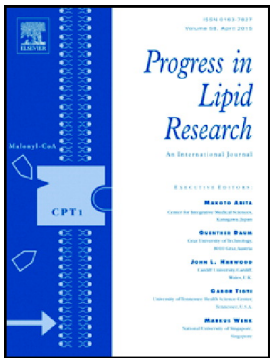


Understanding the nitrolipidome: From chemistry to mass spectrometry and biological significance of modified complex lipids

Bruna Neves, Dolores Pérez-Sala, Helena Beatriz Ferreira, Inês M.S. Guerra, Ana S.P. Moreira, Pedro Domingues, M. Rosário Domingues, Tânia Melo

PII: S0163-7827(22)00031-5

DOI: https://doi.org/10.1016/j.plipres.2022.101176

Reference: JPLR 101176

To appear in: *Progress in Lipid Research*

Received date: 2 December 2021

Revised date: 14 April 2022

Accepted date: 24 May 2022

Please cite this article as: B. Neves, D. Pérez-Sala, H.B. Ferreira, et al., Understanding the nitrolipidome: From chemistry to mass spectrometry and biological significance of modified complex lipids, *Progress in Lipid Research* (2021), https://doi.org/10.1016/j.plipres.2022.101176

Understanding the nitrolipidome: from chemistry to mass spectrometry and biological significance of modified complex lipids

Bruna Neves[1,4] brunabneves@ua.pt**, Dolores Pérez-Sala**[2] dperezsala@cib.csic.es**, Helena Beatriz Ferreira**[1,4] helenabeatrizferreira@ua.pt**, Inês M. S. Guerra**[3,4] ines.guerra@ua.pt**, Ana S. P. Moreira**[1,3] ana.moreira@ua.pt**, Pedro Domingues**[1] p.domingues@ua.pt**, M. Rosário Domingues**[1,4] mrd@ua.pt**, Tânia Melo**[1,4,*] taniamelo@ua.pt

[1]Mass Spectrometry Center, LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Santiago University Campus, 3810-193 Aveiro, Portugal

[2]Department of Structural and Chemical Biology, Centro de Investigaciones Biológicas Margarita Salas, Consejo Superior de Investigaciones Científicas (C.S.I.C.), 28040 Madrid, Spain

[3]CICECO — Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Santiago University Campus, 3810-19 Aveiro, Portugal

[4]CESAM—Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Santiago University Campus, 3810-193 Aveiro, Portugal

[*]Corresponding author.

Abstract

Complex lipids, phospholipids (PLs) and triacylglycerides (TAGs), are prone to modifications induced by reactive nitrated species and reactive oxygen species, generating a range of nitrated, nitrosated or nitroxidized derivatives, as nitro PLs and nitro TAGs. These modified lipids (epilipids) have been reported *in vitro* and *in vivo* using lipidomics approaches. However, their detection in living systems remains a challenge hampered by its complexity, high structural diversity and low abundance. The advances in high-resolution mass spectrometry combined with the higher sensitivity of the instruments like Orbitrap-based mass spectrometers opened new opportunities for the detection of these modified complex lipids. This review summarizes the challenges and findings behind the identification of nitrated, nitrosated and nitroxidized PLs and TAGs fragmentation fingerprints based on collision-induced dissociation (CID) and higher energy CID (HCD) MS/MS approaches. Following what has already been reported for nitrated fatty acids, these complex lipids are found to act as endogenous mediators with potential electrophilic properties and can express bioactivities such as anti-inflammatory and antioxidant actions. This information can be used to design untargeted and targeted lipidomics strategies for these modified complex lipids in biological samples as

well as in pathological, food and industrial settings, further unveiling their biological and signalling roles.

Keywords

Nitration; Nitroxidation; Reactive nitrogen species; Epilipids; Lipidomics

Abbreviations

CID, collision-induced dissociation; CL, cardiolipins; DAG, diacylglyceride; DI, direct infusion; $(NO_2)_2$, dinitro; $(NO)_2(2O)$, dinitroso-hydroperoxy; ESI, Electrospray; FAs, fatty acids; HCD, higher energy collision-induced dissociation; HNO_2, nitrous acid; iNOS, nitric oxide synthase; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NL, neutral loss; NO_2, nitro; NO, nitroso, $NO^{\bullet}$, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; $NO_2^{\bullet}$, nitrogen dioxide ; NO_2^+, nitronium cation; (NO_2) NO), nitronitroso; $(NO_2)O$, nitro-hydroxy; (NO)O, nitroso-hydroxy; $(NO_2)(NO)O$, nitronitroso-hydroxy; $(NO_2)O$, nitro-hydroxy; $(NO_2)(2O)$, nitro-hydroperoxy; (NO)(2O), nitroso-hydroperoxy; $O_2^{\bullet-}$, superoxide anion; $^{\bullet}OH$, hydroxyl radical ; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PLs, phospholipids; PS, phosphatidylserines; PUFAs, polyunsaturated fatty acids; RP, reversed-phase; ROS, reactive oxygen species; RNS,reactive nitrogen species; OA, oleic acid; TAGs, triacylglicerides.

1. Introduction

Phospholipids (PLs) and triacylglycerides (TAGs) are complex lipids commonly present in cell membranes and in lipoproteins. These complex lipids are structural components in membranes and signalling molecules (*e.g*, PLs) but also energy storage (*e.g.*, TAGs) [1,2]. Both PLs and TAGs are susceptible to be modified by reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species are continuously produced under physiological conditions [3,4] and can also be supplied from exogenous dietary food sources (*e.g.*, nitrite (NO_2^-) and nitrate (NO_3^-) can be obtained from green vegetables and cured meats) [5], enabling the generation of endogenous modified species of PLs and TAGs under healthy conditions. However, during inflammation and stressful conditions, like under pathological states, when ROS and RNS are overproduced [6,7], the possibility of PLs and TAGs to undergo modification increase. Unlike the knowledge on the lipid modification by ROS, the effects of RNS induced modifications in complex lipids has been scarcely explored. RNS can accumulate in

hydrophobic environments, such as in cell membranes, or be present in biological compartments, such as the stomach or oral cavity, and can react with polyunsaturated fatty acids (PUFAs) bound to complex lipids or with free fatty acids (FAs). Nitrated free FAs have been extensively studied and are recognized as important endogenous signalling molecules associated with protective roles in living systems [8–11]. More recently, nitrated, nitrosated and nitroxidized complex lipids, such as nitrated PLs and nitrated TAGs have been identified in biomimetic systems [12–18] and few studies have reported the presence of these modified lipids (epilipids) in biological samples [12,13,19], using experimental approaches based on mass spectrometry (MS). Although their biological roles remain to be determined, some nitrated PLs have shown anti-inflammatory [19] and antioxidant activities [14,19]. This raised the interest in the study of the cluster of nitrated, nitrosated and nitroxidized lipid species that can be found in the lipidome under nitrative, nitrosative and nitroxidative stress conditions (nitrolipidome). However, too little attention has been paid to this research topic.

A search of the literature using the platform Web of Science only found 11 primary articles that met the eligibility criteria within the scope of the present review, including the identification and characterization of nitrated, nitrosated and nitroxidized complex lipids using MS-based lipidomics approaches as well as its biological significance (Figure 1). Of the 11 studies considered eligible, 8 of them identified nitrated, nitrosated and/or nitroxidized PLs and 3 identified nitrated and/or nitroxidized TAGs. Moreover, 5 studies reported CID-MS/MS data, 2 reported HCD-MS/MS data and 4 used both collision dissociation techniques to study these epilipids (Figure 1). Additionally, 7 of 11 eligible studies identified modified lipids in *in vitro* and *in vivo* models (Figure 1). Overall, 1 of them discussed both the detection and the biological significance of the modified complex lipids, 8 studies detected epilipids by MS and MS/MS lipidomics approaches, and only 2 study assessed the biological effects. These results highlight the need for research in this area.

2. Understanding the chemistry of nitration of complex lipids

RNS are a heterogeneous group of molecules, radicals and ions that can be generated endogenously through enzymatic and non-enzymatic reactions (Figure 2). These reactions lead

to the formation of distinct species with different reactivity towards lipids, such as nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), nitronium cation (NO_2^{+}) or peroxynitrite ($ONOO^{-}$) or peroxynitrous acid (ONOOH) [20,21]. RNS such as nitrite (NO_2^{-}) and nitrate (NO_3^{-}) can also be obtained from exogenous sources such as food sources, such as meat, vegetables, and fruits. $NO^{\bullet}$ is considered to be the main precursor of other RNS, being produced in a variety of cells such as neuronal, endothelial and smooth muscle cells as well as in macrophages, among others [20,22,23]. $NO^{\bullet}$ is a fairly stable free radical species with a short lifetime and a low reactivity rate [20,24]. However, under oxidative and inflammatory conditions, a higher amount of $NO^{\bullet}$ can be formed, which together with ROS, can accumulate in the hydrophobic environments of lipid membranes. This provides a favorable environment for the conversion of $NO^{\bullet}$ to more reactive RNS such as $ONOO^{-}$ or $NO_2^{\bullet}$ [23,25]. $ONOO^{-}$, which can be formed by the reaction between $NO^{\bullet}$ and the superoxide anion ($O_2^{\bullet -}$) [21,26], can itself induce lipid modifications or be converted into other modifying species. This RNS can react with transition metals such as ferrous ions yielding NO_2^{+}. The decomposition of $ONOO^{-}$ generates $NO_2^{\bullet}$ and NO_3^{-}, while its protonated form, ONOOH, can decompose to yield $NO_2^{\bullet}$ and the hydroxyl radical ($^{\bullet}OH$) [21,25]. $NO_2^{\bullet}$ can also be formed due to the reaction between $ONOO^{-}$ and carbon dioxide or from the auto-oxidation of $NO^{\bullet}$ [21] (Figure 2). In addition, $NO_2^{\bullet}$ can also be synthesized enzymatically, in reactions catalyzed by peroxidases like myeloperoxidase, eosinophil peroxidase and lactoperoxidase (from the oxidation of NO_2^{-} in the presence of hydrogen peroxide)[22,27,28], and cytochrome P450 (from $ONOO^{-}$)[21,24].

The large number of RNS that can be found in living systems can lead to different types of reactions such as nitration, nitrosation or nitroxidation (Figure 3). Nitration and nitrosation reactions can occur under conditions of nitrative and nitrosative stress, corresponding to the covalent addition of a nitro (NO_2) and nitroso (NO) group to lipids, respectively. However, oxidative and nitrative stress conditions can occur simultaneously in biological systems [22,29,30]. Thus, the nitration and oxidation processes mediated by RNS can lead to a wide variety of nitrated, nitrosated and nitroxidized lipids derivatives, both in free unsaturated FAs as well as in FAs esterified in PLs [12–16] or TAGs [17,18,31]. Two main pathways have been

proposed to describe the chemical mechanism of formation of nitrated, nitrosated or nitroxidized complex lipids *in vivo*, namely free-radical mediated nitration (Figure 4) and electrophilic substitution (Figure 5). Biomimetic *in vitro* studies, performed under well defined and controlled conditions, have greatly contributed to the understanding of the mechanism underlying lipid nitration. Such studies have been carried out using pure lipid standards and chemically synthesized RNS such as NO_2^- under acidic conditions [32,33], $ONOO^-$ and $NO_2^\bullet$ [30,34,35].

Free radical-induced nitration mediated by $NO_2^\bullet$ is the most important nitration process *in vivo*, which can occur during digestion [33,36], metabolic stress and inflammation [37]. This nitration mechanism involves the homolytic cleavage of the double bond of unsaturated FAs and the addition of $NO_2^\bullet$ radical yielding a β-nitro-alkyl radical (Figure 4, step 1) which, under low oxygen concentrations, is capable of reacting with a second $NO_2^\bullet$ radical to generate a nitro-nitrite or dinitro intermediate (Figure 4, step 2). Nitro derivatives can be further generated due to the neutral loss (NL) of nitrous acid (HNO_2) (Figure 4, step 3), while hydrolysis can yield the nitro-hydroxy derivative (Figure 4, step 4). At high oxygen concentrations, the reaction between lipids and $NO_2^\bullet$ can generate carbon-centred lipid radicals, which can react with O_2 to form lipid hydroperoxides (Figure 4, step 5), or can yield a β-nitro-alkyl radical, which can further react with O_2 to form a β-nitroperoxyl radical (Figure 4, step 6). This intermediate radical can lead to the generation of nitro-hydroxy derivatives after reduction, nitro-keto derivatives after the loss of water, nitro-epoxy derivatives after deoxidation, or nitro derivatives after elimination of O_2. Therefore, at low concentrations of O_2, the formation of nitrated products predominates, while at high concentrations of O_2, the (nitro)oxidation process will be favoured [30,38,39]. Other factors such as the concentration of RNS versus ROS, the presence of secondary target molecules (scavengers of reactive species, transition metals and thiols), and the partition of reactive species between the hydrophilic and hydrophobic environments [40] will also have an impact on the type of modifying reaction and the derived products. In addition, alternative radical-induced pathways can occur in biological systems leading to the formation of non-electrophilic nitrated products due to the rearrangement of double bonds [41]. Nitrosated

lipids can also be formed through nitrosylation reactions between unsaturated lipids and nitric oxide. Nitric oxide is able to interact with a carbon-centered lipid radical by a radical condensation reaction, with the formation of a nitroso lipid derivative (Figure 4)[30].

The formation of nitro derivatives can also occur through the addition of NO_2^+ by electrophilic substitution at the double bond, without its rearrangement [41,42] (Figure 5, step 1). The nitro derivatives can then react with other RNS, such as $NO_2^\bullet$ (Figure 5, step 2), leading to the generation of other nitrated and nitroxidized derivatives.

Although the NO_2 derivatives of complex lipids can be considered as the main epilipids formed with RNS, and the first step of nitration reactions, further reaction with RNS or ROS can also occur yielding other derivatives such as dinitro ($(NO_2)_2$-complex lipids), nitro-nitroso ($(NO_2)(NO)$-complex lipids), nitro-hydroxy ($(NO_2)O$-complex lipids), among others. Thus, a plethora of structurally different and chemically diverse products, such as nitrated, nitrosated and nitroxidized derivatives, can be generated, under nitrative, nitrosative and nitroxidative conditions (Figure 3).

The identification and detailed characterization of complex epilipids is a remarkable challenge hampered not only by their complexity and great structural diversity but also due to their low abundance in biological samples. In addition, their structural characteristics can have an important impact on their biological significance, similarly to what has been previously reported for nitrated FAs (reviewed in [8]). Thus, the analysis of nitrated, nitrosated and nitroxidized complex lipids is of the utmost importance to understand their potential roles in biological systems. This analysis is mainly performed by lipidomics approaches based on MS as will be described in the next section.

3. Mass spectrometry analysis of nitrated, nitrosated and nitroxidized complex lipids: Discoveries and challenges

The nitrated, nitrosated and the nitroxidized derivatives of complex lipids, PLs and TAGs, have been mainly identified by MS-based lipidomics approaches using direct infusion (DI)-Electrospray (ESI)-MS or liquid chromatography (LC)-ESI-MS in biomimetic systems [12–15]

and biological samples [12,13]. The PLs modified by RNS, namely phosphatidylcholines (PC) [12,13], phosphatidylethanolamines (PE) [12,13] and phosphatidylserines (PS) [14], have been identified in positive-ion mode as $[M+H]^+$ ions, but $[M+Na]^+$ ions could also be observed with a lower abundance (Table 1). Modified PLs were also detected in negative-ion mode, with nitrated, nitrosated or nitroxidized PS and PE detected as $[M-H]^-$ ions, PC as $[M+CH_3COO]^-$ when using carbonate buffers (but $[M+HCOO]^-$ ions can also be detected using formate buffers), and cardiolipins (CL) as $[M-H]^-$ and $[M-2H]^{2-}$ ions (Table 1). The modified TAGs were detected in positive mode as $[M+NH_4]^+$ ions (Table 1).

The modified PLs and TAGs described in the literature either in biomimetic systems or in biological samples using MS and MS/MS-based lipidomics approaches performed in different MS instruments (Tables 2 and 3) included mono-nitro derivatives (NO_2-PLs and NO_2-TAGs), mono-nitroso derivatives (NO-PLs) and nitroxidized derivatives ($(NO_2)O$-PLs and $(NO_2)O$-TAGs). Other multiple nitrated derivatives were also detected (Figure 3). Epilipids (or modified lipids) bearing nitro or nitroso groups with oxygenated moieties (hydroxy or hydroperoxy moieties) have also been identified, as summarized in Table 4. Each type of modification has a specific mass increment compared to unmodified lipid species (Figure 6 and Table 4). Confirmation of the presence of these epilipids, other than mass differences from native lipids, requires analysis of tandem mass spectra (MS/MS) and assignment of typical reporter ions and specific neutral losses (NL) that establish the characteristic fragmentation pattern of nitrated, nitrosated or nitroxidized PLs and TAGs, as will be described in the following section (Table 4). Knowledge of their typical MS/MS fingerprints and characteristic reporter ions is also necessary to design target analysis strategies centered on MS-based lipidomics approaches.

Some isobaric and isomeric species with the same mass shift from native PLs or TAGs have been found, such as the NO_2 and nitroso-hydroxy ($(NO)O$) derivatives of complex lipids, both with a mass increment of 45 Da [13]. Discrimination between isobaric and isomeric complex lipid species can be achieved by specific fragmentation pathways or by reversed-phase (RP)-LC-MS approaches. The RP-LC-MS and MS/MS analysis using a C5 reversed-phase column made possible to identify and discriminate the functional isomers of nitrated PC [13],

PE [13] and PS [14], namely NO_2 and (NO)O derivatives, both with a mass shift of +45 Da (Tables 2 and 3). More recently, C30-RP-LC-ESI-MS and MS/MS allowed to discriminate a higher number of nitrated, nitrosated and nitroxidized derivatives. This methodology enabled the separation and identification of 11 different nitrated, nitrosated and nitroxidized CL products [16] (Tables 2 and 3), namely isomeric structures with a mass shift of +90 Da were identified as dinitro $(NO_2)_2$, nitronitroso-hydroxy ($(NO_2)(NO)O$) and dinitroso-hydroperoxy ($(NO)_2(2O)$) CL derivatives (Table 4). In this study it was also shown that nitrated compounds elute at shorter retention times (RT) than the unmodified species due to their higher polarity compared with nonmodified lipids, and that nitroxidized species elute at shorter RT than the nitrated counterparts. In general, the higher the number of modifications, the shorter the RT. Regarding nitrosated species, nitroso derivatives were shown to elute earlier than the corresponding nitro derivatives.

Although MALDI-MS and particularly MALDI imaging approaches are emerging in the field of lipidomics [43], analysis of nitrated, nitrosated and nitroxidized complex lipids by these techniques was not performed yet but should be considered and explored in future research. These MS approaches are expected to contribute to the screening of these types of epilipids allowing to unveil their role in physiology and pathology, thus in health and disease, both at cellular and tissues levels.

3.1. Tandem MS of nitro PLs (NO_2-PLs) and other modified derivatives of PLs

NO_2-PLs are the most common derivatives formed by the reaction of PLs with RNS. The presence of the NO_2 group provides a specific fragmentation pattern observed in the MS/MS data that allow the identification of this structural feature. The reporter fragmentation pattern identified for NO_2-PLs included: (1) NL of NO_2 moiety ($-HNO_2$, -47 Da); (2) reporter ions of modified FAs; (3) typical fragmentation of the polar headgroup of each PLs class; (4) product ions arising from the combined NL of HNO_2 and the fragmentation of the polar headgroups of PLs (Figure 7).

The typical NL of 47 Da due to loss of HNO_2 allowed the identification of nitrated or nitroxidized PLs *in vitro* and *in vivo* [12–16]. Multiple nitrated products, such as $(NO_2)_2$

derivatives, exhibited multiple losses of HNO_2. The NL of HNO_2 is a product ion abundant in the CID-MS/MS spectra, as indicated in the data acquired in low-resolution instruments like linear ion trap [12,13] (Figure 8 A and C). However, in HCD-MS/MS spectra, this NL of 47 Da is generally observed as a product ion with very low abundance but under certain experimental conditions, such as when higher energy is used, the product ions formed by this NL can be absent [14–16] compromising the identification of NO_2-PLs, as reported for data acquired in high-resolution mass analyzers employing orbitrap technology and with higher-energy collisional dissociation (HCD) as fragmentation method (Figure 8 B and D). However, this can be overcome by the presence of the reporter product ions of the nitro fatty acyl chain, namely the protonated molecules of FAs identified as $[NO_2\text{-}FA+H]^+$ and $[NO_2\text{-}FA\text{-}H_2O+H]^+$ ions, and the carboxylate anions of NO_2-FAs ($[NO_2\text{-}FA{-}H]^-$ and $[NO_2\text{-}FA\text{-}H_2O{-}H]^-$ ions) which are generally observed with high abundance in the HCD-MS/MS data obtained in orbitrap instruments (Figure 8 B and D). The reporter product ions, $[NO_2\text{-}FA+H]^+$ and $[NO_2\text{-}FA\text{-}H_2O+H]^+$ ions are the most abundant ions observed in the HCD-MS/MS of NO_2-PLs, except for NO_2-PC in positive-ion mode. In this case, the presence of the typical product ion of the PC class, at *m/z* 184, suppresses the fragmentation pathway that leads to the formation of the $[NO_2\text{-}FA+H]^+$ ions. Nevertheless, this can be overcome by analysis of NO_2-PC molecules in the negative-ion mode which allows the identification of the carboxylate anions of NO_2-FA with high relative abundance (Figure 9).

Typical fragmentation of polar headgroup of PLs is unequivocally observed in ESI-MS/MS spectra of NO_2-PLs allowing to pinpoint the PLs class [12–16] (Figure 7 and Table 4). Product ion at *m/z* 184 for PC, and NL of 141 Da for PE and NL of 185 Da for PS are observed in the MS/MS spectra of $[M+H]^+$ ions. Other fragmentation can be seen in the MS/MS of spectra of $[M+H]^+$ ions of NO_2-PLs, like the NL of 59 Da and 183 Da in the case of PC, and the NL of 43 Da and 87 Da for PE and PS, respectively. These NL and ions have been only associated with the fragmentation of $[M+Na]^+$ ions of the nonmodified PLs. It must be highlighted that for PC and PE, the fragmentation of negatively charged precursor ions ($[M+CH_3COO]^-$ and

$[M+HCOO]^-$ for PC and $[M-H]^-$ for PE) yields the typical product ions of PC and PE polar head groups at *m/z* 168 and at *m/z* 140, respectively [12].

In the case of ESI-MS/MS of modified CL, the fragmentation observed includes the phosphatidic and lysophosphatidic acids ($[PA-H]^-$ and $[LPA-H]^-$, respectively), the nitro phosphatidic and lysophosphatidic acids ($[NO_2\text{-}PA-H]^-$ and $[NO_2\text{-}LPA-H]^-$, respectively), as well as the $[PA-H]^-$ and $[LPA-H]^-$ product ions with remaining glycerol (+56 Da) or glycerol-phosphate (+136 Da) moieties (Table 5) [16,44]. The NL of HNO_2 is only observed in CID-MS/MS [16]. In ESI-CID-MS/MS spectra, the product ions corresponding to $[NO_2\text{-}PA+56-H]^-$ and $[NO_2\text{-}PA+136-H]^-$ can also be observed.

The typical fragmentation patterns of the polar headgroups of PLs can also be combined with the NL of HNO_2, illustrating the complexity of effects of the nitration of PLs (Figure 7 and Table 4). These combined fragmentation pathways can be observed under both CID and HCD-MS/MS conditions in positive-ion mode and be used as a reporter fragmentation fingerprint, in addition to the previous ones (Figures 8 and 10 and Table 4).

Nitroso (NO)-PLs (Figure 10) also shows typical fragmentation in MS/MS datasets such as (1) NL of the nitrosyl moiety (NL of HNO, -31 Da); (2) reporter ions of modified FAs ($[NO\text{-}FA+H]^+$, $[(NO\text{-}FA\text{-}H_2O+H]^+$, $[NO\text{-}FA\text{-}H]^-$) and $[(NO\text{-}FA\text{-}H_2O\text{-}H]^-$); (3) typical fragmentation of the polar headgroup of each class of PLs and (4) product ions arising from the combined NL of HNO and the fragmentation of the polar headgroups of PLs (Figures 7 and 10 and Table 4).

Tandem MS behaviour of epilipids with a combination of different modifications such as nitro plus nitroso (nitronitroso; $(NO)(NO_2)$) and nitro or nitroso plus oxygenated moieties as hydroxy or hydroperoxy moieties (e.g. nitro-hydroxy, $(NO_2)O$; nitro-hydroperoxy, $(NO_2)(2O)$; nitroso-hydroxy, $(NO)O$ and nitroso-hydroperoxy, $(NO)(2O)$) showed similar trends (Figures 3 and 11 and Table 4). The hydroxy and hydroperoxy groups in the nitroxidized PLs derivatives can be confirmed by the NL of H_2O (-18 Da) and the NL of HOOH (-34 Da) (Table 4). The specific reporter ions and fragmentation patterns of single and multiple nitrated, nitrosated or nitroxidized derivatives of PLs are summarized in Figures 11 and 12.

The dissimilar fragmentation observed in HCD-MS/MS of NO_2-PLs when compared with CID-MS/MS is due to the differences in the ion activation processes under MS/MS. Beam-type HCD-induced fragmentation generally promotes a higher energy flow to the precursor ion allowing the occurrence of multiple collisions of the precursor ions [45] and the product ions generated under these conditions [45,46]. As such, the HCD fragmentation conditions allow a wide range of fragmentation patterns, improving the yield of the product ions with lower *m/z* values. In the case of CID fragmentation conditions, dissociation of precursor ions occurs through a process of resonant-excitation, where multiple collisions can occur and thus increase the internal energy of the ions to induce their dissociation [47]. Until now, this particular behavior was only reported when comparing the MS/MS data of nitrated or nitroxidized PLs acquired using both low- and high-resolution mass spectrometers[14,15] (Figure 12). Thus, data acquired with other mass spectrometry instruments should be interpreted taking into account the parameters of the dissociation method used to induce fragmentation. The reporter fragmentation patterns described have been used primarily for the detection of epilipids in biological samples [12,13,48]. Thus, it is possible to emphasize that the typical NL of HNO_2 is characteristic of nitrated and nitroxidized lipids, mainly under CID-MS/MS conditions and that the product ions of the modified fatty acyl chains are the most suitable reporter ions for targeting nitrated and nitroxidized PLs under HCD-MS/MS conditions (Figure 12).

3.2. Tandem MS of nitro TAG (NO_2-TAG) and nitroxidized derivatives of TAGs

NO_2-TAGs have been identified as ammonium adducts $[NO_2\text{-TAG}+NH_4]^+$ in biomimetic systems, *in vitro* and *in vivo* by LC-MS [17,18,31]. Representative fragmentation patterns of NO_2-TAGs include (1) NL of HNO_2; (2) the NL of modified FAs with the generation of a product ion similar to diacylglyceride (DAG) and (3) the NL of unmodified FAs with the generation of a product ion similar to nitrated DAG (Figure 13).

The CID-MS/MS spectra of $[NO_2\text{-TAG}+NH_4]^+$ showed the NL of HNO_2 as the most abundant product ion (Figure 14 A). In HCD-MS/MS it is possible to see the NL of HNO_2 combined with the NL of NH_3 (NL of (47+17), NL of 64 Da). Product ions arising from the combined NL of NO_2-FAs (as an acid derivative) with loss of NH_3 can be observed with high

relative abundance in HCD-MS/MS (Figure 14 B). In CID-MS/MS, the NL of NO_2-FAs from the precursor ion could be detected with low abundance (Figure 14 A). The loss of unmodified FAs can also be identified, whether or not associated with NH_3 in HCD and CID-MS/MS spectra, respectively (Figure 14). Similar trends are also reported for other nitrated derivatives of TAGs such as dinitro derivative ($(NO_2)_2$-TAGs), as well as for nitroxidized derivatives of TAGs, such as nitro-hydroxy ((NO_2)O-TAGs), nitro-hydroperoxy ((NO_2)(2O)-TAGs) and dinitro-hydroperoxy ($(NO_2)_2$(2O)-TAGs) derivatives. (Figure 14 C and D).

Curiously, for NO_2-TAGs, the typical NL of HNO_2 (plus NL NH_3) is the most suitable reporter ion to identify nitrated or nitroxidized TAGs under both CID-MS/MS and HCD-MS/MS conditions (Figure 14 A and B), unlike those observed for NO_2-PLs (Figure 8 B and D). The information generated can be useful for the detection of nitrated and nitroxidized TAGs in biological samples (Figure 12).

Nitrated TAGs have already been detected in biological samples, specifically in plasma, adipocytes and adipose tissue [17,31], but further studies are needed to disclose important information, namely the position of the modified FAs in the glycerol backbone. In addition, fragmentation from other precursor ions such as sodium and lithium adducts which can be obtained using salts, should also be explored, as they can be analyzed by direct infusion (DI)-ESI-MS.

4. Biological significance of nitrated complex lipids

The nitrated, nitrosated and nitroxidized PLs and TAGs have been identified in biological samples by lipidomics approaches, in particular in cells [12,13,17], tissues [18] and biofluids [17,18]. It has been postulated that these epilipids can be formed endogenously, by direct nitration or by esterification of NO_2-FAs [17,18,31], especially under digestive or inflammatory conditions. The formation of nitrated complex lipids was already reported after gastric digestion (nitrated and nitroxidized triacylglycerides) and under inflammatory conditions (nitrated, nitrosated and nitroxidized phospholipids). Generation of nitrated TAGs was shown *in vitro*, during acid gastric digestion and in adipocytes supplemented with NO_2-FAs, and *in vivo*, in rat

and dog plasma after oral administration of 10-NO_2-OA [17,18,31]. In fact, during digestion, the acidic gastric condition and the physiological oxygen tension in the stomach provides a propitious environment for an extensive nitration of TAGs bearing unsaturated fatty acids. The low pH of the gastric compartment achieved during digestion promotes the formation of nitrous acid (HNO_2) from the protonation of dietary NO_2^-, which can further decompose originating nitrogen dioxide ($^{\bullet}NO_2$) that can lead to the fatty acid nitration [49,50]. However, NO_2-FAs can be uptaken by exogenous dietary sources, namely foodstuffs and plant products, contributing to the presence of endogenous nitrated TAGs. NO_2-FAs have been identified in plants (*Brassica napus*), peas (*Pisum sativum*), rice (*Oryza sativa*), fresh olives and in extra-virgin olive oil [51–54]. After absorption at intestinal level, these exogenous NO_2-FAs can be esterified in TAGs and incorporated into the chylomicrons. These large triacylglyceride-rich lipoproteins can be further catabolized by the liver into very low-density lipoproteins, which can reach the systemic circulation for distribution of nitrated TAGs to distal tissues [55,56]. Modified PLs were detected in biomimetic models of non-communicable diseases, *in vivo* in cardiac mitochondria from diabetic rats [12], and *in vitro* in cardiomyoblasts subjected to starvation [13]. These pathological conditions are characterized by an increase in oxidative stress and inflammation, leading to an enhanced production of ROS and RNS. Both reactive species can induce modifications in PLs with formation of nitrated, nitrosated and/or nitroxidized derivatives. However, modified PLs have been scarcely reported, particularly in plants and foodstuffs. These data demonstrated that esterified forms of NO_2-FA can be endogenously generated, either by direct nitration of the esterified fatty acyl moiety or by the incorporation of free NO_2-FA in more complex lipids, after gastric digestion and inflammatory conditions.

4.1. Detection of nitrated complex lipids in biological samples

Nitrated PLs, namely nitrated PC and PE, have been identified in cardiac mitochondria isolated from the heart of an animal model of type 1 diabetes mellitus (T1DM) [12], comprising 9 species of nitro PC such as NO_2-PC(16:0/18:2), NO_2-PC(16:0/18:1), NO_2-PC(18:2/20:4), NO_2-PC (16:0/22:5), NO_2-PC(18:0/20:5), NO_2-PC(18:0/20:4), NO_2-PC(18:2/20:1), NO_2-

PC(18:0/22:6) and NO_2-PC (18:1/20:4) and also a nitro PE (NO_2-PE(18:0/22:6). Of these modified PLs, only NO_2-PC(18:2/20:1) was reported, with low abundance, in the control group. Nitrated, nitrosated and nitroxidized PC were also reported in cardiomyoblasts (H9c2) under starvation [13], but not in cells under ischemia or under control conditions (Figure 15). The modified PC species detected included 1 nitroso (NO-PC(18:0/18:1)), 1 nitro (NO_2-PC(16:0/18:1)), 2 dinitro (($(NO_2)_2$-PC(16:0/16:1) and $(NO_2)_2$-PC(16:0/18:1)), 1 nitronitroso ((NO_2)(NO)-PC(16:0/20:3)), and 1 nitro-hydroperoxy ((NO_2)(2O)-PC(16:0/18:1)) derivative. Two nitrated derivatives of PE (NO_2-PE(34:1) and NO_2-PE(36:1)) have also been identified. Fazzari and collaborators [18] also described that NO_2-FAs were preferentially incorporated into the PC class in adipocytes supplemented with different NO_2-FAs. It was suggested that this is due to the high abundance of this class of PLs in biological membranes. Nevertheless, the incorporation of NO_2-FAs into PE, PS and PI was also verified.

In these studies, the amount of nitrated, nitrosated and nitroxidized species was higher under disease conditions than under control conditions. This evidence may suggest that, like other electrophilic lipids, nitrated and nitroxidized PLs may be a reservoir for nitro and nitroxidized FAs, which may be further mobilized to perform their pleiotropic signalling actions. Modified species found in higher relative abundance containd NO_2-oleic acid (OA) in their composition, which may be associated with certain health benefits and therapeutic effects. However, as noted above, the number of studies addressing the functional impact of these species is limited.

NO_2-OA can exert its roles by acting as high affinity ligands for peroxisome proliferator-activated receptor gamma (PPARγ), being able to modulate the regulation and maintenance of metabolic homeostasis [57–59], as well as anti-inflammatory and antioxidant pathways [60,61]. In addition, NO_2-OA can also activate PPAR-alpha (PPARα) [35] which is highly expressed in the heart and cardiomyocytes [58,62], where it upregulates the expression of genes encoding the enzymes of oxidation and transport of FAs. Thus, the presence of NO_2-OA-PLs in T1DM and starvation may represent an attempt to increase the uptake of glucose, restore insulin sensitivity, and promote cardioprotection by stimulating cellular adaptation and survival, respectively.

The NO_2 derivatives of PC (16:0/18:1), NO_2-POPC, have also been detected in human adrenal cortex adenocarcinoma cells (SW13/cl.2 cells) treated with 10 μM nitrated POPC [48,63] (Figure 15). In this case, the presence of modified species may occur due to their uptake to become part of the cell membrane or may suggest an increase in nitroxidative stress which leads to the generation of nitrated and nitroxidized species from the PLs that incorporate the membrane.

Nitrated TAGs have been detected in adipocytes supplemented with 10-NO_2-OA and rat plasma after oral administration of 10-NO_2-OA [17]; in the adipose tissue of high-fat diet-fed mice after subcutaneous administration of NO_2-OA [18] and in the plasma of dogs after oral administration of 10-NO_2-OA [31] (Figure 15). In addition, after *in vitro* mimetic nitration under acidic conditions using artificial gastric fluid [17], the generation of nitrated TAGs products, such as NO_2-CLA-TAGs, (NO_2)O-OA-TAGs and (NO_2)(2O)-OA-TAGs has been reported. NO_2-FA-TAGs can shield the electrophilic potential of NO_2-FAs contributing to their systemic distribution regulating cell homeostasis and tissue signalling events. The incorporation of 10-NO_2-OA in the *sn-2* position of TAGs adipocytes fractions has been established to protect the electrophilic nature of NO_2-FAs from inactivation, in particular by PGR-1 [18]. Also, the NO_2-FAs esterified in TAGs can serve as a reservoir of NO_2-FAs for their subsequent mobilization by lipases and return to the free active form or can be a mechanism for their bio-distribution [31]. Dietary unsaturated FAs esterified in TAG, namely from extra virgin olive oil, were also reported to be targets of nitration during digestion conditions [51].

4.2. Biological roles of nitrated complex lipids

Nitrated PLs were reported to have antioxidant potential based on their *in vitro* radical scavenging activity demonstrated by the ability to scavenge 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid cation ($ABTS^{\bullet+}$) radicals [14,19] and through the oxygen radical absorbance capacity (ORAC) assay [19] (Figure 16). Anti-inflammatory potential of nitrated PLs was shown *in vitro* through their ability to inhibit the expression of inducible nitric oxide synthase (iNOS) in RAW 264.7 macrophages activated

by the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), in a well-established model of inflammation [19] (Figure 16). In addition, the effect of nitrated PLs have also been recently studied in cell lines supplemented with exogenous nitro PC(16:0/18:1), NO_2-POPC, at micromolar levels (10 and 30 μM). The results gathered in this study demonstrated that NO_2-POPC can modulate the cytoskeletal organization of actin and vimentin leading to patterns partially overlapping those elicited by nitrated FAs. Indeed, both NO_2-POPC and NO_2-OA induced cell rounding and loss of focal adhesions, and reduced cell adhesion to the substrate, at micromolar concentrations [48] (Figure 16). Thus, certain biological actions of nitrated PLs could be superimposed on those of nitrated FAs, for which there is a great wealth of information and data supporting their bioactive roles [11]. These effects occur mainly through their covalent binding to proteins at critical cysteine residues, much as it has been reported for other electrophilic lipids capable of forming Michael adducts with signalling proteins, such as the cyclopentenone prostaglandins [64,65]. Indeed, numerous transcription factors, metabolic enzymes, signalling proteins, as well as elements of the cytoskeleton and components of cellular degradation pathways, possess critical cysteine residues involved in their regulation [65,66]. Nevertheless, whereas many of the targets for nitrated FAs or cyclopentenone prostaglandins have been identified and the functional consequences of their lipoxidation evaluated [8,65], few studies have identified targets for modification of nitrated PLs. This is due in part to the need for adequate procedures for their detection. Recently, an *in vitro* adduct of glutathione and NO_2-POPC has been detected by MS and characterized by MS/MS [63]. Therefore, it is expected that adducts with cysteine residues in other peptides or proteins will be identified in the future. Indeed, the use of gel-based assays revealed indirect evidence of the interaction of NO_2-POPC with the single cysteine residue of vimentin (C328), as well as with the cysteine residue present in the PPARγ ligand-binding domain (C285), which are known targets of electrophilic lipids [67,68]. In both cases, incubation with NO_2-POPC, but not POPC, blunted the subsequent modification of these cysteine residues by biotinylated iodoacetamide [48]. Moreover, the presence of vimentin C328 appeared to be necessary for the effect of NO_2-POPC on the retraction of vimentin filaments from the cellular periphery in live cells [48], similar to what has

been observed for other electrophilic lipids [67]. As with other electrophilic lipids, it would be important to determine whether nitrated complex lipids can display dual or biphasic actions depending on their levels,the time of action or cellular context. For instance, it is well known that in cellular models, cyclopentenone prostaglandins can amplify certain inflammatory responses when present at submicromolar levels, whereas anti-inflammatory and anti-proliferative responses are primarily observed at micromolar levels [69]. A similar dual action is observed regarding glutathione synthesis [70]. Also, depending on the electrophilic potential, a competition for the binding to the target protein can be observed and thus distinct biological effects may be triggered. In addition to the direct interaction of nitrated PLs with proteins, they can also release $NO^{\bullet}$ [48], which can elicit protein modifications and modulate a plethora of signalling pathways. Therefore, nitrated PLs could act as NO reservoirs. However, their biological actions are not superimposed on those of NO donors [48]. Moreover, as with other electrophilic species, nitrated PLs can induce the generation of secondary reactive species and modifications. In addition, the nitration of PLs in membranes could alter the physicochemical properties of membranes, impacting the function of integral membrane proteins, including many signalling receptors, and potentially peripheral membrane proteins, or increasing membrane permeability [71].

Therefore, more information is needed on their endogenous generation, the levels they reach in biological systems and their subcellular location. In this regard, more *in vitro* and *in vivo* research are necessary to highlight the biochemical mechanisms by which esterified NO_2-FAs in PLs, and TAGs can exert their relevant biologically properties.

Given the wide range of cellular effects reported for NO_2-FAs, it would be of utmost importance to recognize the electrophilic character of esterified NO_2-FAs in complex lipids and their role in the modulation of proteins and enzymes. This will allow to disclose the specificity and reactivity of the different nitrated complex lipid towards target proteins and the mechanisms behind their biologically roles, mirroring what is being done with electrophilic oxidized lipids and nitrated FAs. The identification of nitrated lipoxidation adducts can be achieved using similar analytical strategies, like specific antibodies and bottom-up proteomics, by target or

untargeted approaches. However, there is also a notorious lack of knowledge on the identification and characterization of lipoxidation adducts of esterified NO_2-FAs in complex lipids [48,63]. Recognition of the target proteins of these epilipids may be useful to understand the interplay between protein lipoxidation mediated either by oxidized lipids or nitrated complex lipids. Thus, it is essential to carry out more studies in this area to unveil the potential protein targets of epilipids, especially those having a closer relationship to the membrane. The know-how gained with the detection of the lipoxidation targets of nitrated and nitroxidized complex lipids, in both animal systems and in plants, will may certainly contribute to enhance the knowledge on the pathophysiological mechanisms of disease or maintenance of cell homeostasis. It may also offer new opportunities to better understand the metabolism, pharmacokinetics, and potential biological roles and pharmacological actions of NO_2-FAs, useful for the development of new therapeutic approaches and new drugs candidates.

5. Final remarks and outlook

MS-based lipidomics approaches have been crucial for the detection of nitrated and nitroxidized complex lipids based on the identification of characteristic fragmentation patterns observed under MS/MS conditions, i.e. the typical NL of HNO_2 or HNO and the reporter ions of modified fatty acyl chains. Advances in MS tools, namely the high-resolution and greater sensitivity of the latest mass spectrometers such as HCD-Orbitrap-based instruments have opened up new horizons in the field of lipid nitration in lipidomics, contributing to overcome the challenges of the low quantity and high structural diversity of these epilipids. This can support their accurate identification in complex mixture of lipid compounds present in samples linked to health and disease conditions. Moreover, this underlying information becomes even more important because it can lead to new clues for the discovery of new biomarkers or therapeutic strategies, as proposed for the free NO_2-FAs. However, the literature focusing on high-resolution MS analysis of nitrated and nitroxidized complex lipids is still very scarce. With this in mind, it is necessary to continue research using sensitive analytical lipidomic strategies based on MS as well as the establishment of standardized and reproducible approaches and

guidelines, recognized by the scientific community for a more complete understanding of complex lipids under nitration or nitroxidation conditions. Considering food as an exogenous source of these epilipids, the mapping of nitrated and nitroxidized PLs and TAGs in plants and foodstuffs using the high-resolution MS and MS/MS lipidomics approaches should be considered for future studies.

The few insights available on the biological roles of nitrated complex lipids, mostly focused on the nitrated PLs, lift the tip of the veil on the potential physiological importance of these epilipids, such as by releasing $NO^{\bullet}$ or through the formation of lipoxidation adducts with target protein. However, more knowledge is needed to understand whether these modified lipids can display their biological actions. It is also necessary to transpose this knowledge to physiologically relevant scenarios to screen if the bioactive effects mirror the release of the NO_2-FA moiety.

Overall, there are still several directions for advancement in the field of nitrolipidomics of complex lipids and future work is needed, either to detect nitrated complex lipids and their potential lipoxidation adducts under different conditions and matrices, or to assess their biological effects.

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

Author Contributions: Conceptualization and design: B.N, M.R.D. and T.M.; writing—original draft preparation: B.N, M.R.D. and T.M; writing—review and editing B.N, D.P-.S, H.F, I.M.S.G, A.S.P.M, P.D, M.R.D. and T.M ; supervision, M.R.D. and T.M. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Acknowledgments: The authors would like to thanks to the University of Aveiro and FCT/MCT for the financial support to LAQV/REQUIMTE (UIDP/50006/2020 + UIDB/50006/2020), CESAM (UIDB/50017/2020 + UIDP/50017/2020 + LA/P/0094/2020), CICECO (UIDB/50011/2020+UIDP/50011/2020) and to RNEM, Portuguese Mass

Spectrometry Network, (LISBOA-01-0145-FEDER-402-022125) through national founds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. The authors are also thankful to the COST Action EpiLipidNET, CA19105 - Pan-European Network in Lipidomics and EpiLipidomics. Dolores Pérez-Sala thanks to RTI2018-097624-B-I00 from Agencia Estatal de Investigación, MICINN/FEDER, Spain, and Instituto de Salud Carlos III/FEDER, RETIC ARADyAL RD16/0006/002. Ana Moreira thanks the research contract under the project Coccolitho4BioMat - Coccolithophore microalgae biorefinery: an approach for sustainable biomaterials (POCI-01-0145-FEDER-031032), funded by Centro2020, through FEDER and PT2020. Tânia Melo thanks the Junior Researcher contract in the scope of the Individual Call to Scientific Employment Stimulus 2020 [CEECIND/01578/2020]. Bruna Neves (2021.04602.BD), Helena Beatriz Ferreira (2020.04611.BD) and Inês Guerra (2021.04754.BD) thanks FCT for the PhD grant.

References

[1] Liu Q, Siloto RMP, Lehner R, Stone SJ, Weselake RJ. Acyl-CoA:diacylglycerol acyltransferase: Molecular biology, biochemistry and biotechnology. Prog Lipid Res 2012;51:350–77. https://doi.org/10.1016/j.plipres.2012.06.001.

[2] Bargui R, Solgadi A, Prost B, Chester M, Ferreiro A, Piquereau J, Moulin, M. Phospholipids: Identification and implication in muscle pathophysiology. Int J Mol Sci 2021;22:1-21. https://doi.org/10.3390/ijms22158176.

[3] Juan CA, Pérez de la Lastra JM, Plou FJ, Pérez-Lebeña E. The chemistry of reactive oxygen species (ROS) revisited: Outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. Int J Mol Sci 2021;22:1-21. https://doi.org/10.3390/ijms22094642.

[4] Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS sources in physiological and pathological conditions. Ox Med Cell Long 2016: 1-44. https://doi.org/10.1155/2016/1245049.

[5] Ma L, Hu L, Feng X, Wang S. Nitrate and nitrite in health and disease. Aging Dis 2018;9:938–45. https://doi.org/10.14336/AD.2017.1207.

[6] Nadtochiy SM, Baker PRS, Freeman BA, Brookes PS. Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection. Cardiovasc Res 2009; 82:333–40. https://doi.org/10.1093/cvr/cvn323.

[7] Schopfer FJ, Batthyany C, Baker PRS, Bonacci G, Cole MP, Rudolph V, Groger A, Rudolph S, Nadtochiy PS, Freeman BA. Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives. Free Radic Biol Med 2009;46:1250–9. https://doi.org/10.1016/j.freeradbiomed.2008.12.025.

[8] Melo T, Montero-Bullón J-F, Domingues P, Domingues MRM. Discovery of bioactive nitrated lipids and nitro lipid-protein adducts using mass spectrometry-based approaches. Redox Biol 2019:1–16. https://doi.org/10.1016/j.redox.2019.101106.

[9] Reddy AT, Lakshmi SP, Reddy RC. The nitrated fatty acid 10-nitro-oleate diminishes severity of LPS-induced acute lung injury in mice. PPAR Res 2012:1–12. https://doi.org/10.1155/2012/617063.

[10] Arbeeny CM, Ling H, Smith MM, O'Brien S, Wawersik S, Ledbetter SR, McAlexander A, Schopfer FJ, Willette RN, Jorkasky DK. CXA-10, a nitrated fatty acid, Is renoprotective in decoxycorticosterone acetate-salt nephropathy. J Pharmacol Exp Ther 2019;369:503–10. https://doi.org/10.1124/jpet.118.254755.

[11] Schopfer FJ, Vitturi DA, Jorkasky DK, Freeman BA. Nitro-fatty acids: New drug candidates for chronic inflammatory and fibrotic diseases. Nitric Oxi 2018;79:31–7. https://doi.org/10.1016/j.niox.2018.06.006.

[12] Melo T, Domingues P, Ferreira R, Milic I, Fedorova M, Santos SM, Segundo MA, Domingues MRM. Recent Advances on Mass Spectrometry Analysis of Nitrated Phospholipids. Anal Chem 2016;88:2622–9. https://doi.org/10.1021/acs.analchem.5b03407.

[13] Melo T, Domingues P, Ribeiro-Rodrigues TM, Girão H, Segundo MA, Domingues MRM. Characterization of phospholipid nitroxidation by LC-MS in biomimetic models and in H9c2 Myoblast using a lipidomic approach. Free Radical Bio Med 2017;106:219–27. https://doi.org/10.1016/j.freeradbiomed.2017.02.033.

[14] Neves B, Domingues P, Oliveira M, Domingues MRM, Melo T. Profile of phosphatidylserine modifications under nitroxidative stress conditions using a liquid chromatography-mass spectrometry based Approach. Molecules 2018;24:1–17. https://doi.org/10.3390/molecules24010107.

[15] Neves B, Duarte S, Domingues P, Pérez-Sala D, Oliveira MM, Domingues MRM, Melo T. Advancing Target Identification of Nitrated Phospholipids in Biological Systems by HCD Specific Fragmentation Fingerprinting in Orbitrap Platforms. Molecules 2020;25:1–19. https://doi.org/10.3390/molecules25092120.

[16] Montero-Bullon J-F, Melo T, Rosário M, Domingues MRM, Domingues P. Liquid chromatography/tandem mass spectrometry characterization of nitroso, nitrated and nitroxidized cardiolipin products. Free Radical Bio Med 2019;144:183–91. https://doi.org/10.1016/j.freeradbiomed.2019.05.009.

[17] Fazzari M, Khoo N, Woodcock SR, Li L, Freeman BA, Schopfer FJ. Generation and esterification of electrophilic fatty acid nitroalkenes in triacylglycerides. Free Radical Bio Med 2015;87:113–24. https://doi.org/10.1016/j.freeradbiomed.2015.05.033.

[18] Fazzari M, Khoo NKH, Woodcock SR, Jorkasky DK, Li L, Schopfer FJ, Freeman BA. Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. J Lipid Res 2017;58:375–85. https://doi.org/10.1194/jlr.M072058.

[19] Melo T, Marques SS, Ferreira I, Cruz MT, Domingues P, Segundo MA, Domingues MRM. New insights into the anti-Inflammatory and antioxidant properties of nitrated phospholipids. Lipids 2018;53:117–31. https://doi.org/10.1002/lipd.12007.

[20] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424. https://doi.org/10.1152/physrev.00029.2006.

[21] Szabó C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 2007;6:662–80. https://doi.org/10.1038/nrd2222.

[22] Calcerrada P, Peluffo G, Radi R. Nitric oxide-derived oxidants with a focus on peroxynitrite: molecular targets, cellular responses and therapeutic implications. Curr Pharm Design 2011;17:3905–32. https://doi.org/10.2174/138161211798357719.

[23] Dedon PC, Tannenbaum SR. Reactive nitrogen species in the chemical biology of inflammation. Arch Biochem Biophys 2004;423:12–22. https://doi.org/10.1016/j.abb.2003.12.017.

[24] Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Bio Med 1998;25:434–56. https://doi.org/10.1016/S0891-5849(98)00092-6.

[25] Ramalingam M, Kim S-J. Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases. J Neural Transm 2012;119:891–910. https://doi.org/10.1007/s00702-011-0758-7.

[26] Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol 2002;30:620–50. https://doi.org/10.1080/01926230290166724.

[27] Ricciardolo FLM, Di Stefano A, Sabatini F, Folkerts G. Reactive nitrogen species in the respiratory tract. Eur J Pharmacol 2006;533:240–52. https://doi.org/10.1016/j.ejphar.2005.12.057.

[28] van der Vliet A, Eiserich JP, Halliwell B, Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. J Biol Chem 1997;272:7617–25. https://doi.org/10.1074/jbc.272.12.7617.

[29] Lancaster JR. Nitroxidative, nitrosative, and nitrative stress: kinetic predictions of reactive nitrogen species chemistry under biological conditions. Chem Res Toxicol 2006;19:1160–74. https://doi.org/10.1021/tx060061w.

[30] Spickett CM. Formation of Oxidatively Modified Lipids as the Basis for a Cellular Epilipidome. Front Endocrinol 2020;11:1-18. https://doi.org/10.3389/fendo.2020.602771.

[31] Fazzari M, Vitturi DA, Woodcock SR, Salvatore SR, Freeman BA, Schopfer FJ. Electrophilic fatty acid nitroalkenes are systemically transported and distributed upon esterification to complex lipids. J Lipid Res 2019;60:388–99. https://doi.org/10.1194/jlr.M088815.

[32] Trostchansky A, Souza JM, Ferreira A, Ferrari M, Blanco F, Trujillo M, et al. Synthesis, Isomer Characterization, and Anti-Inflammatory Properties of Nitroarachidonate. Biochem 2007;46:4645–53. https://doi.org/10.1021/bi602652j.

[33] Rocha BS, Gago B, Barbosa RM, Lundberg JO, Radi R, Laranjinha J. Intragastric nitration by dietary nitrite: Implications for modulation of protein and lipid signaling. Free Radical Bio Med 2012;52:693–8. https://doi.org/10.1016/j.freeradbiomed.2011.11.011.

[34] Milic I, Griesser E, Vemula V, Ieda N, Nakagawa H, Miyata N, Galanom JM, Oger C, Durand T, Fedorova M. Profiling and relative quantification of multiply nitrated and oxidized

fatty acids. Anal Bioanal Chem 2015;407:5587–602. https://doi.org/10.1007/s00216-015-8766-3.

[35] Baker PRS, Lin Y, Schopfer FJ, Woodcock SR, Groeger AL, Batthyany C, Sweeney S, Long MH, Baker LMS, Branchaud BP. Fatty Acid Transduction of Nitric Oxide Signaling: Multiple Nitrated Unsaturated Fatty Acid Derivatives Exist in Human Blood and Urine and Serve as Endogenous Peroxisome Proliferator-Activated Receptor Ligands. J Bio Chem. 2005;280:42464-75, https://doi.org/10.1074/jbc.M504212200

[36] Napolitano A, Panzella L, Savarese M, Sacchi R, Giudicianni I, Paolillo L, d'Ischia M. Acid-Induced Structural Modifications of Unsaturated Fatty Acids and Phenolic Olive Oil Constituents by Nitrite Ions: A Chemical Assessment. Chem Res Toxicol 2004;17:1329–37. https://doi.org/10.1021/tx049880b.

[37] Pereira C, Ferreira NR, Rocha BS, Barbosa RM, Laranjinha J. The redox interplay between nitrite and nitric oxide: From the gut to the brain. Redox Biol 2013;1:276–84. https://doi.org/10.1016/j.redox.2013.04.004.

[38] Jain K, Siddam A, Marathi A, Roy U, Falck JR, Balazy M. The mechanism of oleic acid nitration by $^{\bullet}NO_2$. Free Radical Bio Med 2008;45:269–83. https://doi.org/10.1016/j.freeradbiomed.2008.04.015.

[39] O'Donnell VB, Freeman BA. Interactions Between Nitric Oxide and Lipid Oxidation Pathways: Implications for Vascular Disease. Cir Res 2001;88:12–21. https://doi.org/10.1161/01.RES.88.1.12.

[40] Freeman BA, Baker PRS, Schopfer FJ, Woodcock SR, Napolitano A, d'Ischia M. Nitro-fatty acid formation and signaling. J Biol Chem 2008;283:15515–9. https://doi.org/10.1074/jbc.R800004200.

[41] Buchan GJ, Bonacci G, Fazzari M, Salvatore SR, Gelhaus Wendell S. Nitro-fatty acid formation and metabolism. Nitric Oxi 2018;79:38–44. https://doi.org/10.1016/j.niox.2018.07.003.

[42] Trostchansky A, Rubbo H. Nitrated fatty acids: Mechanisms of formation, chemical characterization, and biological properties. Free Radical Bio Med 2008;44:1887–96. https://doi.org/10.1016/j.freeradbiomed.2008.03.006.

[43] Müller WH, De Pauw E, Far J, Malherbe C, Eppe G. Imaging lipids in biological samples with surface-assisted laser desorption/ionization mass spectrometry: A concise review of the last decade. Prog Lipid Res 2021;83:1–15. https://doi.org/10.1016/j.plipres.2021.101114.

[44] Maciel E, Domingues P, Domingues MRM. Liquid chromatography/tandem mass spectrometry analysis of long-chain oxidation products of cardiolipin induced by the hydroxyl radical. Rapid Commun Mass Sp 2011;25:316–26. https://doi.org/10.1002/rcm.4866.

[45] Olsen JV, Schwartz JC, Griep-Raming J, Damoc E, Splendore M, Wouters ER, Senko M, Makarov A, Mann M, Horning S. A dual pressure linear ion trap orbitrap instrument with very high sequencing speed. Mol Cell Proteomics 2009;8:2759–69.

[46] Xia Y, Liang X, McLuckey SA. Ion Trap versus Low-Energy Beam-Type Collision-Induced Dissociation of Protonated Ubiquitin Ions. Anal Chem 2006;78:1218–27. https://doi.org/10.1021/ac051622b.

[47] Bayat P, Lesage D, Cole RB. Low-energy collision-induced dissociation (low-energy CID), collision-induced dissociation (CID), and higher energy collision dissociation (HCD) mass spectrometry for structural elucidation of saccharides and clarification of their dissolution mechanism in DMAc/LiCl. J Mass Spectrom 2018;53:705–16. https://doi.org/10.1002/jms.4205.

[48] Duarte S, Melo T, Domingues MRD, de Dios Alché J, Pérez-Sala D. Insight into the cellular effects of nitrated phospholipids: evidence for pleiotropic mechanisms of action. Free Radical Bio Med 2019;144:192–202. https://doi.org/10.1016/j.freeradbiomed.2019.06.003

[49] DeMartino AW, Kim-Shapiro DB, Patel RP, Gladwin MT. Nitrite and nitrate chemical biology and signalling. Br J Pharmacol 2019;176:228–45. https://doi.org/10.1111/bph.14484.

[50] Schopfer FJ, Khoo NKH. Nitro-fatty acid logistics: formation, biodistribution, signaling, and pharmacology. Trends Endocr Met 2019;30:505–19. https://doi.org/10.1016/j.tem.2019.04.009.

[51] Fazzari M, Trostchansky A, Schopfer FJ, Salvatore SR, Sánchez-Calvo B, Vitturi D, Valderrama R, Barroso JB, Radi R, Freeman BA. Olives and olive oil are sources of electrophilic fatty acid nitroalkenes. PLoS ONE 2014;9:1–9. https://doi.org/10.1371/journal.pone.0084884.

[52] Vollár M, Feigl G, Oláh D, Horváth A, Molnár Á, Kúsz N, Ördög A, Csupor D, Kolbert Z. Nitro-Oleic Acid in Seeds and Differently Developed Seedlings of Brassica napus L. Plants 2020;9:1–15. https://doi.org/10.3390/plants9030406.

[53] Mata-Pérez C, Sánchez-Calvo B, Padilla MN, Begara-Morales JC, Luque F, Melguizo M, Jiménez-Ruiz J, Fierro-Risco J, Peñas-Sanjuán A, Valderrama R. Nitro-Fatty Acids in Plant Signaling: Nitro-Linolenic Acid Induces the Molecular Chaperone Network in Arabidopsis. Plant Physiol 2016;170:686–701. https://doi.org/10.1104/pp.15.01671

[54] Mata-Pérez C, Sánchez-Calvo B, Padilla MN, Begara-Morales JC, Valderrama R, Corpas FJ, Barroso JBl. Nitro-fatty acids in plant signaling: new key mediators of nitric oxide metabolism. Redox Biol 2017;11:554–61. https://doi.org/10.1016/j.redox.2017.01.002.

[55] Alves-Bezerra M, Cohen DE. Triglyceride metabolism in the liver. Compr Physiol 2017;8:1–8. https://doi.org/10.1002/cphy.c170012.

[56] Trevaskis NL, Kaminskas LM, Porter CJH. From sewer to saviour — targeting the lymphatic system to promote drug exposure and activity. Nat Rev Drug Discov 2015;14:781–803. https://doi.org/10.1038/nrd4608.

[57] Coles B, Bloodsworth A, Eiserich JP, Coffey MJ, McLoughlin RM, Giddings JC, Lewis MJ, Haslam RJ, Freeman BA, O'Donnell VB. Nitrolinoleate Inhibits Platelet Activation by Attenuating Calcium Mobilization and Inducing Phosphorylation of Vasodilator-stimulated Phosphoprotein through Elevation of cAMP. J Biol Chem 2002;277:5832–40. https://doi.org/10.1074/jbc.M105209200

[58] Gilde AJ, Van Bilsen M. Peroxisome proliferator-activated receptors (PPARS): regulators of gene expression in heart and skeletal muscle. Acta Physiol Scand 2003;178:425–34. https://doi.org/10.1046/j.1365-201X.2003.01161.x.

[59] Li Y, Zhang J, Schopfer FJ, Martynowski D, Garcia-Barrio MT, Kovach A, Suino-Powell K, Baker PRS, Freeman BA, Chen YE. Molecular recognition of nitrated fatty acids by PPARγ. Nat Struct Mol Biol 2008;15:865–7. https://doi.org/10.1038/nsmb.1447.

[60] Abdelrahman M, Sivarajah A, Thiemermann C. Beneficial effects of PPAR-γ ligands in ischemia-reperfusion injury, inflammation and shock. Cardiovascular Res 2005;65:772–81. https://doi.org/10.1016/j.cardiores.2004.12.008.

[61] Reddy AT, Lakshmi SP, Kleinhenz JM, Cutliff RL, Hart CM, Reddy RC. Endothelial Cell Peroxisome Proliferator–Activated Receptor γ Reduces Endotoxemic Pulmonary Inflammation and Injury. J Immunol 2012;189:5411–20. https://doi.org/10.4049/jimmunol.1201487.

[62] Lopaschuk GD, Ussher JR, Folmes CDL, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. Physiol Rev 2010;90:207–58. https://doi.org/10.1152/physrev.00015.2009.

[63] Montero-Bullon J-F, Melo T, Domingues MRM, Domingues P. Characterization of nitrophospholipid-peptide covalent adducts by electrospray tandem mass spectrometry: a first screening analysis using different instrumental platforms. Eur J Lipid Sci Tech 2018;120:1–6. https://doi.org/10.1002/ejlt.201800101.

[64] Díez-Dacal B, Pérez-Sala D. Anti-inflammatory prostanoids: focus on the interactions between electrophile signaling and resolution of inflammation. Sci World J 2010;10:655–75. https://doi.org/10.1100/tsw.2010.69.

[65] Garzón B, Oeste CL, Díez-Dacal B, Pérez-Sala D. Proteomic studies on protein modification by cyclopentenone prostaglandins: expanding our view on electrophile actions. J Proteomics 2011;74:2243–63. https://doi.org/10.1016/j.jprot.2011.03.028.

[66] Oeste CL, Pérez-Sala D. Modification of cysteine residues by cyclopentenone prostaglandins: Interplay with redox regulation of protein function. Mass Spectrom Rev 2014;33:110–25. https://doi.org/10.1002/mas.21383.

[67] Pérez-Sala D, Oeste CL, Martínez AE, Carrasco MJ, Garzón B, Cañada FJ. Vimentin filament organization and stress sensing depend on its single cysteine residue and zinc binding. Nat Commun 2015;6:7287. https://doi.org/10.1038/ncomms8287.

[68] Shiraki T, Kamiya N, Shiki S, Kodama TS, Kakizuka A, Jingami H. α,β-Unsaturated Ketone Is a Core Moiety of Natural Ligands for Covalent Binding to Peroxisome Proliferator-activated Receptor γ*. J Biol Chem 2005;280:14145–53. https://doi.org/10.1074/jbc.M500901200.

[69] Martínez AE, Sánchez-Gómez FJ, Díez-Dacal B, Oeste CL, Pérez-Sala D. 15-Deoxy-Δ(12,14)-prostaglandin J2 exerts pro- and anti-inflammatory effects in mesangial cells in a concentration-dependent manner. Inflamm Allergy Drug Targets 2012;11:58–65. https://doi.org/10.2174/187152812798889349.

[70] Levonen AL, Dickinson DA, Moellering DR, Mulcahy RT, Forman HJ, Darley-Usmar VM. Biphasic effects of 15-deoxy-delta(12,14)-prostaglandin J(2) on glutathione induction and apoptosis in human endothelial cells. Arterioscl Thromb Vasc Biol 2001;21:1846–51. https://doi.org/10.1161/hq1101.098488.

[71] Pohl EE, Jovanovic O. The Role of Phosphatidylethanolamine Adducts in Modification of the Activity of Membrane Proteins under Oxidative Stress. Molecules 2019;24:1–15. https://doi.org/10.3390/molecules24244545.

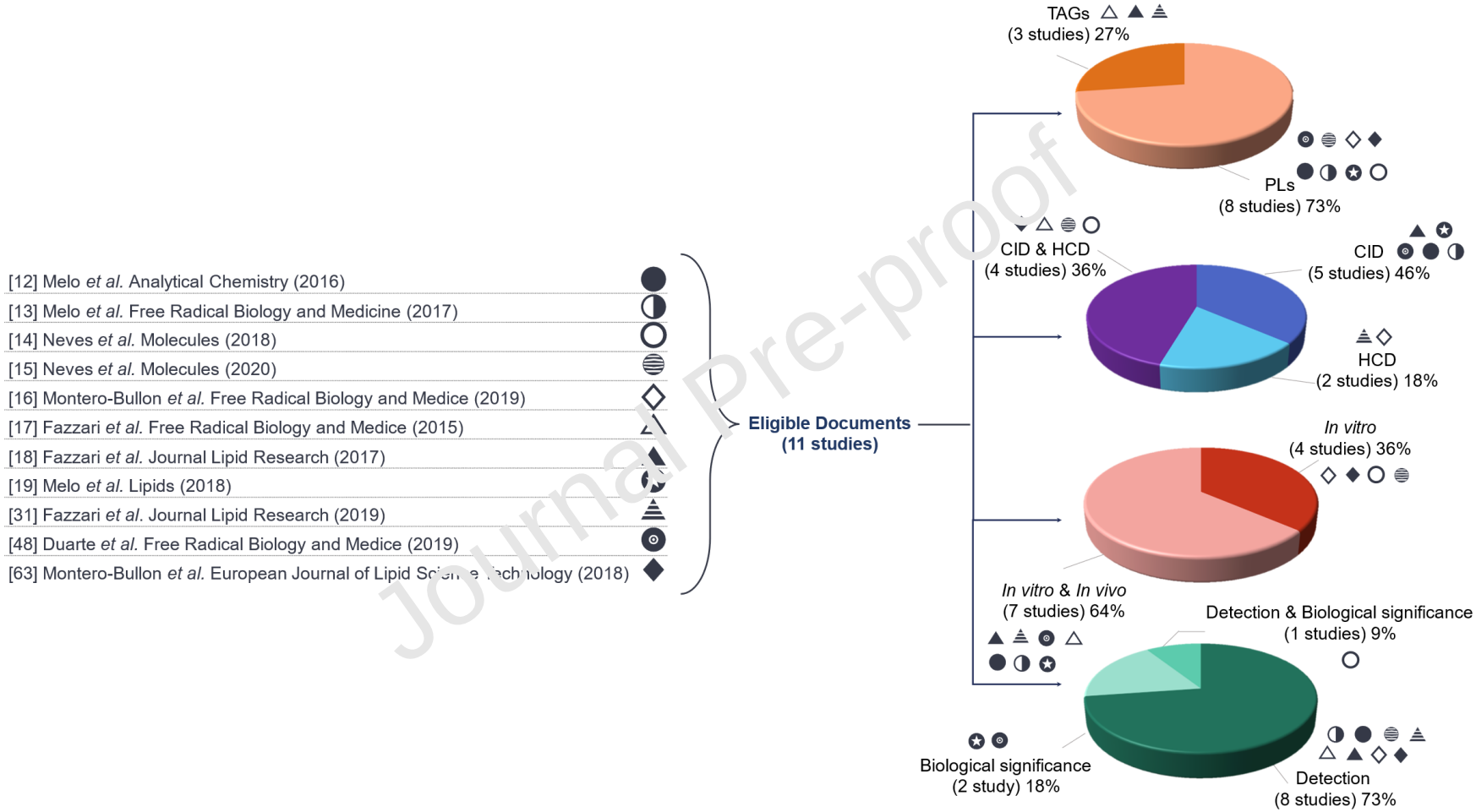


Figure 1

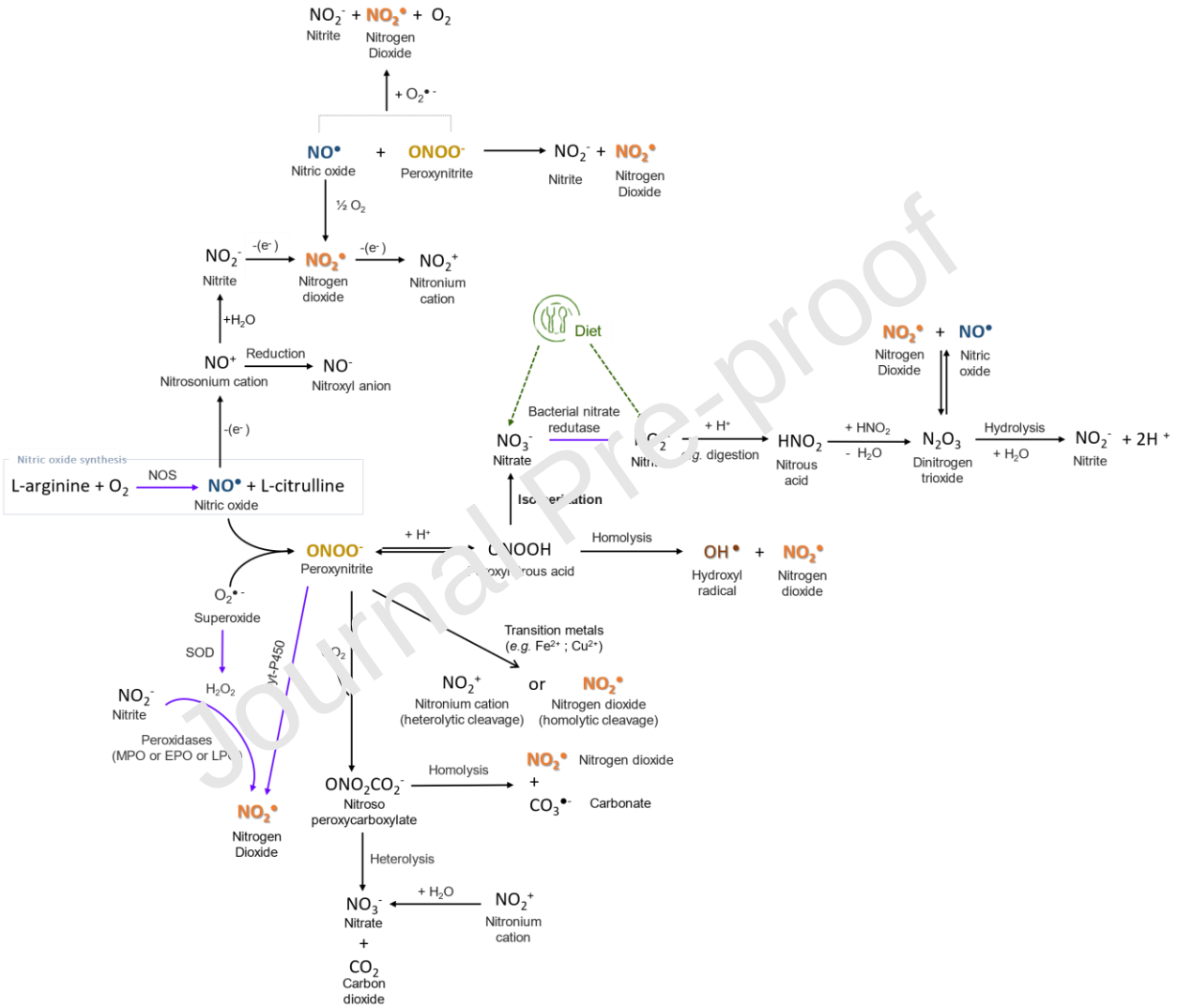


Figure 2

Figure 3

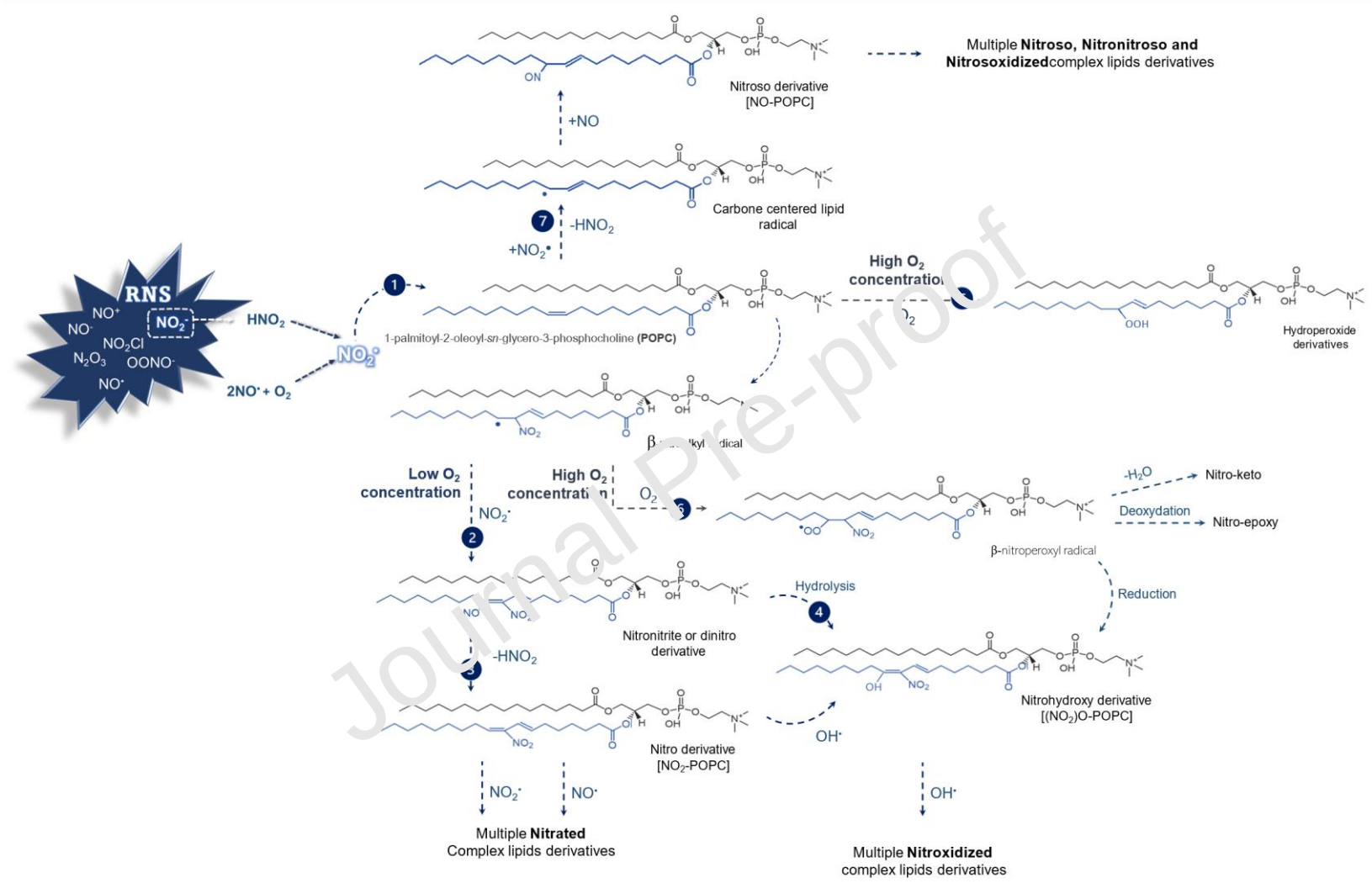


Figure 4

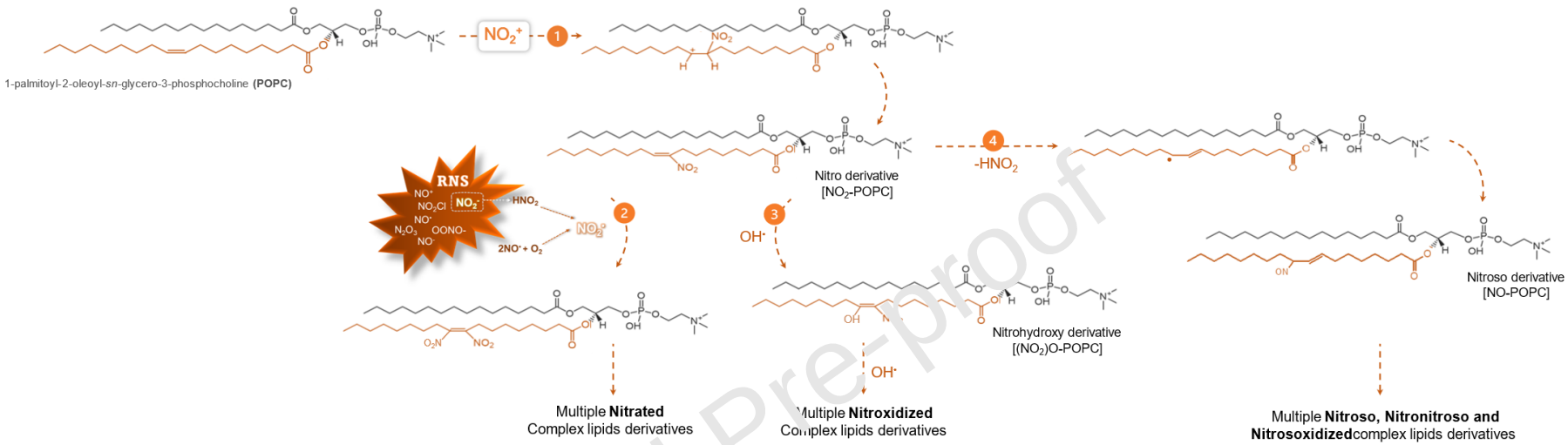


Figure 5

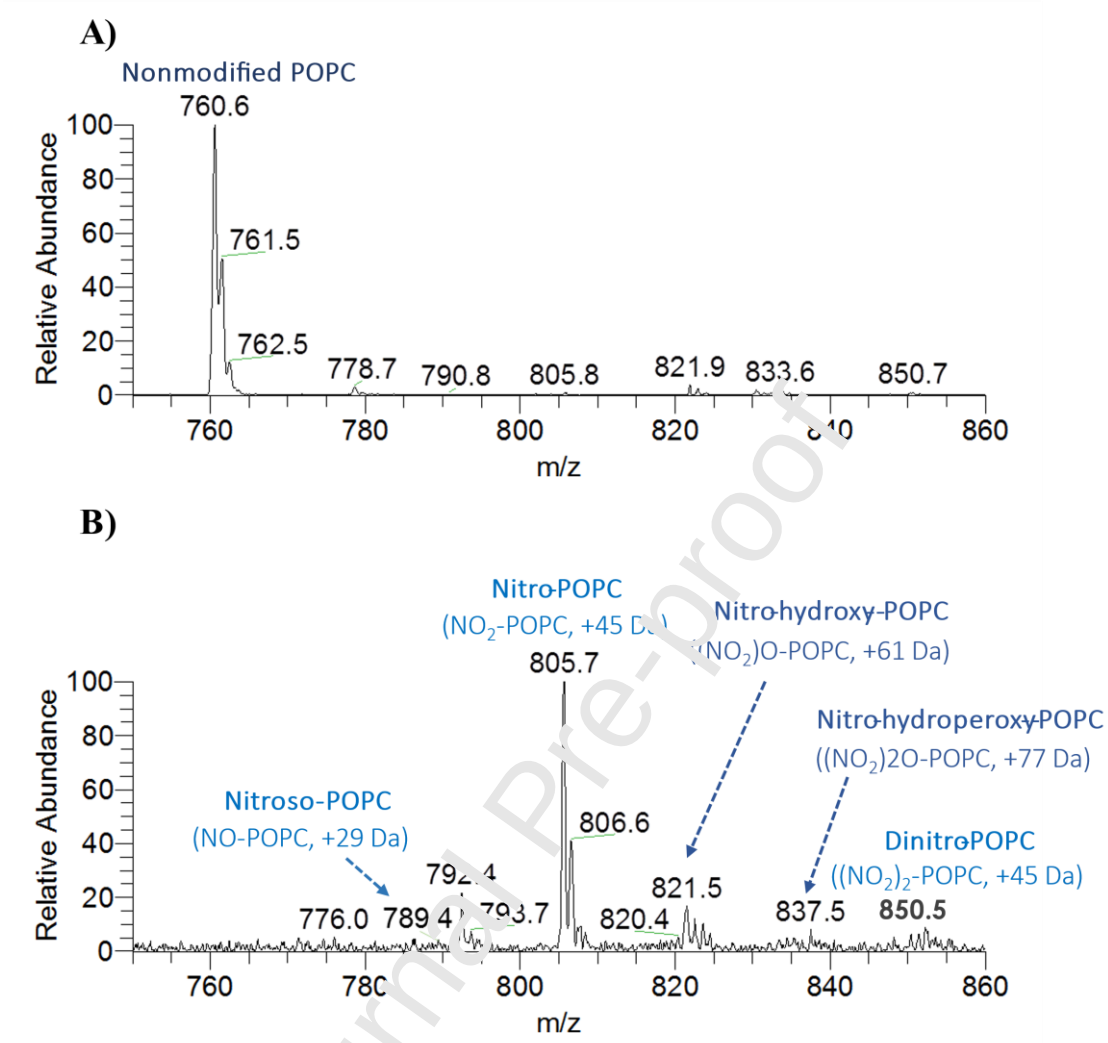


Figure 6

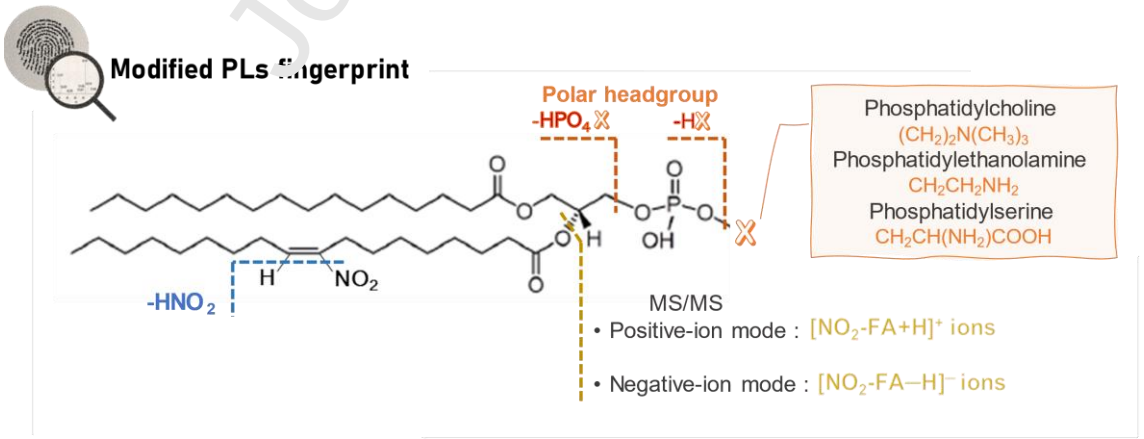


Figure 7

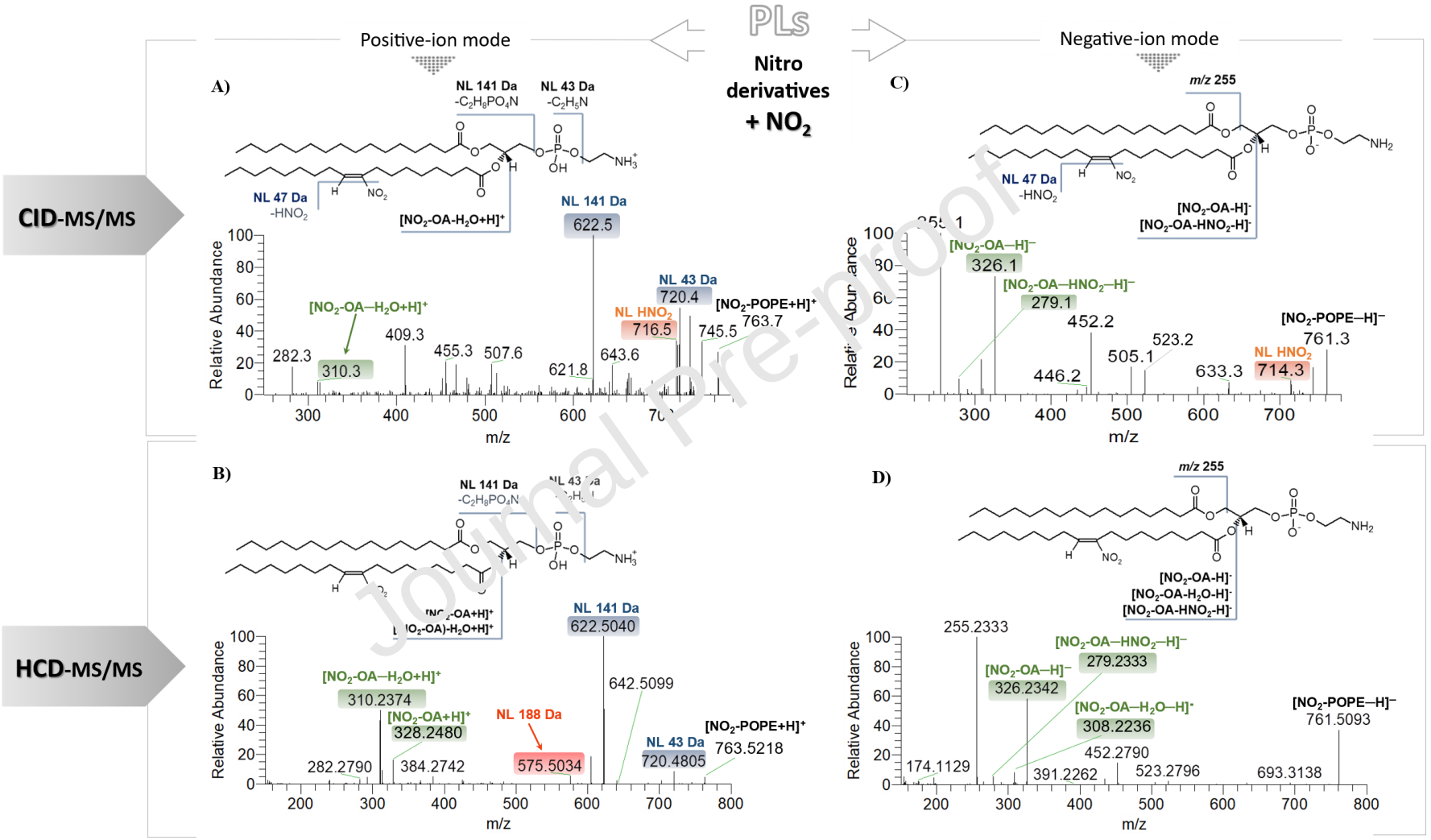


Figure 8

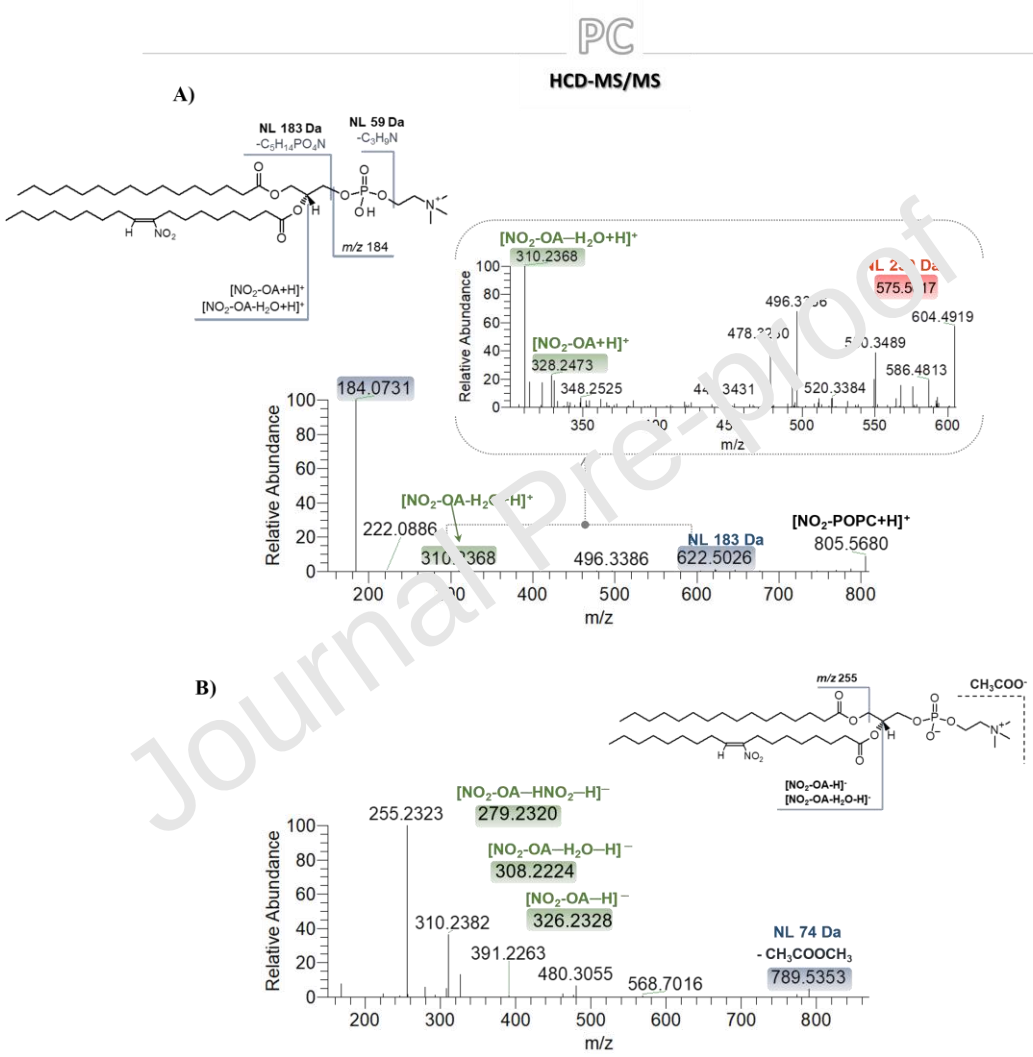


Figure 9

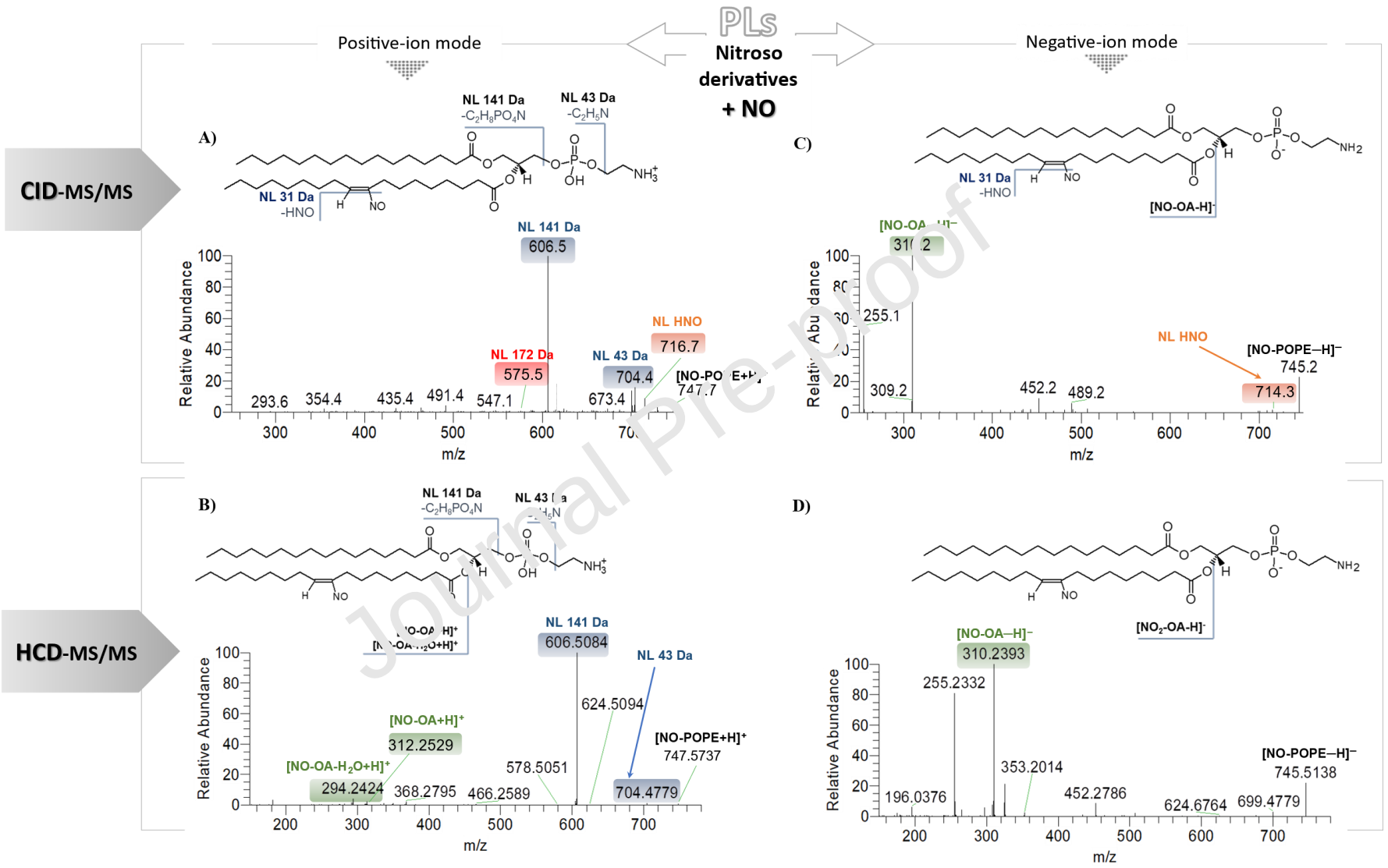
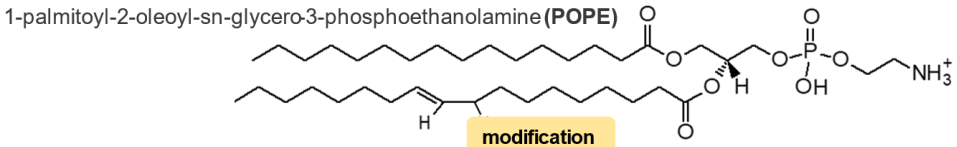


Figure 10



CID

	NL 47 Da $-HNO_2$	NL 31 Da $-HNO$	NL 90 Da $-2HNO_2$	Polar head	Modified FA moieties
Nitro	✓	—	—	✓	✓
Nitroxidized	✓	—	—	✓	✓
Nitroso	—	✓	—	✓	✓
Nitronitroso	✓	✓	—	✓	✓
Dinitro	✓	—	✓	✓	✓
Dinitroxidized	✓	—	✓	✓	✓

HCD

	NL 47 Da $-HNO_2$	NL 31 Da $-HNO$	NL 90 Da $-2HNO_2$	Polar head	Modified FA moieties
Nitro	—	—	—	✓	✓
Nitroxidized	—	—	—	✓	✓
Nitroso	—	—	—	✓	✓
Nitronitroso	—	—	—	✓	✓
Dinitro	—	—	—	✓	✓
Dinitroxidized	—	—	—	✓	✓

Figure 11

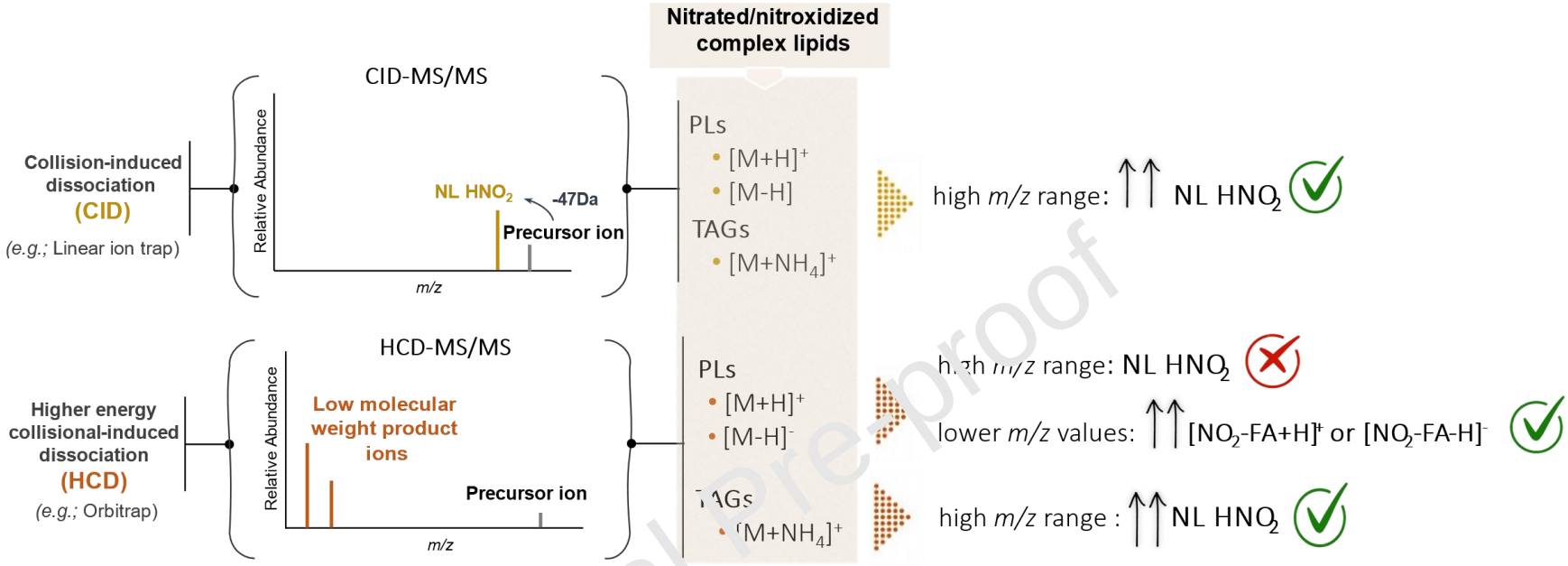


Figure 12

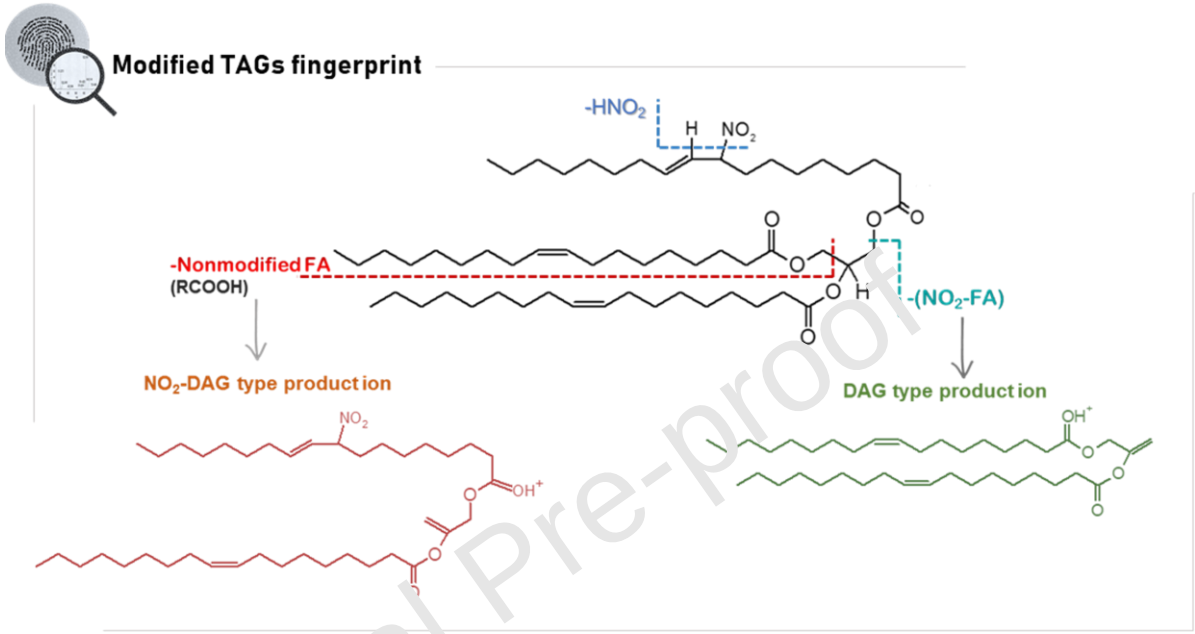


Figure 13

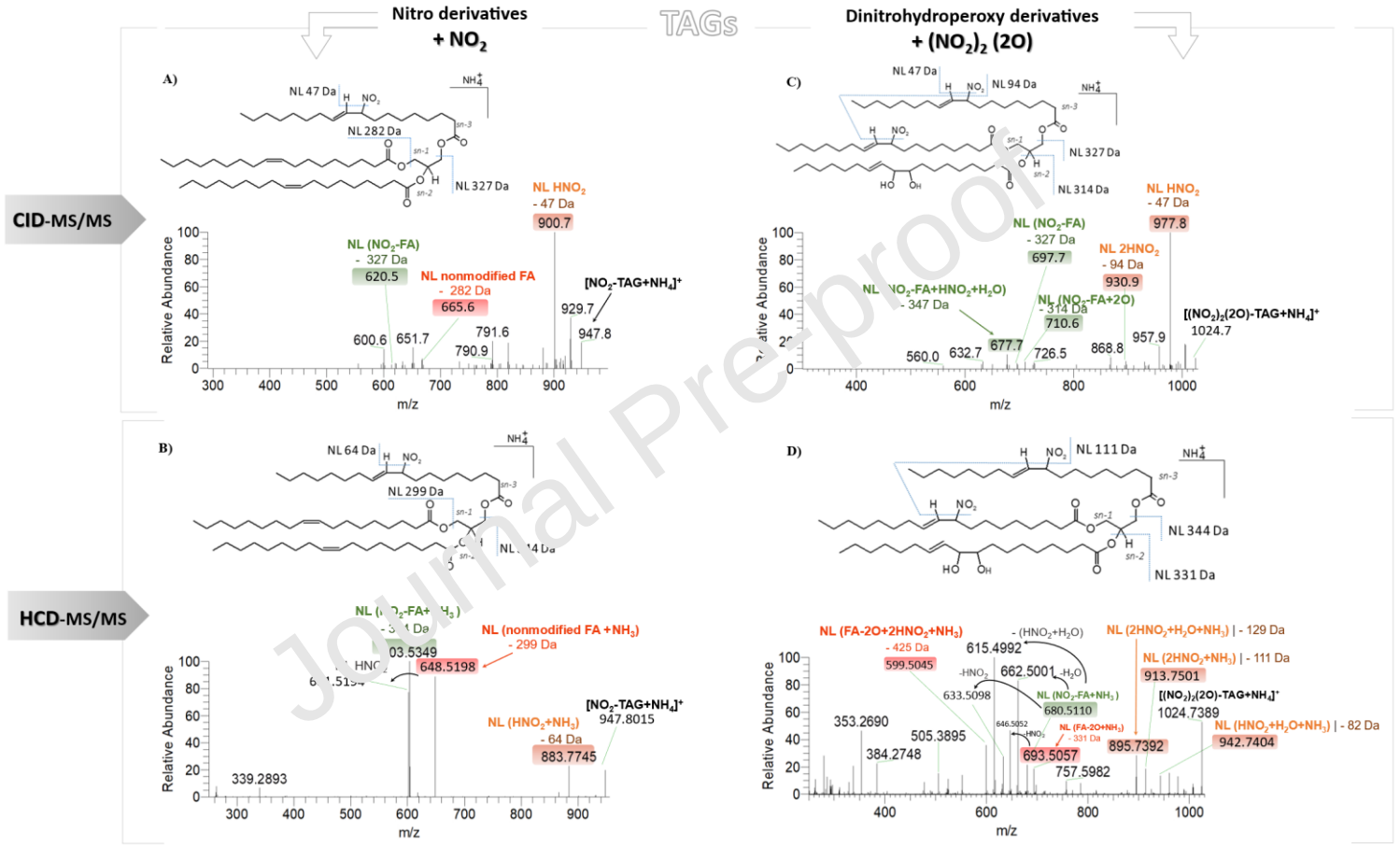

Nitro derivatives
+ NO2
TAGs
Dinitrohydroperoxy derivatives
+ (NO2)2 (2O)
CID-MS/MS
HCD-MS/MS
A)
B)
C)
D)
NL 47 Da
NL 282 Da
NL 327 Da
NL HNO2
- 47 Da
900.7
NL (NO2-FA)
- 327 Da
620.5
NL nonmodified FA
- 282 Da
665.6
[NO2-TAG+NH4]+
929.7
947.8
600.6
651.7
791.6
790.9
Relative Abundance
m/z
NL 64 Da
NL 299 Da
NL (nonmodified FA +NH3)
- 299 Da
648.5198
[NO2-TAG+NH4]+
947.8015
NL (HNO2+NH3)
- 64 Da
883.7745
339.2893
NL 94 Da
NL 314 Da
977.8
NL 2HNO2
- 94 Da
930.9
NL (NO2-FA+2O)
- 314 Da
710.6
[(NO2)2(2O)-TAG+NH4]+
1024.7
697.7
677.7
560.0
632.7
726.5
868.8
957.9
NL 111 Da
NL 344 Da
NL 331 Da
NL (FA-2O+2HNO2+NH3)
- 425 Da
599.5045
615.4992
662.5001
633.5098
680.5110
NL (2HNO2+H2O+NH3) | - 129 Da
913.7501
NL (2HNO2+NH3) | - 111 Da
1024.7389
NL (HNO2+H2O+NH3) | - 82 Da
942.7404
353.2690
505.3895
384.2748
693.5057
757.5982
895.7392

Figure 14

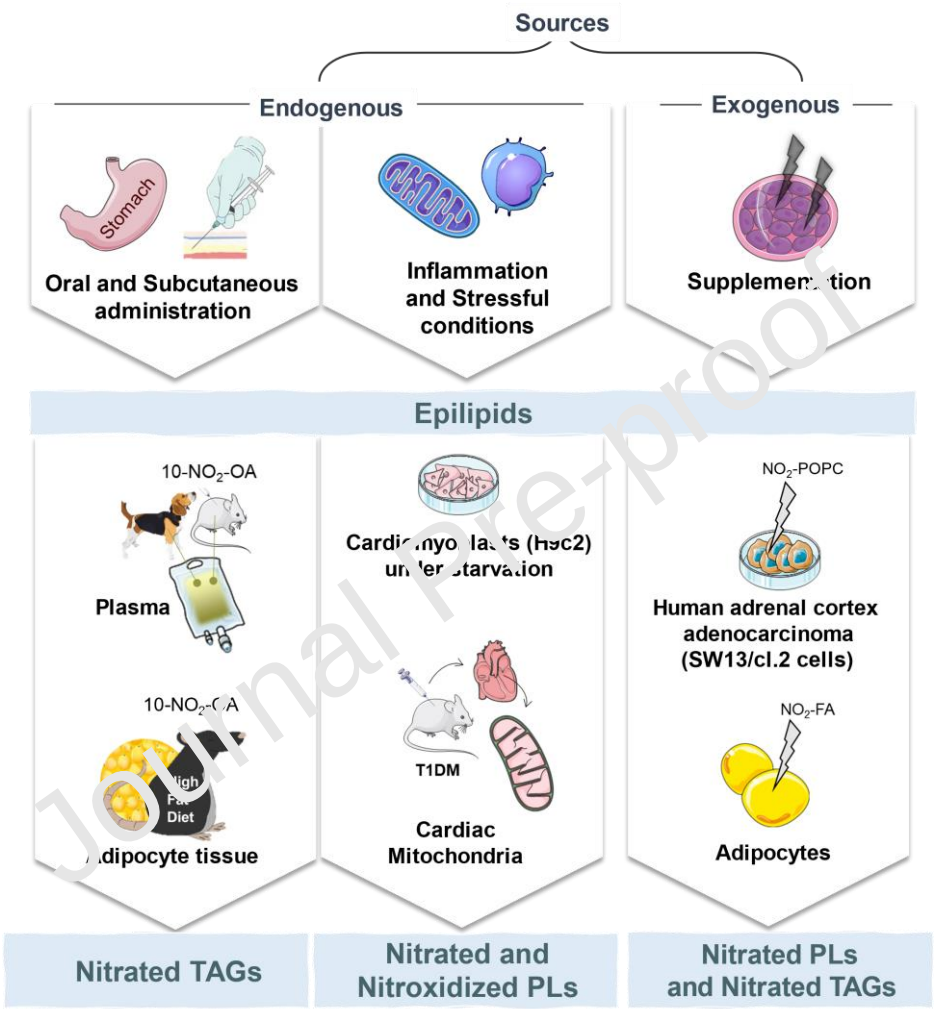


Figure 15

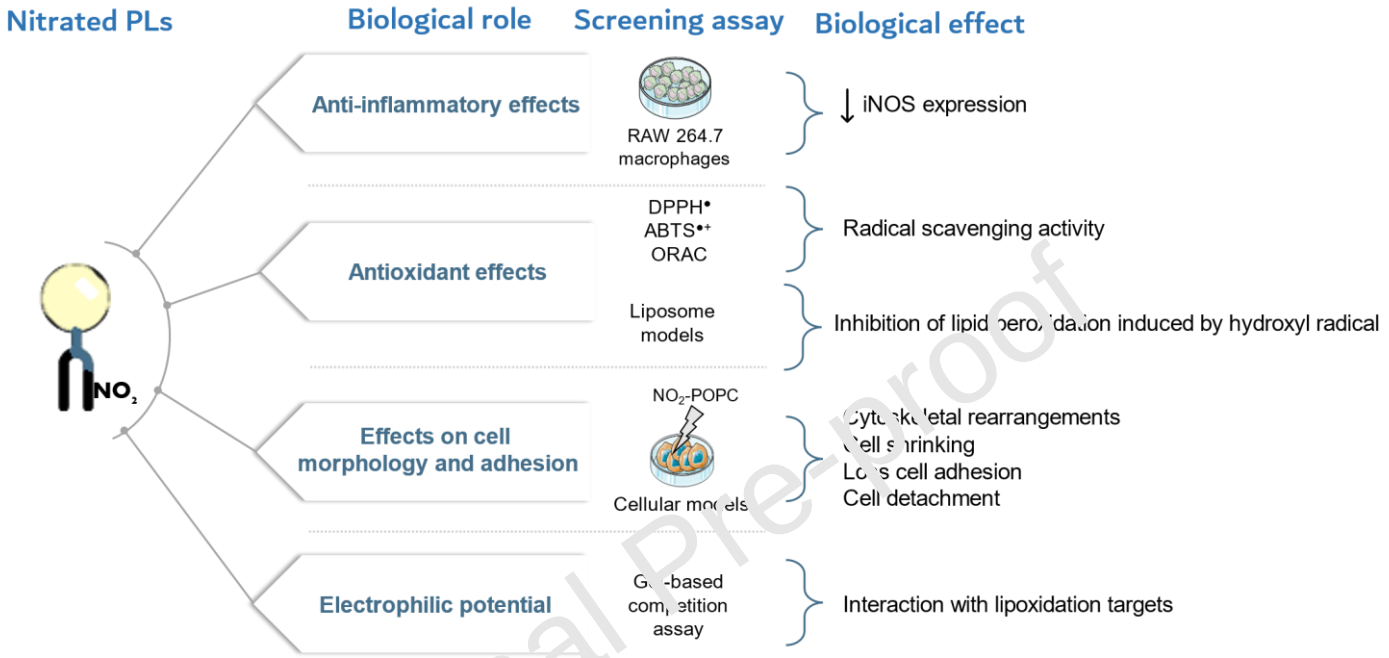


Figure 16

Table 1. Type of ions and adducts observed in the mass spectrum of nitrated or nitroxidized complex lipids, phospholipids and tryacylglicerides (TAGs), in positive- and negative-ion modes. Phosphatidylcholine – PC; phosphatidylserine – PS; phosphatidylethanolamine – PE; cardiolipin – CL.

Nitrated/nitroxidized Complex lipids	Positive-ion mode	Negative-ion mode
PC	$[M+H]^+$; $[M+Na]^+$	$[M+CH_3COO]^-$; $[M+HCOO]^-$
PS	$[M+H]^+$; $[M+Na]^+$	$[M-H]^-$
PE	$[M+H]^+$; $[M+Na]^+$	$[M-H]^+$
CL	---	$[M-H]^-$; $[M-2H]^{2-}$
TAGs	$[M+NH_4]^+$	---

Table 2. Nitrated complex lipids identified using *in vitro* biomimetic model systems. Information on the fragmentation technique used to induce ion activation under tandem mass spectrometry conditions (collision-induced dissociation (CID) or higher energy CID (HCD)), the experimental model employed, and the methodology performed for analysis of the modified complex lipids is also provided in this table.

***In vitro* biomimetic model systems**

Ion activation	Nitrated species	Experimental model	Method	Ref.
CID	Nitro derivative of POPC; PLPC; PAPC; POPE; PLPE; PAPE; POPS; PLPS; and PAPS with identification of positional isomers: 9- and 10-NO_2-OA-PLs; 9-, 10- and 13-NO_2-LA-PLs; and 11-, 12-, 14-, and 15-NO_2-AA-PLs	Incubation of each PLs standard (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	Direct infusion ESI-MS and MS/MS using LXQ-LIT, both in positive- and negative-ion mode Direct infusion ESI-MS using Q-TOF 2 hybrid quadrupole time-of-flight, in positive-ion mode	[12]
	(Di)Nitroso, (di)nitro, nitronitroso, and nitroxidized derivatives of ((NO)O-PLs; (NO_2)O-PLs; (NO_2)(2O)-PLs; $(NO_2)_2$O-PLs; $(NO_2)_2$(2O)-PLs) of POPC; PLPC; PAPC; POPE; PLPE and PAPE	Incubation of each PLs standard (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	Direct infusion ESI-MS and MS/MS using LXQ-LIT, both in positive- and negative-ion mode C5-RP-LC-ESI-MS and MS/MS using Waters Alliance 2690 HPLC system coupled online to LXQ-LIT mass spectrometer, both in positive- and negative-ion mode	[13]
	NO-POPS; NO_2-POPS; (NO_2)O-POPS; (NO_2)(NO)-POPS; (NO_2)(2O)-POPS; $(NO_2)_2$-POPS	Incubation of POPS (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	Direct infusion ESI-MS and MS/MS using LXQ-LIT mass spectrometer, in negative-ion mode. C5-RP-LC-ESI-MS and MS/MS using Waters Alliance 2690 HPLC system coupled online to LXQ-LIT mass spectrometer, in negative-ion mode	[14]
HCD	Nitrated and nitroxidized derivatives of POPC; PLPC; PAPC; POPE; PLPE; PAPE	Incubation of each PL standard (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	Direct infusion ESI-MS and HR-MS/MS using Q-Exactive hybrid quadrupole Orbitrap® mass spectrometer, in positive- and negative-ion mode	[15]
	NO-POPS; NO_2-POPS; (NO_2)O-POPS; (NO_2)(NO)-POPS; (NO_2)(2O)-POPS; $(NO_2)_2$-POPS	Incubation of POPS (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	Direct infusion ESI-MS and HR-MS/MS using Q-Exactive hybrid quadrupole Orbitrap® mass spectrometer, in negative-ion mode	[14]
	NO-TLCL; $(NO)_2$-TLCL; $(NO)_3$-TLCL; $(NO)_4$-TLCL; NO_2-TLCL; $(NO_2)_2$-TLCL; (NO_2)(NO)-TLCL; (NO)O-TLCL; $(NO)_2$(2O)-TLCL; (NO_2)(NO)O-TLCL	Incubation of TLCL (1 mg) with NO_2BF_4 (1 mg) in $CHCl_3$ (1 mL) for 1h, r.t. at 750 rpm	C30-RP-LC-ESI-MS and HR-MS/MS using Ultimate U3000R LC system coupled online to Q-Exactive hybrid quadrupole Orbitrap® mass spectrometer, in negative-ion mode	[16]
	NO_2-cLA-TAG; NO_2-oxo-OA-TAG; NO_2-OH-OA-TAG; NO_2-OOH-OA-TAG	Nitration of 100 µM 3-cLA-TAG or 2-cLA-TAG with 2 mM $NaNO_2$ in artificial gastric fluid, 1h at 37 °C under continuous agitation	C18-HPLC-HR-MS/MS and MS^3 using LTQ Orbitrap Velos equipped with HESI-II source, in positive-ion mode C18-HPLC-APCI-MS/MS using API 4000 Q-trap triple quadrupole, in positive-ion mode	[15]

AA – arachidonic acid; $CHCl_3$ – chloroform; cLA – conjugated linoleic acid; ESI – electrospray ionization; HPLC – high performance liquid chromatography; LA – linoleic acid; LC – liquid chromatography; OA – oleic acid; MS – mass spectrometry; MS/MS – tandem mass spectrometry; $NaNO_2$ – sodium nitrite; NO_2BF_4 – nitronium tetrafluoroborate; PAPC – 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PAPE – 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine; PAPS –1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phospho-L-serine ; PLPC – 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PLPE – 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine; PLPS – 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-L-serine; PLs – phospholipids; POPC – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS – 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; RP – reversed-phase; TAGs – triacylglycerides; TLCL – tetralinoleoyl cardiolipin.

Table 3. Nitrated complex lipids identified in biological samples. Information on the fragmentation technique used to induce ion activation under tandem mass spectrometry conditions (collision-induced dissociation (CID) or higher energy CID (HCD)), the experimental model employed, and the methodology performed for analysis of the modified complex lipids is also provided in this table.

		Biological samples		
Ion activation	**Nitrated species**	**Experimental model**	**Method**	**Ref.**
CID	NO-PC 18:0/18:1; NO_2-PC 16:0/18:1; (NO_2)(NO)-PC 16:0/20:3; $(NO_2)_2$-PC 16:0/16:1; $(NO_2)_2$-PC 16:0/18:1; (NO_2)(2O)-PC 16:0/18:1; NO_2-PE 16:0/18:1; NO_2-PE 18:0/18:1	Lipid extracts of cardiomyoblast cell line H9c2 under starvation conditions obtained using Bligh and Dyer method	HILIC-ESI-MS and MS/MS performed on Waters Alliance 2690 HPLC system coupled online to LXQ-LIT mass spectrometer, in positive-ion mode	[13]
	NO_2-PC 16:0/18:2; NO_2-PC 16:0/18:1; NO_2-PC 18:2/20:4; NO_2-PC 16:0/22:5; NO_2-PC 18:0/20:5; NO_2-PC 18:1/20:4; NO_2-PC 18:0/20:4; NO_2-PC 18:2/20:1; NO_2-PC 18:0/22:6; NO_2-PE 18:0/22:6	Lipid extracts of cardiac mitochondria from a well-characterized animal model of type 1 diabetes mellitus (T1DM) obtained using Bligh and Dyer method	HILIC-ESI-MS and MS/MS performed on Waters Alliance 2690 HPLC system coupled online to LXQ-LIT mass spectrometer, in positive-ion mode	[12]
	NO_2-PC 18:2/20:1	Lipid extracts of cardiac mitochondria from control group obtained using Bligh and Dyer method	HILIC-LC-MS and MS/MS performed on a Waters Alliance 2690 HPLC system coupled online to LXQ-LIT mass spectrometer, in positive-ion mode	[12]
	NO_2-PC; NO_2-PE; NO_2-PS; NO_2-PI; NO_2-FA-TAG; NO_2-FA-MAG; NO_2-FA-DAG; NO_2-Cholestrol esters; NO_2-FA-derived β-oxidation products	Lipid extracts of 3T3-L1-derived adipocytes supplemented with 5 µM of 10-NO_2-OA, NO_2-cLA, NO_2-LA and 10-NO_2-SA for 24h obtained using Bligh and Dyer method Lipid classes fractionation by SPE using NH_2 columns	C18-HPLC-ESI-MS/MS using API 4000 Q-Trap triple quadrupole mass spectrometer, in negative-ion mode, before and after acid hydrolysis of esterified FA	[12]
	NO_2-FA-TAG; NO_2-FA-MAG; NO_2-FA-DAG; NO_2-Cholestrol esters	Lipid extracts of adipose tissue from high-fat diet-fed male C57Bl/6j mice after subcutaneous deliver of 8 mg/Kg/day NO_2-OA for 6.5 weeks obtained using Bligh and Dyer method Lipid classes fractionation by SPE using NH_2 columns	C18-HPLC-ESI-MS/MS using API 4000 Q-Trap triple quadrupole mass spectrometer, in negative-ion mode, before and after acid hydrolysis of esterified FAs	[12]
	(Non)-Electrophilic; NO_2-FA-TAG	Lipid extracts of 3T3-L1-derived adipocyte treated with 5 µM NO_2-OA for 24h obtained using Bligh and Dyer method Lipid classes fractionation by SPE using NH_2 columns	C18-HPLC-ESI-HR-MS/MS and MS^3 using LTQ Orbitrap Velos equipped with HESI-II source, in positive-ion mode, before enzymatic hydrolysis of esterified FA C18-HPLC-ESI-MS/MS using API 4000 Q-Trap triple quadrupole mass spectrometer, in negative-ion mode, after enzymatic hydrolysis of esterified FA	[18]
	(Non)-Electrophilic NO_2-FA-TAG	Lipid extracts of plasma from male Sprague-Dawley rats gavaged with 100 mg/Kg NO_2-OA obtained	C18-HPLC-APCI-MS/MS using API 4000 Q-trap triple quadrupole, in positive-ion mode, before enzymatic	[18]

		using Bligh and Dyer method Lipid classes fractionation by SPE using NH_2 columns	hydrolysis of esterified FA C18-HPLC-ESI-MS/MS using an API 4000 Q-Trap triple quadrupole mass spectrometer, in negative-ion mode, after enzymatic hydrolysis of esterified FA	
HCD	NO_2-PC 16:0/18:1	Lipid extracts from SW13/cl.2 cells (untreated and treated with 10 µmol L -1 of nitrated POPC during culture phase) obtained using Bligh and Dyer method	HILIC-ESI-MS and MS/MS performed on Ultimate 3000 Dionex HPLC system coupled online to Q-Exactive hybrid quadrupole Orbitrap® mass spectrometer, in positive-ion mode	[8]
	(Non)-Electrophilic NO_2-FA-TAG bearing 10-NO_2-OA including: NO_2-FA-TAG 54:3 NO_2-FA-TAG 54:4 NO_2-FA-TAG 54:5 NO_2-FA-TAG 52:2 NO_2-FA-TAG 52:3	Lipid extracts of plasma from male Beagle dogs orally dosed with 31.25 mg/Kg 10-NO_2-OA twice a day and 6h apart, for 14 days obtained using Bligh and Dyer method	C18-HPLC-ESI-HR-MS/MS using Q-Exactive hybrid quadrupole Orbitrap® mass spectrometer equipped with HESI-II source, in positive-ion mode, before and after acid hydrolysis of esterified FA	[21]

cLA – conjugated linoleic acid; DAG – diacylglyceride; ESI – electrospray ionization; FA – fatty acid; HILIC – hydrophilic interaction liquid chromatography; HPLC – high performance liquid chromatography; HR – high resolution; LA – linoleic acid; LC – liquid chromatography; MAG – monoacylglyceride; MS – mass spectrometry; MS/MS – tandem mass spectrometry; OA – oleic acid; PC – phosphatidylcholine; PE – phosphatidyletahnolamine; PI – phosphatidylinositol; PS – phosphatidylserine; POPC – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SA – stearic acid; SPE – solid phase extraction; TAG – triacylglyceride.

Table 4. Typical neutral losses (NL) of nitrosated, nitrated or nitroxidized complex lipids and reporter ions of modified fatty acyl chains observed in tandem mass spectra (MS/MS) in both positive- and negative-ion modes [12–14]. Reporter ions corresponding to the NL of water from the modified fatty acyl chains can also be seen ($[NO_x\text{-}FA–H_2O+H]^+$ or $[NO_x\text{-}FA–H_2O–H]^-$; x=1 or 2) [12–15].

	Complex lipids derivatives		Mass increments	Neutral losses in MS/MS spectra						Reporter ions of modified fatty acyl chain
				NL 31 Da (-HNO)	NL 47 Da ($-HNO_2$)	NL 94 Da ($-2HNO_2$)	NL 18 Da ($-H_2O$)	NL 32 Da ($-O_2$)	NL 34 Da ($-H_2O_2$)	
Nitrosated	Nitroso	NO	+29 Da	✓	---	---	---	---	---	$[NO\text{-}FA+H]^+$ or $[NO\text{-}FA–H]^-$
	Dinitroso	$(NO)_2$	+58 Da	✓	---	---	---	---	---	$[(NO)_2\text{-}FA+H]^+$ or $[(NO)_2\text{-}FA–H]^-$
Nitrated	Nitro	NO_2	+45 Da	---	✓	✓	---	---	---	$[NO_2\text{-}FA+H]^+$ or $[NO_2\text{-}FA–H]^-$
	Dinitro	$(NO_2)_2$	+90 Da							$[(NO)_2\text{-}FA+H]^+$ or $[(NO)_2\text{-}FA–H]^-$
	Nitronitroso	$(NO_2)(NO)$	+74 Da	✓	✓	---	---	---	---	$[(NO_2)(NO)\text{-}FA+H]^+$ or $[(NO_2)(NO)\text{-}FA–H]^-$
Nitroxidized	Nitroso-hydroxy	(NO)O	+45 Da	✓	---	---	✓	---	---	$[(NO)O\text{ -}FA+H]^+$ or $[(NO)O\text{-}FA–H]^-$
	Dinitroso-hydroperoxy	$(NO)_2(2O)$	+90 Da	✓	---	---	✓	✓	✓	$[(NO)_2(2O)\text{-}FA+H]^+$ or $[(NO)_2(2O)\text{-}FA–H]^-$
	Nitro-hydroxy	$(NO_2)O$	+61 Da	---	✓	---	✓	---	---	$[(NO_2)O\text{-}FA+H]^+$ or $[(NO_2)O\text{ -}FA–H]^-$
	Nitro-hydroperoxy	$(NO_2)(2O)$	+77 Da	---	✓	---	✓	✓	✓	$[(NO_2)(2O)\text{-}FA+H]^+$ or $[(NO_2)(2O)\text{-}FA–H]^-$
	Dinitro-hydroxy	$(NO_2)_2O$	+106 Da	---	✓	✓	✓	---	---	$[((NO_2)_2O\text{-}FA+H]^+$ or $[((NO_2)_2O\text{-}FA–H]^-$
	Dinitro-hydroperoxy	$(NO_2)_2(2O)$	+122 Da	---	✓	✓	✓	✓	✓	$[(NO_2)_2(2O)\text{-}FA+H]^+$ or $[(NO_2)_2(2O)\text{-}FA–H]^-$
	Nitronitroso-hydroxy	$(NO_2)(NO)O$	+90 Da	✓	✓	---	✓	---	---	$[(NO_2)(NO)O\text{-}FA+H]^+$ or $[(NO_2)(NO)O\text{-}FA–H]^-$

Table 5. Reporter fragmentation patterns identified in the MS/MS data of nitrated or nitroxidized cardiolipin (CL), allowing the identification of their structural features in biomimetic and biological systems. Lyso phosphatidic acid – LPA; neutral loss – NL; phosphatidic acid – PA.

Phospholipid	NL of modified moiety	Typical fragmentation	Typical fragmentation plus modified moiety
CL	NL of 47 Da (-HNO_2)	$[PA–H]^-$	$[NO_2\text{-}PA–H]^-$
		$[LPA–H]^-$	$[NO_2\text{-}PA+56–H]^-$
	NL of 31 Da (-HNO)	$[PA+56–H]^-$	$[NO_2\text{-}PA+136–H]^-$
		[PA+136–H]	$[NO_2\text{-}LPA–H]^-$

Captions

Figure 1. Information on the eleven studies considered eligible within the scope of the present review. These studies were focused on the identification and characterization of complex nitrated lipids using mass spectrometry (MS)-based approaches as well as on the evaluation of the biological significance of these modified lipids (epilipids). The eleven eligible studies were distributed among complex lipid classes (phospholipids, PLs and triacylglycerides, TAGs), dissociation techniques to induce fragmentation under tandem MS (MS/MS) conditions (collision-induced dissociation (CID) and/or higher energy CID (HCD)), type of study (*in vitro* and/or *in vivo*) and goal of the study (complex lipids detection and/or screening of biological significance).

Figure 2. Generation of the reactive nitrogen species (RNS) found in living systems through enzymatic (purple arrows) and non-enzymatic reactions (black arrows). Nitric oxide ($NO^{\bullet}$) is formed through the oxidation of the amino acid L-arginine to the amino acid L-citrulline in a reaction catalyzed by the enzyme nitric oxide synthase (NOS). $NO^{\bullet}$ can generate the nitrosonium cation (NO^+) after removal of an electron, and then NO^+ can be reduced to yield the nitroxyl anion (NO^-) or can react with water to produce nitrite (NO_2^-). Otherwise, $NO^{\bullet}$ reacts with the superoxide anion ($O_2^{\bullet-}$) to produce peroxynitrite ($ONOO^-$), which can be protonated to generate the peroxynitrous acid (OONOH). The homolysis of OONOH yields the hydroxyl radical ($OH^{\bullet}$) and the nitrogen dioxide radical ($NO_2^{\bullet}$). The $NO_2^{\bullet}$ can also be generated from the autoxidation of $NO^{\bullet}$, from NO_2^- after removal of one electron, or from the reaction between $ONOO^-$ and carbon dioxide (CO_2), through the formation of nitrosoperoxycarbonate anion ($ONOOCO_2^-$) whose homolysis can yield $NO_2^{\bullet}$ and carbonate ($CO_3^{\bullet-}$). The $ONOOCO_2^-$ can also yield nitrate (NO_3^-) and CO_2. NO_2^+ can react with water and further decay to yield NO_3^-, which can also be formed through the isomerization of ONOOH. $ONOO^-$ in the presence of

transition metals (e.g., ferrous (Fe^{2+}) and cupper ions (Cu^{2+})) yields the nitronium cation (NO_2^+), through an heterolytic cleavage, or $NO_2^{\bullet}$, by homolytic rupture. NO_2^+ can also be formed from the $NO_2^{\bullet}$ after removal of an electron. $ONOO^-$ can also react with $NO^{\bullet}$ with the formation of $NO_2^{\bullet}$ and NO_2^-. The reaction between $NO^{\bullet}$, $ONOO^-$, and $O_2^{\bullet-}$ yields NO_2^-, $NO_2^{\bullet}$ and O_2. Additionally, $O_2^{\bullet-}$ can produce hydrogen peroxide in a reaction catalyzed by superoxide dismutase (SOD). The reaction between NO_2^- and H_2O_2 catalyzed by peroxidases (myeloperoxidase (MPO), eosinophil peroxidase (EPO) or, lactoperoxidase (LPO)) generates $NO_2^{\bullet}$. This RNS can also be formed from $ONOO^-$ in a reaction catalyzed by the cytochrome P450. Both NO_3^- and NO_2^- can be obtained directly from dietary sources. However, NO_3^- can also be converted to NO_2^- in the oral cavity in a reaction catalyzed by the enzyme nitrate reductase. At low pH, NO_2^- can be protonated yielding nitrous acid (HNO_2), which can further decompose originating nitrogen dioxide ($NO_2^{\bullet}$) through the formation of dinitrogen trioxide (N_2O_3). The N_2O_3, which can also be formed through the reaction between $NO^{\bullet}$ and $NO_2^{\bullet}$, rapidly hydrolyzes to form NO_2^- in aqueous medium.

Figure 3. Overview of the nitrated, nitrosated and nitroxidized derivatives of complex lipids (phospholipids and/or triacylglicerides) already reported in the literature. These modified species can be generated under nitrative (*i.e.*, nitration reactions that lead to the covalent addition of a nitro group ((NO_2)), nitrosative (*i.e.*, nitrosation reactions that lead to the covalent addition of a nitroso group (NO)) and nitroxidative stress conditions (simultaneous nitration/nitrosation and oxidation reactions), respectively Nitrated lipid derivatives comprise the modified complex lipids with at least one NO_2 group, whereas nitrosated lipid derivatives corresponds to the modified complex lipids with at least one NO group. The modified complex lipids with both NO_2 and NO groups were also reported and correspond to the nitronitroso derivatives. Nitroxidized derivatives correspond to the modified complex lipids having at least one NO and/or one NO_2 group together with hydroxy (O) and hydroperoxy (2O) moieties.

Figure 4. Formation of nitrated, nitrosated and nitroxidazed complex lipids by radical reactions mediated by RNS, as nitrogen dioxide radical ($NO_2^{\bullet}$) or nitric oxide ($NO^{\bullet}$). Phosphatidylcholine (PC) esterified to the fatty acids (FA) 16:0/18:1 (POPC) was selected as an example of complex lipid. At low O_2 concentrations the formation of nitrated derivatives of POPC prevails (step 2 to 4). The β-nitroalkyl radical, generated due to the reaction of $NO_2^{\bullet}$ radical with esterified FA moiety (step 1), can react with other $NO_2^{\bullet}$ radical yielding a nitronitrite or dinitro derivative of POPC (step 2). Through the loss of nitrous acid (HNO_2) it is generated the nitro derivatives (step 3). Otherwise, the hydrolysis of the nitronitrite or dinitro POPC derivative generates the nitrohydroxy derivatives (step 4). At high O_2 concentrations the formation of nitroxidized

derivatives of POPC are favored yielding hydroperoxides derivatives (step 5). The β-nitroalkyl radical can also react with O_2 to form a β-nitroperoxyl radical (step 6) leading to the generation of nitrohydroxy (after reduction), nitro-keto (after loss of water), nitro-epoxy (after deoxidation) or nitro derivatives of complex lipids. The formation of nitroso derivatives of POPC (step 7) can also occur through the nitrosylation reactions between carbon-centered lipid radical and nitric oxide ($NO^{\bullet}$). The nitroso derivative can undergo further modifications to yield multiple nitroso derivatives as well as nitronitroso and nitroxidized derivatives.

Figure 5. Formation of nitrated complex lipids by electrophilic substitution mediated by the RNS nitronium cation (NO_2^+). Phosphatidylcholine (PC) esterified to the fatty acids (FA) 16:0/18:1 (POPC) was selected as an example of complex lipid. The addition of NO_2^+ at the double bond of esterified FA moiety (without its rearrangement) leads to the formation of the nitro derivatives of POPC (step 1). These derivatives can react with other RNS (*e.g.*, $NO_2^{\bullet}$) producing multiple nitrated (step 2) as well as nitroxidized (step 3) and nitronitroso derivatives (step 4).

Figure 6. Comparison of the mass spectra acquired in positive-ion mode of nonmodified **(A)** and modified PC (16:0/18:1), POPC, obtained after reaction with reactive nitrogen species **(B)**. Nitrated and nitroxidized derivatives were identified based on specific mass shifts depending on the type of modification. Modified derivatives comprising distinct mass shifts comparatively to the native POPC and include nitroso-POPC (NO-POPC, + 29Da) nitro-POPC (NO_2-POPC, + 45 Da) and dinitro-POPC ((NO_2)$_2$-POPC, + 90 Da). Nitroxidized derivatives are assigned as nitro-hydroxy-POPC ((NO_2)O-POPC, + 61 Da) and nitro-hydroperoxy-POPC ((NO_2)2O-POPC, +77 Da).

Figure 7. Schematic representation of the fragmentation fingerprint of nitrated/nitroxidized phospholipids (PLs) observed in the tandem mass spectra (MS/MS) in positive ($[M+H]^+$) and negative ($[M-H]^-$) ionization modes of these epilipids. Nitrated derivatives of PLs esterified to one nitro fatty acid (NO_2-FA) were used as example. The typical fragmentation in $[M+H]^+$ data comprise the neutral loss of nitrous acid (HNO_2), the loss of PLs polar headgroup and the presence of the protonated molecules of NO_2-FA ($[NO_2\text{-FA}+H]^+$). The fragmentation observed in $[M-H]^-$ data corresponds to the carboxylate anions of NO_2-FA ($[NO_2\text{-FA}-H]^-$).

Figure 8. Tandem mass (MS/MS) spectra of the nitro derivatives of phospholipids (NO_2-PLs) in both positive (**A** and **B**), as $[M+H]^+$ ions, and negative ionization modes, as $[M-H]^-$ ions (**C** and **D**). The nitro derivative of PE (16:0/18:1), NO_2-POPE, was used as an example of these epilipids. MS/MS spectra were acquired in a low-resolution (**A** and **C**) and a high-resolution (**B**

and **D**) mass spectrometers under collision-induced dissociation (CID; *e.g.*, linear ion trap) and higher energy CID conditions (HCD; *e.g.*, orbitrap), respectively. The schematic representation of the fragmentation patterns obtained from these two different fragmentation techniques used to induce ion activation under MS/MS conditions are also illustrated. In CID-MS/MS spectra, the typical reporter ions commonly used for the identification of NO_2-PLs corresponds to the typical neutral loss (NL) of nitrous acid (HNO_2), yielding a NL of 47 Da. These ions are highlighted ions orange. In contrast, in HCD-MS/MS spectra, the product ions of nitro fatty acyl chains are the most suitable reporter ions used to identify the NO_2-PLs. These ions are highlighted in green. The typical fragmentation of PE polar head group (NL of 43 Da – aziridine and NL of 141 Da – phosphoethanolamine) can be seen in both CID-MS/MS and HCD-MS/MS spectra acquired in positive ionization mode. These ions are highlighted in blue. The ions formed due to combined fragmentation of polar head group and nitro group (NL of 188 Da, combined NL of 141 Da plus 47 Da) are highlighted in light red.

Figure 9. Tandem mass (MS/MS) spectra of nitro derivatives of phosphatidylcholine (NO_2-PC) in positive (**A**), as $[M+H]^+$ ions, and negative ionization modes, as $[M+CH_3COO]^-$ ions (**B**), using as an example the nitro derivative of PC (16:0/18:1), NO_2-POPC. The MS/MS spectra of NO_2-POPC was acquired in a high-resolution mass spectrometer employing orbitrap technology and using higher-energy collisional dissociation (HCD) as fragmentation technique to induce ion activation under MS/MS conditions. In positive ionization mode, the presence of the typical product ion of the PC class (at *m/z* 184 – phosphocholine) suppresses the typical fragmentation pathway of these epilipids, which may hinder the accurate identification of the NO_2-PC derivative (**A**). The complementary analysis of the HCD-MS/MS spectrum of the NO_2-PC derivative in the negative ionization mode allows the identification of the carboxylate anions of the nitro fatty acids, which are highlighted in green (**B**). The ions corresponding to the fragmentation of PC polar head are highlighted in blue (*m/z* 184 – phosphocholine and neutral loss (NL) of 183 Da – NL of phosphocholine in positive mode ($[M+H]^+$ ions); NL of 74 Da – NL of CH_3COOCH_3 in negative ion mode ($[M+CH_3COO]^-$ ions)). The ions formed due to combined fragmentation of polar head group and nitro group (NL of 230 Da, combined NL of 183 Da plus 47 Da) are highlighted in light red.

Figure 10. Tandem mass (MS/MS) spectra of the nitroso derivatives of phospholipids (NO-PLs) in both positive (**A** and **B**), as $[M+H]^+$ ions, and negative ionization modes, as $[M-H]^-$ ions (**C** and **D**). The nitroso derivative of PE (16:0/18:1), NO-POPE, was used as an example of these epilipids. MS/MS spectra were acquired in a low-resolution (**A** and **C**) and a high-resolution (**B** and **D**) mass spectrometers under collision-induced dissociation (CID; *e.g.*, linear ion trap) and higher energy CID (HCD; *e.g.*, orbitrap) conditions, respectively. The schematic

representation of the fragmentation patterns obtained from these two different fragmentation techniques used to induce ion activation under MS/MS conditions are also illustrated. In CID-MS/MS spectra, the typical reporter ions commonly used for the identification of NO-PLs corresponds to the typical neutral loss (NL) of nitroxyl (HNO), yielding a NL of 31 Da. These ions are highlighted ions orange. In contrast, in HCD-MS/MS spectra, the product ions of nitroso fatty acyl chains are the most suitable reporter ions to identify the NO-PLs. These ions are highlighted in green. The typical fragmentation of PE polar head group (NL of 43 Da – aziridine and NL of 141 Da – phosphoethanolamine) can be seen in both CID-MS/MS and HCD-MS/MS spectra acquired in positive ionization mode. These ions are highlighted in blue. The ions formed due to combined fragmentation of polar head group and nitro group (NL of 172 Da, combined NL of 141 Da plus 31 Da) are highlighted in light red.

Figure 11. Summary of the typical reporter ions and fragmenartion patterns of nitrated, nitrosated and nitroxidized phospholipids under collision-induced dissociation (CID) and higher energy CID (HCD) tandem mass (MS/MS) conditions. PE (16:0/18:1), POPE, was selected as an example of PLs. Nitrated, nitrosated and nitroxidized derivatives were described based on the type of modification and, consequently, the specific mass shifts. Nitro and dinitro derivatives correspond to the addition of one (+ 45 Da) and two (+ 90 Da) NO_2 groups to the POPE, respectively. Nitroso is consistent with the addition of one NO (+ 29 Da) to the POPE. Nitroxidized and dinitroxidized corresponds to the addition of one or two NO_2 groups, respectively, combined with the hydroxy or hydroperoxy moieties. The typical neutral loss of nitrou acid (HNO_2) or nitroxyl (HNO) is the most suitable fragmentation to identify nitrated and nitrosated derivatives of PLs under CID-MS/MS, respectively, while under HCD-MS/MS, the carboxylate anions of the modified fatty acids (FA) moieties are more informative.

Figure 12. Schematic representation of the typical features observed in the tandem mass (MS/MS) spectra of nitrated and nitroxidized complex lipids obtained using collision-induced dissociation (CID) and higher energy CID (HCD) that can be used for target analysis. The characteristic fingerprinting is exemplified for the nitro derivatives of complex lipids, namely phospholipids (PLs) and triacylglicerides (TAGs), which can be analyzed in both positive ($[M+H]^+$ ions for PLs, $[M+NH_4]^+$ ions for TAGs) and in negative ionization modes ($[M-H]^-$ ions for PLs). Analysis using low-resolution CID-MS/MS (*e.g.*, linear ion trap-based instruments) and high-resolution HCD-MS/MS experiments (*e.g.*, orbitrap-based instruments) leads to a dissimilar MS/MS fingerprinting pattern. The typical neutral loss (NL) of nitrous acid (HNO_2, NL of 47 Da) is the most suitable reporter ion to identify modified TAGs under both CID-MS/MS and HCD-MS/MS conditions, as well as modified PLs under CID-MS/MS

conditions. The carboxylate anions of modified fatty acids can be used as reporter ions to identify modified PLs under HCD-MS/MS.

Figure 13. Schematic representation of the fragmentation fingerprint of nitrated/nitroxidized triacylglicerides (TAGs) observed in the tandem mass spectra (MS/MS) acquired in positive ionization mode ($[M+NH4]^+$ ions). The typical fragmentation comprises the typical neutral loss (NL) of nitrous acid (HNO_2, NL of 47 Da), the NL of nitrated/nitroxidized fatty acids (FA), NO_2-FA, with the formation of a diacylglyceride (DAG)-like product ion as illustrated by the green structure, and the NL of nonmodified FA with the formation of a nitrated DAG-like product ion as illustrated by the orange structure. Nitro derivatives of TAGs were used as example of these epilipids.

Figure 14. Tandem mass (MS/MS) spectra of the nitrated (A and B) and nitroxidized derivatives (C and D) of triacylglicerides (TAGs) acquired in positive ionization mode ($[M+NH4]^+$). The nitro (NO_2-TAGs) and dinitro-hydroperoxy derivatives ($(NO_2)_2(2O)$-TAGs) of triolein were used as an example of these epilipids. Triolein is a TAGs composed by three oleic acid (OA) moieties. MS/MS spectra were acquired in a low-resolution (**A** and **C**) and a high-resolution (**B** and **D**) mass spectrometers under collision-induced dissociation (CID; *e.g.*, linear ion trap) and higher energy CID (HCD; *e.g.*, orbitrap) conditions, respectively. The schematic representation of the fragmentation patterns obtained from these two different fragmentation techniques used to induce ion activation under MS/MS conditions are also illustrated. The typical fragmentation of the modification is highlighted in orange, while the NL of the fatty acyl chains are highlighted in green (modified) and light red (nonmodified).

Figure 15. Modified complex lipids (epilipids) formed through the reaction of unsaturated complex lipids (triacylglicerides (TAGs) and phospholipids (PLs)) with reactive nitrogen species, which were already identified in biological samples after oral administration of nitro oleic acid, NO_2-OA (modified TAGs), under inflammatory and stressful conditions (modified PLs), or after supplementation with nitro fatty acids (NO_2-FA) or nitrated PLs (modified PLs and TAGs). Nitrated PLs were detected in rat cardiac mitochondria of a model of type 1 diabetes mellitus (T1DM) [5] and in human adrenal cortex adenocarcinoma cells treated with nitrated PC (16:0/18:1), POPC [39,50]**,** while nitrated, nitrosated and nitroxidized derivatives of PLs were identified in cardiomyoblasts (H9c2) under starvation [6]**.** Nitrated TAGs were identified in the plasma of rats[10] and dogs [23] after oral administration of 10-NO_2-OA, in the adipose tissue of high-fat diet-fed mice after subcutaneous administration of 10-NO_2-OA [11] and in adipocytes supplemented with NO_2-FA [10]**.**

Figure 16. Biological roles already reported for nitrated phospholipids, namely nitrated PC (16:0/18:1), NO_2-POPC. Anti-inflammatory effects were evaluated in Raw 264.7 macrophages activated by the toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), in a well-known *in vitro* model of inflammation. Nitrated POPC was able to inhibits the expression of the inducible nitric oxide synthase (iNOS) in the LPS-activated macrophages [19]. Antioxidant effects were shown by the ability of nitrated POPC to scavenge both the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radicals as well as oxygen-derived radicals, which was evaluated through the oxygen radical absorbance capacity (ORAC) assay. Nitrated POPC also inhibited the lipid peroxidation induced by the hydroxyl radical generated through the Fenton reaction by using liposomes as a model of cell membrane [19]. By using cell-based assays, it was demonstrated that NO_2-POPC induced changes in cell morphology, including cytoskeletal rearrangement and cell shrinking, as well as loss of cell adhesion and cell detachment. These effects of NO_2-POPC did not overlap those of NO donors but were comparable to the effects of nitrated fatty acids [48]. In an *in vitro* gel-based competition assay, NO_2-POPC was shown to potentially interact with cysteine residues which are known targets for lipoxidation, namely in vimentin (cysteine C328) and PPARγ constructs (cysteine C285). The C328 residue of vimentin was also suggested as a potential target for direct or indirect modification in NO_2-POPC-treated cells [48].

Table 1. Type of ions and adducts observed in the mass spectrum of nitrated or nitroxidized complex lipids, phospholipids and triacylglicerides (TAGs), in positive- and negative-ion modes. Phosphatidylcholine – PC; phosphatidylserine – PS; phosphatidylethanolamine – PE; cardiolipin – CL.

Table 2. Nitrated complex lipids identified using *in vitro* biomimetic model systems. Information on the fragmentation technique used to induce ion activation under tandem mass spectrometry conditions (collision-induced dissociation (CID) or higher energy CID (HCD)), the experimental model employed, and the methodology performed for analysis of the modified complex lipids is also provided in this table.

Table 3. Nitrated complex lipids identified in biological samples. Information on the fragmentation technique used to induce ion activation under tandem mass spectrometry conditions (collision-induced dissociation (CID) or higher energy CID (HCD)), the experimental model employed, and the methodology performed for analysis of the modified complex lipids is also provided in this table.

Table 4. Typical neutral losses (NL) of nitrosated, nitrated or nitroxidized complex lipids and reporter ions of modified fatty acyl chains observed in tandem mass spectra (MS/MS) in both positive- and negative-ion modes [12–14]. Reporter ions corresponding to the NL of water from

the modified fatty acyl chains can also be seen ($[NO_x\text{-}FA–H_2O+H]^+$ or $[NO_x\text{-}FA–H_2O–H]^-$; x=1 or 2) [12–15].

Table 5. Reporter fragmentation patterns identified in the MS/MS data of nitrated or nitroxidized cardiolipin (CL), allowing the identification of their structural features in biomimetic and biological systems. Lyso phosphatidic acid – PA; neutral loss – NL; phosphatidic acid – PA.