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FRBM Special Issue Editorial:

Redox lipidomics and adductomics - advanced analytical strategies to study oxidized lipids and lipid-protein adducts

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Introduction

As the importance of redox balance in physiology and occurrence of redox imbalance in a variety of pathological conditions is becoming more accepted scientifically [1], so the need to measure the levels of oxidized biomolecules and understand their biological effects is increasingly necessary. Oxidized lipids and proteins are recognized as important indicators of pathological redox imbalance and have potential value as diagnostic biomarkers for oxidative stress related diseases. There is no shortage of evidence that protein oxidation can modulate enzyme activity and signaling pathways, with both beneficial or detrimental effects depending on the circumstances [2-4]. Most attention in this area has focused on thiol-disulfide exchanges on cysteine residues with low pK_as,

which are the ones most susceptible to oxidation by hydrogen peroxide [5]. It is less widely understood that cysteines, as well as the other nucleophilic residues such as histidine and lysine, can also be modified by reactive lipid peroxidation products, which are often produced in parallel with direct protein oxidation products during conditions of oxidative stress such as acute or chronic inflammation [6, 7]. Lipid peroxidation initially involves addition of oxygen into the lipid structure, but subsequent fragmentation close to sites of oxidative damage often results in formation of reactive aldehydes, ketones and α,β-unsaturated species that are able to form covalent adducts with proteins, DNA or nucleophilic headgroups of phospholipids [8]. The process of adduct formation by reactive lipid peroxidation products is referred to as lipoxidation, by analogy with the process of glycoxidation that involves adduction by oxidized carbohydrate products [7]. Furthermore, oxidized lipids themselves are recognized nowadays as active regulators of multiple cellular and physiological functions. Imbalance in the controlled production of oxidized lipids has been associated with different types of cell death [9], as well as induction of immune responses and chronic inflammation [10, 11].

In recent years, interest in oxidized lipids and lipoxidation has been growing, as it is becoming clear that as well as disrupting the function of lipids and proteins, these modifications can cause gain of function through altering biomolecular interactions and subcellular location. Consequently, the European innovative training network MASSTRPLAN (Mass Spectrometry Training Network on Protein Lipid Adduct Analysis) was set up to improve the methodology for identifying both oxidized lipid species and the covalent adducts that result, and to apply these approaches to investigate the variety of oxidized lipid species as well as the cellular targets of lipoxidation. This Special Issue brings together reviews and original articles presenting the latest developments on these methods and their application to key pathological conditions from researchers within the MASSTRPLAN network and worldwide. It forms a companion to a Special Issue in the sister journal Redox Biology, entitled “Lipoxidation targets: From basic mechanisms to pathophysiology”, which focuses on the more biological question of what proteins are targets for lipoxidation and how this modification alters their function in health and disease. The FRBM Special Issue is divided into 4 sections: 1) advances in analysis of oxidized lipids; 2) advances in analysis of nitrated lipids; 3) advances in analysis of oxidized and lipoxidized proteins; and 4) oxidized lipids and proteins in diseases. Together, they provide a broad overview of recent developments in this trending field.

1. Advances in analysis of oxidized lipids

Two manuscripts in this section cover the analysis and diagnostic potential of oxysterols, which are cholesterol derivatives formed via enzymatic and free radical driven pathways. The review by Sottero *et al*. describes analytical challenges and advances in oxysterol lipidomics [12], and provides a comprehensive overview on the state-of-the-art applications of oxysterol analysis in a variety of human pathologies including cancer, diseases associated with defects in cholesterol metabolism (e.g. neurodegenerative and hereditary disorders), as well as hypercholesterolemia accompanying cardiovascular diseases and metabolic syndrome. The current knowledge in the field allows us to propose that modulation of the most frequently formed oxysterols might be a generic indicator of redox imbalance and inflammation, and can also be influenced by external factors such as inappropriate diet. However, the authors suggest that some oxysterol patterns might be specific to the onset and progression of certain diseases, including Smith-Lemli-Opitz syndrome and breast cancer. This aspect of specificity of certain oxysterol patterns and their metabolites in lysosomal storage diseases is further developed by Griffiths *et al*. (2019) in an original contribution where they describe a novel branch of the acidic pathway of bile acid biosynthesis [13]. Using blood plasma of patients characterized by high concentrations of non-enzymatically formed 7-oxocholesterol, 7β-hydroxycholesterol and 3β,5α,6β-triol, and an LC-MS method based on the charge-tagging approach, the authors were able to identify most of the intermediates of this new pathway branch converting oxysterols to the corresponding bile acids, which could potentially serve as a diagnostic markers for lysosomal storage diseases.

Within the high diversity of oxidized lipids, oxygenated polyunsaturated fatty acids or oxylipins represent the most studied sub-class. The review by Gladine *et al*. provides an excellent overview of the oxylipin structural heterogeneity and associated biological functions [14]. Although current analytical methods allow coverage of over 170 oxylipins derived from up to six PUFAs, the authors highlight high variances in the reported concentrations for commonly measured oxylipins. Preanalytical variability (sample collection, storage, extraction, enrichment) and analytical variability (LC separation, availability of isotopically labelled standards and MS methods), which are both crucial for accurate quantification of oxygenated PUFAs in biological matrixes, are reviewed and discussed, as well as important aspects of inter-individual variances associated with age, gender, life style and even circadian regulation of the level of circulating oxylipins. Dasilva and Medina further develop the topic of oxylipin analysis in biological samples with a specific focus on ω-3 and ω-6 lipid mediators in nutritional research in their review [15]. In addition to a comprehensive overview of the synthesis, modes of action and analysis of oxylipins formed from arachidonic, eicosapentaenoic and docosahexaenoic fatty acids, the authors consider the therapeutic options for regulating oxylipin formation as well as nutritional intervention approaches based on

increased consumption of ω-3 PUFA rich food. Despite great advances in the analytical strategies for oxylipin research, several challenges such as isomer separation still require careful optimization of the analytical protocols. Consequently, Ianni *et al*. reviewed application of chiral stationary phases for HPLC analysis of oxygenated PUFAs [16]. They provide a detailed overview of available analytical systems used for the separation of oxygenated PUFA enantiomers with a special focus on the successful applications of polysaccharide-based chiral phases. Furthermore, to increase the coverage of analytes classes and provide simultaneous detection of PUFAs, their oxygenated derivates, main vitamin E forms and their metabolites, Giusepponi *et al*. provide a validated protocol based on the optimized sample preparation, RP chromatographic separation and multiple reaction monitoring (MRM) quantification [17].

In comparison to the analysis of oxidized PUFAs, detection and identification of their phospholipid (PL) esterified forms is so far less common. However, advanced mass spectrometry-based methods have also become a valuable tool in this area of research. Thus, Philippova et al. compared MRM based quantification of eight oxidized phosphatidylcholine (oxPC) lipids in human blood plasma with relative quantification based on E06-oxPC and oxLDL ELISA assays and found only weak or no correlation between MS- and antibody-based methods [18]. However, the results of LC-MS methods showed significant correlation with presence of hypertension in the cohort studied. The authors indicate the lack of commercially available isotopically labelled standards necessary for absolute quantification as one of the limitations for the wide-spread application of LC-MS based methods for analysis of oxidized phospholipids. Indeed, many studies evaluating the levels and biological effects of oxPL rely on standards prepared in-house, of which oxidized PAPC is the most often used. Therefore, Ni et al. performed a multi-laboratory evaluation of oxidized molecular species present in oxPAPC preparations generated by air oxidation at four different location and report the results [19]. Using RP separation and tandem mass spectrometry they identified over 50 lipid peroxidation products in each of the samples, but the relative quantities of formed oxidization products varied significantly. The authors propose the use of a “truncation score”, which is an abundance ratio of short to long chain oxidation products, to characterize and standardize oxPAPC preparations used by different groups.

Lipid modifications might occur not only via peroxidation of polyunsaturated acyl chains present in PL but also at their polar head group. In this issue, Shadyro et al review the mechanisms of free-radical and biochemical reactions involving polar headgroups of PLs, for instance after the cleavage by different phospholipases [20]. They emphasize the importance of hydroxy groups as necessary for such ROS-induced destruction and highlight biological activities of the products formed. An example of another type of PL head group modification is provided by Colombo et al

[21]. Here, modification of phosphatidylethanolamine (PE) lipids via oxidation on unsaturated acyl chains and glycation and glycoxidation on the terminal amino group of the head group moiety were characterized by means of reverse phase chromatography and tandem mass spectrometry. Specifically, a C30 stationary phase was used to separate modified lipids, which allowed high resolution capacity for multiple positional and structural isomers.

Finally in this section, two reviews highlight the great variety of modern LC and MS techniques as well as computational solutions for data processing available for the analysis modified lipids. Li et al describe diversity and biological activities of possible oxidation products formed within different lipid classes, including free PUFA, PUFA-containing PLs, glycerolipids and cholesteryl esters, followed by the review of the state-of-the-art analytical methods for the detection and identification including common (ESI and APCI) and more specific ionization techniques (e.g. silver ion coordination ionspray) as well as MS methods and application of ion mobility [22]. The large datasets obtained by modern analytical methods, especially from complex biological samples, require high-throughput data analysis tools, and an informative overview is provided by Ni et al. [23]. They introduce a new term, “epilipidome”, to describe the subset of the natural lipidome that is formed by lipid modifications via enzymatic and non-enzymatic reaction (e.g. oxidation, nitration, sulfation, halogenation) required to regulate complex biological functions and provide the review of computational tools available to perform accurate and robust high-throughput identification of modified lipids from LC-MS/MS datasets.

2. Advances in analysis of nitrated lipids

Over the past two decades, nitric oxide and reactive nitrogen species (RNS) have been recognized as major regulators of redox signalling. These species can elicit various modifications of macromolecules, with impact in several cellular processes. Nitrated lipid derivatives are now considered important mediators in several physiopathological processes, remarkably, inflammation. In this issue, Wood *et al* reviewed the interactions of prostaglandin endoperoxide H synthase (PGHS) with nitro-arachidonic acid (NO_2AA), as a mechanism of regulation of the activity of PGHS [24]. Based on a review of the literature and on new results, they suggested that PGHS-2 activity is inhibited by NO_2AA via a free radical-dependent inhibitory mechanism. Mass spectrometry has been the method of choice for identification and quantification of nitrated lipid derivatives, and its application for analysis of nitrated lipids has been further developed by the group of Domingues in a study characterizing higher-energy C-trap dissociation (HCD) fragmentation patterns of nitrated cardiolipins [25]. Based on defined fragmentation mechanisms, they developed a new LC-MSMS method for the identification of nitroso, nitrated and nitroxidized

cardiolipin derivatives involving separation by a C30 column, which has previously been reported to be very effective for oxidized lipids [26]. Nitrated phospholipids have also been identified in biological systems, and associated with antioxidant and anti-inflammatory properties. An original article by Duarte *et al*. describes the effects *in vitro* of nitrated 1-palmitoyl-2-oleyl-phosphatidylcholine (NO_2-POPC) in cellular models [27]. Surprisingly, NO_2-POPC induced cellular changes that led to the loss of cell adhesion or impaired cell attachment. The results suggested that NO_2-POPC interacted with protein targets through cysteine residues, thus inducing effects different from those of NO donors or nitrated oleic acid, and should be considered as a different class of electrophilic lipid mediator acting via protein lipoxidation.

3. Advances in analysis of oxidized and lipoxidized proteins

Understanding the effects of lipid peroxidation products on proteins is also an important area of research. In their review in this issue, Shibata and Uchida introduce several important concepts, such as the exposome (i.e. the factors that an individual is exposed to that that may alter biomolecules), which in the case of reactive electrophiles leads to the adductome, or the complete profile of adducts formed on biomolecules, including advanced glycation products (AGEs) and advanced lipoxidation products (ALEs) [28]. Analysis of the adducts (adductomics) is most commonly studied by mass spectrometry. The application to DNA adducts is mentioned briefly, but the main focus of the article is on the analysis of lipoxidation adducts on serum proteins including albumin, hemoglobin and lipoproteins. The authors explain their development of an LC-MSMS method for separating, identifying and quantifying adducts on histidine and lysine residues, which led to the discovery several novel adducts. This represents a powerful approach that can be applied to clinical samples, but has the limitation that information on the site of modification in the protein is lost. Thus methods involving sequencing of lipoxidized peptides, such as reported previously [29], are complementary. Sousa et al now report the application of this approach to study the effect of lipoxidation on pyruvate kinase *in vitro* and in cultured cells, and were able to identify a number of differentially susceptible residues that could be adducted by the lipid oxidation products acrolein and 4-hydroxyhexanal, with concomitant loss of enzymatic activity [30]. Martinez Fernandez et al. also applied mass spectrometry to quantify the levels of adducts of proteins, but in this case the formation of glycated albumin in the plasma of patients with heart failure was monitored on the native protein, without digestion and sequencing [31]. In this original article, they report that glycated albumin was significantly increased in class IV heart failure, and also that it had pro-inflammatory effects on HL-1 cardiomyocytes, altering its secretome and increasing release of inflammatory cytokines IL-6 and TNFα.

The theme of adducts on human serum albumin is continued in the original article by Campos-Pinto et al, who describe work to develop an antibody with specificity to albumin-HNE adducts [32]. Antibody-based techniques are well-established as important approaches to detecting and investigating the effects of lipoxidation [33]. In order to investigate sites on modified albumin recognized by an in-house antiserum and compare it with a commercial polyclonal antibody, Campo-Pintos et al. devised a novel approach using a control and HNE-modified array of overlapping 13-mer peptides, and showed that the epitopes recognized by both polyclonals were similar, but not all sites in albumin found by LC-MSMS to be modified by HNE were immunogenic. The application of in-situ modified arrays offer good potential for investigating both antibody specificity but also interactions with other biomolecules.

Zorrilla et al. expand our knowledge on the effects of lipoxidative adduct formation in their review on the effects of lipoxidation on protein protein interactions [34]. They emphasize the need for multidisciplinary approaches such as ultrafiltration, size exclusion chromatography, affinity purification methods, fluorescence spectroscopy methods and proximity assays, and genetic approaches, although LC-MSMS methods undoubtedly have value in the identification of interactors. They provide details on a variety of proteins that have been investigated using these methods and found to interact with other proteins, membranes or DNA, which include structural protein (vimentin, surfactant protein A), many signaling proteins (H-ras, JNK, Pin-1, RhoP21) and transcription factors (Nrf2, PPARγ, c-Jun) as well as heat shock proteins. Analysing interactions in simple model systems readily provides information on mechanisms or sites of interactions but may not exactly reflect the situation in cells, where other biomolecules may affect the interactions. Zorrilla et al. also explain the importance of lipoxidation-induced interactions, giving examples of its role in altering subcellular localization and contributing to the formation of amyloid structures.

4. **Oxidized lipids and proteins in diseases**

For several years now researchers have been working to clinical translation of lipidomics and redox lipidomics. Applications have been described in the investigation of biomarkers and mechanistic aspects of human ailments as well as in the identification of therapeutic targets, drug development and validation. In this issue, Zhong et al comment that while the oxidative modification theory of LDL in atherosclerosis was proposed 30 years ago, our understanding of the action of oxidized lipids in CVDs is far from being complete [35]. Oxidized lipids have been shown to be involved in all stages of atherosclerosis, from uptake of oxidized LDL by macrophages, foam cell formation, recruitment of numerous immune cells to the formed plaque and role of oxPLs in modulation of platelet function and thrombosis. The authors review current knowledge on the

association between oxidized phospholipids and oxidized cholesterol esters with several cardiovascular diseases, and significance of different subpopulations of oxidized lipids pools based on their association with lipoproteins (LDL or Lp(a)) or plasminogen. They propose that not only lipid-lowering therapy, but also Lp(a) lowering interventions, might provide a significant reduction the levels of circulating oxPL and oxCE, especially in myocardial infarction and calcific aortic valve stenosis.

Another human disease closely associated with lipid metabolism dysfunction is non-alcoholic fatty liver disease (NAFLD). Svegliati-Baroni *et al*. present a comprehensive overview of the mechanisms and analytical strategies to investigate lipotoxicity in NAFLD and non-alcoholic steatohepatitis (NASH) [36]. NAFLD is the most common form of chronic liver disease worldwide and its progression to NASH strongly increases the risk of developing cirrhosis and hepatocellular carcinoma (HCC) [37]. NAFLD and NASH are also associated with insulin resistance and development of metabolic syndrome. Lipotoxicity is a consequence of the lipid excess and a leading cause of hepatocellular damage and inflammation in chronic steatosis. This review article describes the toxicity of specific lipid classes and their role in liver damage, focusing on cellular mechanisms and their association with oxidative stress and lipid peroxidation. The role of gut microbiota, which provides signals through the intestine, in lipotoxicity is also described in this article, together with an overview of lipidomic strategies available to explore the liver lipidome, its modifications in NALFD and NASH, and the efficacy of nutritional and pharmacological interventions, for example those recently investigated in clinical trials on vitamin E and omega-3 fatty acids.

Dermatology is another topical field where evidence of the role of lipid oxidation is emerging. Gruber et al. have reviewed the literature on recent translations of redox lipidomic technology to this field, focusing on homeostatic aspects and skin development, as well as on inflammatory diseases of the skin, such as environmental and irritant stress, acne, atopic dermatitis and psoriasis [38]. They describe general lipidome alterations, as well as increased production of enzymatically generated oxylipins such as HETE, HODE, prostaglandins and thromboxanes; accumulation of non-enzymatically formed oxidized phospholipids upon UV irradiation and their possible role in downstream transcriptional activation is also acknowledged. Furthermore, the importance of knowledge of skin (redox)-lipidome remodelling in the development and efficacy assessment of dietary intervention and skin care protocols is explained.

The last two review articles in this issue address the role of redox imbalance and associated processes in brain ageing and different forms of neurodegeneration. Redox metabolism and signaling regulate CNS pathophysiology at different levels and brain aging, one of the major risk factors for neurodegeneration and loss of cognitive function, is closely associated with the

accumulation of oxidized biomolecules and cellular senescence [39]. Nevertheless, the molecular mechanisms that sustain the preservation or deterioration of neurons and other cell components in the brain areas are still poorly understood. For instance, Parkinson disease is one of the main forms of age-related neurodegeneration, characterized by an aberrant redox metabolism in dopaminergic neurons of the substantia nigra, but the underlying molecular mechanisms of pathogenesis remain enigmatic. Iron dysregulation and accumulation as well as mitochondria damage are now reported as key players in neurodegeneration and dopaminergic cell death, according to the review article of Artyukhova et al. [40]. In this stimulating review article the application of the genome editing tool CRISPR/Cas9 in deciphering molecular changes driving PD-related impairments is considered with a focus on redox metabolism and lipid peroxidation of neuronal cells, their molecular and functional relationship to mishandling of iron, aggregation and oligomerization of alpha-synuclein and mitochondrial injury, mitophagy and programmed cell death by apoptosis and ferroptosis. Identification of new targets for therapeutic interventions and innovative approaches to genome editing are also discussed. In contrast, Pamplona *et al*. focus on the role of lipids in the human prefrontal cortex (PFC), a recently evolutionarily-emerged brain region involved in cognitive functions [41]. Specific lipid profiles characterized by the presence of a high content of PUFAs appear to sustain human PFC degeneration and cognitive decline during aging. Oxidation of polyunsaturated substrates results in generation of large number of reactive carbonyl species inducing lipoxidation damage on PFC proteins and leading to the decline in energy metabolism, cytoskeletal alterations, changes in efficiency of neurotransmission and proteostasis. The authors also discuss an array of possible intervention strategies that might help to delay aging, including calorie restriction and intermediate fasting.

5. Perspectives

The combination of reviews and original articles presented in this Special Issue, together with those in the Special Issue in Redox Biology, represent a very comprehensive view of the current knowledge and recent developments on the analysis and biological roles of oxidized lipids and the resulting modified proteins, through to applications of this information in medical science. However, it is clear that there are still many outstanding questions on molecular mechanisms and translational potential, so without doubt the field will continue to expand in coming years.

Abbreviations

CVD, cardiovascular disease; HPLC, high performance liquid chromatography; LC-MS(MS), liquid chromatography (tandem) mass spectrometry; NO_2AA, nitro-arachidonic acid; NO_2-POPC,

nitrated 1-palmitoyl-2-oleyl-phosphatidylcholine; non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH); oxCE, oxidized cholesterol ester; oxPAPC, oxidized 1-palmitoyl-2-arachidonyl-sn- glycero-3-phosphorylcholine; oxPC, oxidized phosphatidylcholine; oxPL, oxidized phospholipid; PUFA, polyunsaturated fatty acid; RP, reverse phase.

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