homeostatic control between mitochondrial aerobic metabolism, associated to energy demand, and capability to deal with the unavoidable “collateral damage”.

Acknowledgements

This article did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.02.027.

References

[1] L. Galluzzi, I. Vitale, S.A. Aaronson, J.M. Abrams, D. Adam, P. Agostinis, E.S. Alnemri, L. Altucci, I. Amelio, D.W. Andrews, M. Annicchiarico-Petruzzelli, A.V. Antonov, E. Arama, E.H. Baehrecke, N.A. Barlev, N.G. Bazan, F. Bernassola, M.J.M. Bertrand, K. Bianchi, M.V. Blagosklonny, K. Blomgren, C. Borner, P. Boya, C. Brenner, M. Campanella, E. Candi, D. Carmona-Gutierrez, F. Cecconi, F.K. Chan, N.S. Chandel, E.H. Cheng, J.E. Chipuk, J.A. Cidlowski, A. Ciechanover, G.M. Cohen, M. Conrad, J.R. Cubillos-Ruiz, P.E. Czabotar, V. D'Angiolella, T.M. Dawson, V.L. Dawson, V. De Laurenzi, R. De Maria, K.M. Debatin, R.J. DeBerardinis, M. Deshmukh, N. Di Daniele, F. Di Virgilio, V.M. Dixit, S.J. Dixon, C.S. Duckett, B.D. Dynlacht, W.S. El-Deiry, J.W. Elrod, G.M. Fimia, S. Fulda, A.J. Garcia-Saez, A.D. Garg, C. Garrido, E. Gavathiotis, P. Golstein, E. Gottlieb, D.R. Green, L.A. Greene, H. Gronemeyer, A. Gross, G. Hajnoczky, J.M. Hardwick, I.S. Harris, M.O. Hengartner, C. Hetz, H. Ichijo, M. Jaattela, B. Joseph, P.J. Jost, P.P. Juin, W.J. Kaiser, M. Karin, T. Kaufmann, O. Kepp, A. Kimchi, R.N. Kitsis, D.J. Klionsky, R.A. Knight, S. Kumar, S.W. Lee, J.J. Lemasters, B. Levine, A. Linkermann, S.A. Lipton, R.A. Lockshin, C. Lopez-Otin, S.W. Lowe, T. Luedde, E. Lugli, M. MacFarlane, F. Madeo, M. Malewicz, W. Malorni, G. Manic, J.C. Marine, S.J. Martin, J.C. Martinou, J.P. Medema, P. Mehlen, P. Meier, S. Melino, E.A. Miao, J.D. Molkentin, U.M. Moll, C. Munoz-Pinedo, S. Nagata, G. Nunez, A. Oberst, M. Oren, M. Overholtzer, M. Pagano, T. Panaretakis, M. Pasparakis, J.M. Penninger, D.M. Pereira, S. Pervaiz, M.E. Peter, M. Piacentini, P. Pinton, J.H.M. Prehn, H. Puthalakath, G.A. Rabinovich, M. Rehm, R. Rizzuto, C.M.P. Rodrigues, D.C. Rubinsztein, T. Rudel, K.M. Ryan, E. Sayan, L. Scorrano, F. Shao, Y. Shi, J. Silke, H.U. Simon, A. Sistigu, B.R. Stockwell, A. Strasser, G. Szabadkai, S.W.G. Tait, D. Tang, N. Tavernarakis, A. Thorburn, Y. Tsujimoto, B. Turk, T. Vanden Berghe, P. Vandenabeele, M.G. Vander Heiden, A. Villunger, H.W. Virgin, K.H. Vousden, D. Vucic, E.F. Wagner, H. Walczak, D. Wallach, Y. Wang, J.A. Wells, W. Wood, J. Yuan, Z. Zakeri, B. Zhivotovsky, L. Zitvogel, G. Melino, G. Kroemer, Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018, Cell Death Differ. 25 (3) (2018) 486–541, https://doi.org/10.1038/s41418-017-0012-4.
[2] L. Ernster, Oxygen as an enviromental poison, Chem. Scripta 26 (4) (1986) 525–534.
[3] B. Halliwell, J.M. Gutteridge, Oxygen toxicity, oxygen radicals, transition metals and disease, Biochem. J. 219 (1) (1984) 1–14, https://doi.org/10.1042/bj2190001.
[4] W.S. Yang, B.R. Stockwell, Ferroptosis: death by lipid peroxidation, Trends Cell Biol. 26 (3) (2015) 165–176, https://doi.org/10.1016/j.tcb.2015.10.014.
[5] W.S. Yang, R. SriRamaratnam, M.E. Welsch, K. Shimada, R. Skouta, V.S. Viswanathan, J.H. Cheah, P.A. Clemons, A.F. Shamji, C.B. Clish, L.M. Brown, A.W. Girotti, V.W. Cornish, S.L. Schreiber, B.R. Stockwell, Regulation of ferroptotic cancer cell death by GPX4, Cell 156 (1–2) (2014) 317–331, https://doi.org/10.1016/j.cell.2013.12.010.
[6] H. Imai, F. Hirao, T. Sakamoto, K. Sekine, Y. Mizukura, M. Saito, T. Kitamoto, M. Hayasaka, K. Hanaoka, Y. Nakagawa, Early embryonic lethality caused by targeted disruption of the mouse PHGPx gene, Biochem. Biophys. Res. Commun. 305 (2) (2003) 278–286, https://doi.org/10.1016/s0006-291x(03)00734-4.
[7] J.P. Friedmann Angeli, M. Schneider, B. Proneth, Y.Y. Tyurina, V.A. Tyurin, V.J. Hammond, N. Herbach, M. Aichler, A. Walch, E. Eggenhofer,

D. Basavarajappa, O. Radmark, S. Kobayashi, T. Seibt, H. Beck, F. Neff, I. Esposito, R. Wanke, H. Forster, O. Yefremova, M. Heinrichmeyer, G.W. Bornkamm, E.K. Geissler, S.B. Thomas, B.R. Stockwell, V.B. O'Donnell, V.E. Kagan, J.A. Schick, M. Conrad, Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice, Nat. Cell Biol. 16 (12) (2014) 1180–1191, https://doi.org/10.1038/ncb3064.

[8] W.S. Hambright, R.S. Fonseca, L. Chen, R. Na, Q. Ran, Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration, Redox Biol. 12 (C) (2017) 8–17, https://doi.org/10.1016/j.redox.2017.01.021.

[9] T. Ueta, T. Inoue, T. Furukawa, Y. Tamaki, Y. Nakagawa, H. Imai, Y. Yanagi, Glutathione peroxidase 4 is required for maturation of photoreceptor cells, J. Biol. Chem. 287 (10) (2012) 7675–7682, https://doi.org/10.1074/jbc.M111.335174.

[10] L. Chen, W.S. Hambright, R. Na, Q. Ran, Ablation of ferroptosis inhibitor glutathione peroxidase 4 in neurons results in rapid motor neuron degeneration and paralysis, J. Biol. Chem. 290 (47) (2015) 28097–28106, https://doi.org/10.1074/jbc.M115.680090.

[11] B.A. Carlson, R. Tobe, E. Yefremova, P.A. Tsuji, V.J. Hoffmann, U. Schweizer, V.N. Gladyshev, D.L. Hatfield, M. Conrad, Glutathione peroxidase 4 and vitamin E cooperatively prevent hepatocellular degeneration, Redox Biol. 9 (2016) 22–31, https://doi.org/10.1016/j.redox.2016.05.003.

[12] R. Kang, L. Zeng, S. Zhu, Y. Xie, J. Liu, Q. Wen, L. Cao, M. Xie, Q. Ran, G. Kroemer, H. Wang, T.R. Billiar, J. Jiang, D. Tang, Lipid peroxidation drives gasdermin D-mediated pyroptosis in lethal polymicrobial sepsis, Cell Host Microbe 24 (1) (2018) 97–108, https://doi.org/10.1016/j.chom.2018.05.009 e104.

[13] M. Wortmann, M. Schneider, J. Pircher, J. Hellfritsch, M. Aichler, N. Vegi, P. Kolle, P. Kuhlencordt, A. Walch, U. Pohl, G.W. Bornkamm, M. Conrad, H. Beck, Combined deficiency in glutathione peroxidase 4 and vitamin e causes multiorgan thrombus formation and early death in mice, Circ. Res. 113 (4) (2013) 408–417, https://doi.org/10.1161/CIRCRESAHA.113.279984.

[14] H. Yin, L. Xu, N.A. Porter, Free radical lipid peroxidation: mechanisms and analysis, Chem. Rev. 111 (10) (2011) 5944–5972, https://doi.org/10.1021/cr200084z.

[15] L. Tang, Y. Zhang, Z. Qian, X. Shen, The mechanism of Fe(2+)-initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical, Biochem. J. 352 Pt 1 (2000) 27–36.

[16] S. Benson, Kinetics of pyrolysis of alkyl hydroperoxides and their O–O bond dissociation energies, J. Chem. Phys. 40 (1964) 1007–1012, https://doi.org/10.1063/1.1725239.

[17] G.W. Burton, K.U. Ingold, Vitamin E as an in vitro and in vivo antioxidant, Ann. N. Y. Acad. Sci. 570 (1989) 7–22, https://doi.org/10.1111/j.1749-6632.1989.tb14904.x.

[18] S. Miyamoto, G.R. Martinez, M.H. Medeiros, P. Di Mascio, Singlet molecular oxygen generated by biological hydroperoxides, J. Photochem. Photobiol., B 139 (2014) 24–33, https://doi.org/10.1016/j.jphotobiol.2014.03.028.

[19] K. Bersuker, J. Hendricks, Z. Li, L. Magtanong, B. Ford, P.H. Tang, M.A. Roberts, B. Tong, T.J. Maimone, R. Zoncu, M.C. Bassik, D.K. Nomura, S.J. Dixon, J.A. Olzmann, The CoQ oxidoreductase FSP1 acts parallel to GPX4 to inhibit ferroptosis, Nature 575 (2019) 688–692, https://doi.org/10.1038/s41586-019-1705-2.

[20] S. Doll, F.P. Freitas, R. Shah, M. Aldrovandi, M.C. da Silva, I. Ingold, A.G. Grocin, T.N. Xavier da Silva, E. Panzilius, C. Scheel, A. Mourao, K. Buday, M. Sato, J. Wanninger, T. Vignane, V. Mohana, M. Rehberg, A. Flatley, A. Schepers, A. Kurz, D. White, M. Sauer, M. Sattler, E.W. Tate, W. Schmitz, A. Schulze, V. O'Donnel, B. Proneth, G.M. Popowicz, D. Pratt, J.P.F. Angeli, M. Conrad, FSP1 is a glutathione-independent ferroptosis suppressor, Nature 575 (2019) 693–698, https://doi.org/10.1038/s41586-019-1707-0.

[21] L. Ernster, F. Navazio, Soluble diaphorase in animal tissues, Acta Chem. Scand. 12 (1958) 595–602, https://doi.org/10.3891/acta.chem.scand.12-0595.

[22] M. Maiorino, M. Coassin, A. Roveri, F. Ursini, Microsomal lipid peroxidation: effect of vitamin E and its functional interaction with phospholipid hydroperoxide glutathione peroxidase, Lipids 24 (8) (1989) 721–726, https://doi.org/10.1007/bf02535211.

[23] G. Miotto, M. Rossetto, M.L. Di Paolo, L. Orian, R. Venerando, A. Roveri, A.M. Vuckovic, V. Bosello Travain, M. Zaccarin, L. Zennaro, M. Maiorino, S. Toppo, F. Ursini, G. Cozza, Insight into the mechanism of ferroptosis inhibition by ferrostatin-1, Redox Biol 28 (2019) 101328, https://doi.org/10.1016/j.redox.2019.101328.

[24] H.J. Forman, K.J. Davies, F. Ursini, How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo, Free Radic. Biol. Med. 66 (2014) 24–35, https://doi.org/10.1016/j.freeradbiomed.2013.05.045.

[25] A. Seiler, M. Schneider, H. Forster, S. Roth, E.K. Wirth, C. Culmsee, N. Plesnila, E. Kremmer, O. Radmark, W. Wurst, G.W. Bornkamm, U. Schweizer, M. Conrad, Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death, Cell Metab. 8 (3) (2008) 237–248, https://doi.org/10.1016/j.cmet.2008.07.005.

[26] G. Scott, Antioxidant in Science, Technology, Medicine and Nutrition, Albion Publishing International Publishers, Chichester, West Sussex, England, 1997.

[27] A. Panday, M.K. Sahoo, D. Osorio, S. Batra, NADPH oxidases: an overview from structure to innate immunity-associated pathologies, Cell. Mol. Immunol. 12 (1) (2015) 5–23, https://doi.org/10.1038/cmi.2014.89.

[28] C.L. Quinlan, I.V. Perevoshchikova, M. Hey-Mogensen, A.L. Orr, M.D. Brand, Sites of reactive oxygen species generation by mitochondria oxidizing different substrates, Redox Biol. 1 (2013) 304–312, https://doi.org/10.1016/j.redox.2013.04.005.

[29] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417 (1) (2009) 1–13, https://doi.org/10.1042/BJ20081386.

[30] G. Loschen, A. Azzi, C. Richter, L. Flohé, Superoxide radicals as precursors of mitochondrial hydrogen peroxide, FEBS Lett. 42 (1) (1974) 68–72, https://doi.org/10.1016/0014-5793(74)80281-4.

[31] M. Hayyan, M.A. Hashim, I.M. AlNashef, Superoxide Ion: generation and chemical implications, Chem. Rev. 116 (5) (2016) 3029–3085, https://doi.org/10.1021/acs.chemrev.5b00407.

[32] F. Ursini, M. Maiorino, P. Hochstein, L. Ernster, Microsomal lipid peroxidation: mechanisms of initiation. The role of iron and iron chelators, Free Radic. Biol. Med. 6 (1) (1989) 31–36, https://doi.org/10.1016/0891-5849(89)90156-1.

[33] A.D. De Grey, HO2*: the forgotten radical, DNA Cell Biol. 21 (4) (2002) 251–257, https://doi.org/10.1089/104454902753759672.

[34] J. Kehrer, J. Robertson, C. Smith, C. McQueen (Ed.), Free Radicals and Reactive Oxygen Species, Comprehensive Toxicology Elsevier Ltd, Oxford, UK, 2010, pp. 277–307.

[35] I. Klapper, R. Hagstrom, R. Fine, K. Sharp, B. Honig, Focusing of electric fields in the active site of Cu-Zn superoxide dismutase: effects of ionic strength and amino-acid modification, Proteins 1 (1) (1986) 47–59, https://doi.org/10.1002/prot.340010109.

[36] M. Gao, J. Yi, J. Zhu, A.M. Minikes, P. Monian, C.B. Thompson, X. Jiang, Role of mitochondria in ferroptosis, Mol Cell 73 (2) (2019) 354–363, https://doi.org/10.1016/j.molcel.2018.10.042 e353.

[37] S.J. Dixon, K.M. Lemberg, M.R. Lamprecht, R. Skouta, E.M. Zaitsev, C.E. Gleason, D.N. Patel, A.J. Bauer, A.M. Cantley, W.S. Yang, B. Morrison 3rd, B.R. Stockwell, Ferroptosis: an iron-dependent form of nonapoptotic cell death, Cell 149 (5) (2012) 1060–1072, https://doi.org/10.1016/j.cell.2012.03.042.

[38] M.M. Gaschler, F. Hu, H. Feng, A. Linkermann, W. Min, B.R. Stockwell, Determination of the subcellular localization and mechanism of action of ferrostatins in suppressing ferroptosis, ACS Chem. Biol. 13 (4) (2018) 1013–1020, https://doi.org/10.1021/acschembio.8b00199.

[39] C.L. Quinlan, R.L. Goncalves, M. Hey-Mogensen, N. Yadava, V.I. Bunik, M.D. Brand, The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I, J. Biol. Chem. 289 (12) (2014) 8312–8325, https://doi.org/10.1074/jbc.M113.545301.

[40] M. Gao, P. Monian, N. Quadri, R. Ramasamy, X. Jiang, Glutaminolysis and transferrin regulate ferroptosis, Mol. Cell 59 (2) (2015) 298–308, https://doi.org/10.1016/j.molcel.2015.06.011.

[41] H. Liang, S.E. Yoo, R. Na, C.A. Walter, A. Richardson, Q. Ran, Short form glutathione peroxidase 4 is the essential isoform required for survival and somatic mitochondrial functions, J. Biol. Chem. 284 (45) (2009) 30836–30844, https://doi.org/10.1074/jbc.M109.032839.

[42] C. Godeas, G. Sandri, E. Panfili, Distribution of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in rat testis mitochondria, Biochim. Biophys. Acta 1191 (1) (1994) 147–150, https://doi.org/10.1016/0005-2736(94)90242-9.

[43] D. Shin, J. Lee, J.H. You, D. Kim, J.L. Roh, Dihydrolipoamide dehydrogenase regulates cystine deprivation-induced ferroptosis in head and neck cancer, Redox Biol. 30 (2020) e101418, , https://doi.org/10.1016/j.redox.2019.101418.

[44] H. Kühn, S. Banthiya, K. van Leyen, Mammalian lipoxygenases and their biological relevance, Biochim. Biophys. Acta 1851 (4) (2015) 308–330, https://doi.org/10.1016/j.bbalip.2014.10.002.

[45] W.S. Yang, K.J. Kim, M.M. Gaschler, M. Patel, M.S. Shchepinov, B.R. Stockwell, Peroxidation of polyunsaturated fatty acids by lipoxygenases drives ferroptosis, Proc. Natl. Acad. Sci. U. S. A. 113 (34) (2016) E4966–E4975, https://doi.org/10.1073/pnas.1603244113.

[46] V.E. Kagan, G. Mao, F. Qu, J.P. Angeli, S. Doll, C.S. Croix, H.H. Dar, B. Liu, V.A. Tyurin, V.B. Ritov, A.A. Kapralov, A.A. Amoscato, J. Jiang, T. Anthonymuthu, D. Mohammadyani, Q. Yang, B. Proneth, J. Klein-Seetharaman, S. Watkins, I. Bahar, J. Greenberger, R.K. Mallampalli, B.R. Stockwell, Y.Y. Tyurina, M. Conrad, H. Bayir, Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis, Nat. Chem. Biol. 13 (1) (2017) 81–90, https://doi.org/10.1038/nchembio.2238.

[47] H. Arai, A. Nagao, J. Terao, T. Suzuki, K. Takama, Effect of d-alpha-tocopherol analogues on lipoxygenase-dependent peroxidation of phospholipid-bile salt micelles, Lipids 30 (2) (1995) 135–140, https://doi.org/10.1007/bf02538266.

[48] R. Shah, M.S. Shchepinov, D.A. Pratt, Resolving the role of lipoxygenases in the initiation and execution of ferroptosis, ACS Cent. Sci. 4 (3) (2018) 387–396, https://doi.org/10.1021/acscentsci.7b00589.

[49] O. Zilka, R. Shah, B. Li, J.P. Friedmann Angeli, M. Griesser, M. Conrad, D.A. Pratt, On the mechanism of Cytoprotection by ferrostatin-1 and Liproxstatin-1 and the role of lipid peroxidation in ferroptotic cell death, ACS Cent. Sci. 3 (3) (2017) 232–243, https://doi.org/10.1021/acscentsci.7b00028.

[50] S. Khanna, S. Roy, H. Ryu, P. Bahadduri, P.W. Swaan, R.R. Ratan, C.K. Sen, Molecular basis of vitamin E action: tocotrienol modulates 12-lipoxygenase, a key mediator of glutamate-induced neurodegeneration, J. Biol. Chem. 278 (44) (2003) 43508–43515, https://doi.org/10.1074/jbc.M307075200.

[51] S.M. Rapoport, T. Schewe, R. Wiesner, W. Halangk, P. Ludwig, M. Janicke-Hohne, C. Tannert, C. Hiebsch, D. Klatt, The lipoxygenase of reticulocytes. Purification, characterization and biological dynamics of the lipoxygenase; its identity with the respiratory inhibitors of the reticulocyte, Eur. J. Biochem. 96 (3) (1979) 545–561, https://doi.org/10.1111/j.1432-1033.1979.tb13068.x.

[52] J. Zhang, M. Kundu, P.A. Ney, Mitophagy in mammalian cells: the reticulocyte model, Methods Enzymol. 452 (2009) 227–245, https://doi.org/10.1016/S0076-6879(08)03615-X.

[53] K. Schnurr, J. Belkner, F. Ursini, T. Schewe, H. Kühn, The selenoenzyme

phospholipid hydroperoxide glutathione peroxidase controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity of the oxygenation products, J. Biol. Chem. 271 (9) (1996) 4653–4658, https://doi.org/10.1074/jbc.271.9.4653.
[54] S. Altamura, N.M. Vegi, P.S. Hoppe, T. Schroeder, M. Aichler, A. Walch, K. Okreglicka, L. Hultner, M. Schneider, C. Ladinig, C. Kuklik-Roos, J. Mysliwietz, D. Janik, F. Neff, B. Rathkolb, M. Hrabe de Angelis, C. Buske, A.R. da Silva, K. Muedder, M. Conrad, T. Ganz, M. Kopf, M.U. Muckenthaler, G.W. Bornkamm, Glutathione peroxidase 4 and vitamin E control reticulocyte maturation, stress erythropoiesis and iron homeostasis, Haematologica 104 (2019), https://doi.org/10.3324/haematol.2018.212977.
[55] R.G. Snodgrass, B. Brune, Regulation and functions of 15-lipoxygenases in human macrophages, Front. Pharmacol. 10 (2019) 719, https://doi.org/10.3389/fphar.2019.00719.
[56] S.H. Brutsch, C.C. Wang, L. Li, H. Stender, N. Neziroglu, C. Richter, H. Kühn, A. Borchert, Expression of inactive glutathione peroxidase 4 leads to embryonic lethality, and inactivation of the Alox15 gene does not rescue such knock-in mice, Antioxid Redox Signal. 22 (4) (2015) 281–293, https://doi.org/10.1089/ars.2014.5967.
[57] T. De Saussure, Recherches chimiques sur la végétation, Chez la Ve, Nyon Libraire, Paris, 1804.
[58] P. Hochstein, L. Ernster, Adp-activated lipid peroxidation coupled to the tpnh oxidase system of microsomes, Biochem. Biophys. Res. Commun. 12 (1963) 388–394.
[59] K. Fukuzawa, T. Seko, K. Minami, J. Terao, Dynamics of iron-ascorbate-induced lipid peroxidation in charged and uncharged phospholipid vesicles, Lipids 28 (6) (1993) 497–503, https://doi.org/10.1007/bf02536080.
[60] D.D. Gibson, K.R. Hornbrook, P.B. McCay, Glutathione-dependent inhibition of lipid peroxidation by a soluble, heat-labile factor in animal tissues, Biochim. Biophys. Acta 620 (3) (1980) 572–582, https://doi.org/10.1016/0005-2760(80)90149-6.
[61] F. Ursini, M. Maiorino, M. Valente, L. Ferri, C. Gregolin, Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides, Biochim. Biophys. Acta 710 (2) (1982) 197–211, https://doi.org/10.1016/0005-2760(82)90150-3.
[62] F. Ursini, M. Maiorino, C. Gregolin, The selenoenzyme phospholipid hydroperoxide glutathione peroxidase, Biochim. Biophys. Acta 839 (1) (1985) 62–70, https://doi.org/10.1016/0304-4165(85)90182-5.
[63] R. Brigelius-Flohé, K.D. Aumann, H. Blöcker, G. Gross, M. Kiess, K.D. Klöppel, M. Maiorino, A. Roveri, R. Schuckelt, F. Ursini, E. Wingender, L. Flohé, Phospholipid-hydroperoxide glutathione peroxidase. Genomic DNA, cDNA, and deduced amino acid sequence, J. Biol. Chem. 269 (10) (1994) 7342–7348.
[64] I. Ingold, C. Berndt, S. Schmitt, S. Doll, G. Poschmann, K. Buday, A. Roveri, X. Peng, F. Porto Freitas, T. Seibt, L. Mehr, M. Aichler, A. Walch, D. Lamp, M. Jastroch, S. Miyamoto, W. Wurst, F. Ursini, E.S.J. Arner, N. Fradejas-Villar, U. Schweizer, H. Zischka, J.P. Friedmann Angeli, M. Conrad, Selenium utilization by GPX4 is required to prevent hydroperoxide-induced ferroptosis, Cell 172 (3) (2018) 409–422, https://doi.org/10.1016/j.cell.2017.11.048 e421.
[65] S. Toppo, L. Flohé, F. Ursini, S. Vanin, M. Maiorino, Catalytic mechanisms and specificities of glutathione peroxidases: variations of a basic scheme, Biochim. Biophys. Acta 1790 (11) (2009) 1486–1500, https://doi.org/10.1016/j.bbagen.2009.04.007.
[66] L. Orian, P. Mauri, A. Roveri, S. Toppo, L. Benazzi, V. Bosello-Travain, A. De Palma, M. Maiorino, G. Miotto, M. Zaccarin, A. Polimeno, L. Flohé, F. Ursini, Selenocysteine oxidation in glutathione peroxidase catalysis: an MS-supported quantum mechanics study, Free Radic. Biol. Med. 87 (2015) 1–14, https://doi.org/10.1016/j.freeradbiomed.2015.06.011.
[67] S.H. Hong, D.H. Lee, Y.S. Lee, M.J. Jo, Y.A. Jeong, W.T. Kwon, H.A. Choudry, D.L. Bartlett, Y.J. Lee, Molecular crosstalk between ferroptosis and apoptosis: emerging role of ER stress-induced p53-independent PUMA expression, Oncotarget 8 (70) (2017) 115164–115178, https://doi.org/10.18632/oncotarget.23046.
[68] S. Doll, B. Proneth, Y.Y. Tyurina, E. Panzilius, S. Kobayashi, I. Ingold, M. Irmler, J. Beckers, M. Aichler, A. Walch, H. Prokisch, D. Trumbach, G. Mao, F. Qu, H. Bayir, J. Fullekrug, C.H. Scheel, W. Wurst, J.A. Schick, V.E. Kagan, J.P. Angeli, M. Conrad, ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition, Nat. Chem. Biol. 13 (1) (2017) 91–98, https://doi.org/10.1038/nchembio.2239.
[69] G.P. Drummen, L.C. van Liebergen, J.A. Op den Kamp, J.A. Post, C11-BODIPY (581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro) spectroscopic characterization and validation of methodology, Free Radic. Biol. Med. 33 (4) (2002) 473–490, https://doi.org/10.1016/s0891-5849(02)00848-1.
[70] Y. Chen, Y. Liu, T. Lan, W. Qin, Y. Zhu, K. Qin, J. Gao, H. Wang, X. Hou, N. Chen, J.P. Friedmann Angeli, M. Conrad, C. Wang, Quantitative profiling of protein carbonylations in ferroptosis by an aniline-derived probe, J. Am. Chem. Soc. 140 (13) (2018) 4712–4720, https://doi.org/10.1021/jacs.8b01462.
[71] G. Nohammer, E. Schauenstein, P. Weber, Microspectrometric quantification of 4-hydroxypentenal binding to protein thios in Ehrlich ascites tumor cells, Acta Hystocchem Suppl 16 (1976) 189–196.
[72] P. Winkler, W. Lindner, H. Esterbauer, E. Schauenstein, R.J. Schaur, G.A. Khoschsorur, Detection of 4-hydroxynonenal as a product of lipid peroxidation in native Ehrlich ascites tumor cells, Biochim. Biophys. Acta 796 (3) (1984) 232–237, https://doi.org/10.1016/0005-2760(84)90122-x.
[73] Z.Z. Shi, J. Osei-Frimpong, G. Kala, S.V. Kala, R.J. Barrios, G.M. Habib, D.J. Lukin, C.M. Danney, M.M. Matzuk, M.W. Lieberman, Glutathione synthesis is essential for mouse development but not for cell growth in culture, Proc. Natl. Acad. Sci. U. S. A. 97 (10) (2000) 5101–5106.
[74] T.P. Dalton, Y. Chen, S.N. Schneider, D.W. Nebert, H.G. Shertzer, Genetically altered mice to evaluate glutathione homeostasis in health and disease, Free Radic. Biol. Med. 37 (10) (2004) 1511–1526, https://doi.org/10.1016/j.freeradbiomed.2004.06.040.
[75] R. Franco, J.A. Cidlowski, Glutathione efflux and cell death, Antioxid Redox Signal. 17 (12) (2012) 1694–1713, https://doi.org/10.1089/ars.2012.4553.
[76] S. Orrenius, V. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 143–183, https://doi.org/10.1146/annurev.pharmtox.47.120505.105122.
[77] H. Forman, Glutathione, L. Flohé (Ed.), Biosynthesis of Glutathione and its Regulation, CRC Press, Boca Raton, FL, 2018, pp. 3–34 33487-2742.
[78] J. Fujii, J.I. Ito, X. Zhang, T. Kurahashi, Unveiling the roles of the glutathione redox system in vivo by analyzing genetically modified mice, J. Clin. Biochem. Nutr. 49 (2) (2011) 70–78, https://doi.org/10.3164/jcbn.10-138SR.
[79] M. Yamamoto, T.W. Kensler, H. Motohashi, The KEAP1-NRF2 system: a thiol-Based Sensor-Effector Apparatus for maintaining redox homeostasis, Physiol. Rev. 98 (3) (2018) 1169–1203, https://doi.org/10.1152/physrev.00023.2017.
[80] K. Itoh, K.I. Tong, M. Yamamoto, Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles, Free Radic. Biol. Med. 36 (10) (2004) 1208–1213, https://doi.org/10.1016/j.freeradbiomed.2004.02.075.
[81] V. Vollrath, A.M. Wielandt, M. Iruretagoyena, J. Chianale, Role of Nrf2 in the regulation of the Mrp2 (ABCC2) gene, Biochem. J. 395 (3) (2006) 599–609, https://doi.org/10.1042/BJ20051518.
[82] C.M. Mahaffey, H. Zhang, A. Rinna, W. Holland, P.C. Mack, H.J. Forman, Multidrug-resistant protein-3 gene regulation by the transcription factor Nrf2 in human bronchial epithelial and non-small-cell lung carcinoma, Free Radic. Biol. Med. 46 (12) (2009) 1650–1657, https://doi.org/10.1016/j.freeradbiomed.2009.03.023.
[83] A. Meister, On the enzymology of amino acid transport, Science 180 (4081) (1973) 33–39, https://doi.org/10.1126/science.180.4081.33.
[84] N.P. Curthoys, R.P. Hughey, Characterization and physiological function of rat renal gamma-glutamyltranspeptidase, Enzyme 24 (6) (1979) 383–403, https://doi.org/10.1159/000458694.
[85] T.J. Woodlock, R. Brown, M. Mani, L. Pompeo, H. Hoffman, G.B. Segel, R. Silber, Decreased L system amino acid transport and decreased gamma-glutamyl transpeptidase are independent processes in human chronic lymphocytic leukemia B-lymphocytes, J. Cell. Physiol. 145 (2) (1990) 217–221, https://doi.org/10.1002/jcp.1041450205.
[86] J. Lewerenz, S.J. Hewett, Y. Huang, M. Lambros, P.W. Gout, P.W. Kalivas, A. Massie, I. Smolders, A. Methner, M. Pergande, S.B. Smith, V. Ganapathy, P. Maher, The cystine/glutamate antiporter system x(c)(-) in health and disease: from molecular mechanisms to novel therapeutic opportunities, Antioxid Redox Signal. 18 (5) (2013) 522–555, https://doi.org/10.1089/ars.2011.4391.
[87] S. Tan, D. Schubert, P. Maher, Oxytosis, A novel form of programmed cell death, Curr. Top. Med. Chem. 1 (6) (2001) 497–506, https://doi.org/10.2174/1568026013394741.
[88] J. Lewerenz, G. Ates, A. Methner, M. Conrad, P. Maher, Oxytosis/Ferroptosis-(Re-) emerging roles for oxidative stress-dependent non-apoptotic cell death in diseases of the central nervous system, Front. Neurosci. 12 (2018), https://doi.org/10.3389/fnins.2018.00214.
[89] A. Tarangelo, L. Magtanong, K.T. Bieging-Rolett, Y. Li, J. Ye, L.D. Attardi, S.J. Dixon, p53 suppresses metabolic stress-induced ferroptosis in cancer cells, Cell Rep. 22 (3) (2018) 569–575, https://doi.org/10.1016/j.celrep.2017.12.077.
[90] L. Jiang, N. Kon, T. Li, S.J. Wang, T. Su, H. Hibshoosh, R. Baer, W. Gu, Ferroptosis as a p53-mediated activity during tumour suppression, Nature 520 (7545) (2015) 57–62, https://doi.org/10.1038/nature14344.
[91] A. Hafner, M.L. Bulyk, A. Jambhekar, G. Lahav, The multiple mechanisms that regulate p53 activity and cell fate, Nat. Rev. Mol. Cell Biol. 20 (4) (2019) 199–210, https://doi.org/10.1038/s41580-019-0110-x.
[92] V. Kalatzis, S. Cherqui, C. Antignac, B. Gasnier, Cystinosin, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter, EMBO J. 20 (21) (2001) 5940–5949, https://doi.org/10.1093/emboj/20.21.5940.
[93] G.J. McBean, The transsulfuration pathway: a source of cysteine for glutathione in astrocytes, Amino Acids 42 (1) (2012) 199–205, https://doi.org/10.1007/s00726-011-0864-8.
[94] M.H. Stipanuk, I. Ueki, Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur, J. Inherit. Metab. Dis. 34 (1) (2011) 17–32, https://doi.org/10.1007/s10545-009-9006-9.
[95] D. Hayes, M. Wiessner, T. Rauen, G.J. McBean, Transport of L-[14C]cystine and L-[14C]cysteine by subtypes of high affinity glutamate transporters over-expressed in HEK cells, Neurochem. Int. 46 (8) (2005) 585–594, https://doi.org/10.1016/j.neuint.2005.03.001.
[96] M. Hayano, W.S. Yang, C.K. Corn, N.C. Pagano, B.R. Stockwell, Loss of cysteinyl-tRNA synthetase (CARS) induces the transsulfuration pathway and inhibits ferroptosis induced by cystine deprivation, Cell Death Differ. 23 (2) (2016) 270–278, https://doi.org/10.1038/cdd.2015.93.
[97] O. Kakhlon, Z.I. Cabantchik, The labile iron pool: characterization, measurement, and participation in cellular processes(1), Free Radic. Biol. Med. 33 (8) (2002) 1037–1046, https://doi.org/10.1016/s0891-5849(02)01006-7.
[98] C. Chen, B.H. Paw, Cellular and mitochondrial iron homeostasis in vertebrates, Biochim. Biophys. Acta 1823 (9) (2012) 1459–1467, https://doi.org/10.1016/j.bbamcr.2012.01.003.
[99] T.A. Rouault, The indispensable role of mammalian iron sulfur proteins in function

and regulation of multiple diverse metabolic pathways, Biometals 32 (3) (2019) 343–353, https://doi.org/10.1007/s10534-019-00191-7.
[100] N. Santana-Codina, J.D. Mancias, The role of NCOA4-mediated ferritinophagy in health and disease, Pharmaceuticals 11 (4) (2018), https://doi.org/10.3390/ph11040114.
[101] S.K. Chiang, S.E. Chen, L.C. Chang, A dual role of heme oxygenase-1 in cancer cells, Int. J. Mol. Sci. 20 (1) (2018), https://doi.org/10.3390/ijms20010039.
[102] W. Hou, Y. Xie, X. Song, X. Sun, M.T. Lotze, H.J. Zeh 3rd, R. Kang, D. Tang, Autophagy promotes ferroptosis by degradation of ferritin, Autophagy 12 (8) (2016) 1425–1428, https://doi.org/10.1080/15548627.2016.1187366.
[103] X. Fang, H. Wang, D. Han, E. Xie, X. Yang, J. Wei, S. Gu, F. Gao, N. Zhu, X. Yin, Q. Cheng, P. Zhang, W. Dai, J. Chen, F. Yang, H.T. Yang, A. Linkermann, W. Gu, J. Min, F. Wang, Ferroptosis as a target for protection against cardiomyopathy, Proc. Natl. Acad. Sci. U. S. A. 116 (7) (2019) 2672–2680, https://doi.org/10.1073/pnas.1821022116.
[104] E.L. Klett, S. Chen, A. Yechoor, F.B. Lih, R.A. Coleman, Long-chain acyl-CoA synthetase isoforms differ in preferences for eicosanoid species and long-chain fatty acids, J. Lipid Res. 58 (5) (2017) 884–894, https://doi.org/10.1194/jlr.M072512.
[105] E.A. Killion, A.R. Reeves, M.A. El Azzouny, Q.W. Yan, D. Surujon, J.D. Griffin, T.A. Bowman, C. Wang, N.R. Matthan, E.L. Klett, D. Kong, J.W. Newman, X. Han, M.J. Lee, R.A. Coleman, A.S. Greenberg, A role for long-chain acyl-CoA synthetase-4 (ACSL4) in diet-induced phospholipid remodeling and obesity-associated adipocyte dysfunction, Mol. Metab. 9 (2018) 43–56, https://doi.org/10.1016/j.molmet.2018.01.012.
[106] S.J. Dixon, G.E. Winter, L.S. Musavi, E.D. Lee, B. Snijder, M. Rebsamen, G. Superti-Furga, B.R. Stockwell, Human Haploid cell genetics Reveals roles for lipid metabolism genes in nonapoptotic cell death, ACS Chem. Biol. 10 (7) (2015) 1604–1609, https://doi.org/10.1021/acschembio.5b00245.
[107] D. Hatfield, B.J. Lee, L. Hampton, A.M. Diamond, Selenium induces changes in the selenocysteine tRNA[Ser]Sec population in mammalian cells, Nucleic Acids Res. 19 (4) (1991) 939–943, https://doi.org/10.1093/nar/19.4.939.
[108] C. Muller, K. Wingler, R. Brigelius-Flohé, 3'UTRs of glutathione peroxidases differentially affect selenium-dependent mRNA stability and selenocysteine incorporation efficiency, Biol. Chem. 384 (1) (2003) 11–18, https://doi.org/10.1515/BC.2003.002.
[109] L. Latreche, O. Jean-Jean, D.M. Driscoll, L. Chavatte, Novel structural determinants in human SECIS elements modulate the translational recoding of UGA as selenocysteine, Nucleic Acids Res. 37 (17) (2009) 5868–5880, https://doi.org/10.1093/nar/gkp635.
[110] K. Wingler, M. Böcher, L. Flohé, H. Kollmus, R. Brigelius-Flohé, mRNA stability and selenocysteine insertion sequence efficiency rank gastrointestinal glutathione peroxidase high in the hierarchy of selenoproteins, Eur. J. Biochem. 259 (1–2) (1999) 149–157, https://doi.org/10.1046/j.1432-1327.1999.00012.x.
[111] M. Maiorino, F.F. Chu, F. Ursini, K.J. Davies, J.H. Doroshow, R.S. Esworthy, Phospholipid hydroperoxide glutathione peroxidase is the 18-kDa selenoprotein expressed in human tumor cell lines, J. Biol. Chem. 266 (12) (1991) 7728–7732.
[112] B.R. Cardoso, D.J. Hare, A.I. Bush, B.R. Roberts, Glutathione peroxidase 4: a new player in neurodegeneration? Mol. Psychiatry 22 (3) (2017) 328–335, https://doi.org/10.1038/mp.2016.196.
[113] M. Maiorino, M. Scapin, F. Ursini, M. Biasolo, V. Bosello, L. Flohé, Distinct promoters determine alternative transcription of gpx-4 into phospholipid-hydroperoxide glutathione peroxidase variants, J. Biol. Chem. 278 (36) (2003) 34286–34290, https://doi.org/10.1074/jbc.M305327200.
[114] M. Schneider, H. Forster, A. Boersma, A. Seiler, H. Wehnes, F. Sinowatz, C. Neumuller, M.J. Deutsch, A. Walch, M. Hrabe de Angelis, W. Wurst, F. Ursini, A. Roveri, M. Maleszewski, M. Maiorino, M. Conrad, Mitochondrial glutathione peroxidase 4 disruption causes male infertility, Faseb. J. 23 (9) (2009) 3233–3242, https://doi.org/10.1096/fj.09-132795.
[115] M. Conrad, S.G. Moreno, F. Sinowatz, F. Ursini, S. Kolle, A. Roveri, M. Brielmeier, W. Wurst, M. Maiorino, G.W. Bornkamm, The nuclear form of phospholipid hydroperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability, Mol. Cell Biol. 25 (17) (2005) 7637–7644, https://doi.org/10.1128/MCB.25.17.7637-7644.2005.
[116] H. Imai, M. Saito, N. Kirai, J. Hasegawa, K. Konishi, H. Hattori, M. Nishimura, S. Naito, Y. Nakagawa, Identification of the positive regulatory and distinct core regions of promoters, and transcriptional regulation in three types of mouse phospholipid hydroperoxide glutathione peroxidase, J. Biochem. 140 (4) (2006) 573–590, https://doi.org/10.1093/jb/mvj186.
[117] C. Ufer, A. Borchert, H. Kühn, Functional characterization of cis- and trans-regulatory elements involved in expression of phospholipid hydroperoxide glutathione peroxidase, Nucleic Acids Res. 31 (15) (2003) 4293–4303, https://doi.org/10.1093/nar/gkg650.
[118] B. Speckmann, H.J. Bidmon, A. Pinto, M. Anlauf, H. Sies, H. Steinbrenner, Induction of glutathione peroxidase 4 expression during enterocytic cell differentiation, J. Biol. Chem. 286 (12) (2011) 10764–10772, https://doi.org/10.1074/jbc.M110.216028.
[119] R. Brigelius-Flohé, L. Flohé, Regulatory Phenomena in the Glutathione Peroxidase Superfamily, Antioxid Redox Signal, 2019, https://doi.org/10.1089/ars.2019.7905.
[120] A.M. Vučković, V. Bosello Travain, L. Bordin, G. Cozza, G. Miotto, M. Rossetto, S. Toppo, R. Venerando, M. Zaccarin, M. Maiorino, F. Ursini, A. Roveri, Inactivation of the glutathione peroxidase GPx4 by the ferroptosis-inducing molecule RSL3 requires the adaptor protein 14-3-3epsilon, FEBS Lett. 594 (2019) 611–624, https://doi.org/10.1002/1873-3468.13631.
[121] A. Roveri, M. Maiorino, F. Ursini, Enzymatic and immunological measurements of soluble and membrane-bound phospholipid-hydroperoxide glutathione peroxidase, Methods Enzymol. 233 (1994) 202–212, https://doi.org/10.1016/s0076-6879(94)33023-9.
[122] S.K. Wang, J.D. Weaver, S. Zhang, X.G. Lei, Knockout of SOD1 promotes conversion of selenocysteine to dehydroalanine in murine hepatic GPX1 protein, Free Radic. Biol. Med. 51 (1) (2011) 197–204, https://doi.org/10.1016/j.freeradbiomed.2011.03.018.
[123] S. Ma, R.M. Caprioli, K.E. Hill, R.F. Burk, Loss of selenium from selenoproteins: conversion of selenocysteine to dehydroalanine in vitro, J. Am. Soc. Mass Spectrom. 14 (6) (2003) 593–600, https://doi.org/10.1016/S1044-0305(03)00141-7.
[124] F. Ursini, S. Heim, M. Kiess, M. Maiorino, A. Roveri, J. Wissing, L. Flohé, Dual function of the selenoprotein PHGPx during sperm maturation, Science 285 (5432) (1999) 1393–1396, https://doi.org/10.1126/science.285.5432.1393.
[125] H.-Y.J. W, N.J. Shelley, S.W. Amina, Direct MALDI-MS analysis of cardiolipin from rat organ sections, J. Am. Soc. Mass Spectrom. 18 (3) (2006) 567–577, https://doi.org/10.1016/j.jasms.2006.10.023.
[126] K. Nomura, H. Imai, T. Koumura, T. Kobayashi, Y. Nakagawa, Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis, Biochem. J. 351 (Pt 1) (2000) 183–193, https://doi.org/10.1042/0264-6021:3510183.
[127] G. Cozza, M. Rossetto, V. Bosello-Travain, M. Maiorino, A. Roveri, S. Toppo, M. Zaccarin, L. Zennaro, F. Ursini, Glutathione peroxidase 4-catalyzed reduction of lipid hydroperoxides in membranes: the polar head of membrane phospholipids binds the enzyme and addresses the fatty acid hydroperoxide group toward the redox center, Free Radic. Biol. Med. 112 (2017) 1–11, https://doi.org/10.1016/j.freeradbiomed.2017.07.010.