Lipoproteins are targets and markers of lipoxidation

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ABSTRACT

Lipoproteins are essential systemic lipid transport particles, composed of apolipoproteins embedded in a phospholipid and cholesterol monolayer surrounding a cargo of diverse lipid species. Many of the lipids present are susceptible to oxidative damage by lipid peroxidation, giving rise to the formation of reactive lipid peroxidation products (rLPPs). In view of the close proximity of the protein and lipid moieties within lipoproteins, the probability of adduct formation between rLPPs and amino acid residues of the proteins, a process called lipoxidation, is high. There has been interest for many years in the biological effects of such modifications, but the field has been limited to some extent by the availability of methods to determine the sites and exact nature of such modification. More recently, the availability of a wide range of antibodies to lipoxidation products, as well as advances in analytical techniques such as liquid chromatography tandem mass spectrometry (LC-MSMS), have increased our knowledge substantially. While most work has focused on LDL, oxidation of which has long been associated with pro-inflammatory responses and atherosclerosis, some studies on HDL, VLDL and Lipoprotein(a) have also been reported. As the broader topic of LDL oxidation has been reviewed previously, this review focuses on lipoxidative modifications of lipoproteins, from the historical background through to recent advances in the field. We consider the main methods of analysis for detecting rLPP adducts on apolipoproteins, including their advantages and disadvantages, as well as the biological effects of lipoxidized lipoproteins and their potential roles in diseases.

1. Introduction to lipoproteins and lipid oxidation

Lipoproteins are a family of complex particles consisting of amphipathic apolipoproteins that carry a wide range of lipids, and therefore have important physiological functions in systemic lipid transport. The basic structure is a shell formed of a monolayer of phospholipids and free cholesterol surrounding a core of cholesteryl esters and triacylglycerides [1], with one or more proteins associated with the surface phospholipids and partially embedded in the hydrophobic lipid core. The role of the proteins is thus to solubilize and stabilize the lipoproteins in an aqueous environment such as plasma, which is a major location of lipoproteins. Lipoproteins are classified according to their size and density, which is determined by the ratio of protein to lipid, and therefore depends on the lipid and apolipoprotein composition. The plasma lipoproteins are typically divided into 7 classes, which are chylomicrons, chylomicron remnants, VLDL, IDL, LDL, HDL, and Lp(a). The chylomicrons are responsible for transport of dietary lipids from the intestine to lipid-metabolizing tissues muscle and adipose, and the resulting chylomicron remnants deliver remaining lipid to the liver. VLDL, IDL, LDL are involved in the second phase of triglyceride and cholesterol delivery from the liver to peripheral tissues, whereas HDL particles are responsible for reverse cholesterol transport from cells in the peripheral tissues to the liver to lipoproteins of the endogenous pathway. The functions and activities of the lipoprotein particles are...
largely determined by the combination of apolipoproteins they contain, which direct their interaction with receptors as well as containing a variety of enzymatic activities. The lipid cargo is very diverse, and in addition of several classes of phospholipid, cholesterol and cholesteryl esters, it can include free fatty acids, sphingolipids, ceramides, and sulfolipids [2].

Many of the components of lipoproteins are susceptible to oxidative damage by free radicals of non-radical oxidants, and this has been an energetic field of study since pioneering studies in the 1980s demonstrated that oxidation of LDL contributed to changes in its biological properties [3–5] as described in Section 4. Lipids containing unsaturated fatty acyl chains (especially poly-unsaturated ones) can be peroxidized following hydrogen abstraction by radical attack and subsequent addition of di-oxygen; the downstream reactions are complex and lead to a plethora of short- and long-chain oxidation products; this topic has been extensively reviewed previously [6–9]. Peroxidation at bis-allylic sites in the fatty acyl chains tends to result in cleavage of the hydrocarbon backbone by Hock rearrangement or other mechanisms, resulting in formation of an aldehyde on one or both chains [10]. Intrachain cyclization reactions can also occur, for example leading to formation of isoprostanes or isoevulangldins. Secondary radical attack and addition of oxygen can occur at other sites, resulting in complex structures with multiple reactive groups including aldehydes, ketones, epoxides and cyclopentenone rings. A common motif that rises following cleavage is the γ-substituted α,β-alkenal, as in 4-hydroxynonenal (HNE) or 4-oxo-nenenal, although a variety of analogous structures are possible [6,8,11]. The headgroups of amine-containing phospholipids, e.g phosphatidylserine, can be modified by oxidative deamination, which also generates an aldehyde [12]. Alternatively, unsaturated lipids can be attacked by electrophilic agents such as hypochlorous acid (HOCl), which causes the formation of chlorohydrins (hydroxyl and chlorine groups on adjacent carbons) on fatty acyl chains, or cleavage of the vinyl ether bonds of plasmalogens to yield chloro-aldehydes. Radical nitrogen species such as nitrogen dioxide can cause peroxidation via hydrogen abstraction, but may also undergo addition reactions leading to nitrate or nitrosoylated fatty acyl chains, some of which also contain motifs similar to the substituted alkenals mentioned above. The key point about lipid oxidation products containing aldehydes, ketones, epoxides and substituted alkenals is that they are electrophilic and moderately to highly reactive with nucleophilic groups, which endows them with unusual properties through the ability to react with biological molecules. While several different collective names for such compounds exist, in this review we have used the term “reactive lipid peroxidation products”, or rLPPs. Fig. 1 shows the structure of the various rLPPs discussed in this review.

Lipid oxidation in lipoproteins has been extensively investigated and the reader is directed to reviews on this topic for more detail [13,14]. Consequently, lipid oxidation is not the main focus of this review, which instead addresses the more specific issue of how the protein moieties of lipoproteins are modified by the resulting rLPPs, a process known as lipoxidation and explained in more detail in Section 2. In view of the close proximity of protein and lipids within lipoproteins, the probability of adduct formation between rLPPs and residues of the proteins is relatively high, but to date no lipid-protein reviews have focused specifically on lipoxidation, despite the fact that there is growing awareness of the signaling importance of this modification [15–18]. This review addresses mainly HDL and LDL lipoxidation, as the majority of research has investigated these lipoproteins, and aims to provide a concise overview of lipoprotein modification by oxidized lipid adducts, from early pioneering studies to recent advances in molecular knowledge.

2. Protein oxidation and lipoxidation

In parallel to lipid oxidation, the apoprotein moieties of lipoproteins can also be oxidized directly by a range of oxidants; usually the basic side chains (lysine, arginine and histidine), and sidechains containing sulfur (cysteine, methionine) or aromatic groups (tyrosine, tryptophan, phenylalanine) are most readily modified. Several studies have investigated the relative susceptibility of lipoprotein residues to oxidants, both in vitro and ex-vivo, in an attempt to identify markers of damage as well as to understand changes in lipoprotein function [19–22]. Nucleophilic sidechains, i.e. those containing amine or thiol groups, can also be modified by reaction with rLPPs. Protein lipoxidation can occur by one of two chemical reactions: Michael addition occurs when the electrophilic β-carbon of a reactive lipid oxidation product reacts with the amino group of histidine and lysine or the thiol group of a cysteine; Schiff’s base formation involves reaction of the free amino group of a lysine, arginine or N-terminal amine with the carbonyl group of an aldehyde or ketone. Schiff’s base adducts are less stable than Michael adducts, and both are potentially reversible depending on the chemical environment. Moreover, depending on the electrophilic species involved, secondary reactions such as cyclization, dehydration, oxidation, or condensation with an additional electrophile may take place, leading to formation of more stable adducts that are referred to as advanced lipoxidation end products, or ALEs, by analogy with the better-known advanced glycation end products (AGEs). For example, the HNE Michael adducts tend to cyclize to hemiacetals, while the HNE Schiff’s base with lysine can generate a stable 2-pentapyrrole derivative [23]; the Michael adduct of acrolein can rearrange to form formyl-dehydroperidino lysine (FDP-lysine) [24]. The process of lipoxidation has been very well explained previously [23,25,26], and consequently will not be covered in depth here, although the structures most commonly discussed in the review are shown in Fig. 2.

Many approaches have been used to study lipoprotein oxidation and lipoxidation [10,16,27]. The ultimate goal is to be able to detect and identify lipoxidation under physiological conditions and in biological or clinical samples, but many studies of modification in vitro have been carried out as a stepping stone to this stage, to develop methodology or establish the most likely products. Oxidation of lipoproteins in vitro is often achieved by treatment with transition metal ions, mostly commonly Cu²⁺, but occasionally Fe²⁺ in combination with hydrogen peroxide as a Fenton reagent to generate hydroxyl radicals is used. The use of iron also represents a model for ferretin-induced oxidation of LDL. Other methods for oxidation of lipoproteins include radical initiators, such as 2,2’-Azobis(2-methylpropionamide) dihydrochloride (AAPH), or treatment with UV light. These are all able to initiate lipid peroxidation, and therefore lead to lipoxidation of the apolipoprotein. More physiological approaches include enzymes such as lipoxygenases and cytochrome P450 enzymes, which catalyse radical oxidations and hydroxylations of fatty acids, or myeloperoxidase, which is well-known for its ability to generate hypohalous acids such as HOCl, but is also able to catalyse direct oxidations of some substrates [28]. There have also been many studies in vitro where lipoproteins, especially LDL, have been modified by direct treatment with reactive lipid peroxidation products; most work has been carried out with small unsaturated aldehydes, such as acrolein, malondialdehyde (MDA) and HNE, but a small number of studies with less well-known rLPPs and even esterified oxidized phospholipids have been reported, as described in the following sections.

3. Approaches to the analysis of LDL and HDL lipoxidation

The analysis of LDL and HDL is dependent on the separation of these lipoproteins from whole plasma. A common method for separation of these particles is by gradient ultracentrifugation. Using different salt concentrations and centrifugation at high g-forces, the lipoproteins of different density migrate in the gradient, allowing their separation and forming bands that can be collected and analysed further. This methodology, which dates back to 1955, was developed by Havel et al. [29] and has been used extensively for the study of lipoproteins [30,31]. There are other methods that take advantage of different characteristics
of lipoprotein particles for their separation, such as size exclusion chromatography, which depends on the size difference between the lipoproteins, and gradient gel electrophoresis, which uses both size and charge for its separation [30,32,33].

All the studies included in this review used a form of the gradient ultracentrifugation method to separate lipoproteins, followed by the analysis of oxidized lipoproteins by one or more of the methods reviewed in this section. The analytical methods can be broadly divided into (1) biochemical methods, involving colorimetric or fluorescence reagents and labeling with or without additional separation by liquid chromatography; (2) antibody-based methods, including enzyme-linked immunosorbent assay (ELISA) and immunostaining; and (3) mass spectrometric approaches, which usually require the combination of liquid chromatography with tandem mass spectrometry. Each of these approaches have advantages and disadvantages in terms of the complexity of the methodology and the quality or depth of information generated, two aspects that are usually directly related (Fig. 3). Much early work used simple biochemical assays until the generation of antibodies specific for lipoxidation products allowed the application of immunoassays. Mass spectrometry techniques require complex and expensive instruments as well as extensive data handling, and consequently are less widely accessible, although very valuable as they yield site-specific information that cannot be obtained by the other approaches. Ultimately, all the techniques provide complementary information, and are most powerful if used in combination.

### 3.1. Biochemical methods with chromophore or fluorophore labeling

In this section, techniques involving the use of reagents that generate a chromophore or fluorescent signal on reaction with the lipoxidized protein or lipoxidation agent are considered. However, it is important to bear in mind that not all methods are specific for lipoxidation: in fact, the most used reagents are ones that react with aldehydes, which could also be formed by direct oxidation of amino acid residues. Moreover, some of the methods used were indirect, for example, deducing the amount of modification by changes in amount of free rLPP or unmodified residues.

For example, in one of the earliest studies of lipoprotein lipoxidation [27], involving the direct reaction of HNE with LDL, the formation of HNE-LDL adducts was determined indirectly by comparison of the amount of unreacted HNE in the supernatant of treated and untreated lipoproteins, which was quantified by high-performance liquid chromatography on an octadecyl silica (ODS) column with detection at 220 nm; the HNE incorporation was found to increase with increased aldehyde concentration, with 78% bound to the protein moiety, 21% to the lipids, and 1% as free aldehyde within the lipid fraction. To investigate more specifically the residues modified, the apolipoprotein B-100 (ApoB-100) was delipidated, reacted with dinitrofluorobenzene to dinitrophenylate and stabilize free hydroxyl and amine groups before total hydrolysis and quantitation using a standard amino acid analyser. This approach was adopted as it was noted that the adducts were not stable during the acid hydrolysis. Comparison of the levels of dinitrophenylated amino acids (i.e. those not modified by HNE) suggested that HNE attacked mostly lysine and tyrosine, and to a lesser extent, cysteine, serine and histidine [27]. The observation that tyrosine and serine could be modified is interesting as these residues are not widely considered as targets of hydroxyalkenals, but could simply have been an artefact of the experimental design with dinitrofluorobenzene.

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**Fig. 1.** Structures of the reactive lipid peroxidation products (rLPPs) reported to form adducts with lipoproteins. Note that Ne-(3-methylpyridinium) is shown as the lysine adduct form.
Fig. 2. Structures of common advanced lipoxidation end products (ALEs) or adducts detected on lipoproteins. The type of rLPP causing adduct formation is written in bold above the structure(s) and the resulting adduct names are give in plain text below. Trans-HHP-lysine is trans-N^3-(hept-1-enyl)-4-hexylpyridinium]lysine.

Fig. 3. The most used techniques for the analysis of lipoxidized lipoproteins, and their advantages and disadvantages. TBArs, thiobarbituric acid reactive substance assay; TNBS, 2,4,6-trinitrobenzene sulfonic acid; UV, Ultraviolet; IHC, Immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; LC-MS, liquid chromatography-mass spectrometry; GC-MS, Gas chromatography-mass spectrometry.
alternative reagent that is more specific for detection of free amine groups, i.e., lysines, arginines and the N-terminal of proteins, is 2,4,6-
trinitrobenzene sulfonic acid (TNBS). This has been used in several
studies to monitor indirectly protein modification by lipid peroxidation
products [34–40].

Amino acid analysis was also used by Uchida et al. to investigate the
sites of HNE modification in Cu²⁺-treated LDL, but in this case the
adducts were stabilized by treatment with sodium borohydride (NaBH₄)
to prevent the issues noted by Jürgens et al. [27], and the amino acids
were labeled with o-phthalaldehyde for fluorescence detection during
HPLC [41]. This enabled the quantification of approximately 40 lysines
and 50 histidines (mol/mol LD L) modified in HNE-treated LDL, of
which 49% and 80% respectively were identified as Michael adducts,
with the remainder as Schiff’s base adducts. However, this study also
relied on complementary findings from the use of specific antibodies, as
described in the next section.

A commonly used reagent for detecting the presence of protein
aldehydes is 2,4-dinitrophenylhydrazine (DNPH) [42–44]. It reacts
readily with protein-bound aldehydes or ketones to form a hydrazone
derivative with a characteristic absorbance at 365 nm, and despite its
mutagenic properties and explosive nature (most now only available
as a 0.2 M solution), continues to be widely used in studies of protein
oxidation. However, its application to oxidation of lipoproteins has
been limited. The formation of protein carbonyls in HOCI-treated LDL
was investigated by Yang et al. using DNPH labeling followed by deli-
pidation and tryptic cleavage of the ApoB-100; modified peptides were
identified on HPLC by the absorbance at 365 nm, isolated and sequenced
using a gas phase automated sequencer [45]. The modified peptides
contained cysteine, lysine, tryptophan and methionine residues; it was not clear from this study whether the modifications were
due to direct oxidation of the residues by HOCI, or formation of rLPPs
that reacted with the cysteine to give Michael adducts. In the use of
DNPH as a labeling reagent for lipidoxidation, it is also important to bear
in mind that Schiff’s base adducts of hydroxylalkenals do not contain a
free aldehyde to react with DNPH; instead, DNPH may react via Mi-
chael addition with them to generate 2,4-dinitrophenyl pyrazolines,
which have a slightly shifted absorption maximum compared to hy-
drazoneS. Moreover, when using DNPH to study lipidoxidation in Cu-
oxidized LDL it has been observed from the judicious use of controls
in the absence of DNPH that the tryptophan oxidation products N-formyl
kynurenine and kynurenine have significant absorbance at 365 nm,
which can confound the detection of protein carbonyls, so careful
profiling of the labeled peptides is necessary [46].

Alternatively, the thiobarbituric acid reactive substances (TBARS)
assay can be used to measure malondialdehyde (MDA) equivalents
present in the sample, including MDA adducts on the protein, which are
reversible under the conditions used for the assay. The reaction be-
tween thiobarbituric acid and MDA yields a pink chromophore with an
absorbance maximum at 535 nm. In its simplest form, the TBARS assay
has been widely criticized as a measure of lipid peroxidation in complex
samples (reviewed by Halliwell and Chirico [47] and Spickett et al.
[48]), but under stringent conditions, especially when combined with
HPLC separation, it can be used to monitor LDL oxidation [38,40,41,49–52]. Hoff et al. used the TBARS assay combined with
TNBS analysis of free amines to show that, similarly to the HNE-LDL
modification reported previously [27], MDA modification of ApoB-100
increased with increased MDA treatment concentration, and appeared to
correlate with the decrease of available amines [53]. This is in
accordance with previous reports of decreases in lysine availability
upon treatment of LDL with MDA [36].

While these colorimetric assays may now seem somewhat simplistic,
they were nevertheless crucial in building evidence on the occurrence
of covalent modifications of LDL by short chain non-esterified alde-
hydes. In contrast, a rather different approach was adopted by
Karakatsani et al. to investigate lipoxidation by phospholipid-esterified
rLPPs [54]. They used both a phosphorus assay and ³¹P nuclear
magnetic resonance (NMR) to measure the phosphorus in the delipi-
dated ApoB-100 fraction of Cu-oxidized LDL. They identified an NMR
peak that appeared to correspond to the phospholipids bound to the
protein by hydrophobic bonds, which disappeared upon treatment with
phospholipase A2 (PLA₂), showing that even in the delipidated protein
there is still a fraction of bound phospholipid. Furthermore, they
identified a second peak, not hydrolyzed by PLA2, which appeared to
correspond to the oxidized phosphatidylcholine fraction that formed
covalent adducts with the proteins. This finding was not corroborated
until several years later by complementary techniques involving anti-
bodies and LC-MSMS analysis, described in later sections.

3.2. Antibody-based detection of apolipoprotein lipoxidation

Antibodies are an extremely useful tool for biomolecular and bio-
chemical techniques used throughout the biosciences and biomedical
sector. From detection methods to therapeutics, they are utilized in a
variety of fields owing to their ease of use and, at least for polyclonal
antibodies, relatively quick and inexpensive production. These char-
acteristics also make them very attractive for commercial development.
The generation of antibodies against specific aldehydes, usually in the
form of protein adducts, has allowed the identification of lipoxidized
lipoproteins by an array of immunotechniques, and without doubt has
underpinned many key advances in this field [10,16]. Several groups
have worked on the development of such antibodies, characterized
them, and used them to investigate possible biological effects of oxi-
dized lipoproteins, and their role in certain pathological conditions,
mainly atherosclerosis.

3.2.1. Development and application of anti-MDA Antibodies

The first application of antibodies against rLPP-modified lipopro-
teins were using either previously existing antibodies against MDA-
lysine adducts [55], or polyclonal antibodies raised against MDA-treated
LDL [56]. The MDAlys monoclonal IgG2A was selected for binding
ability to MDA-modified LDL but not to native human LDL, and was
able to detect MDA-modified proteins by immunocytochemistry and
their colocalization with ApoB-100 in atheromas from rat [55]. In
contrast, an ELISA was developed using the polyclonal anti-MDA-LDL
that appeared to bind to MDA-ApoB adducts in MDA-treated LDL, while
not recognizing native LDL, acetyl-LDL or MDA-HDL, suggesting that
the antibody was both modification- and protein-specific [56]. Both
studies were important for the establishment of this approach, which
has subsequently been used extensively for lipoproteins. Anti-MDA
monoclonal and polyclonal (e.g. MAL-2) antibodies produced using
either rabbits or guinea pigs were later used to identify the presence of
adducts of this aldehyde bound to ApoB100 in copper-oxidized LDL,
using techniques such as western blotting using the monoclonal anti-
bodies MDA2 and OLF4-3C10 [36,57] and a solid-phase fluorescence
assay [58].

3.2.2. Development and application of anti-HNE Antibodies

In view of the identification of HNE as a major cytotoxic lipid
peroxidation product, it is not surprising that much effort was invested
in producing anti-HNE antibodies, with both polyclonal sera and
monoclonal antibodies being produced against HNE-treated LDL, or
HNE bound to other proteins or peptides. In parallel with their work on
MDA-LDL antibodies, Palinski et al. produced an antiserum (HNE-6)
and a monoclonal antibody (NAS9) against HNE-LDL, which they
reported were specific for HNE-lysine adducts [57]. The monoclonal
and polyclonal antibodies were used to identify the presence of HNE-LDL
adducts in copper-oxidized LDL and atherosclerotic lesions, and did not
recognize unmodified LDL [36]. Polyclonal antiserum to HNE-modified
LDL were also raised in rabbits by Esterbauer’s group [51,59]. It was
found that the antiserum recognized LDL treated with either HNE or
CuSO₄, but not MDA, hexanal, 2,4-heptadienal or 4-hydroxyhexenal,
while there was a small cross-reactivity with 4-hydroxyoctenal. This
demonstrated the specificity of the polyvalent serum to the nine carbon hydroxyalkenal, but interestingly it was also shown that copper-oxidized Lip(a) and VLDL both cross-reacted with the antisem, indicating that the specificity was due to the adduct and not the lipoprotein. The work also provided confirmation that oxidation of these lipoproteins resulted in HNE formation and lipoxidation [51]. Later the group prepared a further polyclonal antisem to HNE-LDL and reported weaker results than expected. The group later provided confirmation that oxidation of these lipoproteins also demonstrated the specificity of the polyvalent serum to the nine carbon hydroxyalkenal, but interestingly it was also shown that copper-oxidized LDL or bovine serum albumin, but not other amino acids, suggesting that HHE bound to the histidine residues of ApoB-100 in copper oxide LDL [50]. The same approach was used to raise an antibody against 4-hydroperoxynonenal (HPNE) adducts (clone PM9), and it was found that 4-hydroperoxide group leads to formation of structurally unusual lysine adducts that were specifically recognized by the antibody aldehydes [63]. It was used to demonstrate the occurrence of HPNE lipoxidation in copper-oxidized LDL, which was verified by western blot analysis, as well as in lesions in samples of the aortic wall of patients with generalized arteriosclerosis, demonstrated by immunohistochemistry [63]. More recently, monoclonal antibodies were developed against protein adducts of the twelve carbon compound 4-hydroxy-2E,6Z-dodecadienal (HDDE); these were used to identify adducts in human aortic section, but as yet have not been demonstrated to bind to lipoproteins or used in specific studies of their oxidation [64].

3.2.4. Development and application of antibodies against oxidized lipoproteins

Besides the development of antibodies against specific aldehydes described above, several groups also developed antibodies against copper-oxidized LDL [36,57,58,65]. Further characterization of two antibodies developed (OB/04 and OB/09) uncovered their reaction not only with copper-oxidized LDL, but also with azo-initiator oxidized LDL and copper-oxidized VLDL, while not reacting with the oxidized HDL or any of the native forms of the lipoproteins [65]. This suggested that the epitopes of these antibodies were generated against modified portions of apolipoproteins present in LDL and VLDL, but not in HDL.

An alternative approach that has proved very valuable is the isolation of autoantibodies produced during a diseased state; for example, autoantibodies present in mice with aortic atherosclerosis caused by a high fat diet, designated E0 antibodies, were found to bind to a variety of modified forms of LDL, including LDL modified by copper, acrolein, MDA and HNE [66–68]. One of the autoantibodies, E06, was subsequently extensively studied, and was found to bind to the phosphocholine head group of oxidized phosphatidylcholines [35], thus binding to both oxidized phospholipids and oxidized phospholipid-protein adducts. As it is not ApoB-specific, it is also able to recognize copper-oxidized HDL, demonstrating the existence of phospholipid-esterified aldehyde adducts in lipoproteins [66]. Antibody E06 [68] is now commercially available and has been used for detection of oxLDL in clinical samples, as discussed in Section 5. It is worth noting that in early publications this antibody was referred to as E06 [68], but now the name E06 is more commonly used, including by the commercial supplier Avanti Polar Lipids.

3.2.5. Considerations in the utilization of antibodies against lipoxidized proteins

It is important to bear in mind that there has been considerable variability in the methods of production of the antibodies discussed above, although much of the testing was carried out against human lipoproteins or tissue samples. As a consequence, there are substantial differences in specificity and sensitivity between the antibodies that must be borne in mind when interpreting the results, and which explain the different cross-reactivities reported.

Nevertheless, antibodies show great potential as a sensitive technique for the identification of lipoxidation in lipoproteins and have facilitated the development of methods for detecting and identifying lipoxidation, both in plasma and tissue samples. This has supported our understanding of the biological effects of these modifications [62], which is explored further in Section 4, as well as helping to evaluate the potential of therapeutic techniques [69], discussed in Section 5. A key advantage of immunoassays is their simplicity and accessibility, with most biological laboratories well set-up for ELISAs, western blotting and immunocyto/histochemistry. The potential for quantitative analysis is also important, although dependent on well-characterized standards. On the other hand, these methods provide limited information on the site of modifications within the lipoproteins. Where the antibody is specific for a type of adduct, the residues modified can be

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inferred, and has for example been used to calculate that oxidized LDL contains 3 nmol HNE-histidine per mg protein [60]. However, identification of the precise residues that are modified requires the application of advanced protein analysis by LC-MSMS, as explained in the next section.

3.3. Advances in mass spectrometry approaches for analysis of lipoxidation

3.3.1. LC-ESI-MS(MS) analysis of amino acid lipoxidation (non-site-specific)

With the development of LC-ESI-MS, later followed by tandem mass spectrometry and peptide sequencing, their application for the identification of modified lipoproteins was inevitable. One of the first reported uses of mass spectrometry for the direct measurement of aldehyde adducts identified MDA and HNE adducts to the lysine residues of copper oxidized LDL, by protein hydrolysis and GC-MS [38]. The study reports not only the identification of adducts of MDA (3-(Nε-lysino) propan-1-ol) and HNE (3-(Nε-lysino)-4-hydroxynonan-1-ol), but also a lysine-MDA-lysine iminopropene crosslink (1,3-di(Nε-lysino)propane). While lysine-MDA adducts were found even in LDL from healthy subjects, their increase and the formation of lysine-HNE adducts during copper oxidation of LDL was found to account for the modification of less than 1% of the lysine amino acids present in LDL [38]. This emphasizes the challenges of studying such modifications [70].

GC-MS has been used in the identification of several fatty acids adducted to copper oxidized LDL [49]. Acid hydrolysis was performed on delipidated apolipoproteins, and the fatty acids released were analysed by selected ion monitoring-GS-MS (SIM-GC-MC), together with a phosphorus assay. The carboxylic acids found to be increased in oxidized LDL included pentanedioic (glutaric) acid (PDA), nonanedioic (azelaic) acid (NDA), hexanoyl, palmitate, stearate and oleate; the latter three showed a 10–20-fold increase. These results were interpreted as the formation of adducts between proteins and phospholipids, which had also been suggested previously by antibody studies [49], although it is known that total delipidation of lipoproteins is very difficult and non-covalently bound lipids often remain [54–71]. LC-MS of hydrolyzed samples was also used to demonstrate that pyridoxamine could inhibit the formation of several advanced lipoxidation end products, specifically Nε-(carboxymethyl)lysine (CML), Nε-(carboxyethyl)lysine (CEL), malondialdehyde-lysine, and 4-hydroxynonenal-lysine, in the copper-catalysed oxidation of LDL [72].

Uchida’s group in Japan have worked intensively in this area using the approach of total protein digestion by 6 N HCl followed by LC-MSMS analysis of modified amino acids. Lipoxidation adducts of trans-2-nonenal [73], 4-oxo-2(E)-nonenal [74], and acrolein [75] were detected in LDL oxidized in vitro. More recently, they have developed a methodology to investigate the lipid peroxidation “adductome” of LDL [76], based on the fact that different lipoxidation adducts of, for example, lysine have different retention times in the LC run and different mass-to-charge ratios. In LC-MSMS, a precursor ion scan for diagnostic ions of the peptides and precursor ion scanning (PIS) of a fragment ion at m/z 268, which corresponds to the reduced HNE-modified histidine immonium ion and allows peptides containing this modification to be found [80]. The group attributed most of the parent ion peaks resulting from the PIS to theoretical HNE-modified ApoB-100 peptides, tentatively localizing the modifications to six histidine containing peptides [79]. Later they used this approach to study the effect of HDL on LDL oxidation. By carrying out a quantitative analysis of specific histidines, they were able to demonstrate that the presence of human HDL, which contains the anti-oxidative enzyme paraoxonase, abrogated the lipoxidation of LDL histidines, whereas avian HDL, which lacks paraoxonase, did not [80]. Modifications in ApoB-100 induced by copper oxidation of LDL were also investigated using an untargeted LC-MSMS approach by Obama et al., and a variety of amino acid oxidations were observed (mono-oxidations of histidine and tryptophan and kynurenine), as well as HNE-histidine and Nε-(3-methylpyridinium)-lysine lipoxidations resulting from acrolein modification [81]. A key novelty in this work was the testing of both in-gel and on-membrane (polyvinylidene difluoride; PVDF) trypsin digestion, which enabled much smaller amounts of lipoprotein to be used [81], compared to the previous work by Bolgar et al. where milligram amounts were used [79].

3.3.4. Application of proteomic approaches to identification of sites of HDL modification by small rLPPs

HDL is a particularly challenging lipoprotein to work with for MS-based proteomic analysis, as ApoB-100 is a very large protein consisting of 4536 amino acid residues with a molecular weight of over 500 kDa. More recently, tandem mass spectrometric methods have become more common, such as MALDI-MS/MS and LC-ESI-MS/MS. The basic principles of the methods applied to the analysis of oxidized and lipoxidized proteins have been explained in previous reviews [77] and will not be covered here. Most work has been carried out by the “bottom-up” approach, which involves enzymatic digestion of proteins to peptides, which can be separated by LC, and then fragmented within the mass spectrometer to enable sequencing. This approach is not trivial for the localization of any oxidative modification [77], and analysis of lipoxidation is even more challenging [70]. For lipoxidation adducts, which are not particularly stable (Schiff’s base adducts are readily reversible and even Michael adducts can degrade), it is important to “fix” the adducts by a reduction step before commencing the proteolysis steps. A smaller number of studies have used a “top-down” approach, which analyses the intact protein complete with modifications, and then partially fragments it to provide further information. Some of these techniques have also been discussed by Colzani et al. in a review on mass spectrometric approaches for the analysis of protein adducts with reactive carbonyl species, which provides a detailed consideration of the approaches for the analysis of more complex biological samples, including lipoproteins, and the use of derivatization with DNP for the MS identification of acrolein, HNE and MDA adducts with proteins [24]. The derivatization approaches can also be carried out with alternative labeling agents such as the hydroxylamine-functionalized biotin-containing probe, aldehyde reactive probe (ARP), and have been tested by several groups in general redox proteomic studies [16,70,78], but have not been applied to studies of lipoproteins.
In contrast, the proteins from HDL present a more amenable target: approximate molecular weight for ApoA-I is 28 kDa, ApoA-II is 18 kDa, and apoC forms are 8–9 kDa. Consequently, in parallel other groups have studied the oxidative modifications of HDL, with reasonable success. Heinecke’s group carried out several investigations of apolipoprotein A-I (ApoA-I) using targeted LC-MSMS methods, and succeeded in obtaining 80% sequence coverage, enabling a fairly thorough assessment of the modifications. While their focus was mainly on the chlorination and nitration of tyrosine residues by myeloperoxidase [82], they also investigated the reaction of acrolein with ApoA-I [83].

Using the endoproteasines GluC to digest the protein, they obtained 82% sequence coverage and were able to identify eight specific lysine residues modified to Nε-(3-methylpyridinium)lysine (MP-lysine); Nε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) did not appear to be formed and no cysteine modifications were detected because ApoA-I lacks this amino acid. Interestingly, using the monoclonal antibody mAb5F6 mentioned in Section 3.2.3, they were able to show that ApoA-I colocalized with acrolein-lysine adducts in human atherosclerotic lesions. The modification of both ApoA-I and ApoA-II by acrolein has been investigated by Chadwick et al. to determine its role in HDL cross-linking and impairment of the reverse cholesterol transport pathway in peripheral tissues [84]. This demonstrated the occurrence of mass shifts corresponding to the addition of acrolein molecules by both Michael addition and Schiff base on intact ApoA-I and ApoA-II proteins, and related it to the formation of crosslinks seen in western blots, but as only intact protein analysis was carried out, the sites of adduct formation were not identified.

Lipoxidation of the HDL protein component ApoC-II has also been studied, using a more unusual rLPP derived from the ozonolysis of cholesterol, 3β-hydroxy-5-oxo-5,6-secocholan-6-al (3-HOSCA) [85]. There is evidence that co-localization of ApoC-II and serum amyloid P in atherosclerotic plaques can lead to the formation of fibrils, and previous data from studies in vitro demonstrated that treatment of ApoC-II with 3-HOSCA accelerates fibril formation. To investigate the formation of covalent adducts, ApoC-II was incubated with 3-HOSCA, digested with GluC, separated by HPLC and then analysed by MALDI-TOF-MS to identify modified peptides; however, as the peptides were large (~40 residues), the likely site of modification within them could only be inferred. Nevertheless, the study demonstrated that ApoC-II could be randomly modified at six different lysine residues, typically resulting in one 3-HOSCA attached per ApoC-II molecule, and it was concluded that the presence of this adduct in HDL leads to the formation of fibrils by both covalent Schiff base formation, and other non-covalent mechanisms [85].

Acrolein adducts have also been identified and located in rat ApoE, which is a 294 amino acid protein with a molecular weight of 34 kDa, and about 74% sequence homology with human ApoEIII. The protein was digested with endoproteasides AspN and GluC to improve sequence coverage, before analysis by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI TOF/TOF MS). Using this untargeted approach, the researchers were able to find acrolein modifications yielding an aldime adduct at K149 and K155; a propanal adduct at K135 and K138; MP-lysine at K64, K67, and K254, and an FDP-lysine derivative at position K68. These lipoxidations were concluded to contribute to impairment of binding to the LDL receptor and heparin, and may also be responsible for protein unfolding [86].

### 3.3.5. Application of proteomic approaches to localization of adducts formed by phospholipid-esterified rLPPs

The research described above has focused on adducts formed by small, non-esterified alkenals, but it is well-established that phospholipid-esterified alkanals or alkenals are also major products of lipid oxidation, and antibody-based studies described in Section 3.2 suggested that they can also form adducts with proteins. To confirm their formation and identify the sites of modification, studies of LDL oxidation were carried out by the group of Spickett [71]. LDL was treated with the esterified nine-carbon alkanal 1-palmitoyl-2-(9-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PONPC), delipidated and in gel-trypsin digestion carried out, followed by LC-MS/MS; approximately 70% sequence coverage of ApoB-100 was obtained, which was good considering the size of the protein. In this work a novel targeted mass spectrometry approach was developed, involving the use of narrow-window extracted ion chromatograms (XICs) to pinpoint the presence of characteristic fragments of certain modifications. These included the m/z 184 ion, an intense fragment commonly formed during fragmentation of phosphatidylcholines, corresponding to the head group. They were able to identify two different peptides modified with PONPC and one modified with the analogous 5-carbon alkanal, 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC). This was the first published report to identify directly and localize lipoxidation adducts of chain-shortened phospholipids.

There is also substantial evidence that adducts of oxidized PC are present on Lp(a), a lipoprotein formed by covalent linking of ApoA to ApoB-100 [87]. The adducts are formed as Schiff bases to two lysine residues within Kringle IV or Kringle V domains of the ApoA. Re-combinant Apo(a)s were used to investigate the lysine binding site (LBS) responsible for adduct formation, using both E06 antibody binding and analysis of tryptic peptides by LC-MSMS. Precursor ion scanning for m/z 184 was again employed to show the presence of a number of prominent peptide peaks containing PC, and analysis of the lipid phase of the lipoprotein demonstrated the presence of the short chain aldehyde POVPC, which is E06-detectable [88].

Shortly afterwards, an alternative method for identifying oxidized phospholipid adducts was developed and applied to using the modification of ApoA in HDL [89]. A key advantage in this study was the use of an enrichment process consisting of two separations steps. The first one separated the phospholipid-modified peptide, which is highly hydrophobic, from less hydrophobic molecules using a C18 column. Subsequently aminolysis was carried out; this is a specific reaction that breaks the bond between the phosphoglycerol backbone and the fatty acyl chain at the sn-2 position, thus separating the PC head group and intact long hydrophobic chain from the modified peptide, making the latter more hydrophilic and allowing it to be eluted from the column. Using this method, the group was able to identify a plethora of peptides of ApoA-I, II and III from copper-oxidized HDL [89] and myeloperoxidase-oxidized HDL [90], demonstrating the modification with several different oxidized phospholipids.

It can be seen that mass spectrometry analysis of lipoxidation is a challenging but evolving field: the rate at which papers are published in this area is increasing, the data obtained on the modifications is getting more detailed, and the development of the technology is allowing the analysis of more complex samples. The different approaches: amino acid analysis versus peptide sequencing are quite complementary in the information produced; the former is very good for quantitative studies, whereas the latter is able to identify specific residues that are modified, and therefore enables the mechanisms of function or dysfunction to be elucidated. From the peptide sequencing methods, a substantial body of evidence on the sites of modification of several apolipoproteins has now been obtained and is illustrated in Fig. 4. A key challenge is now to understand the functional effects of these modifications.

## 4. Biological effects of lipoxidized lipoproteins

Since the discovery of the role of oxidized LDL in pathologies like atherosclerosis, some effort has been put into understanding the biological effects this lipoprotein can have, especially in pathology. Many reviews have been published on the subject, from its role in atherosclerosis [13,91–93], apoptosis [94] and inflammation [95], its growth and proliferation promoting effects [96], its involvement on endothelial dysfunction and ageing [97], among others. Although for oxidized LDL not as much research has been done, some of its effects have also been uncovered, and it is clear that the beneficial effects of this lipoprotein...
can be lost following covariant modification. Oxidation of this lipoprotein induces the loss of its protective effect against oxidized LDL [98], and that in turn induces further oxidative stress and cytotoxicity (although to a lower extent than oxLDL) [99]. The effect of oxHDL has also been shown on the dysfunction of endothelial progenitor cells (EPCs) and human renal proximal tube epithelial cells (HK-2), through activation of CD-36 receptors and mitogen-activated protein kinase (MAPK) pathways [100,101], as well as on the induction of adipogenesis in females with high body mass index (BMI) [102].

While a lot has been done to uncover the biological effects of oxidized lipoproteins, relatively little of this work is on the effect of lipoxidized lipoproteins specifically. Fig. 5 summarizes the main biological effects that have been reported to date. Much of the work has focused on MDA- and HNE-modified LDL. As early as 1980, MDA-treated LDL was used as a model system and was shown to be taken up by macrophages, but the mechanisms were poorly understood; it was suggested that this resulted from the link between AhR and p21 which could be suppressed by polycyclic aromatic hydrocarbons (PAHs) in an arylhydrocarbon receptor (AhR)-dependent manner; it was suggested that this resulted from the link between AhR and p21 leading to cell cycle arrest [109]. An association between the levels of circulating MDA-LDL and vascular inflammation has been reported, using 18F-FDG PET/CT imaging [110], and it was suggested that part of this inflammation might be due to activation of the complement system, by the binding of MDA and malondialdehyde acetaldehyde (MAA) adducts in treated LDL to the complement anaphylatoxin C3a [111]. HNE-modified LDL is also thought to have altered functionality; it has been reported that HNE-containing oxLDL can induce cell proliferation at low levels in smooth muscle cells, while at higher levels it caused apoptosis in various cell types [112,113]. This oxLDL-induced apoptosis might be linked to the derivatization of proteins such as tyrosine kinase receptors by HNE [112,113] and possibly other oxidized lipids, and to the inhibition of the ubiquitin-proteasome pathway [114].

Oxidation of LDL has also been shown to stimulate the production of antibodies against oxidized PC species. Structural and functional similarities between the E06 antibody and T15, an anti-oxPC secreted by B-1 cells and involved in the immune response provoked by bacterial infection with S. pneumoniae, have been uncovered [115]. They suggested that oxidation of PC species, and/or possibly their addition to protein residues, alters their conformation, exposing the PC headgroup and making it accessible to the antigen binding site of E06/T15 fractions that mediated the high affinity binding of oxLDL to the receptor CD36 [106,107].

Other studies showed that in oxidized LDL and MDA-treated LDL with similar degrees of modification, oxLDL showed higher binding to macrophages and degradation rate when compared to MDA-LDL [108]. It has been reported that MDA-treated LDL induced THP-1 cell growth, which could be suppressed by polycyclic aromatic hydrocarbons (PAHs) in an arylhydrocarbon receptor (AhR)-dependent manner; it was suggested that this resulted from the link between AhR and p21 leading to cell cycle arrest [109]. An association between the levels of circulating MDA-LDL and vascular inflammation has been reported, using 18F-FDG PET/CT imaging [110], and it was suggested that part of this inflammation might be due to activation of the complement system, by the binding of MDA and malondialdehyde acetaldehyde (MAA) adducts in treated LDL to the complement anaphylatoxin C3a [111]. HNE-modified LDL is also thought to have altered functionality; it has been reported that HNE-containing oxLDL can induce cell proliferation at low levels in smooth muscle cells, while at higher levels it caused apoptosis in various cell types [112,113]. This oxLDL-induced apoptosis might be linked to the derivatization of proteins such as tyrosine kinase receptors by HNE [112,113] and possibly other oxidized lipids, and to the inhibition of the ubiquitin-proteasome pathway [114].

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antibodies. The production of such antibodies has an important biological effect, since they have the ability to inhibit the uptake of oxLDL by macrophages, as mentioned previously [68]. At this time, it was still unclear whether they were autoantibodies, i.e. antibodies produced by the body against part of “self”, or if the modified proteins and lipids were being recognized as external factors [115]. Similarly, antibodies found in human plasma that bound to oxidized and MDA modified LDL have been tested, and results show that one in particular, IK17, binds to MDA-LDL, MDA-HDL and Cu-oxidized LDL, whilst not binding to LDL treated with HNE or other proteins modified with MDA, suggesting a specific affinity to protein-bound MDA [116]. This antibody was also able to block the uptake of oxLDL by macrophages, which is important as it could potentially be used in therapeutics. In contrast, another study reported that when human LDL was injected into immune-competent and immune-deficient mice (lacking B and T cells), extensively oxidized LDL was cleared faster than native LDL but with similar clearance rates in the two mouse types, suggesting that the clearance of oxLDL is not simply mediated by antibody production [117]. However, there is now extensive evidence that covalent adducts such as ALEs or AGEs constitute “damage associated molecular patterns” (DAMPs) and are recognized by the immune system. They are often referred to as oxidation-specific epitopes, or OSEs; they occur on the surfaces of lipoproteins, apoptotic cells [118] or microvesicles [119], and can be recognized by a wide variety of cell surface pattern recognition receptors central in innate immunity, such as scavenger receptors and some Toll-like receptors (reviewed by [120,121]). The lipoprotein

Fig. 5. Physiological effects of lipoxidized lipoproteins, with consequences for the immune system, inflammation, apoptosis and HDL function. (1) Oxidized LDL stimulates the production of antibodies against itself and oxidized phospholipids, that can be recognized by the immune system; (2) oxLDL and MDA-LDL bind to scavenger receptors, leading to their internalization and formation of foam cells; oxPC-Lp(a) promotes the expression of IL-8 by macrophages, increasing inflammation; (3) HNE-LDL leads to apoptosis by both inhibition the ubiquitin proteasome pathway and by modification of tyrosine kinase receptors, leading to their inhibition; (4) Acrolein-HDL inhibits the increase of HDL caused by 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and HDL uptake and disassembly due to the decreased binding to the LDL receptor.
modifications most typically studied in this context are MDA, HNE and oxPCs, probably because these are most readily analysed, but it is highly likely that modifications by other rLPPs also contribute but have not yet been identified. The autoantibodies (also called natural antibodies or Nabs) produced downstream of the immune activation, and reported in patients with a variety of inflammatory diseases, are often IgMs although IgGs also occur [121].

Another lipoprotein that has been quite well-studied owing to its proatherogenic effects is Lp(a), which is an ApoA covalently linked to ApoB-100 by a disulfide bond [122]. It appears to be quite well-established that Lp(a) can bind covalently to oxidized phosphatidylcholines via lysine residues in the Kringle V domain [123], and it has been found that Lp(a) is the preferred carrier of oxidized phospholipids (oxPLs) in plasma [124]. Lp(a) has been reported to have various pro-inflammatory effects that appear to depend on the presence of the oxPL adduct. For example, Edelstein et al. reported that human apo(a) induced the production of interleukin 8 by cultured THP-1 macrophage-like cells [123]; subsequently this was confirmed in THP-1 and U937 cell lines and the use of siRNA indicated that both CD36 and TLR2 contributed to the effect. Inhibitors of MAPKs, Jun N-terminal kinase and ERK1/2 were found to abolish IL-8 gene expression, suggesting that these signaling pathways are involved in the response downstream of the receptors [87]. Overall, there is mounting evidence that a Schiff base adduct on lysine in the Kringle IV type 10 is responsible for the pro-inflammatory and pro-atherogenic properties of this lipoprotein. There has also been interest in anti-neoplastic effects of Lp(a), which may be caused by degradation products of the lipoprotein, although convincing evidence is only just emerging [122] and the importance of oxPL adducts in this effect is not established.

Some studies of HDL have reported altered biological activity following lipoxidation by acrolein. In particular, acrolein modification of ApoE was found to destabilize the protein, and caused a significant decrease in its ability to bind 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and support cholesterol efflux from cholesterol-loaded J774 mouse macrophages. The modified HDL also showed decreased binding to the LDL receptor in a co-immunoprecipitation assay and decreased binding affinity on a HiTrap heparin-sepharose column [86]. Similar findings were reported by Chadwick et al. [84], who showed that HDL cross-linked by acrolein was less able to act as an acceptor of free cholesterol from COS-7 cells, compared to native HDL. In fact, acrolein-HDL increased the neutral lipid uptake into macrophages. Thus it can be seen that acrolein modification caused substantial disruption of HDL structure, leading to a dysfunctional particle with impaired ability to support the reverse cholesterol transport pathway, with consequent atherogenic effects.

5. Lipoxidized lipoproteins as markers of disease and their use in therapeutics discovery

The altered biological effects described in the previous section are in the main pro-inflammatory, and therefore can be expected to contribute to a variety of diseases with underlying inflammatory etiology. It has long been accepted that LDL oxidation and ApoB-100 modification lead to impaired and altered biological functions that contribute to the progression and pathology of atherosclerosis [125], but lipoproteins can also be involved in the pathophysiology of other diseases. For example, dyslipidemia is associated with chronic kidney disease, diabetes mellitus and metabolic syndrome, and is characterized by lipoprotein abnormalities including an increase of triacylglyceride-rich lipoproteins such as VLDL and chylomicrons, LDL particles that are smaller and more dense, and an overall decrease in HDL cholesterol [126,127]. HDL deficiency or dysfunction have also been implicated in neurodegenerative disorders: high levels of this lipoprotein appear to correlate with increased cognitive function and memory in the senior demographic [128], but in Alzheimer’s disease a certain genotype of ApoE, allele APOE-e4, has been shown to predict an accelerated decline of cognitive function in carriers [128], although there is no evidence that it is related to increased susceptibility to lipoxidation.

The development of techniques to localize and identify lipoxidation, reviewed in Section 3, has allowed its analysis in clinical samples. A strong focus, going back more than twenty years, has been on lipid-protein modification in samples from atherosclerotic plaques. The presence of MDA and HNE adducts and the presence of oxPLDL was reported in atheroma tissue by immunocytochemistry with antibodies against HNE, MDA and copper-oxidized LDL [57]. The binding was found to be specific for the lipid-rich region of the atherosclerotic lesion and this pattern was maintained across the different antibodies [57], consistent with the expected localization of the oxidized LDL. Several other groups also reported similar results using anti-HNE-LDL antibodies [129], OB/04 and OB/09 antibodies against oxLDL [65], an autoantibody against MDA-LDL [116], or using E06 and an antibody against MDA-LDL [130]. A review from 2000 summarized additional identifications of different advanced lipoxidation end-products found in atherosclerotic lesions, including MDA-lysine [38], HNE-lysine [37,38], and levuglandin E2 [131], which were analysed by both immunohistochemical and chemical techniques [132]. Antibodies raised against less studied aldehydes have also been tested in tissue from atherosclerotic lesions: the antibodies developed against HNE-histidine adducts [50] and HNE-lysine adducts [63], which were shown to recognize copper-oxidized LDL, were also used to identify the presence of these adducts in human atherosclerotic aorta. An antibody to 4-HDDE-protein adducts showed increased staining in abdominal aorta of a cardiovascular patient with atherosclerosis and versus aorta from a healthy normotensive 41-year-old male, with no apparent atherosclerosis [64]. Thus despite the strong focus on smaller aldehyde adducts [133], evidence is emerging that other adducts also occur in disease, and may become useful markers in the future.

Anti-HNE antibodies have also been used to demonstrate that HNE-treated LDL could promote the HNE modification of other proteins, such as HSP60, a protein that is a target of autoimmune adaptive responses, and in its modified form a ligand to scavenger receptors alongside oxidized LDL, suggesting a synergetic effect in the progression of atherosclerosis [134]. The mechanisms by which this happens are not well understood, but it is possible that HNE treatment results in solubilization of HNE molecules in the LDL particles, and can then modify HSP60 when cells are treated with HNE-LDL. Another option is that as Michael additions are reversible, HNE bound to the ApoB100 in HNE-LDL could be released and modify other proteins.

The development of a well-validated ELISA assay to detect oxidized phospholipids on ApoB-100 containing LDL, using the E06 antibody, has enabled a substantial number of studies on the presence of oxidized phospholipids on LDL particles with several different cardiovascular diseases (CVD), mostly carried out by the groups of Tsimikas and Witzum [135]. For example, a reclassification of cardiovascular event in subjects from the Bruneck study followed over 15 years, which reported that the highest tertile of OxPL/ApoB was associated with higher risk of cardiovascular disease and stroke [136]. A study of stable subjects with coronary artery disease showed that oxidized phospholipids on ApoB-100 (oxPL-apoB) and plasminogen (oxPL-PLG) in plasma correlated positively with D-dimer, an end product of fibrin degrada-tion that indicates a pro-thrombotic state [137]. The relationship of oxPLs in Lp(a) to calcific aortic valve disease (CAVD) has also been investigated in the Copenhagen General population study, and showed that oxPL-apoB, oxPL-apo(a) and lipoprotein(a) levels all associated with risk of CAVD, suggesting that they may be causal risk factors for the condition [138]. These are recent studies that expand the earlier work reviewed in [135]. The simplicity, high-throughput and easy analysis of results of this assay makes it suitable for clinical studies, and although the technique does not give residue-specific information on the sites of lipoxidation, knowing the association with these pathologies should promote more detailed research on these modifications.

Mass spectrometry techniques to detect lipoxidation markers have
already been used to study cardiovascular disease and atherosclerosis. The Uchida group used targeted LC-MSMS to identify an rLPP adduct, N’-(8-carboxyoctanoyl)lysine (COL), in oxidized LDL. They applied this method to investigate its occurrence in sera from atherosclerosis-prone mice as well as from patients with hyperlipidemia, and found that the modification was specifically associated with the lipoprotein fraction of the sera. A significantly higher amount of COL was detected in both disease conditions compared with the controls [76]. It was interesting that in hyperlipidemic humans, the COL levels were tightly clustered while the healthy controls showed more variability, whereas in mice it was the other way round. Despite the relatively small patient and animal numbers, this study shows the potential of MS analysis for novel biomarker discovery.

The detection of autoantibodies against known lipoxidation adducts, namely MDA, MAA, MDA-LDL, and MAA-LDL, has been used to evaluate their potential as biomarkers of atherosclerosis [136,139,140]. The MAA-protein addsucts specifically were previously shown to be present in aortic tissue of rabbits on a high fat diet [141] and atherosclerotic rats [142], and led to an innate and acquired immune response, provoking the production of antibodies against it. One study developed an ELISA assay capable of binding the anti-MAA and anti-MAA-LDL antibodies from the plasma of human patients with non-obstructive coronary artery disease (CAD), acute myocardial infarction and obstructive multi-vessel CAD [139]. While the data for the MDA-LDL and MAA-LDL adducts did not show a significant difference between the different conditions, which is consistent with published results from other groups [136,140], the results on the circulating anti-MAA IgG, IgM and IgA antibodies showed a significant increase in the diseased states when compared with the controls. The IgG/IgM/IgA profile between the different conditions also showed variability, information that could be used as a potential biomarker.

Other conditions besides atherosclerosis have also been subject of lipoprotein lipoxidation studies. One study investigated the association between systemic lupus erythematosus (SLE) and both arterial and renal disease [143]. SLE is an autoimmune condition more prevalent in women, known to be linked with an early onset of atherosclerosis. The group looked at the presence of oxidized LDL using the E06 antibody, and determined the levels of autoantibodies against oxLDL, MDA-LDL and cardiolipin in patients with SLE. The results showed an increase of the E06 binding levels in the patients with the condition compared with the controls, and the levels of autoantibodies against oxLDL, MDA-LDL and cardiolipin were also significantly increased in the patients with SLE. It was suggested that the results obtained and the prevalence of premature atherosclerosis can relate to excess lipid peroxidation as playing an important role in SLE [143]. Antibodies against MDA-LDL and HNE-LDL were also used to stain multiple sclerosis plaques at different stages of disease, and these showed a localization of staining on the foam cells present [112], suggesting that the plasma LDL that enters the foam cells present [112], which is thought to contribute to its protective functions [149–151]. While the use of carnosine itself presents some limitations, recent work has led to the development of (2S)-2-(3-amino propanoylamino)-3-(1H-imidazol-5-yl)propanol (carnosinol), which is a derivative of carnosine with improved oral bioavailability and resistance to carnosinases [24,152,153]. The compound was tested in both rat and mouse models of diet-induced obesity and metabolic syndrome, and was found to reduce HNE adduct formation in liver and skeletal muscle, while also improving typical symptoms of metabolic syndrome, namely dyslipidemia, insulin resistance, steatohepatitis and inflammation. Although as yet these scavenging compounds have not been tested specifically with lipoproteins, the likelihood is that formation of adducts will also be attenuated, although the non-polar environment may reduce the effectiveness. Another compound group, the kavalactones (including kawain, methysticin and dihydromethysticin) has been identified as both advanced glycation and lipid peroxidation inhibitors. The latter was shown by the inhibition of TBARS formation in LDL and linoleic acid, which, in conjunction with its metal chelating properties, indicate a possible indirect inhibition of ALE formation [154].

A somewhat different strategy has been developed by Nankar and Pande [155]. They synthesized 11 peptides from conserved regions of human ApoE, which is known to have oPL binding activity. The peptides showed differential oxPL binding and native PL binding, and those that bound exclusively to the oxPLs also were able to inhibit IL-8 secretion in human blood, thus demonstrating anti-inflammatory activity.

6. Summary and perspectives

In conclusion, interest in the formation of rLPP adducts with lipoproteins is growing steadily, with many publications reporting their formation in a wide variety of conditions. Lipoproteins are already considered as biomarkers in some conditions, but understanding of lipoxidative modifications will add an additional layer of specificity to improve their value. Development of a range of antibodies has been invaluable for immunohistochemistry, and has provided substantial evidence for formation of certain adducts in vivo, frequently with increased levels in inflammatory diseases. The current limitation is that antibodies are only available for a relatively small number of rLPP adducts, compared to the number that exist and have been found by other methods, but work is ongoing to improve this. Despite the challenging nature of the analysis, improvements in mass spectrometry technologies are yielding very detailed qualitative and quantitative information on the sites of modification, which is important both in understanding the biological effects of the modifications, and designing therapeutic interventions. It is essential to appreciate the complementary nature of the different techniques, in terms of the types of information that they offer. It is also worth noting that currently the interest in rLPPs and lipoxidation is largely skewed towards pathology, as such modifications have widely been considered as deleterious, but the concept of rLPPs as signaling molecules with a role in hormesis is gaining favor. Ultimately, the understanding in this area will depend on the sensitivity of methods for detecting the modifications, and specific inhibitors to unravel their outcomes.
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Declarations of interest

None.

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