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Review

"Redox lipidomics technology: Looking for a needle in a haystack"

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ABSTRACT

Aerobic life is based on numerous metabolic oxidation reactions as well as biosynthesis of oxygenated signaling compounds. Among the latter are the myriads of oxygenated lipids including a well-studied group of polyunsaturated fatty acids (PUFA) - octadecanoids, eicosanoids, and docosanoids. During the last two decades, remarkable progress in liquid-chromatography-mass spectrometry has led to significant progress in the characterization of oxygenated PUFA-containing phospholipids, thus designating the emergence of a new field of lipidomics, redox lipidomics. Although non-enzymatic free radical reactions of lipid peroxidation have been mostly associated with the aberrant metabolism typical of acute injury or chronic degenerative processes, newly accumulated evidence suggests that enzymatically catalyzed (phospho)lipid oxygenation reactions are essential mechanisms of many physiological pathways. In this review, we discuss a variety of contemporary protocols applicable for identification and quantitative characterization of different classes of peroxidized (phospho)lipids. We describe applications of different types of LC–MS for analysis of peroxidized (phospho)lipids, particularly cardiolipins and phosphatidylethanolalmines, in two important types of programmed cell death - apoptosis and ferroptosis. We discuss the role of peroxidized phosphatidylserines in phagocytotic signaling. We exemplify the participation of peroxidized neutral lipids, particularly tri-acylglycerides, in immuno-suppressive signaling in cancer. We also consider new approaches to exploring the spatial distribution of phospholipids in the context of their oxidizability by MS imaging, including the latest achievements in high resolution imaging techniques. We

Abbreviations: Cyt c, cytochrome c; PUFA, polyunsaturated fatty acids; oxPUFA, oxygenated polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPI, lyso-phosphatidylinositol; LPS, lyso-phosphatidylserine; LPG, lyso-phosphatidylglycerol; LPA, lyso-phosphatidic acid; mCL, mono-lyso-cardiolipin; oxPLs, oxygenated phospholipids; oxCL, oxygenated cardiolipin; oxPE, oxygenated phosphatidylethanolamine; OxPS, oxygenated phosphatidylserine; oxPC, phosphatidylcholine; TAG, triglyceride; CE, cholesteryl ester; FFA, free fatty acids; LA, linoleic acid; AA, arachidonic acid; AdA, adrenic acid; HpETE-PE, 15-hydroperoxyeicosatetraenoic acid-PE; HNE, 4-hydroxynonenal; ONE, 4-oxononenal; HHE, 4-hydroxyhexenal; OHE, 4-oxohexenal; PEBP1, phosphatidylethanolamine binding protein 1; GPX4, glutathione peroxidase 4; GSH, glutathione; ASCL4, acyl-CoA synthase 4; APT, aminophospholipid translocase; ROS, reactive oxygen species; COX, cyclooxygenase; LOX, lipoxygenase; MS, mass spectrometry; LC, liquid chromatography; LC–MS, liquid chromatography-mass spectrometry; SPE, solid phase extraction; HPTLC, high performance thin-layer chromatography; GC–MS, gas chromatography-mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; DESI, desorption electrospray ionization; SIMS, secondary ion mass spectrometry; GCIB-SIMS, gas cluster ion beam secondary ion mass spectrometry; MSI, mass spectrometry imaging; IHC, immunohistochemistry; TBI, traumatic brain injury; IMM, inner mitochondrial membrane; OOM, outer mitochondrial membrane; ALS, amyotrophic lateral sclerosis; ETC, electron transport chain; NAO, nonyl acridine orange; DC, dendritic cells; LD, lipid droplet; AMVN, 2,2′-Azobis(2,4-dimethylvaleronitrile); H&E, hematoxylin and eosin

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present innovative approaches to the interpretation of LC–MS data, including audio-representation analysis. Overall, we emphasize the role of redox lipidomics as a communication language, unprecedented in diversity and richness, through the analysis of peroxidized (phospho)lipids.

"And above all, watch with glittering eyes the whole world around you because the greatest secrets are always hidden in the most unlikely places." — Roald Dahl

1. Introduction

Structural and functional/signaling competence of membranes as essential elements of cell organization is achieved, to a large extent, through a remarkable diversification of phospholipids (PLs), their major constitutive components. Appreciation of this huge molecular assortment of PLs has come with the development of contemporary liquid chromatography mass spectrometry (LC–MS)-based lipidomics and its part dealing with the characterization of oxidatively modified (phospho)lipids, redox lipidomics (Maguire et al., 2017). While detailed information has been obtained with regards to phospholipid composition of biomembranes, the approaches to identification and quantitative analysis of peroxidized PLs are only beginning to emerge. Among the major challenges are the extreme low abundance of these oxidatively modified compounds combined with the inherent transient nature of their presence in membranes and difficulties in obtaining standards for accurate LC–MS-based analyses. In spite of this, significant progress in redox (phospho)lipidomics has been made and the major advancements are the focus of this review presented along with many technological issues that still remain to be resolved.

2. Diversity of polyunsaturated phospholipids and their vulnerability to oxidation

PLs are lipids composed of fatty acid(s), an alcohol, a phosphate and a polar group (Gordon, 2003). Glycerophospholipids are the major phospholipid class in which one or two fatty acids are attached at the *sn*-1, *sn*-2 positions of the glycerol backbone through an ester or ether bond. A polar group occupies the *sn*-3 position via a phosphodiester bond. Glycerophospholipids are further classified based on the nature of their polar groups (Fahy et al., 2005). Saturated and mono-unsaturated fatty acids are usually present at both *sn*-1 and *sn*-2 positions, whereas polyunsaturated fatty acids (PUFA) preferably occupy the *sn*-2 position (Fig. 1). PUFA contain two or more methylene-interrupted *cis*-double bonds. Uncommon PUFA can have ethylene or other poly-methylene interrupted *cis*-double bonds (Zakhartsev et al., 1998). In eukaryotic cells, PUFA are usually synthesized from saturated fatty acids by two major classes of enzymes, elongases and desaturases. Elongases add an ethylene group and desaturases insert a double bond in the fatty acids (Jakobsson et al., 2006; Zhang et al., 2016). There are 7 classes of each of the elongases and desaturases reported in the literature (Jakobsson et al., 2006; Kanehisa and Goto, 2000). A combination of these enzymes produces multiple PUFAs with lengths between 16–28 carbons and 2 to 6 double bonds. The KEGG's pathway for the biosynthesis of unsaturated fatty acids lists a total of 30 PUFA (Kanehisa and Goto, 2000). Combined with the other possible saturated and unsaturated fatty acids with at least 12 carbons, each subclass of "two-legged" PLs can have 1980 species with at least one PUFA. These numbers are much greater (about 5.2 million) for cardiolipins (CL) in which two diacylglyceryl-groups are connected by a 1,3-bis-phosphoglyceryl-group, thus having 4 acyl chains. PUFA are highly susceptible to oxidation due to the presence of a weaker C—H bond at the *bis*-allelic position. These hydrogen atoms are easily abstracted to form the lipid radical (Pratt et al., 2003) - the first intermediate of enzymatic and non-enzymatic lipid peroxidation (Yin et al., 2011).

3. Oxidized lipids as signals – diversity of lipid mediators (oxPUFA vs oxPL)

PUFA-containing lipids of ω-6 and ω-3 series and their oxidation products are essential signaling molecules, lipid mediators, orchestrating many metabolic processes and cell responses, including inflammation (Dennis and Norris, 2015; Levy, 2005; Levy et al., 2001; Serhan, 2014, 2017; Serhan et al., 2018). LC–MS protocols are widely used for identification, quantification and monitoring of multiple different PUFA as well as their oxidatively modified products (Dennis and Norris, 2015; Serhan, 2014; Harkewicz and Dennis, 2011; Dumlao et al., 2011). By using electrospray MS coupled with reverse phase liquid chromatography (LC) many of these lipid mediators – oxygenated derivatives of PUFA (eg, leukotrienes, lipoxins, hepoxilins, protectins, resolvins, maresins) - have been identified and their specific roles in recruitment of innate immune cells (eg, neutrophils and macrophages) and resolution of inflammation is well established (Serhan, 2014; Lewis et al., 1990; Levy et al., 2012; Serhan et al., 2009).

The role of oxygenated PLs (oxPLs) in the development and resolution of inflammation as well as in the pathogenesis of different diseases is widely discussed (Bochkov et al., 2010; Spickett and Pitt, 2015; O'Donnell and Murphy, 2012; Anthonymuthu et al., 2016; Balasubramanian et al., 2015; Dar et al., 2018; Salomon, 2012; Thomas and O'Donnell, 2012). OxPLs include a highly diversified group of oxygenated PUFA-lipids and their structure is essential for their biological role, as determined by the polar head, the length of the fatty acid, the number of double bonds and oxygen-containing functional groups such as hydroperoxy-, hydroxy-, epoxy- and keto- that are positioned on the fatty acid residues (Fig. 1). OxPLs can be formed non-enzymatically (Bochkov et al., 2010) and enzymatically via reactions catalyzed by diffident metalloproteins such as iron-containing lipoxygenases (LOXs)

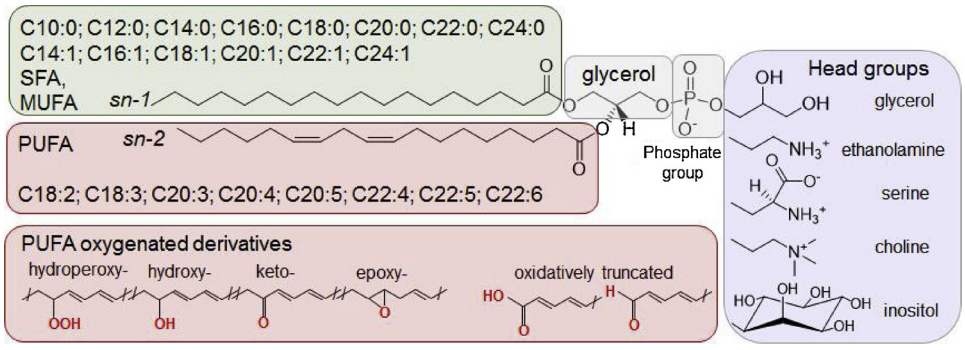

Fig. 1. Structural formulae of major glycerophospholipids and their oxidation products. Glycerophospholipids have two fatty acid residues that are attached at the *sn*-1, *sn*-2 positions of the glycerol backbone and polar groups that occupy the *sn*-3 position. PUFA are usually present at the *sn*-2 position. OxPLs include a group of oxygenated PUFA-lipids where oxygen-containing functional groups such as hydroperoxy-, hydroxy-, epoxy- and keto- are positioned on the fatty acid in the *sn*-2 position.

(Chaitidis et al., 1998; Kuhn et al., 2015), cytochromes P450 (Spector, 2009) and cytochrome c (cyt c)/CL peroxidase complex (Kapralov et al., 2007). Some of the phospholipid oxidation products generated in these enzymatic reactions have been identified and their signaling roles established. For example, oxygenated CL (oxCL) has been documented to act as a required mitochondrial signal for the execution of the intrinsic apoptotic program (Kagan et al., 2005). In ferroptosis, oxygenated phosphatidylethanolamine (oxPE) is generated as a predictive biomarker of cell death (Kagan et al., 2017). Oxygenated phosphatidylserine (oxPS) is a potent enhancer of phagocytosis of apoptotic cells by macrophages (Tyurin et al., 2014). Finally, oxygenated phosphatidylcholine (oxPC) formation has been associated with signaling in chronic inflammation (Bochkov et al., 2010).

4. Low abundance of lipid oxidation products; spreading/diversification of oxygenated products over numerus PUFA

Different polar (phospho)lipids as well as neutral lipids with esterified PUFA-residues can be enzymatically metabolized to yield the majority of lipid mediators (Tyurina et al., 2014, 2017; Burke and Dennis, 2009). Oxygenated PUFA are known to play multi-functional roles as essential signals coordinating metabolism and physiology (Dennis and Norris, 2015; Wenzel et al., 2017; Hauck and Bernlohr, 2016; Ramakrishnan et al., 2014). Although reactions of lipid peroxidation are widespread and found in most of tissues in a variety of organisms including humans, the absolute amounts/concentrations of lipid oxidation products detectable in vivo are quite low, on the order of 0.03–3.0 mol% of total non-oxidized lipids (Bochkov et al., 2010; Kagan et al., 2005, 2017; Tyurin et al., 2014; Tyurina et al., 2018). Oxidation of lipids produces oxygenated derivatives of PUFA residues as well as secondary products with shortened hydrocarbon chains (Bochkov et al., 2010; Kagan et al., 2005, 2017; Tyurina et al., 2018; Salomon and Gu, 2011; Gugiu et al., 2006; Veglia et al., 2017; Marathe et al., 2000). One of the important characteristic features of these oxidatively-truncated products is their high electrophilicity and reactivity towards nucleophilic targets in essential macromolecules (Salomon and Gu, 2011; Veglia et al., 2017; Barayeu et al., 2019). Consequently, peroxidized lipids may form adducts with many intracellular molecules of non-lipidic nature resulting in very low steady-state concentrations of peroxidized lipids per se (see below) (Fig. 2.)

5. Enzymatic vs non-enzymatic lipid oxidation mechanisms and their products – selectivity and specificity of lipid oxidation

There are two schools of thought about the mechanisms involved in the production of oxPLs. In the 1940–50's, an aggressive transfer of the chemical concepts on free radical mechanisms of liquid-phase oxidation of organic hydrocarbons into biology had occurred leading to the formulation of new disciplines of free radical biology and medicine (Radi, 2018; Levonen et al., 2014; Gutteridge and Halliwell, 2000). It has been assumed that under conditions of the so-called oxidative stress, non-enzymatic reactions of free radical oxidation overwhelm the well-controlled low molecular weight antioxidants and proteins with antioxidant action processes, thus leading the initiation of cell and tissue injury (Sies, 2015; Harman, 2006). Following this chain of logic, significant research efforts have concentrated on the search for new

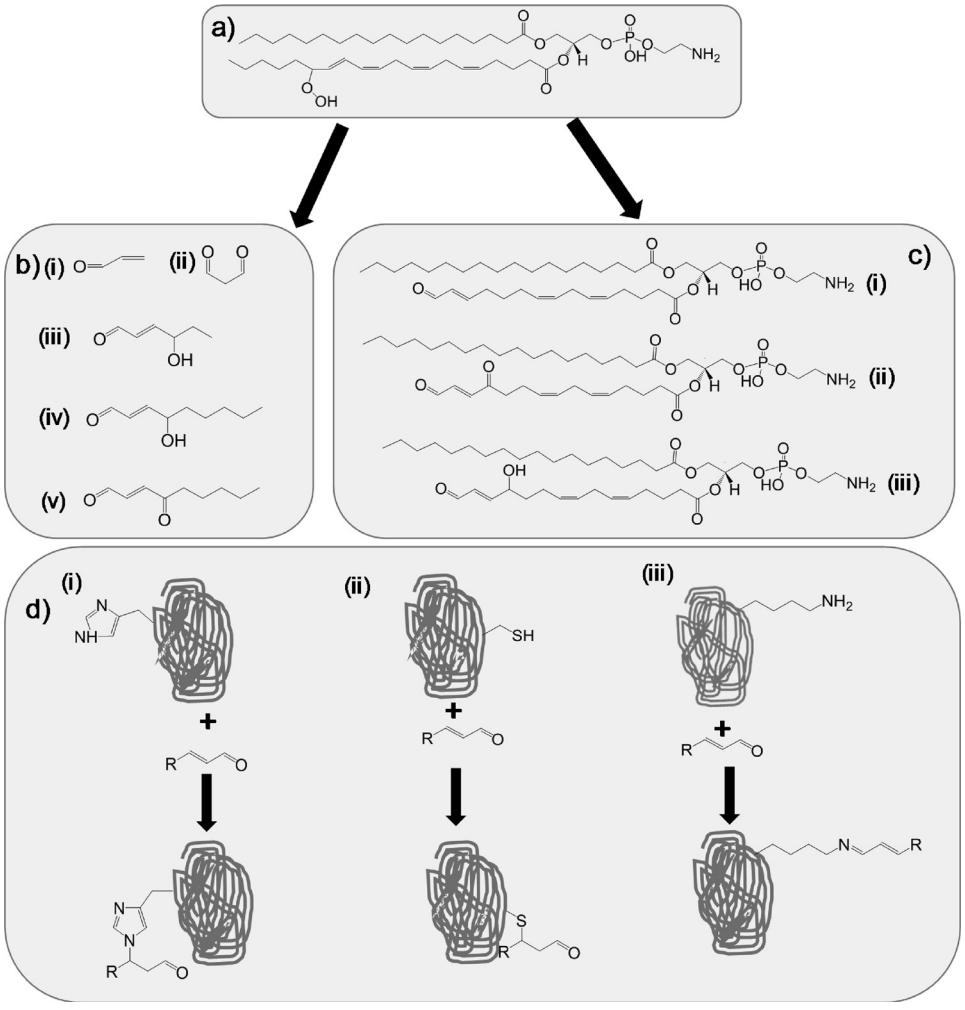

Fig. 2. Schema showing truncation of oxPE and its reaction with nucleophilic amino acids. (**a**) structure of 1-octadecanoyl-2-(15-hydroperoxy-5E,8E,11E,13E-eicosatetraenoyl)-sn-glycero-3- phosphoethanolamine and possible leaving groups [**b**; (i) acrolein, (ii) malondialdehyde, (iii) acrolein, (iv) 4-hydroxyhexaenal, (v) 4-hydroxynonenal, (vi) 4-oxononenal] and remaining groups [**c**; (i) 1-octadecanoy-2-(15-oxo-5Z,8Z,13E-pentadecatrienoyl)-*sn*-glycero-3-phosphoethanolamine, (ii) 1-octadecanoy-2-(15, 12-di-oxo-5Z,8Z,13E-pentadecatrienoyl)-*sn*-glycero-3-phosphoethanolamine, (iii) 1-octadecanoy-2-(15-oxo-12-hydroxy-5Z,8Z,13E-pentadecatrienoyl)-*sn*-glycero-3-phospho-ethanolamine] electrophiles. (**d**) reaction schema showing the reaction of oxidized-PE derived electrophiles with neucleophilic amino acid side chains (i) histidine, (ii) cysteine and (iii) lysine

natural and synthetic free radical scavengers capable of maintaining and/or re-establishing control over free radical oxidation reactions, particularly as they relate to lipid peroxidation (Halliwell and Gutteridge, 2015). Based on the established role of transition metals and their interactions with intermediates of univalent oxygen reduction – reactive oxygen species (ROS) - in the catalysis of chemical oxidations, the ideas of dis-coordinated metabolism of iron and copper along with excessive ROS production were introduced as the major mechanisms of tissue injury in biomedicine (Halliwell and Gutteridge, 1984a, 1986; Halliwell and Gutteridge, 1984b). In spite of the initial enthusiasm and encouraging results (Albanes et al., 1996; Wilson et al., 1973; Lohr et al., 1988), subsequent detailed experimental studies and clinical trials of a variety of antioxidants were disappointing (Ashor et al., 2019; Celik et al., 2010; Dotan et al., 2009).

One of the major features of these free radical oxidation reactions is the strong dependence of their rates on the number of double bonds and *bis*-allylic sites with readily abstractable hydrogens in PUFA-lipids rather than selectivity and specificity (Aliwarga et al., 2017; Kanner et al., 1987). In parallel with these explorations of random free radical reactions in bio-systems, traditional biochemistry revealed a number of enzymatic processes where “caged” free radical mechanisms were engaged in highly selective as well as regio- and stereo-specific oxidation of PUFA lipids (Suardiaz et al., 2013; Anthonymuthu et al., 2018). These studies have identified several families of oxygenases and (per) oxidases utilizing PUFA-lipids as their substrates. Among them are three major groups represented by: i) di-oxygenases and LOXs common for prokaryotic and eukaryotic cells (Kuhn et al., 2015), ii) mono-oxygenases, CYP450 (Spector, 2009), and iii) peroxidases, cyclooxygenases (COXs) (Marnett, 2000). In the context of this review, the important feature of these enzymes is their involvement in the production of signaling lipd mediators, oxygenated free PUFA, acting via specialized receptors (Dennis and Norris, 2015; Tilley et al., 2001; Erridge et al., 2008; Hazen, 2008). These signaling molecules include a variety of octadecanoids, eicosanoids, docosapentanoids and docosahexanoids - oxygenated derivatives of PUFA with 18, 20, and 22 carbons (Dennis and Norris, 2015). In addition to these well explored lipid mediators described over the last six decades (Dennis and Norris, 2015; Levy, 2005; Levy et al., 2001; Serhan et al., 2018, 2009; Dennis, 2016; Serhan et al., 2002), lately a significant focus has been placed on enzymatic peroxidation of different classes of glycerophospholipids (O’Donnell and Murphy, 2012; Kagan et al., 2017; Tyurina et al., 2018; Kagan et al., 2015). Because of the huge variety of different positional, regio- and stereo-isomers of PUFA PLs, they may represent a signaling language with essentially unlimited diversification encompassing millions of individual molecular species (Tyurina et al., 2018; Kagan et al., 2015). As much as this may be important for the myriads of their signaling functions, these same features represent huge analytical difficulties in their characterization.

6. Technological challenges of redox lipidomics

The analysis of oxidized lipids has become a formidable task in mass spectrometry. This is primarily due to the plethora and heterogeneity of oxidized products that can be formed. Oxidized lipids are present in low abundance and in some cases, their chemical instability and thermolabile nature make them susceptible to degradation. While hydroperoxides are the primary products of lipid oxidation, secondary products such as hydroxy, aldehyde, ketone and epoxide products add to the complexity/heterogeneity of the analysis. In addition, one has to consider not only full-length oxidation products but those with potential cyclization/rearrangement reactions, truncated PLs and fragments of oxidized fatty acyl chains as well (Bochkov et al., 2010; Breusing et al., 2010; Niki, 2009; Niki et al., 2005; Reis and Spickett, 2012).

A variety of upfront biochemical methods aimed at derivatization of oxidized products have been used but many of the assays for hydroxy-derivatives, aldehydes and ketones are not specific for individual oxidized products (Jessup et al., 1994; Miyazawa et al., 1994; Moore and Roberts, 1998; Ungvari et al., 2011; Wolff, 1994; Yamamoto, 1994). Immunoassays and ELISA assays for oxidized lipids have been reported, and while more specific, depend on available antibodies, which are limited (Li et al., 2014; Wang et al., 1995). While all of the above assays are helpful, most cannot identify specific oxidized species of PLs and are only valid for classes of oxidized lipid products allowing for only a generalized overview of the oxidation process.

Many different methods have been developed for the separation and quantification of different lipid classes and their oxidation products starting with specific protocols for sample preparation, time of analysis, type of detectors, ability to quantitate with increased sensitivity, including solid phase extraction (SPE), high performance thin-layer chromatography (HPTLC)-phosphorus analysis, HPLC-UV, light scattering analysis, gas chromatography-mass spectrometry (GC–MS) analysis, etc (Jurowski et al., 2017; Peterson and Cummings, 2006; Barden and Mori, 2018). However, these methods are not suitable for the simultaneous analysis of complex lipid mixtures due to their cumbersome procedures as well as insufficient sensitivity and specificity. For example, HPTLC as well as SPE, were frequently applied for lipid analyses in the past, but at the present time, these approaches are mostly used for the preparative separation of selected lipids classes (Jurowski et al., 2017; Fauland et al., 2013). GC–MS analysis provides sufficient information about oxygenated products of PUFA but information about the intact lipid moiety is lost. In addition, GC–MS usually requires derivatization of samples (Serhan et al., 2009).

Over the past three decades, LC-ESI-MS/MS has emerged as the premier method to structurally characterize and quantitate complex lipids, including lipid oxidation products, from living cells and tissues (Pulfer and Murphy, 2003; Hayakawa and Okabayashi, 2004; Ikeda et al., 2009; Tyurina et al., 2015; Nakanishi et al., 2009; Lydic and Goo, 2018; Holcapek et al., 2018). These new methodologies gave birth to a new sub-discipline, oxidative (or redox) lipidomics, a rapidly growing field dealing with the identification and characterization of the totality of oxygenated lipids and understanding their role under normal physiological and disease conditions (Dennis and Norris, 2015; Spickett and Pitt, 2015; Kagan et al., 2005, 2017; Tyurina et al., 2017; Veglia et al., 2017; Dennis, 2016; Pulfer and Murphy, 2003; Hayakawa and Okabayashi, 2004; Ikeda et al., 2009; Tyurina et al., 2015; Nakanishi et al., 2009; Xia and Budge, 2018).

Various types of mass-spectrometry (MS) have become, by far, the most sensitive, accurate and quantitative methodology for studies of lipid peroxidation products. Both matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) have been widely used for the analysis of PLs and their oxidized products (Lewis et al., 1990; Levy et al., 2012). Recent technological improvements in mass spectrometric platforms allow for enhanced sensitivity, resolution and scan speed of mass ions. Both ESI and MALDI are considered soft ionization techniques. These platforms have been used for many years for the studies of intact biomolecules including lipids. However, more often than not, the use of ESI is chosen over MALDI due to its ability to be interfaced directly to a LC system. Coupled with LC and using a targeted analysis approach with confirmation by fragmentation, ESI techniques allow for further sensitivity and specificity. However, even with these improvements, some oxidized species may be isobaric, making structural identification and quantitation more difficult. In addition, standards for the large abundance of oxidized lipids species are scarce. While MS allows the direct detection of oxidized lipid species, in some cases, such as in oxygenated derivatives of arachidonic acid, further fragmentation (MS^n) or hydrolysis of oxPLs and further analysis of the hydrolyzed fatty acids is needed for distinguishing the position of oxygenated groups. LC–MS and LC–MS/MS can be set up for quantitative analysis using internal standards and calibration curves established with reference standards (Murphy and Gaskell (2011)). LC-ESI-MS/MS strategies have been developed and optimized for the species-selective analysis of multiple oxidized species of PLs and triglicerides

(TAGs) using normal-phase and reversed-phase chromatography.

Both normal phase, hydrophilic interaction liquid chromatography (HILIC) and reverse-phase have been used for the separation of lipids, with the latter being the most popular. Normal phase separation is based on adsorption and polarity of the lipids to the stationary surface whereas in reverse-phase, hydrophobicity plays the major role. Both techniques have their advantages and disadvantages. Normal phase chromatography allows separation of lipid classes although both oxidized and non-oxidized species may overlap and the signals for the low abundant (oxidized) species may be suppressed. While reverse- phase separation allows for the separation of individual molecular species, multiple lipid classes may overlap. However, any type of up-front chromatographic separation can dramatically reduce the complexity of the lipid sample, which aids in the identification and quantitation of oxidized lipids, including the low abundant oxidized species. Some groups have utilized supercritical fluid chromatography for the separation of peroxy-, hydroxy-, and epoxy-lipid species, however, specialized equipment, software and personalized databases are needed for this type of approach (Bochkov et al., 2010). We and others have utilized a two-dimensional chromatography approach whereby lipids are separated by class in the first dimension under normal phase or HILIC conditions with further separation of oxidized and non-oxidized lipid species by revere-phase in the second dimension (Spickett and Pitt, 2015; O'Donnell and Murphy, 2012). While this procedure is somewhat labor intensive and some sample loss may occur, it offers a powerful alternative to a single chromatographic run when low-level oxidized lipid species are sought.

Accurate identification of oxPLs can be achieved by using recently developed high resolution MS orbitrap instruments such as Thermo's Q-Exactive and Fusion Lumos. Unlimited fragmentation capacities in the ion-trap part of the Fusion Lumos spectrometer combined with the high mass accuracy of its Orbitrap platform, increased speed of spectral acquisition coupled with reverse phase liquid chromatography, permits detection, unequivocal identification and quantitative characterization of oxPLs in cells as well as in tissues in vivo as exemplified by the data shown in Fig. 3.

7. General approaches to the analysis of oxidized lipids – shotgun lipidomics vs LC–MS

The analysis of lipids by mass spectrometry has been conducted on a variety of mass spectrometry platforms including GC–MS, MALDI-MS and ESI-MS. While a variety of ionization techniques have been used in the field of lipidomics (including ESI, APCI, APPI, DESI and SIMS), ESI is most widely utilized (Yang and Han, 2016). ESI-MS employs two approaches, a direct infusion approach (shotgun lipidomics) and a

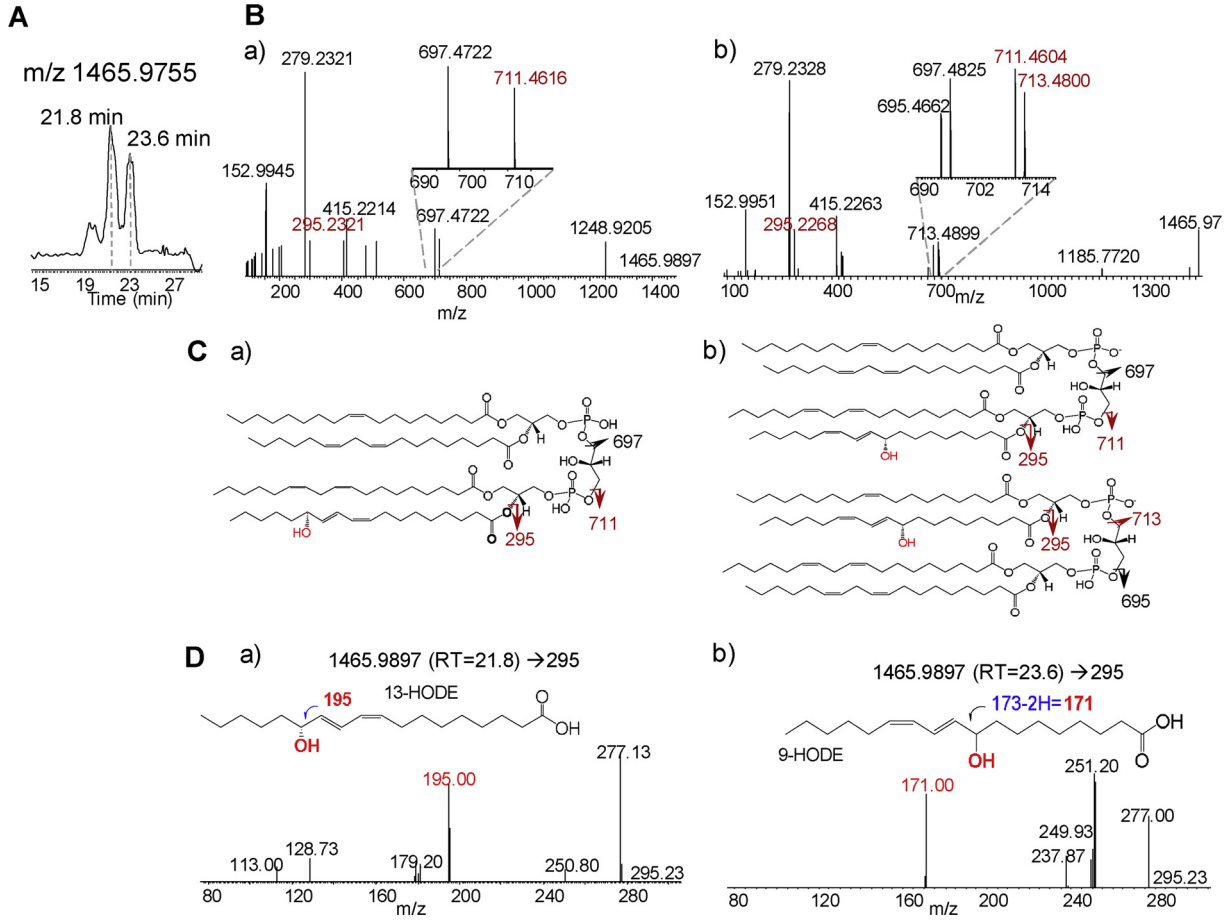

Fig. 3. Structural characterization of oxCL molecular species obtained from ileum of mice exposed to total body irradiation (9.5 Gy) using LC–MS/MS Fusion Lumos spectrometer (Thermo Fisher, San Jose, CA).

A. Typical base peak profile of CL molecular species with *m/z* 1465.9755 in the range of retention time (RT) from 15 to 30 min. **B**. Full MS (Gordon, 2003)) spectra of CL molecular species with *m/z* 1465.9755 at RT 21.8 min (a) and 23.6 min (b). Inserts; MS (Gordon, 2003) spectra in the *m/z* range from 690 to 720 **C**. Structural formulas and MS (Gordon, 2003) fragmentation pattern of 13 – HODE-CL (a) and 9 – HODE-CL (b). **D**. Full MS (Fahy et al., 2005)) spectra of CL molecular species with *m/z* 1465.9755 →295 at RT 21.8 min (a) and 23.6 min (b). Structural formulae and fragmentation patterns of 13 – HODE (insert – a) and 9 – HODE (insert – b). Mice were exposed to total body irradiation at dose of 9.5 Gy. Two days after exposure mice were sacrificed and lipids were extracted by SPE. CLs molecular species were separated by reverse phase chromatography (C18 column was used) and analyzed by ESI-MS/MS using Fusion Lumos (ThermoFisher, San Jose, CA). Two distinct peaks at retention time 21.8 and 23.6 min with *m/z* 1465.9755 were identified as 13 – HODE-CL (left panels) and 9 – HODE-CL (right panels)

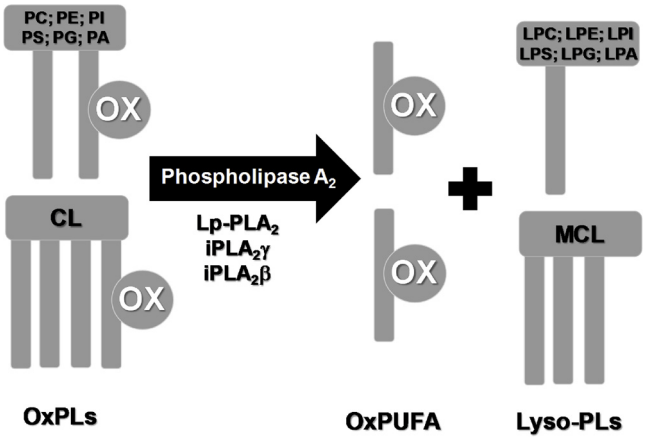

Fig. 4. A schema illustrating hydrolysis and re-esterification of phospholipids and their oxidation products by PLA_2.
Several A_2 phospholipases such as $LpPLA_2$, calcium independent $iPLA_2\gamma$ and $iPLA_2\beta$ release oxidatively modified fatty acids. This approach can significantly reduce the diversification and simplify the initial analysis of oxPL species. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPI, lyso-phosphatidylinositol; LPS, lyso-phosphatidylserine; LPG, lyso-phosphatidylglycerol; LPA, lyso-phosphatidic acid; mCL, mono-lyso-cardiolipin.

LC–MS approach. Both types of analyses have their own inherent advantages and disadvantages.

7.1. Liquid chromatography-mass spectrometry (LC–MS)

A mass spectrometer coupled to an up-front liquid chromatography system separates lipids based on polarity (normal phase and HILIC columns) or hydrophobicity (reverse-phase columns) and thereby greatly reduces the complexity of the lipid sample which generally increases sensitivity (Cajka and Fiehn, 2014). This may aid in the detection of low abundance lipids whereby the chances of suppression from other highly abundant lipids is greatly reduced (Astarita et al., 2015). LC provides the important parameter of retention time, which aids in the structural identification of the lipid. Normal phase and HILIC columns have the ability to separate lipids by class based on polarity and this selectivity carries over to the standards/internal standards used. Thus, the standards will have a similar retention time aiding in the quantification of specific lipid species. However, separation of fatty acyl-chain isomers by HILIC and normal phase chromatography is not usually possible although some exceptions may exist.

Reverse phase chromatography, on the other hand, separates lipid species based on their hydrophobicity, which is reflected mostly in their hydrocarbon chains. Therefore, this chromatographic method has the ability to separate lipids based on acyl chain length and number of double bonds (Ovčačíková et al., 2016; Sandra and Sandra, 2013). This method also allows the separation of isomeric lipid species with different acyl chains such as PE (18:0/18:2) and PE (18:1/18:1). Unlike normal phase chromatography, standards and internal standards may potentially elute at different retention times. This could complicate quantification, since the lipids of interest and standards may be subjected to different matrix effects and solvent mixtures, affecting their overall ionization (Krautbauer et al., 2016). On the other hand, each lipid species is usually associated with a specific retention time window for a given solvent system, which aids in the identification of a particular lipid species. A number of different scanning routines (see below) may also be used in conjunction with LC—MS/MS analyses, which increases confidence in lipid species identification.

7.2. Shotgun lipidomics

In shotgun lipidomics, no prior up-front separation of lipids is performed and the lipid extract is infused directly into the mass spectrometer in an appropriate solvent. The identification of lipids is carried out utilizing specific scanning routines (for example, product ion, neutral loss and precursor ion scanning) making this type of analysis simple and rapid (Yang and Han, 2016; Ejsing et al., 2009; Han et al., 2012). In addition, since standards/internal standards "see" the same solvent system as the lipid extracts, matrix and ion suppression effects are the same for all the lipid species. This makes quantification relatively straightforward and comprehensive. Low abundance ions, however, may suffer in this approach, due to the suppressive effects of highly abundant ions. In addition, shotgun lipidomics is limited in its ability to separate isomeric lipid species as well as in sensitivity as compared to LC–MS. Structural analysis may also be complicated by the fact that MS/MS or MS^n analysis may involve fragmentation of more than one precursor ion in the case of isobaric species.

7.3. Simplifying the analytical task–hydrolyzing phospholipids to reduce the vast number of oxidized species

LC–MS analysis of oxPLs is still a difficult task mainly due to their low abundance, huge diversification and absence of appropriate standards. In contrast, the LC–MS protocols for detection and identification of fatty acids and their oxidation products are very well developed (Astarita et al., 2015; Massey and Nicolaou, 2011; Quehenberger et al., 2018) and standards for many oxygenated fatty acids and their metabolites are commercially available. Given that the diversity of phospholipid oxidation products is 2–3 orders of magnitude greater than that of oxygenated PUFA, enzymatic hydrolysis of oxPLs by phospholipases A_2 have been utilized which significantly reduces the diversification and simplifies the initial analysis of oxPL species (Fig. 4). Clearly, this approach is associated with a loss of information regarding the phospholipid origin of the oxygenated PUFA. Porcine pancreatic phospholipase A_2 was successfully used to liberate identifiable hydrolysis products – oxygenated and non-oxygenated PUFA occupying the *sn*-2 position of oxCL and non-oxidized CL (Tyurina et al., 2014; Kim and Hoppel, 2011). However, its low specificity for *sn*-2 oxPUFA-residues is associated with high yields of non-oxygenated fatty acids *vs* orders of magnitude lower concentrations of oxygenated fatty acids (Tyurina et al., 2014). This complication can be avoided by using phospholipases A_2 with a higher specificity towards oxygenated PUFA-residues. Several phospholipases A_2 have been identified as enzymes that easily release oxidatively modified fatty acids such as low-density lipoprotein-associated phospholipase A_2 ($LpPLA_2$VIIA or PAF-acetylhydrolase), calcium independent $iPLA_2\gamma$ (Liu et al., 2017) and $iPLA_2\beta$ (Song et al., 2014). $LpPLA_2$ releases PAF or PAF-like oxidatively truncated fatty acids from phosphatidylcholine (PC). It can also hydrolyze oxPUFA from CL (Buland et al., 2016) and phosphatidylserine (PS) (Tyurin et al., 2014, 2012). $iPLA_2\gamma$ predominantly liberates oxygenated PUFA from CL (Liu et al., 2017). Using an enzymatic digestion protocol followed by MS analysis, the major oxygenated molecular species of CL formed in a model system (Tyurina et al., 2014) as well as in dysfunctional mitochondria from rotenone exposed human lymphocytes have been identified, characterized and quantitatively assessed (Tyurina et al., 2013).

8. Redox phospholipidomics of cell death signaling – enzymatic nature, selectivity, specificity

8.1. Oxidation of cardiolipins in apoptosis

Enzymatic oxidation of CL by cyt c in mitochondria is one of the early stages of intrinsic apoptosis (Kagan et al., 2005). Redistribution of CLs from the inner mitochondrial membrane (IMM) to the outer

mitochondrial membrane (OMM) creates conditions for the physical contact between the phospholipid facing the intermembrane space and cyt c resulting in the formation of the cyt c/CL complex. This complex has a peroxidase activity and utilizes ROS generated by the discoordinated ETC to generate superoxide radicals and their dismutation product, H_2O_2. The latter can be used as a source of oxidizing equivalents for the cyt c/CL complexes to catalyze oxidative modifications of PUFA-CLs that can act as apoptotic signals (Kagan et al., 2005). By using mass spectrometry, several species of hydroperoxy-CL and hydroxy-CL have been identified as major oxidation products formed by the complexes in vitro and in in vivo studies (Tyurina et al., 2015; Tyurin et al., 2008, 2009; Tyurina et al., 2011a, 2008; Tyurina et al., 2011b, 2010). However, the low level of oxCL, less than 0.01% of all PLs, and a wide number of generated oxygenated species in cells and tissues (Kagan et al., 2005; Tyurin et al., 2008; Bayir et al., 2007) make identification and quantitative assessments of individual oxCL molecular species difficult and requires specific efforts. The huge diversification of oxCL species can be reduced by enzymatic hydrolysis with phospholipases such as PLA_1 plus PLA_2. The hydrolysis yields a limited number of identifiable oxygenated and non-oxygenated fatty acids as well as lyso-CLs (Tyurina et al., 2014). Recently, a new redox optolipidomics approach using acridine 10-nonyl bromide (nonyl acridine orange, NAO) to detect CL and its oxygenated species has been developed (Mao et al., 2016). Mono-oxygenated derivatives of C18:2-containing CLs in cells were identified as pro-apoptotic cell death signals.

8.2. Oxidation of phosphatidylserine in apoptosis and its role in the clearance of apoptotic cells

The presentation of essential "eat-me" signals, PS and oxPS, on the cell surface is a unique and critical signal for the engulfment of apoptotic or damaged cells by professional phagocytes - macrophages and microglia (Tyurin et al., 2014; Kagan et al., 2002; Tyurina et al., 2004a, b). Early accumulation of oxCL in mitochondria results in the release of pro-apoptotic factors, including cyt c, into the cytosol (Kagan et al., 2005), where the peroxidase function of released cyt c can be realized via the interaction with PS localized in the inner leaflet of the plasma membrane (Jiang et al., 2004). The apoptotic program includes externalization of PS and oxPS to the cell surface mainly due to inhibition of the aminophospholipid translocase (APT) (Tyurina et al., 2004c, 2007) (Fig. 5).

An elegant LC/MS study in combination with cell biology demonstrated that this action resulted in a dramatic increase in oxPUFA-containing PS species and their externalization on cell surface that enabled phagocytic clearance of apoptotic cells by macrophages. It has been demonstrated that oxygenated molecular species of PS containing two oxygens (hydroperoxy-, dihydroxy-, and hydroxy-epoxy-) in the PUFA chain of PS were the dominant PS oxidation products (Tyurin et al., 2014). Additionally, the relevance of oxPS to phagocytosis was demonstrated by using Lp-PLA_2 – an enzyme with documented selectivity towards hydrolysis of oxidatively modified (but not of non-oxidized) PS species (Tyurin et al., 2014, 2012). OxPS species were detected in conditions associated with apoptotic cell death in vivo and in vitro (Tyurin et al., 2008, 2009; Tyurina et al., 2008, 2011b; Tyurina et al., 2010). The presence of oxPS on the surface of apoptotic cells is important for the interaction with the receptors (Arroyo et al., 2002; Tyurina et al., 2002). Thus, acyl chains of oxPS can be recognized by different receptors including CD36, scavenger receptor A (SRA, MSR1, CD204), and SR-oxPS (scavenger receptor for PS and oxidized lipoproteins, CXCL16) (Fadeel et al., 2007; Shimaoka et al., 2000; Zhang et al., 2008; Greenberg et al., 2006).

8.3. Oxidation of phosphatidylethanolamines in ferroptosis

Phosphatidylethanolamines (PE) are the second most abundant class of glycerophospholipids and the major lipid class containing ω-3 arachidonic acid (AA) and adrenic acid (AdA) residues (Anthonymuthu et al., 2018; Kenny et al., 2019). During ferroptosis, the preformed 15-LOX/PEBP1 complexes selectively oxidize AA-PE and AdA-PE (Kagan et al., 2017; Wenzel et al., 2017). Binding of PEBP1 allosterically modifies 15-LOXes to modify their substrate selectivity from *free* AA and AdA towards AA/AdA-PE. Three independent factors: 1) relative abundance of AA containing PE, 2) hexagonal phase membrane structure and 3) 15LOX/phosphatidylethanolamine binding protein 1 (PEBP1) complex synergistically act towards the selective and specific production of 15-hydroperoxyeicosatetraenoic acid-PE (HpETE-PE) (Anthonymuthu et al., 2018). A decrease in the phospholipid hydroperoxide reduction capacity due to either inactivation/low expression of glutathione peroxidase 4 (GPX4) or by the decline in glutathione (GSH) levels leads to the accumulation of HpETE-PE and induction of ferroptosis (Stockwell et al., 2017; Angeli et al., 2014). Notably, manipulations of acyl CoA synthase-4 (ACSL4) expression are associated with a markedly decreased sensitivity of the cells to pro-ferroptotic stimulation (Doll et al., 2017). The amazing feature of this pro-ferroptotic reaction is its remarkable selectivity in preferring AA-PE out of thousands of other oxidizable PUFA-PLs along with the regio-specificity

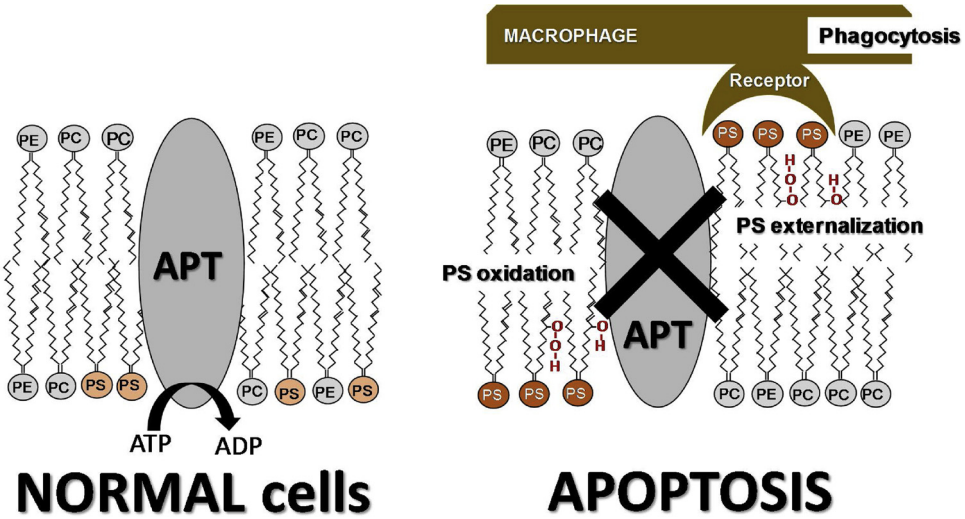

Fig. 5. A schema illustrating oxidation and externalization of PS on the surface of apoptotic cells and their engulfment by macrophages. In normal cells, non-oxidized PS is localized exclusively in the inner leaflet of plasma membrane. APT, aminophospholipid translocase.

of the oxidation products - 15-HpETE-PE. It should be noted, however, that these particular propensities of 15-LOX/PEBP1 complexes have been established in cells highly enriched with AA-PLs, due to the growing of cells in the presence of AA (Wenzel et al., 2017). It is possible that other PUFA-PLs may undergo peroxidation yielding pro-ferroptotic signals in cells and tissues enriched with other oxidizable PLs.

9. Proximate death signals - adducts of oxidized lipids with proteins - how much do we know about them

PUFA and PUFA-containing PLs are extremely vulnerable to oxidative attack by reactive oxygen species. Once oxidized, either by enzymatic or non-enzymatic means, a variety of reactive electrophilic oxidation products can be formed (Esterbauer et al., 1991; Fritz and Petersen, 2011, 2013; Schopfer et al., 2011). While the initial oxidation product is a hydroperoxide, further decomposition can lead to a reactive electrophile, resulting in reactive fragments of PUFAs, reactive PUFA fragments from PLs or a reactive, truncated phospholipid (Esterbauer et al., 1991; Fritz and Petersen, 2011, 2013; Schopfer et al., 2011; Ayala et al., 2014; Pizzimenti et al., 2013; Stemmer and Hermetter, 2012). By far, the most widely studied lipid derived electrophiles are the reactive fragments from PUFAs or PUFA containing PLs (Fig. 2). These include α, β-unsaturated aldehydes, malondialdehyde as well as hydroxy-, oxo- and epoxy-alkenals and γ − ketoaldehydes (Pizzimenti et al., 2013; Maier et al., 2010). Several species that have been studied in detail include the well-known lipid-derived electrophiles such as 4-hydroxynonenal (HNE), 4-oxononenal (ONE), 4-hydroxyhexenal (HHE) and 4-oxohexenal (OHE) (Tyurin et al., 2009; Stemmer and Hermetter, 2012; Maier et al., 2010; Domingues et al., 2013).

These electrophiles are chemically reactive, can covalently modify protein targets and their small size makes them readily diffusible. The amino acids Cys, His and Lys are typically modified by the lipid-derived electrophiles although direct oxidation of a protein's amino acids (carbonylation of Pro, Arg, Lys and Thr) can also occur (Ayala et al., 2014; Dalle-Donne et al., 2003). The abundance, reactivity and half-life of these products usually determines the extent of the protein modification event. Since their half-life tends to be short, the electrophiles usually react with proteins at or near the site of formation. Most lipid-adducted proteins result in deleterious/cytotoxic effects on the cell resulting in a modification of proteins leading to decreased gene expression and cell death (Hauck and Bernlohr, 2016; Dalle-Donne et al., 2003; Catalá and Díaz, 2016; Zhong and Yin, 2015). However, some may modify a protein's function in an adaptive way by stimulating gene expression and cell survival (Dalle-Donne et al., 2003; Zhong and Yin, 2015).

Lipid-derived protein adducts have been associated with various processes affected by oxidative stress, including inflammation, degenerative diseases and tumor formation. Indeed, lipid-derived protein adducts have been detected in Alzheimer's, Huntington's and Parkinson's disease, Amyotrophic lateral sclerosis, Down's Syndrome, cancer, atherosclerosis and various autoimmune diseases (Dalle-Donne et al., 2003; Zhong and Yin, 2015). Since these adducts tend to be in low abundance, a variety of enrichment methods coupled to mass spectrometry have been devised. These include biotin hydrazide affinity capture as well as various click chemistry approaches (Maier et al., 2010; Chen et al., 2018).

In addition to the well-known, short-chain lipid derived electrophiles, lipid aldehydes and their truncated products can also act as potential protein adducts. The majority of oxPL aldehydes studied thus far appear to be associated with choline PLs and display a complex reactivity toward proteins via Schiff base formation and Michael addition and can covalently modify lipoproteins and various proteins in cells (Domingues et al., 2013).

We have identified various oxidized species of PEs as proximate death signals in ferroptosis (Kagan et al., 2017). In addition, other

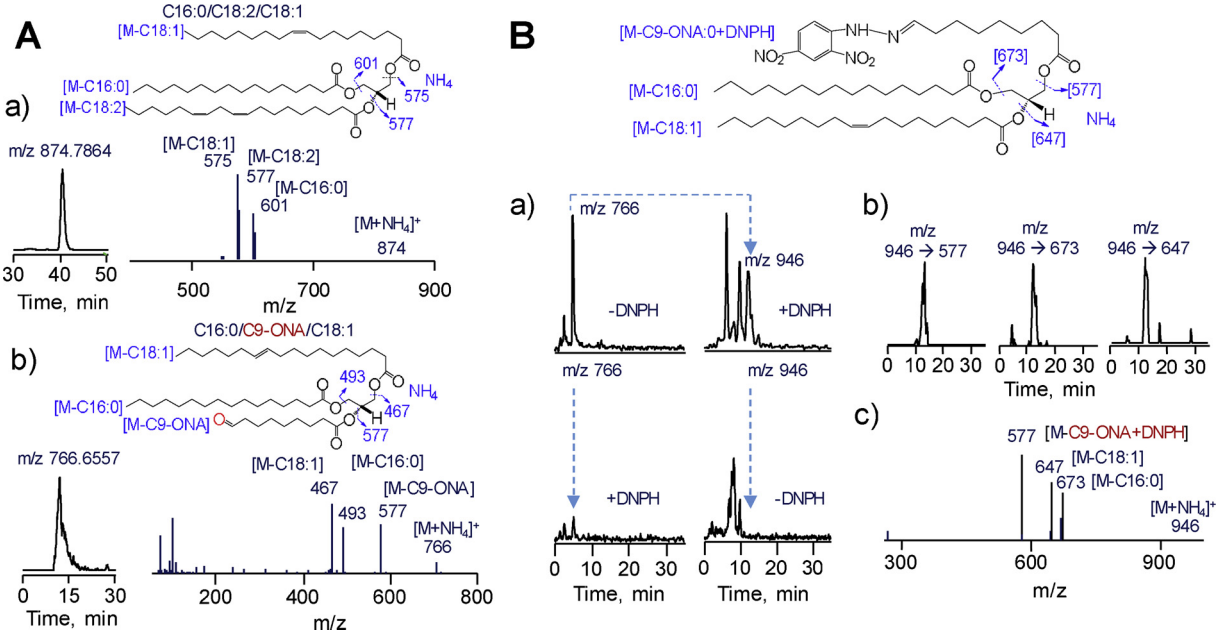

Fig. 6. Detection and identification of triglycerides and their oxidatively truncated species.
A. LC–MS profiles and LC–MS/MS spectra of TAG individual molecular species C16:0/C18:2/C18:1 (a) and their oxidatively truncated derivatives C16:0/C9-ONA/C18:1(b). **B.** Typical LC–MS profiles and LC–MS/MS spectrum of truncated TAG (C16:0/C9-ONA/C18:1) species before and after incubation with 2,4-dinitrophenylhydrazine, DNPH (a). After derivatization with DNPH the peak at *m/z* 766 almost disappears (see lower left panel, a) and a new product at *m/z* 946 is recorded (right upper panel, a). Before TAG derivatization (at the same retention time) the peak at *m/z* 946 was not observed (right lower panel, a). Structural LC–MS/MS analysis of truncated TAG (C16:0/C9-ONA/C18:1) is shown in panels b and c. Possible structure of the DNPH modified product is inserted. LC-ESI-MS/MS analysis of TAGs/CEs was performed on a Dionex LC system (UltiMate 3000 auto sampler) that was coupled to a Q-Exactive hybrid-quadrupole-orbitrap mass spectrometer (ThermoFisher, Inc. San Jose, CA). TAGs/CEs were separated on a reverse phase column (Luna 3 μm C18 (Gordon, 2003) 100A, 150 x 1.0 mm, (Phenomenex)) at a flow rate of 0.065 mL/min. The analysis was performed using gradient solvents (methanol and propanol) containing 0.1% NH_4OH.

studies have identified forms of oxPE as potential signals in exacerbating the effects of IL-13-induced secretion of mucins in asthmatics (Zhao et al., 2009). A recent study has described a proteomic method for the detection of protein carbonylation produced by lipid-derived electrophiles (mostly HNE and ONE) during ferroptotic death (Chen et al., 2018). Several protein targets with potential links to ferroptosis have been identified. Truncated, reactive phospholipid aldehydes, if they participate in the ferroptotic death pathway, still remain to be determined. What remains unclear for all reactive oxidized lipid species is whether or not the adducted oxidized lipid products (either the small lipid-derived electrophiles or truncated phospholipid products) that covalently modify proteins are truly associated with the pathogenic/signaling process or are generated as a consequence of the particular signaling/death pathway.

10. Oxidized neutral lipids in immune cells of the tumor microenvironment

During cell-to-cell communications, tumor cells constantly modify their microenvironment that includes cancer-associated fibroblasts, endothelial cells and cells of the immune system (T cell, leukocytes, macrophages, neutrophils etc.) with a variety of secretory factors released from all cellular components (Gupta et al., 2017). Immuno-surveillance plays a critical role in the control of tumor progression whereby dendritic cells (DC) are the most potent antigen presenting cells responsible for the development of immune responses. Accumulation of cells with immune suppressive propensities, particularly myeloid-derived suppressor cells (MDSCs), and expansion of immature DCs with aberrant cross-presentation capacities – are important immunological abnormalities in cancer (Gabrilovich, 2017). Interactions between these types of cells are recognized as essential contributors to the failed anti-tumor immunity (Ostrand-Rosenberg et al., 2012).

Excessive formation of lipid droplets (LDs) - neutral lipid storage organelles - has been identified as a potent regulator of DC functions (Herber et al., 2010). DCs isolated from tumor-bearing mice and cancer patients or treated with tumor explant supernatants (EL4, MC38) contained considerable amounts of oxygenated neutral lipids: TAGs, cholesteryl esters (CEs) and free fatty acids (FFAs). LC-ESI-MS/MS analysis revealed increased amounts of mono- and di-oxygenated TAGs molecular species containing linoleic (LA) and arachidonic acid (AA) residues (16:0/18:2 + [O]/18:1; 16:0/20:4 + [O]/18:1; 18:1/18:2 + 2[O]/18:1; 16:0/20:4 + 2[O]/18:1) (Fig. 6). Moreover, oxygenated TAGs were represented by a wide spectrum of truncated molecular species 16:0/9-ONA/16:0; 16:0/9-ONA/18:2; 16:0/9-ONA/18:1; 18:1/9-ONA/18:1, containing mostly 9-oxo-nonanoic acid (Ramakrishnan et al., 2014; Veglia et al., 2017; Mohammadyani et al., 2014). Additionally, the content of oxygenated CE 18:2 + [O] molecular species, containing hydroxy-, epoxy- groups and oxidatively truncated CE 9-ONA was detected (Veglia et al., 2017). Oxidative truncation of LA in TAGs/CEs leads to the formation of electrophilic products, which may form adducts with -SH, -NH_2 groups of heat shock protein 70 (Hsp70) which are critically important for DC cross-presentation (Mohammadyani et al., 2014; Dannenberg, 1997). Accumulation of oxFFAs and oxTAGs/oxCEs in DCs, mediated via the Msr1 receptor, may be responsible for the loss of their immune-regulatory functions in cancer. While supplementation of DCs with non-oxidized LA revealed the accumulation of TAG/CEs, no oxygenated TAG/CE species were found under these conditions. Notably, the transfer of peptide-MHC class I complexes to the DC surface was not affected (Ramakrishnan et al., 2014). In contrast, a lipid-soluble azo-initiator of peroxyl radicals, AMVN, added to LA supplemented DCs caused the accumulation of oxidized lipids in LDs and blocked antigen cross-presentation by inhibiting the transfer of peptide-MHC class I complexes to the DC surface (Ramakrishnan et al., 2014).

11. Imaging mass-spectrometry of lipids and its potential in redox lipidomics

While soft ionization methods such as ESI-MS drastically improve structural quantitative lipidomic analysis, lipid extraction *en bloc* loses spatial localization information. There are several soft ionization techniques that allow direct mass spectrometric analysis of locations on the surface of a native sample (Mass Spectrometry Imaging or MSI) (Spengler, 2015; Norris and Caprioli, 2013). Samples are usually cultured cells or thin slices of frozen tissue, but other kinds are possible such as insects (Khalil et al., 2015; Phan et al., 2017) and plants (Qin et al., 2018). Many locations ("pixels") are analyzed in a grid pattern to generate an image based on the MS data. The size of a single pixel can range from tens of microns to tens of nanometers, and is dependent on the instrument type, analyte abundance and sample preparation (Bodzon-Kulakowska and Suder, 2016). MSI is different from fluorescent microscopy and immunohistochemistry (IHC) in that it is an untargeted approach that does not require custom antibodies or other probes. One MSI experiment can potentially detect hundreds of different lipid molecular species from a single sample. Comparison of MSI images with other histology techniques (Porta Siegel et al., 2018; Kriegsmann et al., 2019) allows lipid abundances to be mapped to specific features (Amoscato et al., 2014; Sparvero et al., 2016; Tian et al., 2017).

There are many soft ionization techniques for MSI, and two of the commonly used ones are MALDI (Matrix-Assisted Laser Desorption/Ionization) and SIMS (Secondary Ion Mass Spectrometry) (Spengler, 2015; Norris and Caprioli, 2013; Bodzon-Kulakowska and Suder, 2016; Mohammadi et al., 2016). MALDI is excellent not just for lipids but for proteins and other analytes. SIMS is capable of much higher spatial resolution than MALDI, although sub-cellular resolution of some lipids is possible with MALDI (Barré et al., 2018; Bakker et al., 2017; Zavalin et al., 2012; Lanni et al., 2014). The high beam energies of most SIMS primary ion sources cause extensive fragmentation of analytes larger than 1000 Da (Winograd, 2015; Bich et al., 2014). However, Gas

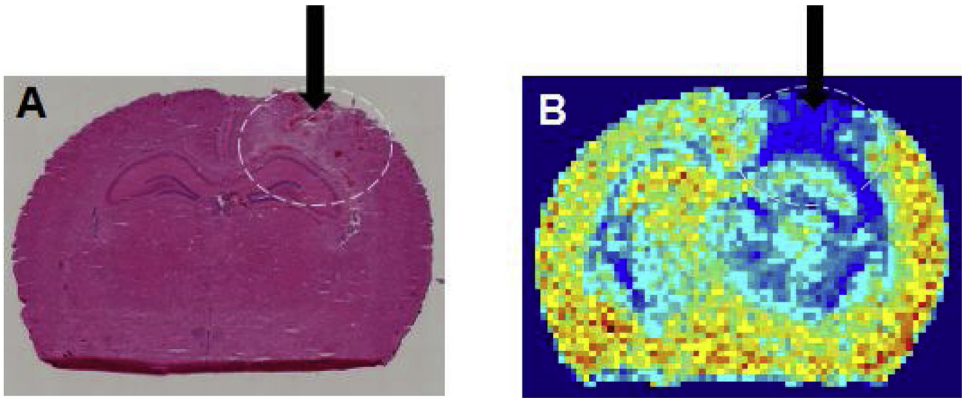

Fig. 7. MALDI detection and analysis of molecular species of CL in freshly frozen tissue sections from brains of control rats and after traumatic brain injury (TBI).
A. Optical image of an H&E stained coronal rat brain section 3 h after TBI. Point of impact is marked with an arrow. **B**. MALDI imaging of a serial section from the same brain showing the loss of CL(74:7) from the contusional and pericontusional areas.

Cluster Ion Beam SIMS (GCIB-SIMS) uses a lower energy primary ion beam and allows improved analysis of lipids above 1000 Da (Mahoney, 2013; Winograd, 2018; Tian et al., 2016; Angerer et al., 2015; Sämfors et al., 2019). Since SIMS sources ablate ("sputter") the surface, they can provide depth profiling information by repeated analyses on the same region (Winograd, 2018).

MS Imaging of oxPLs is challenging for many of the same reasons as ESI-MS, such as the low abundance and broad diversity of oxidized products (Sparvero et al., 2010, 2012). A single lipid precursor could generate numerous primary and secondary oxidation products, each of which could be in very low abundance. These signals might be suppressed by more abundant ions. LC–MS gives a separation step prior to analysis, which helps to mitigate signal suppression, but this is not possible with MSI. Therefore, alternative approaches can be used for redox MSI, such as using chemical or enzymatic methods to remove suppressing signals (Amoscato et al., 2014; Sparvero et al., 2016; Tian et al., 2017). For example, MALDI detection and analysis of molecular species of CL - which are present in very low abundance - until recently was not practically possible. However, treatments of the samples with: i) cross-linking reagents, (to eliminate the signals from amino-containing PLs, PE and PS) and ii) phospholipase C (to eliminate the signals from most abundant and suppressive PC) "unmasks" CLs and allows for quantitative analysis of its distribution and relative presence in tissues and cells (Amoscato et al., 2014). This protocol, applied to freshly frozen tissue sections from brains of control rats and those after traumatic injury, revealed significant depletion of PUFA-containing CL species in the area of direct impact as well as other parts of the brain where the spreading lesion was reliably detectable (Sparvero et al., 2016; Tian et al., 2017) (Fig. 7). These results are interpretable in terms of lower amounts of oxidizable PUFA-CL species present as a result of their peroxidative modification (Ji et al., 2012). In other words, if the primary oxidation product is not directly detectable, then measuring the loss of its precursor material is an alternative (Sparvero et al., 2016; Tian et al., 2017; Hankin et al., 2011; Woods et al., 2013; Roux et al., 2016; Barbacci et al., 2017). Chemical modification of the primary oxidation product into a derivative that is more easily detectable is another approach (Stübiger et al., 2014). Sometimes the short chain truncated product or various lyso-products from the redox reactions are detectable (Sparvero et al., 2012). Of note, contemporary protocols of MALDI and GCIB-SIMS imaging provide high spatial resolution (slightly over 1 μm) thus permitting single cell and subcellular imaging of individual lipids (Tian et al., 2019).

12. Audio-representation of redox lipidomics data – "rancid sounds" of oxidized lipids

Sonification is the auditory counterpart of data visualization, and one of the earliest examples is the Geiger counter, which detects the presence of radioactive decay (alpha particles) via an "auditory readout" (the characteristic clicking sound) (Reuter et al., 1990; Brazil and Fernstrom, 2011). There are many examples of audio-representation or sonification of data in the physical sciences (Dubus and Bresin, 2013). Sonification can provide new insights into data, such as discerning similarities or differences when comparing protein folding sequences (Bywater and Middleton, 2016). It also can ease the difficulty of visualizing multivariate, time-series datasets (Hegg et al., 2018). MS data is a series of peaks representing the *m/z* and intensity of each ion, and high-resolution MS can analyze hundreds or thousands of different ions in a single experiment. Musical information is a series of pitches (frequencies) and silences organized in the framework of time (Clendinning and Marvin, 2016). MS data would seem to be excellent for being depicted by sonification to musical serialism. However there has been very little work in sonifying mass spectral data (Tomlinson et al., 2012). Depicting redox lipidomic data by musical serialism could complement the visual display and provide new insights in understanding the differences between two datasets.

To this end, we embarked on a project using the Nyquist programming language (Dannenberg, 1997) to sonify CL mass spectral data from naïve rat brains and those from a traumatic brain injury (TBI)-induced animal model.[1] We also utilized the web-based resource Music Algorithms,[2] which allows any type of data to be sonified (Middleton and Dowd, 2008). The intensities from the mass spectra were converted into whole number integers as a prerequisite. For Music Algorithms, we utilized the intensities from the *m/z* range of 1500 to 1600 from control and TBI samples, since this is the region of the spectrum where the PUFA-CL substrates (and potentially their oxidation products) are detected. The TBI and control data each produced a separate one-voice audio file using the custom pitch input option. In order to set a minimum/maximum dynamic range for the pitch input, a value of 0 (no pitch) and the value 14320 (the maximum intensity from the two data sets) was placed at the beginning of each control and trauma data set. The pitch mapping was set to division, 1-88. The pitch input was then modified using the inversion function. This allows the higher intensity values to be displayed/sonified as lower notes and the lower intensity values as higher notes on a piano keyboard. For the duration input, the data were entered in the same way as for the pitch input, but without modification by inversion. The duration mapping was set to division, 1-9. A chromatic scale was chosen for both control and TBI samples, using 180 beats per minute as the tempo. The piano was chosen as the instrument, and the audio outputs were saved as MIDI files and converted to MP3 format.

When comparing the two audio files (see supplementary information), it becomes apparent that the TBI samples resulted in a greater number of lower tones/notes (higher intensity peaks) in the data from the 1500-1600 *m/z* region, indicating more oxidizable (or oxidized) CL species. The limitations of this program are tonal dynamics, (i.e. each note is played at the same volume) and dynamic range (for example, a data set may have more values than notes available on a piano keyboard). If data are mapped entirely arbitrarily, the result can be unpleasant and lacking in context (Reuter et al., 1990). By its nature, mapping data by arbitrary musical serialism removes the context of the data. Much as examining a spectrum for key information (such as +16 Da mass differences indicative of oxygen addition), applying a small degree of "color" to the sonification can help highlight important information in the data.[3]

13. Integration of redox lipidomics data into bioinformatics and systems biomedicine

The ultimate effectiveness of redox phospholipidomics analysis depends on the translation of obtained lipidomics data into the corresponding metabolic and other normal biological or aberrant disease-associated responses. Similar to other omics, redox lipidomics also depends on bioinformatics and system biology approaches to translate and interpret the data obtained. These packages primarily focus on building an oxidized lipid database and automatically identifying them in the LC–MS data after pre-processing for peak alignment and integration. LipidMatch includes 214 potential oxidized fatty acids, phospholipid species that have 126 oxidized fatty acids and 88 potential short chain oxidized fatty acids in its *in silico* library (Koelmel et al., 2017). LipidPioneer, an interactive template developed for the generation of lipid libraries can generate oxidized and oxidatively truncated lipid species (Ulmer et al., 2017). LipidFinder: a computational lipidomics work-flow was used to identify oxidized phosphatidylinositol in platelets (O'Connor et al., 2017; Fahy et al., 2019). LOBSTAHS exploits the

[1] www.cs.cmu.edu/~rbd/algocompbook/sonification-examples/sonification.html Accessed on 1 December 2018.

[2] www.musicalgorithms.org/3.2/ Accessed on 1 December 2018.

[3] www.cs.cmu.edu/~rbd/algocompbook/sonification-examples/sonification.html Accessed on 1 December 2018.

unique tendency of adduct ions of lipids, which remain relatively consistent across samples, to discover and identify redox oxPLs and oxylipins (Collins et al., 2016). LPPtiger incorporates algorithms to predict the redox lipidome, generate spectral and fragmentation libraries, and identify oxPLs (Ni et al., 2017). The continuous improvements in the understanding of redox lipidomics and analytical methods prompted the development of various bioinformatics packages for redox lipidomics data analysis. However, the bioinformatics-based interpretation of the redox lipidomics results is still limited to specific areas and has not been translated globally for use in all research fields. As a result, there are only a few examples of the use of custom in-house pipelines for redox lipidomic analysis (eg, in platelets and in ferroptosis) (Kagan et al., 2017; Wenzel et al., 2017; O'Donnell et al., 2014).

The complexity of the redox alterations in the lipidome and its involvement in biological processes necessitates the need for more detailed system biology approaches. At present, many studies used simple mathematical correlations and multivariate analysis for the data integration. Attempts have been made to integrate the redox biology data into pathway analysis. Currently, the KEGG pathway contains information on the role of various oxidized products in various pathways (Kagan et al., 2017). These pathways include, but are not limited to: fatty acid oxidation products in inflammation, lipid peroxidation in nonalcoholic fatty liver disease, linoleic acid metabolism, arachidonic acid metabolism, PPAR signaling pathway, AA/AdA-PE in ferroptosis (Kanehisa et al., 2011). Using the oxPL data we have constructed a model for predicting ferroptotic events using a simplified network of metabolic reactions. This model reproduced the major ferroptotic reaction pathways and the elements involved in such pathways. However, more detailed models and network analyses are needed for a better understanding and translation of redox lipidomics data.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

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