



Formation of Oxidatively Modified Lipids as the Basis for a Cellular Epilipidome

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While often regarded as a subset of metabolomics, lipidomics can better be considered as a field in its own right. While the total number of lipid species in biology may not exceed the number of metabolites, they can be modified chemically and biochemically leading to an enormous diversity of derivatives, many of which retain the lipophilic properties of lipids and thus expand the lipidome greatly. Oxidative modification by radical oxygen species, either enzymatically or chemically, is one of the major mechanisms involved, although attack by non-radical oxidants also occurs. The modified lipids typically contain more oxygens in the form of hydroxyl, epoxide, carbonyl and carboxylic acid groups, and nitration, nitrosylation, halogenation or sulfation can also occur. This article provides a succinct overview of the types of species formed, the reactive compounds involved and the specific molecular sites that they react with, and the biochemical or chemical mechanisms involved. In many cases, these modifications reduce the stability of the lipid, and breakdown products are formed, which themselves have interesting properties such as the ability to react with other biomolecules. Publications on the biological effects of modified lipids are growing rapidly, supporting the concept that some of these biomolecules have potential signaling and regulatory effects. The question therefore arises whether modified lipids represent an “epilipidome”, analogous to the epigenetic modifications that can control gene expression.

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INTRODUCTION

The oxidation of lipids and lipid-like substances has been known for centuries, and has been widely regarded as an undesirable effect: in foods, lipid oxidation leads to the development of rancidity and acrid flavors, while in materials such as rubber it causes loss of elasticity and perishing (1). In biology, where lipids have important structural, nutritional, and signaling roles, the adventitious, radical oxidation of lipids in cells and tissues was for many years also be regarded as a detrimental process, for example disrupting cell membranes and causing cytotoxicity (**Figure 1A**) (2, 3). On the other hand, in the 1950s the structure of prostaglandins was elucidated and found to result from peroxidation of arachidonic acid [reviewed by (4)]; subsequently, thromboxanes and leukotrienes were also realized to be derived from hydroperoxyeicosatetraenoates (HPETEs) (5).

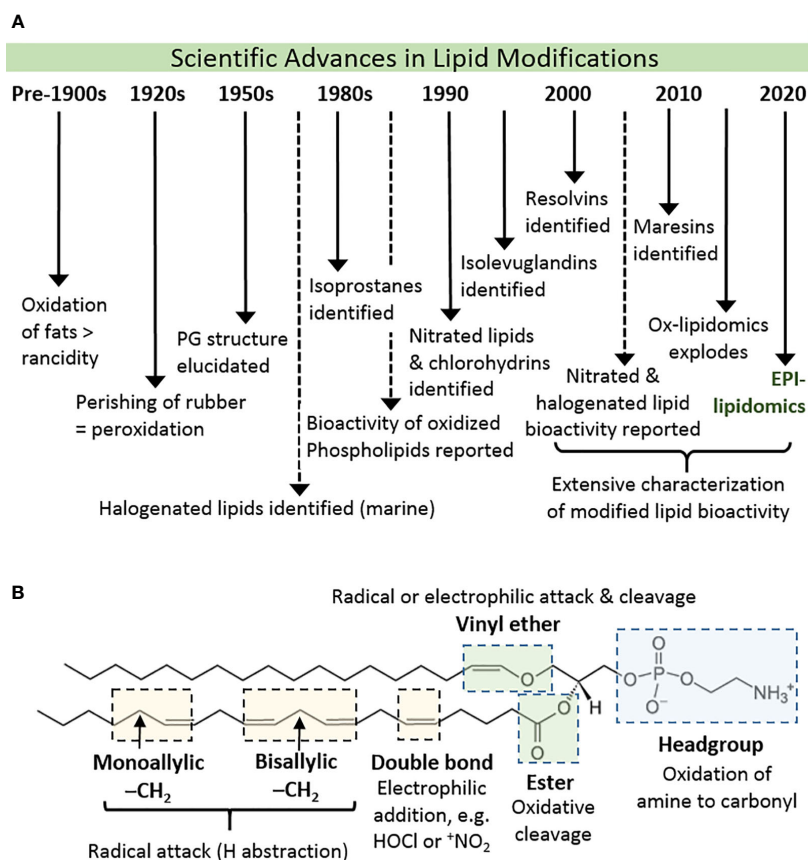


FIGURE 1 | History and basics of lipid oxidation. **(A)** Diagrammatic timeline of research into lipid oxidation identifying key discoveries and concepts. **(B)** Major sites of attack in phospholipids and types of reaction that can occur there, using 1-(1-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphoethanolamine as an example. Other phospholipids containing these or analogous chemical groups show similar susceptibility.

These enzymatically generated non-esterified lipid products were recognized as important signalling molecules in the cardiovascular and immune systems, and therefore as important therapeutic targets (6). Consequently, there was much interest in their enzymatic production by cyclooxygenases, lipoxygenases, and cytochrome P450-dependent enzymes (7), a topic that continues to be of interest and is reviewed elsewhere in this issue. Later, the non-enzymatic formation of analogous compounds (F_2 -isoprostanes) was discovered (8) and, in parallel, evidence began to emerge that non-enzymatic oxidation products of fatty acids esterified in phospholipids also had biological activities (9). While initial studies reported detrimental effects in atherosclerosis, soon it was noted that some of these compounds were able to block immune receptors and prevent damaging immune responses, e.g. in sepsis (10). The years from 2000 onwards witnessed an explosion in the identification of non-enzymatic lipid modifications and resulting biological effects. A wide variety of additional oxidation product families were identified, including isolevuglandins, nitrated and halogenated fatty acids or phospholipids, oxysterols and halogenated sterols, as well as the discovery of resolvins (11) and maresins (12) from oxidation products of the omega-3 fatty acids eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA)

and docosahexaenoic acid (DHA). In most cases, a strong driver in their discovery has been the elucidation of biological signalling effects and, as the field has evolved, it has become clear that certain modified lipid species have beneficial effects in specific circumstances; in many cases, we also have an understanding of the mechanisms involved. Thus, oxidatively modified lipids are now well-established as mediators of biological processes (2, 13–16).

CHEMICAL PROPERTIES OF LIPIDS THAT ENABLE MODIFICATIONS

Lipids are a hugely diverse chemical group, but the lipid species most prevalent in biological systems, and especially in mammalian cells, are free fatty acids, ceramides, phospholipids (including phosphatidylglycerols and sphingomyelin), mono-, di- and tri-acylglycerols, and sterols. The lipid structure determines the nature and likelihood of oxidative modifications to it, but reactive oxidizing compounds also demonstrate different specificities (17). The chemical moieties most typically susceptible to oxidative attack and modifications are shown in

Figure 1B. In general, these are electron-dense regions of the molecules (double bonds), or ones where the bonds are polarized and can be broken with lower energy input.

The site of attack that leads to the widest range of modifications and oxidation products is the fatty acyl chain. Although fully saturated hydrocarbon chains can be attacked by high energy oxidants, e.g. ozone and triplet oxygen, higher numbers of double bonds increase the susceptibility to radical attack, as hydrogen atoms can more easily be abstracted from bis-allylic carbon atoms (17). On the other hand, mono-unsaturated fatty acyl chains react readily with non-radical oxidants, such as hypochlorous acid (18). In sphingomyelins, the sphingosine moiety appears to be the main site of modification, at least by hydroxyl radicals, reflecting the presence of a C-C double bond (19). Likewise, in cholesterol the mono-unsaturated B ring is readily oxidized, although enzymatic oxidation of the tail also occurs (20–22).

In phospholipids, fatty acyl chains are connected to the glycerol backbone by 3 different types of bond: ester bonds, ether bonds (in alkanyl phospholipids), or vinyl ether bonds (in alkenyl phospholipids, also called plasmalogens). The ether or vinyl ether bonds occur mostly commonly at the SN-1 position of the glycerol. The ester bonds are most common biologically and can be hydrolyzed enzymatically, for example by phospholipase A₁ or A₂, which results in formation of lysophospholipids. These have altered biological properties and can be considered as biological mediators. In contrast, vinyl ether bonds are susceptible to attack by radicals (23) and electrophilic oxidants (24), forming oxidant-dependent products. Phospholipid headgroups containing an amine group can also undergo oxidation, although the quaternary ammonium structure of phosphocholine is resistant; changes in headgroup structure are likely to impact significantly on the phospholipid function within the cell membrane (25, 26).

TYPES OF LIPID MODIFICATIONS

The variety of sites of modification in lipids present the basis for the large range of products that can be formed (27), but this is expanded by the type of oxidant that causes the modification and the stability or otherwise of the initial product. This aspect will be explored in the following sections to illustrate the potential for diversity in modified lipids. **Figure 2** provides an overview of the key types of products.

Peroxidation of Fatty Acyl Chains Caused by Free Radical Attack

Whether enzymatic or non-enzymatic lipid modification is considered, radical attack leads to the widest range of products, largely because of the unstable nature of the initial oxidation products, their potential for rearrangement, and subsequent breakdown or fragmentation. For hydrocarbon chains, radical attack involves the abstraction of a hydrogen to form a carbon-centered radical, and leads to formation of a peroxide by incorporation of molecular oxygen (28). The potential for rearrangement at carbon radical stage depends on the degree

and nature of unsaturation in the local area; for example, whether it is a conjugated system.

Hydrogen abstraction at bis-allylic carbons is favored, although it can also occur at allylic sites. This makes polyunsaturated fatty acyl chains such as linoleate (1 bis-allylic carbon); linolenate acid (2 bis-allylic carbons), arachidonate (3 bis-allylic carbons), eicosapentenoate (4 bis-allylic carbons), and docosahexenoate (5 bis-allylic carbons) increasingly susceptible to peroxidation, which can occur at multiple sites (3, 17). As the extent of modification by oxygen increases, the complexity of the oxidation product set increases, and their stability decreases. The initial product is a peroxy radical, which can either react intramolecularly to form an endoperoxide in which the molecule retains an unpaired electron, or it can abstract a hydrogen from an adjacent molecule to form a hydroperoxide, concomitantly initiating the chain reaction of lipid peroxidation.

Endoperoxide formation is central in the formation of a number of bioactive oxidized lipid families, including the isoprostanes (29). Rearrangement of the endoperoxide results in formation of 5-membered ring structures, such as cyclopentenone rings, which are present in isoprostanes and their enzymatic analogues prostaglandins (30). Alternatively, the hydrocarbon chain can be cleaved to form the highly reactive compounds isolevuglandins, which are di-aldehydes (31, 32). Similar reactions also result in formation of the lipid oxidation breakdown product malondialdehyde.

In contrast, the hydroperoxides are relatively stable, and can be detected in biological samples following organic extraction and storage at -20°C or lower (33). Hydroperoxides can be reduced through the action of phospholipid-dependent glutathione peroxidase (GPx4), which converts the hydroperoxide to an alcohol (34, 35), although a mechanism for removing the -OH moiety to regenerate the hydrocarbon chain is not currently known. Hydroperoxides can also be converted to epoxides through homolytic cleavage of the hydroperoxide to form an alkoxyl radical, which attacks the adjacent carbon atom (36).

Either peroxy radical, endoperoxides, or hydroperoxides can undergo intra-molecular reactions leading to the fragmentation of the carbon chain, which usually generates an aldehyde at one or both sides of the cleavage site. This process is responsible for the formation of a variety of lipid peroxidation breakdown products, of which the best-known example is 4-hydroxynonenal, in parallel with the corresponding chain-shortened phospholipid (37). These products can subsequently be metabolized by enzymes of the aldoketoreductase (AKR) and aldehyde dehydrogenase families, involving either reduction to alcohols or oxidation to carboxylic acids (38–40), thus generating further product diversity. An idea of the extent of the possible diversity can be obtained by considering that addition of two molecular oxygens to arachidonate can yield a family of 64 F₂-isoprostanes, when stereoisomers are included (41). Moreover, fragmentation of oxidized phospholipids can yield multiple breakdown products, and analysis is challenging as ones from different parent lipids may be isomeric or isobaric, as observed by liquid chromatography tandem mass spectrometry (42).

Analogous reactions can also take place on cholesterol and sphingolipid chains. Radical oxidation of cholesterol yields a

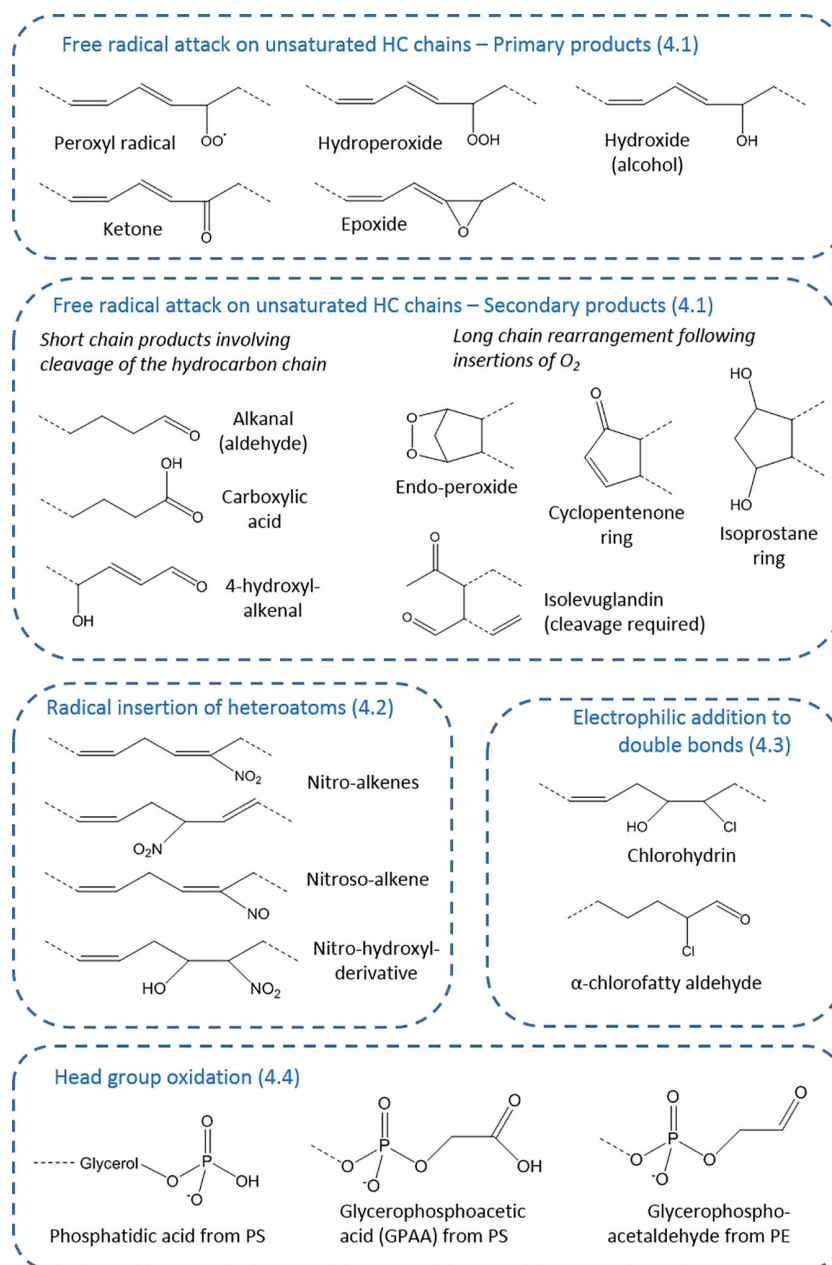


FIGURE 2 | Types of oxidative modifications on fatty acyl chains. The products are organized according to section of the article (numbered), showing the wide variety of chemical structures possible. These chemical moieties can occur on esterified or non-esterified fatty acyl chains, or cholesterol, and for each generic structure many distinct compounds (isomers and stereoisomers) may exist—for example, 64 in the case of isoprostanes—as well as analogous compounds from starting lipids with different chain length and unsaturation.

family of oxysterols modified on the B-ring, including 7-hydroxycholesterol, 7-keto cholesterol, 5-hydroperoxycholesterol, 5,6-epoxycholesterol and cholestane-3,5,6-triol (43). In contrast, enzymatic oxidation catalyzed by cytochrome P450 enzymes (e.g. CYP27A1, CYP46A1) tends to hydroxylate the saturated hydrocarbon tail, although 7 α -hydroxycholesterol is formed by CYP7A1 (20). Carotenoids contain conjugated polyunsaturated chains and are also highly susceptible to oxidative attack; carotene

oxidation products reported include cyclized hydroxy- and keto-containing as well as aldehydes resulting from chain cleavage (44, 45). Oxidation of sphingosylphosphorylcholine has been observed to form hydroxyl and keto derivatives on the sphingosine chain (19).

The ability of radicals to initiate hydrogen abstraction varies. Hydroxyl radical (OH \cdot) is one of the most reactive radicals formed in biological systems, and readily causes lipid

peroxidation (46). In contrast, superoxide, a radical produced by certain NADPH oxidases, is relatively poor at initiating lipid peroxidation, and hydrogen peroxide is unable to do this in the absence of transition metal ions that support Fenton chemistry to generate hydroxyl radicals (17); transition metals such as copper, manganese and iron readily undergo one-electron (radical) reactions. Similarly, the non-radical anion peroxyxynitrite (ONOO^-) does not cause hydrogen abstraction directly, although it is reactive and can be converted to nitrogen-containing radicals such as nitrogen dioxide that do, and also reacts with carbon dioxide to form carbonate radicals (CO_3^-) that enhance peroxidation (47). Although a radical, nitric oxide (NO) is a better reductant than oxidant in biological systems (48). Radical nitrogen species can also be generated by the neutrophil enzyme myeloperoxidase; as well as its conventional non-radical product hypochlorous acid, it is able to oxidize nitric oxide to form the radical NO_2 , and can also oxidize other compounds, for example tyrosine to yield tyrosyl radicals (49, 50). Oxygen itself is a di-radical and can initiate the direct peroxidation of dry lipid monolayers *in vitro* (auto-oxidation), but this process may not be biologically relevant as the oxygen concentration in cell membranes is much lower than air.

Modification of Fatty Acyl Chains by Radical Nitrogen Species

As well causing peroxidation, reactive nitrogen radicals can also cause nitration and nitroxidation of unsaturated fatty acyl chains, and the resulting nitrated lipids have important biological functions, for example as anti-inflammatory agents and stress signaling molecules in both animals and plants (51–53). The formation of nitrogen-containing oxidized lipid derivatives was first documented in the mid-1990s (54) and was rapidly followed by further mechanistic studies of nitration reactions (55). Radical-initiated nitration can occur by two distinct mechanisms. The first requires hydrogen abstraction by a radical followed by addition of NO_2 at the carbon-centred radical, in a mechanism analogous to lipid peroxidation. Under acidic conditions, peroxyxynitrite is converted to peroxyxynitrous acid (ONOOH), which decomposes to form OH^\bullet and NO_2 ; thus hydroxyl radical initiates the hydrogen abstraction followed by addition of NO_2 to nitrate the hydrocarbon chain, forming a nitro-lipid (51, 56). The radical NO^\bullet could also undergo a radical condensation with the carbon-centred radical, which would result in lipid nitrosylation. NO_2 can also react directly with one of the carbons in the double bond to form a nitroalkane radical, and if the NO_2 concentration is high a second nitration can occur to yield a di-nitro species. Subsequent loss of nitrous acid (HNO_2) leads to nitro-alkenes, and substitution with water can form nitrohydroxy lipids (51). As with oxidation products resulting from free radical attack, the molecular rearrangements of nitro-lipids allow a wide variety of positional and stereochemical isomers to be formed, for example on phosphatidylserine (57), cardiolipin (58), phosphatidylcholine, and phosphatidylethanolamine (59). Nitrated fatty acids have been detected in human plasma, suggesting that they are

biologically relevant lipid products (60). Nitration of unsaturated fatty acids can also occur by non-radical electrophilic substitutions, as described in the following section.

Electrophilic Attack by Non-Radical Species

Unsaturated fatty acids and fatty acyl chains of phospholipids can be oxidatively modified in a non-radical manner *via* electrophilic addition of oxidants to double bonds. For example, addition of the reactive nitronium ion (NO_2^+), usually from a polarized nitronium carrier such as nitronium hexafluorophosphate, generates nitroalkenes (51), although it is not clear that such a mechanism is biologically relevant. In contrast, electrophilic addition of hypohalous acids to unsaturated lipids is better established, with more evidence for its occurrence *in vivo*. Hypohalous acids include hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypoiodous acid (HOI) and are produced mainly by phagocytes (18). The main source of HOCl is the neutrophil enzyme myeloperoxidase; this enzyme has a higher K_m for bromide than chloride, but the higher biological chloride levels mean that HOCl is the major product (61, 62). Eosinophil peroxidase is a related enzyme that is highly selective for HOBr production (61).

Hypohalous acids can add across double bonds in unsaturated fatty acyl chains to form halohydrins: the products on mono-unsaturated chains (e.g. mono-chlorohydrins) are fairly stable, but reaction with poly-unsaturated chains leads to a large number of products through rearrangement by loss of water or loss of chlorine, with the possibility of further reactions in the presence of high concentrations of HOCl (7). Chlorohydrins of fatty acids (adjacent hydroxy and chloro groups) have been detected in clinical conditions such as acute pancreatitis and sepsis (63, 64). Hypohalites can also attack vinyl ether bonds in plasmalogen phospholipids, which causes cleavage to form a lysophospholipid and releases an α -halo-fatty aldehyde (24, 65). This contrasts with radical attack of plasmalogens, which yields fatty aldehydes (23). It has most commonly been reported for HOCl, and α -chloro hexadecanal and α -chloro octadecanal have been detected in plasma of patients with cardiovascular disease (66) and sepsis (67, 68), but bromo-fatty aldehydes can also be formed (69). HOCl can react with the double bond in cholesterol to form 5-chloro-6-hydroxy-cholesterol and its isomer (70); these were reported in cell membranes (71) and subsequently myeloperoxidase-derived chlorine was reported to form a family of chlorinated sterols (72). HOCl can react with β -carotene and shows overlap in the products formed by free radical cleavage (73). Thus although the variety of halogenated products is less than that from radical oxidation, it still adds substantially to the modified lipid family.

Modifications of Phospholipid Headgroups

Although attention tends to focus on hydrocarbon chain oxidation, amine-containing phospholipid head groups can be attacked both by radicals and electrophilic oxidants. The photooxidation of phosphatidylethanolamines (PE) has been demonstrated to cause loss of ethanolamine to form

phosphatidic acid; interestingly, glycation by reaction with the amine enhanced the propensity for oxidation and led to oxidative cleavages in the glucose unit (Figure 2) (74, 75). The ethanolamine head group can also be modified by reaction with isolevuglandins (76), illustrating the complexity of effects of phospholipid oxidation, and such products have been detected in cells (77). Radical oxidation of phosphatidylserine (PS) typically yields glycerol-3-phosphoacetic acid (GPAA) via oxidative deamination (78, 79), whereas glycerol-3-phosphoacetaldehyde and glycerol-3-phosphonitrile were observed following reaction with HOCl (80). These modifications are important as the head groups play key roles in membrane structure and function, as well as cell signaling.

DISCUSSION

It is clear that oxidative modifications of lipids are legion, resulting a substantial expansion in the variety and properties of lipids. Many of the oxidized, nitrated, and chlorinated products show altered biological activities, including toxicity, altered proliferation, differentiation, pro-inflammatory, anti-inflammatory and barrier protective effects, via diverse signalling pathways to affect gene expression or other regulatory processes. In this sense, the modifications offer a chemical/biochemical mechanism to alter cell behaviour in both beneficial and deleterious ways, and to some extent meet the concept of an epilipidome. There is a close analogy to the recent shift in thinking on “reactive oxygen species (ROS)” as potentially beneficial signalling compounds, rather than agents

of destruction (81, 82). On the other hand, the modifications underlying epigenetics are reversible and enzyme-catalyzed, offering clear evidence that they are a regulatory process. The recent concept of epi-proteomics also depends on the principle of reversibility: many post-translational modifications are enzymatically controlled and reversible, e.g. phosphorylation, and histone acetylation (83, 84). In contrast, the same cannot be said of lipid oxidation. While some enzymes are specific for lipid oxidation products, such as GPx4, aldoketo reductases and aldehydes dehydrogenases, these constitute metabolism rather than direct reversibility. On this basis, the epilipidome would function in the sense of a metabolic loop, involving formation and degradation via distinct pathways. It is also worth bearing in mind that reactive lipid oxidation products exert at least some of their effects via covalent interactions with proteins in the form of post-translational modification known as lipoxidation, and these reactions are chemically reversible (85). In view of the wide variety of cellular effects reported for modified lipids, as well as its role in ferroptosis (86, 87) and inflammatory diseases (88), it is important to continue to explore their potential as an epilipidome, including aspects of reversibility and enzyme interaction. This will require development of new technologies to handle the large datasets of modified lipids that form the epilipidome (89).

AUTHOR CONTRIBUTIONS

CMS is the sole author and therefore responsible for all aspects of this article.

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