Atherosclerosis 288 (2019) 101–111

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Oxylipin profile of human low-density lipoprotein is dependent on its extent of oxidation

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HIGHLIGHTS

- Oxylipin profile of LDL changes dramatically during copper oxidation.
- Oxidation of LDL generates new oxylipins, with both pro- and anti-inflammatory properties.
- Oxylipin profile of LDL can determine the level of oxidation of the LDL particle.

ARTICLE INFO

Keywords:
Oxylipins
LDL
Atherosclerosis
Mass spectrometry
Lipids

ABSTRACT

Background and aims: Atherosclerosis is usually the underlying cause of heart attacks, strokes and peripheral vascular diseases – collectively known as cardiovascular diseases. Oxidation of low density lipoprotein (LDL) and its lipid content has an important role in the formation of lipid-laden atherosclerotic plaques. Not much is known about the impact of oxidative stress on bioactive oxylipin molecules present in LDL. The aim of this study is to understand the changes in oxylipin molecules present in LDL characterized by varying degrees of LDL oxidation.
Methods: LDL was isolated from the pooled plasma of healthy normolipidemic volunteers and was subjected to *in vitro* copper-catalyzed oxidation for varying time intervals (0 h, 6 h, 12 h, 24 h and 30 h). At each time interval, oxylipins were isolated through solid phase extraction and quantified using a targeted LC/-MS/MS approach employing stable isotope dilution method.
Results: Our results demonstrate that different forms of oxidized LDL (OxLDL) are characterized by specific oxylipin distribution and concentration. Compared to non-oxidized LDL, there is a significant increase in oxylipin generation ($p \leq 0.05$) in OxLDL subjected to 12 h and 24 h of oxidation. Though linoleate derived oxylipins are the most abundant in OxLDL extracts, the concentration of particular oxylipin species differed with different degrees of oxidation. Specifically, two pro-inflammatory linoleate-derived triols, namely 9,10,13-triHOME and 9,12,13-triHOME, exhibited a concentration increase of ~25 fold in 12h-OxLDL compared to non-oxidized LDL. Moreover, Partial least squares Discriminant Analysis (PLS-DA) identified 10 oxylipins, primarily prostaglandins, which could serve as additional biomarkers for oxidative stress or cardiovascular risk assessment.
Conclusions: Our data suggests that oxidative stress induces profound changes in the oxylipin content of LDL and the pattern of change is based on the extent of oxidation.

1. Introduction

Low density lipoprotein (LDL) has been implicated in the pathogenesis of atherosclerosis and hence has been studied extensively [1]. It is the exposure of the LDL molecule to free radicals that results in functional changes of the particle to a proatherogenic molecule leading to plaque initiation and progression [2]. Oxidized LDL (OxLDL) is more atherogenic compared to native LDL (n-LDL) and its enhanced atherogenic activity has been attributed to its lipid oxidation, specifically phospholipids and cholesterol esters [3]. OxLDL is also known to exist in different forms, depending upon the extent of oxidation and different mixtures of bioactive compounds it contains [4]. We have recently

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https://doi.org/10.1016/j.atherosclerosis.2019.07.018
Received 3 January 2019; Received in revised form 2 July 2019; Accepted 17 July 2019
Available online 18 July 2019

shown that not only the phosphatidylcholine species in LDL, but also the phosphatidylinositol species are prone to oxidation, resulting in the generation of fragmented and non-fragmented lipid oxidation products with powerful biological activities [5]. Another class of potent lipid oxidation products present within lipoproteins is oxylipins [6]. These oxylipins are primarily generated by one of three enzymatic pathways: cyclooxygenases (COXs), which generate the prostanoids and thromboids; lipoxygenases (LOXs), which generate leukotrienes, resolvins, hepoxilins and mid-chain alcohols; and cytochrome p450s (CYPs), which generate epoxides and omega-terminal alcohols [6]. The precursors to oxylipins are the abundant polyunsaturated fatty acids (PUFAs) present within lipoproteins. This enzymatic process results in the generation of an important superclass of lipid mediators, which includes the arachidonic acid (AA)-derived eicosanoids. There are also small fractions of the oxylipin family that are produced non-enzymatically. Recent advancements in analytical techniques have unraveled the presence of esterified oxylipins in the circulation [7,8], formed by attaching to other functional groups, including cellular phospholipids and cholesterol esters. Though their physiological significance there is not well understood, studies show that lipoproteins are the carriers of these circulating oxylipins [9]. Recent studies by Newman et al. [9,10] have shown that the oxylipin profile is different among the various plasma lipoproteins and that dietary interventions change the lipoprotein oxylipin profile in specific patterns.

The most commonly used model system to generate OxLDL is copper oxidation. In the original work by Steinbrecher et al. [11], it was found that copper oxidized LDL allowed it to bind to macrophage receptors, resulting in foam cell formation, similar to LDL incubated with endothelial cells. It was later shown that LDL, recovered from advanced lesions, contains products that resemble copper catalyzed oxidation [12,13]. Moreover, the principal copper containing protein in serum, ceruloplasmin (Cp), is a potent catalyst of LDL oxidation *in vitro* [14] and prospective studies show that serum Cp may be an independent risk factor for cardiovascular disease [15], indicating the physiological role of copper in the generation of OxLDL *in vivo*. Even though other methods of LDL oxidation have been investigated, by far the most commonly utilized OxLDL in atherosclerosis research is through copper oxidation, since copper oxidized LDL has been shown to have potent atherogenic properties such as platelet aggregation, macrophage foam cell formation, and increase in TNF-α release from endothelial cells [16,17]. Given the pro-inflammatory activity of oxylipins, it is important to understand how the LDL oxylipin profile changes during copper oxidation. In this report, we show the most comprehensive oxylipin analysis of LDL during copper oxidation.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade solvents were obtained from VWR International (Mississauga, ON, Canada); deuterated oxylipin internal standards were purchased form Cayman Chemical (Ann Arbor, MI, USA) and all other chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The solid phase extraction (SPE) cartridges (Strata X and X-AW, 100 mg/3 mL) were purchased from Phenomenex (Torrance, California, USA).

2.2. Isolation and oxidation of human LDL

The blood samples were drawn from healthy normolipidemic volunteers (n = 5, male = 3, female = 2), aged between 18 and 40 years, as part of a clinical study approved by the University of Manitoba Research Ethics board. Both verbal and written consent was obtained from all the volunteers prior to their participation in the study. The blood samples were obtained by venipuncture after the subjects had fasted overnight. The blood was collected in EDTA-coated vacutainers (BD Vacutainer, NJ, USA). The samples were centrifuged (1000*g* × 10 min) at 4 °C to isolate the plasma. Equal volume of plasma from each individual was pooled to get the 'pooled plasma sample'.

From the pooled plasma, LDL (density 1.019–1.063 g/mL) was isolated by density-gradient ultracentrifugation using a modification of the short-run-ultracentrifugation (SRU) method of Kleinveld et al. [18]. Desalting of the LDL fraction was accomplished by passing it through a Sephadex G-25 column (GE Healthcare, UK). The concentrated LDL was diluted in PBS (phosphate-buffered saline, pH 7.4) and filtered (0.22 μm, sterile). The LDL-protein content was determined using a BCA protein kit (Thermo Scientific Micro BCA Protein Assay Kit) and adjusted to 500 μg/mL with PBS. This diluted LDL with PBS at 500 μg/mL was exposed to 5 μM Cu++ as previously described [19,20] to generate the OxLDL. In short, triplicates of 3 mL of diluted LDL solution were oxidized with 5 μM CuSO4 in the dark at room temperature for 30 h, with 200 μL aliquots removed at 0, 6, 12, 24 and 30 h.

Analysis of oxylipin levels in LDL was carried out by HPLC-MS/MS multiple-reaction monitoring as previously described [21–23], based on methods developed by Deems et al. [24]. Briefly, 200 μL of LDL or oxidized LDL solution was used for oxylipin analysis. The LDL solution was spiked with 10 μL of deuterated internal standard solution (Cayman Chemical, MI, USA) and 3.1 μL of antioxidant cocktail [0.2 g/L BHT, 0.2 g/L EDTA, 2 g/L triphenylphosphine, and 2 g/L indomethacin in methanol/ethanol/water (2:1:1,by vol)]. Samples were then adjusted to pH < 3 and loaded onto preconditioned Strata-X solid phase extraction columns (Phenomenex, CA, USA). Columns were preconditioned with methanol and pH 3 water before loading with sample. Samples were loaded onto the columns, rinsed with 10% methanol, and eluted with methanol. The solution obtained by elution was dried down in a nitrogen evaporator and resuspended in the mobile phase (water/acetonitrile/acetic acid, 70/30/0.02, v/v/v) for analysis by HPLC-MS/MS (QTRAP 6500; Sciex, Ontario, Canada). Lower limit of detection (LLOD) and quantitation (LLOQ) were set at 3 and 5 levels above the background, respectively. Quantification of oxylipins was determined by using the stable isotope dilution method [25] and expressed as nanogram per milligram of LDL protein. To better approximate the true concentrations of oxylipins, the response factors were determined from dose response curves and applied to all oxylipins, unless otherwise noted when primary standards were unavailable [26,27]. Further details of oxylipins scanned for, but below the limit of detection (< 3 times above baseline) or below the limit of quantitation (< 5 times above baseline), as well as retention times, mass transitions, internal standards and standard curve slopes, are described in Refs. [21–23].

2.3. Statistical analyses

All data are presented as mean ± SD, unless otherwise stated. When differences were observed, Tukey *post-hoc* test multiple comparison tests were used. Statistical analysis was performed using SPSS Software (version 24, IBM Corporation, Armonk, NY, USA) and *p*-values ≤0.05 were considered statistically significant. Additional statistical analysis was performed using an online tool, MetaboAnalyst 3.0 [28].

3. Results

3.1. Oxylipin amount differs with the extent of human LDL oxidation

Of the 162 plasma oxylipins scanned, 78 were quantified according to the set quantification limit. Table 1 provides the details of these quantifiable oxylipins. Fig. 1A demonstrates the marked difference in the quantity of oxylipins produced with varying degrees of LDL oxidation. The average amount of oxylipin production exhibited a sharp increase with 12 h of oxidation, with further increment observed for the next 12 h. But beyond 24 h of LDL oxidation, the total amount of oxylipin generated declined. Based on Tukey HSD, there was a significant increase ($p \leq 0.05$) in oxylipin amount in 12h-OxLDL and 24h-OxLDL

Table 1
Oxylipins[a] quantified after screening for source PUFA, Q1 mass (Da), Q3 mass (Da), deuterated internal standards[b] (IS) and retention times (min).

Sl.No	Source PUFA	Analyte ID	Q1 mass (Da)	Q3 mass (Da)	Internal standard	Retention time (min)
LOX pathway						
1	AA	5-HETE	319	115	(d8) 5-HETE	17.13
2	AA	8-HETE	319	155	(d8) 5-HETE	16.66
3	AA	9-HETE[c]	319	123	(d8) 5-HETE	16.8
4	AA	11-HETE[c]	319	167	(d8) 12-HETE	16.39
5	AA	12-HETE	319	135	(d8) 12-HETE	16.56
6	AA	15-HETE	319	175	(d8) 15-HETE	16.05
7	AA	5,6-diHETE	335	115	(d4) LTB4	15.28
8	AA	5,15-diHETE	335	201	(d4) LTB4	11.55
9	AA	8,15-diHETE	335	235	(d4) LTB4	11.08
10	AA	LTB4	335	195	(d4) LTB4	12.01
11	AA	6t,12-epi-LTB4	335	195	(d4) LTB4	11.66
12	AA	12-epi-LTB4	335	195	(d4) LTB4	11.95
13	AA	6S-LXA4	351	115	(d4) LTB4	8.91
14	AA	5-oxoETE	317	203	(d7) 5-oxoETE	17.5
15	AA	12-oxoETE	317	153	(d7) 5-oxoETE	16.54
16	AA	15-oxoETE	317	113	(d7) 5-oxoETE	16.11
17	ALA	9-HOTrE	293	171	(d4) 9-HODE	14.46
18	ALA	13-HOTrE	293.4	195	(d4) 13-HODE	14.6
19	ALA	9-oxoOTrE	291	185	(d7) 5-oxoETE	14.96
20	DHA	4-HDoHE	343	101	(d8) 5-HETE	17.32
21	DHA	7-HDoHE	343	141	(d8) 5-HETE	16.66
22	DHA	8-HDoHE[c]	343	109	(d8) 5-HETE	16.73
23	DHA	10-HDoHE	343	153	(d8) 12-HETE	16.35
24	DHA	11-HDoHE	343	149	(d8) 12-HETE	16.48
25	DHA	13-HDoHE	343	221	(d8) 12-HETE	16.17
26	DHA	14-HDoHE	343	205	(d8) 15-HETE	16.29
27	DHA	16-HDoHE[c]	343	233	(d8) 15-HETE	15.97
28	DHA	17-HDoHE	343	245	(d8) 15-HETE	16.03
29	DHA	10(S),17(S)-DiHDoHE	359	153	(d5) RvD1	11.27
30	DHA	7(R)-Maresin-1	359	177	(d4) LTB4	11.41
31	DγLA	8-HETrE	321	157	(d8) 5-HETE	17.07
32	DγLA	15-HETrE	321	221	(d8) 15-HETE	16.7
33	EPA	RvE1	349	195	(d5) RvD1	5.72
34	EPA	5-HEPE	317	115	(d8) 5-HETE	15.79
35	EPA	8-HEPE	317	155	(d8) 5-HETE	15.33
36	EPA	9-HEPE[c]	317	149	(d8) 5-HETE	15.47
37	EPA	12-HEPE	317	179	(d8) 12-HETE	15.32
38	LA	9-HODE	295	171	(d4) 9-HODE	15.93
39	LA	13-HODE	295	195	(d4) 13-HODE	15.78
40	LA	9-oxoODE	293	185	(d7) 5-oxoETE	16.26
41	LA	13-oxoODE	293	167	(d7) 5-oxoETE	15.95
42	LA	9,10,13-triHOME	329	171	(d4) 9,10 diHOME	7.44
43	LA	9,12,13-triHOME	329	211	(d4) 12,13 diHOME	7.38
44	DPA	17k-DPA	343	247	(d7) 5-oxoETE	16.86
CYP pathway						
45	AA	16-HETE	319	189	(d8) 15-HETE	15.5
46	AA	20-HETE	319	245	(d6) 20-HETE	15.01
47	AA	5,6-DiHETrE	337	145	(d11) 8,9 DiHETrE	15.55
48	AA	8,9-DiHETrE	337	127	(d11) 8,9 DiHETrE	14.85
49	AA	11,12-DiHETrE	337	167	(d11) 11,12 DiHETrE	14.27
50	AA	14,15-DiHETrE	337	207	(d11) 14,15 DiHETrE	13.56
51	AA	11,12-EpETrE	319	167	(d11) 11,12 DiHETrE	17.64
52	AA	14,15-EpETrE	319	175	(d11) 14,15 DiHETrE	17.19
53	ALA	12,13-EpODE	293	183	(d4) 12,13 diHOME	16.17
54	DHA	20-HDoHE[c]	343	241	(d6) 20-HETE	15.7
55	EPA	18-HEPE	317	215	(d8) 15-HETE	14.49
56	EPA	14,15-EpETE	317	207	(d11) 14,15 DiHETrE	16.26
57	LA	9,10-EpOME	295	171	(d4) 9,10 diHOME	17.35
58	LA	12,13-EpOME	295	195	(d4) 12,13 diHOME	17.17
59	LA	9,10-diHOME	313	201	(d4) 9,10 diHOME	13.24
60	LA	12,13-diHOME	313	183	(d4) 12,13 diHOME	12.71
COX pathway						
61	AA	12-HHTrE	279	217	(d8) 12-HETE	13.54
62	AA	PGB2	333	271	(d4) 15d-PGJ2	10
63	AA	PGD2	351	271	(d4) PGD2	7.91
64	AA	15d-PGD2	333	271	(d4) 15d-PGJ2	11.86
65	AA	dhk-PGD2	351	207	(d4) PGD2	9.25
66	AA	PGE2	351	271	(d4) PGE2	7.59
67	AA	dhk-PGE2	351	207	(d4) PGE2	8.55
68	AA	11β-PGE2	353	335	(d4) PGF2a	6.91
69	AA	15k-PGE2	349	235	(d4) PGE2	7.91
70	AA	PGJ2	333	189	(d4) 15d-PGJ2	9.91
71	AA	PGF2α	353	193	(d4) PGF2α	7.45

(*continued on next page*)

Table 1 (*continued*)

Sl.No	Source PUFA	Analyte ID	Q1 mass (Da)	Q3 mass (Da)	Internal standard	Retention time (min)
72	AA	15k-PGF2α	351	219	(d4) PGF2α	7.81
73	AA	6k-PGE1	367	331	(d4) PGE2	5.85
74	AA	tetranor-PGEM	327	291	(d4) PGE2	2.44
75	DγLA	PGE1	353	235	(d4) PGE2	7.8
76	DγLA	TXB1	371	171	(d4) TXB2	6.59
Non-enzymatic products						
77	AA	5-iso-PGF2αVI	353	115	(d4) 8-iso PGF2α	7.22
78	AA	8-iso-PGF2αIII	353	193	(d4) 8-iso PGF2α	6.7

[a] AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DγLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; LA, linolenic acid; DPA, docosapentaenoic acid; HETE, hydroxy-eicosatetraenoic; diHETE, dihydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; LT, leukotriene; LX, lipoxin; oxoETE, oxo-eicosatetraenoic acid; EpETrE, epoxy-eicosatrienoic acid; PG, prostaglandin; dhk, dihydroketo; HOTrE, hydroxy-octadecatrienoic acid; oxoOTrE, oxo-octadecatrienoic acid; EpODE, epoxy-octadecadienoic acid; HDoHE, hydroxy-docosahexaenoic acid; DiHDoHE, dihydroxy-docosahexaenoic acid; TX, thromboxane; HETrE, hydroxy-eicosatetrienoic acid; Rv, resolvin; HEPE, hydroxy-eicosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; oxoODE, oxo-octadecadienoic acid; EpOME, Epoxy-octadecenoic acid; diHOME, dihydroxy-octadecenoic acid; triHOME, trihydroxy-octadecenoic acid.

[b] Concentration of all IS was 1 ng/μL, except PGD_2 (2 ng/μL).

[c] Many oxylipins (9- and 11-HETE, 9-HEPE and 8-,16- and 20-HDoHE) could be generated both enzymatically and non-enzymatically [23,50–54].

compared to the oxylipin amount in n-LDL (LDL in basal state with no Cu^{++} oxidation).

3.2. Oxylipin concentrations in n-LDL vs. OxLDL

The heat map (Fig. 1B) illustrates the mean concentration of individual oxylipin molecules within the various OxLDL extracts over 30 h. From 0 to 12 h of $Cu++$ mediated oxidation, a large proportion of oxylipins exhibited a gradual increase in their concentration compared to baseline values. The arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) derived oxylipins were present predominantly in OxLDL extracts and were absent in n-LDL. Notably, out of the 40 detectable oxylipins derived from AA, 16 were absent in n-LDL and were detected only after continuous oxidation for 12 h. These include 12 prostaglandins, namely prostaglandin B2 (PGB2), PGD2, PGE2, PGF2α, PGJ2, 11β-PGE2, 15d-PGD2, 15k-PGE2, 15k-PGF2α, 8-iso-PGF2αIII, di-hydroketo(dhk)-PGD2, dhk-PGE2, 2 leukotrienes, namely leukotriene B4 (LTB4) and 12-epi-LTB4, 12-hydroxy-heptadecatrienoic acid (12-HHTrE) and 11,12- dihydroxy-eicosatrienoic acid (11,12-DiHETrE). In addition, amongst the 7 measurable EPA derived oxylipins, two oxylipins, namely Resolvin E1 (RvE1) and 14,15-epoxy-eicosatetraenoic acid (14,15-EpETE), were not detected in n-LDL and their levels surged above the limit of detection only after extensive oxidation for up to 12 h. A similar trend is observed for DPA derived oxylipin 17k-DPA. Between LDL and OxLDL extracts, there was loss in concentration of non-linoleic acid (LA) derived oxylipins beyond 24 h of extensive oxidation.

3.3. Oxidation modifies LDL oxylipin profile

To investigate the association between parent PUFA and their respective oxylipins with oxidation, the oxylipin molecules were grouped by parent fatty acid. Fig. 2A shows the distribution of oxylipins by parent fatty acid as a percent of total oxylipin amount. Between n-LDL and 6h-OxLDL, the oxylipin profiles were similar, in which they were mainly comprised of AA-oxylipins followed by LA-oxylipins. Together, they contributed to more than 90% of the total oxylipin content in n-LDL, in which AA-oxylipins alone comprised around 60% of total oxylipin amount. The docosahexaenoic acid (DHA), dihomo- γ-linolenic acid (DγLA), eicosapentaenoic acid (EPA) and α-linoleic acid (ALA) derived oxylipins collectively constituted nearly 5% of the total oxylipin amount. However, LDL oxidation beyond 6 h greatly enhanced the fractional abundance of LA-oxylipins. In 12h-OxLDL, oxylipins were primarily comprised of LA-oxylipins (~60%) followed by AA-oxylipins (~25%). Fig. 2B shows the oxylipin amount within LDL and OxLDL by parent fatty acid expressed as nanogram per milligram of LDL. Total AA-oxylipins averaged 21.79 ± 2.45 ng/mg in n-LDL (Supplemental Table 1). For AA-oxylipins, there was a continuous increase in oxylipin production up until 12 h of LDL oxidation, where their average rose to 132.81 ± 52.97 ng/mg, but dropped with extensive oxidation beyond 12 h. By comparison, total LA-oxylipins averaged 10.66 ± 2.91 ng/mg in n-LDL and their average amount mounted to 885.08 ± 166.62 ng/mg in 24h-OxLDL, but dropped with extensive oxidation beyond 24 h.

Fig. 2C and D shows the distribution of linoleate-derived and arachidonate-derived oxylipin species within LDL and OxLDL during the 30 h time course of LDL oxidation. Among the linoleate-derived oxylipins (Fig. 2C), the most abundant regioisomers were two triols, namely 9,10,13-triHOME and 9,12,13-triHOME. Compared to n-LDL, the amount of 9,10,13-triHOME and 9,12,13-triHOME was greatly increased (nearly 25 fold) in 12h-OxLDL. They continued to increase after 12 h, with maximum abundance observed at 24 h (Supplemental Table 2).

Next, to evaluate the potential impact of oxidation on the different oxygenases involved in oxylipin production, we classified the oxylipins by the four major known routes/pathways through which they are generated from PUFAs; (i) cyclooxygenases (COXs) pathway, (ii) lipoxygenases (LOXs) pathway, (iii) cytochrome P450 (CYP450) pathway and (iv) non-enzymatic auto-oxidation of PUFA. Fig. 2E shows the distribution of oxylipins by these major pathways as a percent of total oxylipin amount. As shown in Fig.2E, n-LDL was dominated by LOX and COX pathways associated oxylipins followed by CYP450 and non-enzymatic oxylipins. With oxidation beyond 6 h, LOX associated oxylipins alone contributed to almost 80% of the total oxylipin production. Fig. 2F depicts the concentrations within LDL and OxLDL by major pathways, expressed as nanogram per milligram of LDL. As displayed in Fig. 2F, total COX and LOX associated oxylipins averaged 17.96 ± 3.66 ng/mg and 15.36 ± 3.57 ng/mg, respectively in the n-LDL (Supplemental Table 4). There was a progressive increase in both COX and LOX associated oxylipin production up to 24 h of LDL oxidation, where they in turn averaged 112.92 ± 24.88 ng/mg and 899.23 ± 172.10 ng/mg, respectively. The average oxylipin concentration of CYP450 associated oxylipins in n-LDL was 1.04 ± 0.07 ng/mg. With subsequent oxidation, the amount of CYP450 associated oxylipins showed a continuous increase until 12 h of LDL oxidation, where they averaged 6.21 ± 3.79 ng/mg. The non-enzymatic products (5-iso-PGF2αVI, 8-iso-PGF2αIII) were absent in n-LDL and they were detected only after extensive oxidation for up to 12 h.

Next, we examined whether the profiles of oxylipins were different within each oxylipin sub-classes and between LDL and OxLDL extracts. In both LDL and OxLDL extracts (Fig. 3A), the three most abundant classes of oxylipins were prostaglandins, alcohols and triols. The fractional abundance of these classes varied with the different stages of LDL

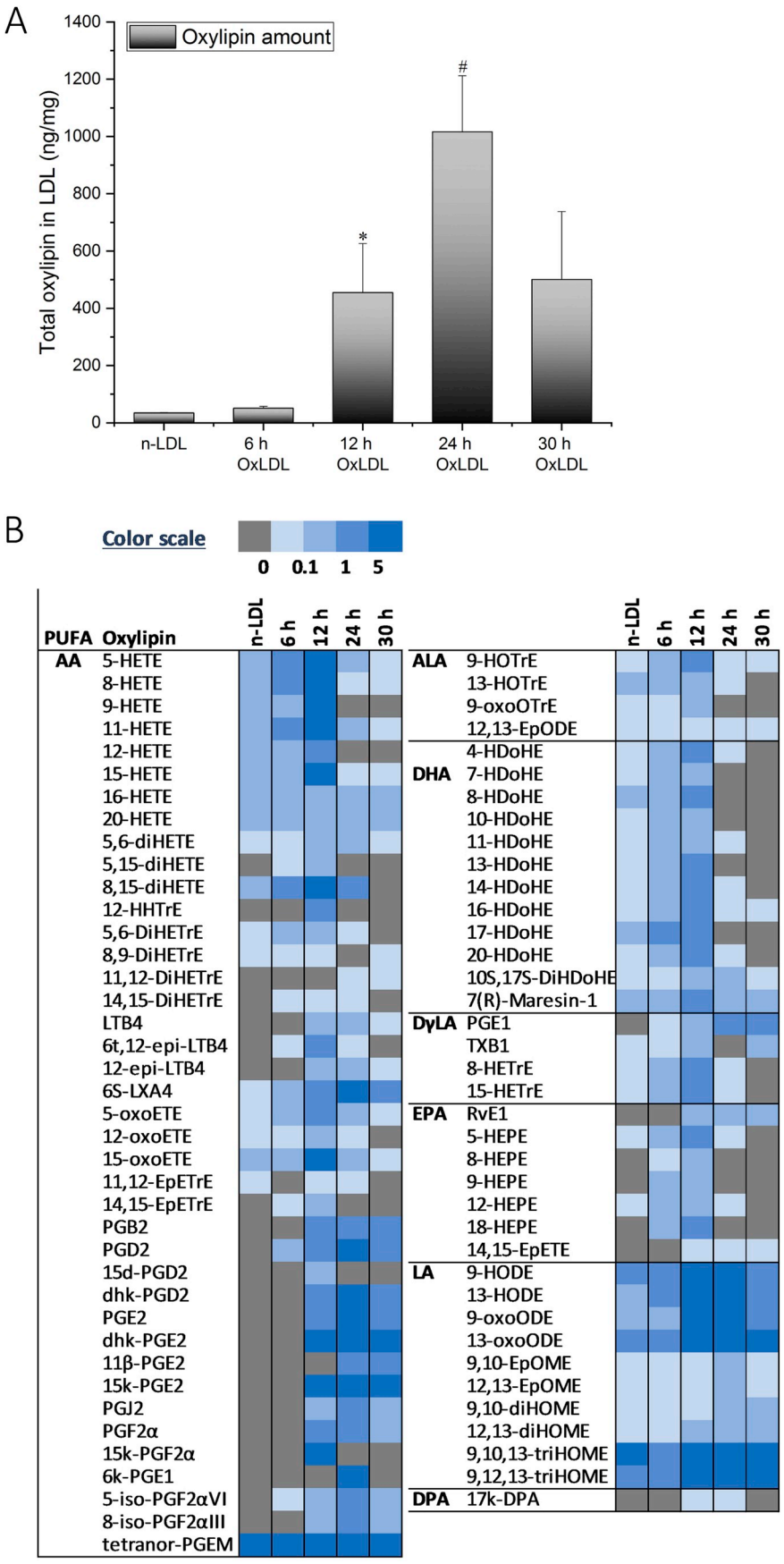

Fig. 1. (A) Effect of oxidation on oxylipin production. Total oxylipin amount within LDL and OxLDL extracts expressed as ng mg^{-1} of LDL. Significant differences at $p \leq 0.05$ were determined by Tukey HSD (* 12h-OxLDL *vs*. n-LDL, # 24h-OxLDL *vs*. n-LDL). (B) Scaled oxylipin concentrations in LDL and OxLDL extracts. The heat map shows the average oxylipin concentration in LDL and OxLDL extracts. The grey colour indicates the absence of oxylipin and the blue shade indicates the presence of oxylipin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

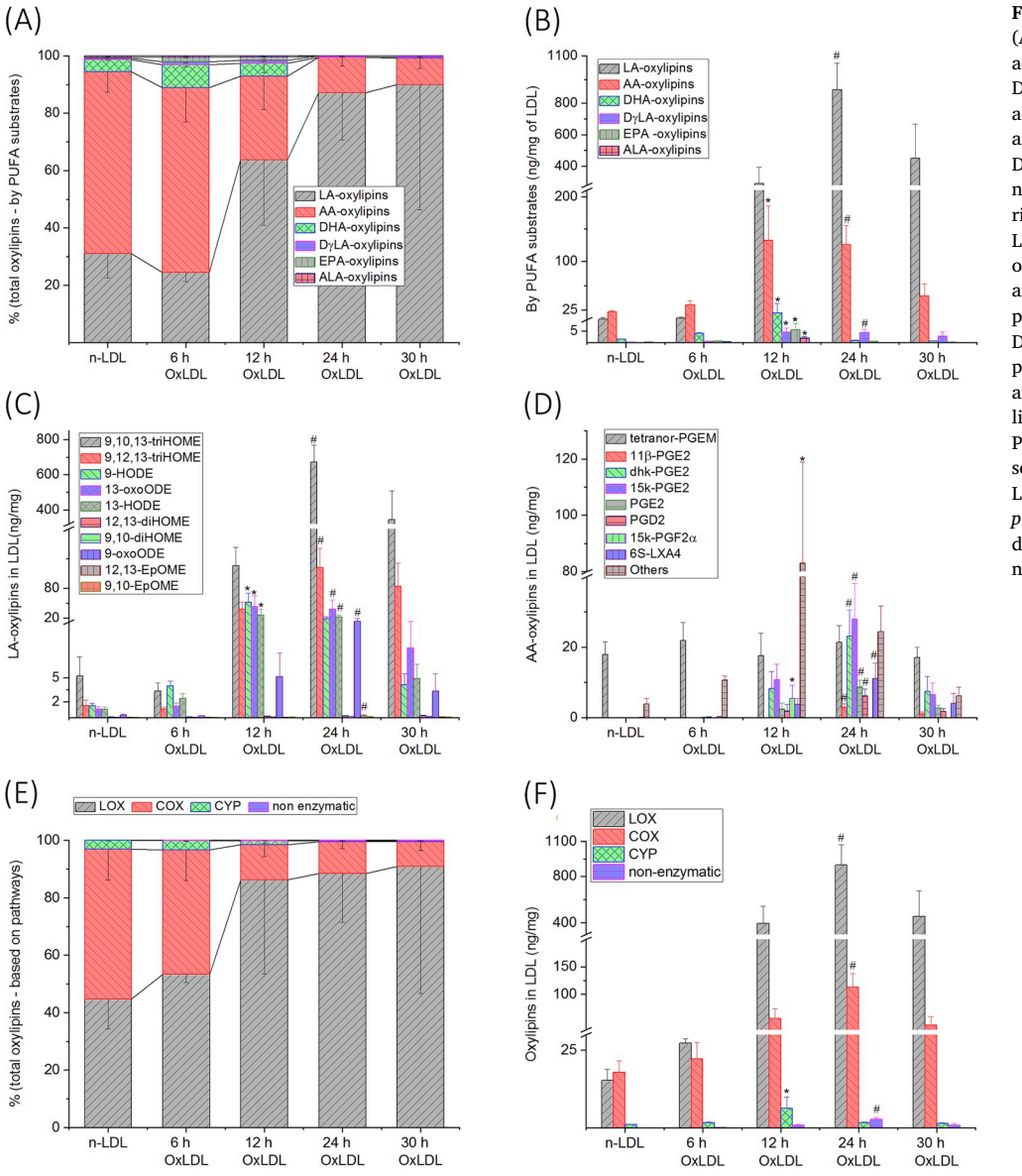

Fig. 2. LDL oxylipin profiles.
(A–D) LDL oxylipin profile by parent fatty acids in LDL and OxLDL extracts. (A) Distribution of oxylipins by parent fatty acids as a percent of whole total oxylipin amount, (B) total amount of LA, AA, DHA, DγLA, EPA and ALA oxylipins expressed as ng mg^{-1} of LDL; (C) levels of linoleate-derived oxylipins expressed as ng mg^{-1} of LDL, (D) levels of arachidonate-derived oxylipins expressed as ng mg^{-1} of LDL. (E and F) LDL oxylipin profile by associated pathways in LDL and OxLDL extracts. (E) Distribution of oxylipins by associated pathways as a percent of total, (F) total amount of (i) cyclooxygenases (COXs), (ii) lipoxygenases (LOXs), (iii) cytochrome P450 (CYP450) and (iv) non-enzymatic associated oxylipins expressed as ng mg^{-1} of LDL. Significant *post-hoc* differences at $p < 0.05$ after Tukey's adjustment are indicated at each time point (* 12h-OxLDL *vs.* n-LDL, # 24h-OxLDL *vs.* n-LDL).

oxidation. The fractional abundance of prostaglandins decreased from n-LDL to 30h-OxLDL. For alcohols, they were comparatively more abundant in n-LDL, 6h-OxLDL and 12h-OxLDL extracts. Though the fractional abundance of triols declined initially with 6 h of oxidation; their abundance again increased notably from 12 h onwards. Among the prostaglandins (Fig. 3B), the tetranor-PGEM remained a prominent portion of both n-LDL and OxLDL extracts. Among alcohols (Fig. 3C), the levels of 13-HODE (hydroxy-octadecadienoic acid), 9-HODE, 11-HETE (hydroxy-eicosatetraenoic), 5-HETE and 8-HETE were significantly higher in 12h-OxLDL compared to n-LDL. Triols were dominated mainly by two molecules namely 9,10,13-triHOME and 9,12,13-triHOME in both n-LDL and OxLDL extracts and their levels were significantly elevated in 24h-OxLDL compared to n-LDL (Fig. 3D).

3.4. Prostaglandins and triols as biomarkers of oxidative stress

To access the major changes in LDL oxylipin profile with oxidation, the oxylipin profiles at baseline and OxLDL extracts were comprehensively examined using the Partial least squares Discriminant Analysis (PLS-DA). PLS-DA analysis revealed substantial variations in oxylipin profile (Fig. 4A), with varying degree of LDL oxidation. The PLS-DA plot discovered three separate clusters, which have been categorized as follows: C1, the oxylipin profiles of n-LDL and 6h-OxLDL; C2, the oxylipin profile of 12h-OxLDL and C3, the oxylipin profiles of 24h-OxLDL and 30h-OxLDL. The C2 cluster was well-separated from C1 and C3 clusters, indicating that oxylipin profile at 12h-OxLDL is very different from the oxylipin profiles at other time intervals. The n-LDL and 6h-OxLDL groups revealed a great resemblance in their oxylipin profile, which is evident from their tight clustering. Likewise, the 24h-OxLDL and 30h-OxLDL groups had similarity in their oxylipin profile.

Most importantly, PLSDA analysis revealed that a total of 10 oxylipins with VIP values of > 1.0 have contributed to characterize their differences between the three clusters (Fig. 4B). Among these 10 oxylipins, 8 were prostaglandins, namely prostaglandin E1 (PGE1), PGE2, dhk-PGE2, dhk-PGD2, PGF2α, PGJ2, 15k-PGE2 and 11β-PGE2, and 2 were triols, namely 9,10,13-triHOME and 9,12,13-triHOME. The *p* values of the above-mentioned 10 oxylipins, obtained by one-way repeated measures ANOVA with *post-hoc* Tukey HSD between different time intervals, are listed in Supplemental Table 9. Of these, the top nine metabolites based on VIP score (VIP > 1.50) displayed a similar pattern; the concentration of all these metabolites showed a gradual increase up to 24 h, with maximum concentration observed for 24h-OxLDL, and then decreased slightly with further oxidation. Their detailed change trends are presented as a boxplot in Fig. 5.

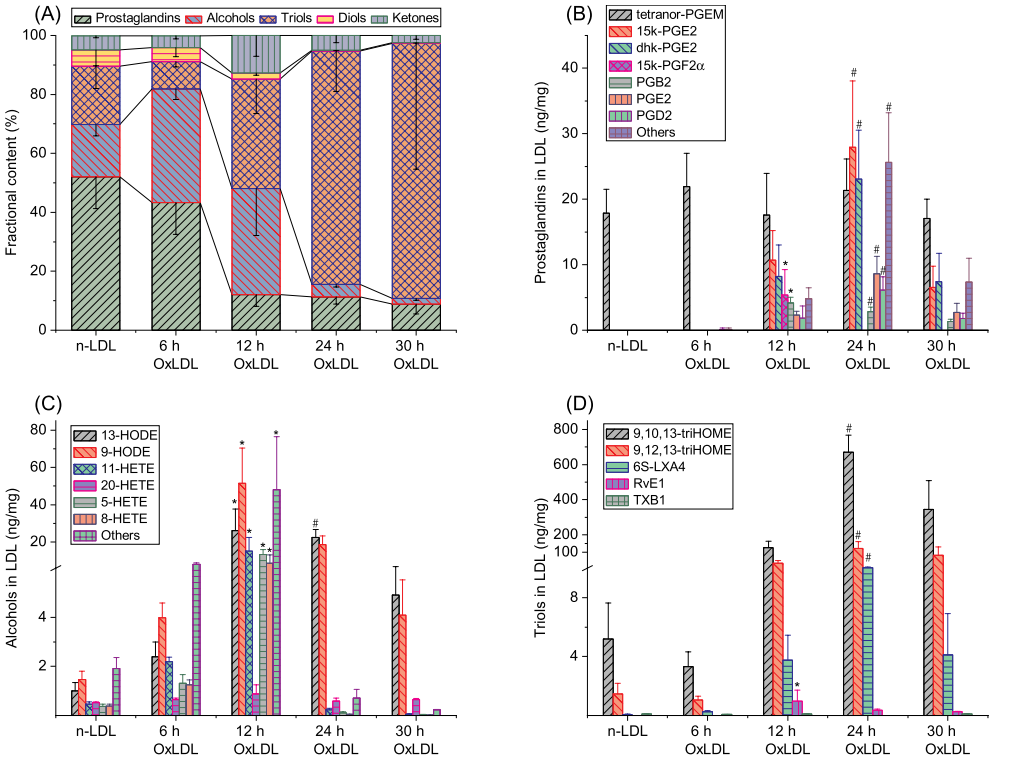

Fig. 3. The fractional abundance by oxylipin class between LDL and OxLDL extracts. (A) Distribution of oxylipins by different classes as a percent of total oxylipin content. (B–D) Total amount of oxylipin regioisomers within prostaglandins, alcohols and triols expressed as ng mg^{-1} of LDL. Significant *post-hoc* differences at $p < 0.05$ after Tukey's adjustment are indicated at each time point (* 12h-OxLDL *vs.* n-LDL, # 24h-OxLDL *vs.* n-LDL).

4. Discussion

It is well established that oxidative modification of LDL plays a proatherogenic role in the development of atherosclerosis. The effect of oxidation on oxylipin content in LDL, as well as its link with biomarkers related to oxidative stress, is poorly understood, and largely remains elusive. This work presents a controlled study on the impact of oxidative stress on the oxylipin content in LDL due to *in vitro* oxidation with copper ions. This study is the first lipidomic characterization of human LDL-oxylipins subjected to varying degrees of LDL oxidation. The results of the present study demonstrate that the LDL oxylipin content varied considerably between non-oxidized and oxidized LDL fractions. However, the pattern of change is substrate specific and depends upon the extent of LDL oxidation. Additionally, classification based on PLS-DA analysis identified 10 oxylipins that may be useful as potential biomarkers of oxidative stress or inflammation. These include 8 prostaglandins and 2 triols.

12h-OxLDL and 24h-OxLDL had a significantly higher amount of oxylipins compared to n-LDL (Fig. 1A). This indicates that a higher degree of LDL oxidation induced a proportionally higher amount of oxylipins. Recently, Shearer and colleagues [7,10] had shown that oxylipins are carried around the blood stream in acylated forms by lipoproteins and these esterified oxylipins are released by the action of lipolytic enzymes in tissues such as heart, and skeletal muscles and mediate endothelial dysfunction. They have also reported that the enzyme lipoprotein lipase (LpL) can release oxygenated lipids from very low-density lipoproteins (VLDL), suggesting that the triglyceride fraction also contains oxylipins. Oxylipins are also known to be esterified in cellular phospholipids [7,29,30], suggesting that lipoprotein phospholipids may also carry oxylipins.

Oxylipins appear in the circulation as both free and esterified forms. Several new studies [7–10] have shown that with the exception of linoleate-derived compounds, oxylipins are primarily found in esterified form and lipolytic enzymes release these oxylipins from lipoproteins. Also, a previous work by Tsoukatos DC et al. [31] demonstrated that polyunsaturated oxidized phospholipids in the sn-2 position are substrates for lipolytic enzymes and that an increase in oxidative stress increases the activity of these enzymes. Given that the esterified pool of oxylipins is by far the largest fraction of the total LDL oxylipins pool, the observed increase in oxylipin amount with copper oxidation suggests a conversion from esterified to free oxylipins.

Unlike VLDL, where the primary lipid component is triglycerides, in LDL, the primary lipid component is cholesterol ester followed by phospholipids and triglycerides. Therefore, the observed significant increase in oxylipin production with LDL oxidation up to 24 h suggests enhanced LpL activity and release of esterified oxylipins from OxLDL compared to n-LDL. Additionally, previous *in vitro* studies had demonstrated that mild oxidation of LDL increases its binding to LpL while extensive oxidation decreases it [31–33]. In another study employing x-ray scattering techniques, Cristiano et al. [34] had shown that LDL particles, oxidized for 18 h incubation with copper, exhibited significant loss of biological activity when compared to nonoxidized particles. This may explain why we observed a decrease in oxylipin production with extensive oxidation beyond 24 h.

n-LDL oxylipins were primarily represented by AA-oxylipins, secondly by LA-oxylipins then by DHA-oxylipins (Fig. 2A). This observed oxylipin composition at baseline is in accordance with the results published by Newman et al. [9] though they did not consider LA-oxylipins. We reason that this observed oxylipin composition does not reflect the true representation of the abundance of PUFA in n-LDL, since dominance of AA-oxylipins is contributed by a prostaglandin metabolite, tetranor-PGEM, which alone appeared to make up a greater fraction of the total oxylipin amount in the basal state (17.87 ± 3.68 ng/mg). This metabolite was found at similar concentrations in non-oxidized and oxidized LDL extracts (Supplemental Table 3). 12 h of oxidation led to a pronounced shift in the overall composition of oxylipins from baseline. In 12h-OxLDL, the composition order is changed as follows; LA-oxylipins > AA-oxylipins > DHA-oxylipins (Fig. 2A). This suggests that though lipase activity was observed irrespective of the parent fatty acid up to 24 h, LA-oxylipins were the most abundant oxylipins liberated by LpL. Moreover, this concentration order correlates well with the reported three to seven times increased ratio of linoleic acid to arachidonic acid in LDL particle [35,36]. Hence, it can be concluded that release of these oxylipins from LDL is primarily

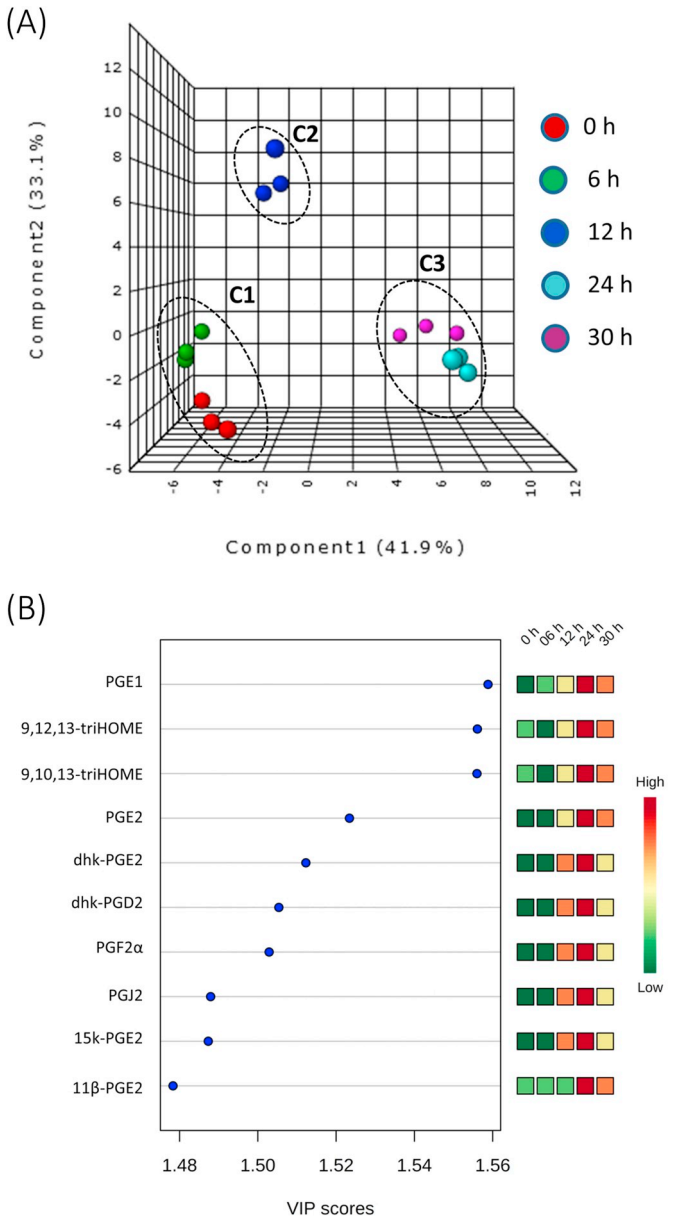

Fig. 4. The LDL oxylipin profile differs between different time intervals.
(A) Score-plot of PLS-DA analysis showing differentiation between different clusters, (B) important metabolites selected on the basis of Variables Important in Projection (VIP) score identified by PLS-DA. The coloured boxes on the right indicate the relative concentrations of the corresponding oxylipins at each time interval.

dependent on the availability of the substrate.

OxLDL had notably higher concentrations of two regioisomers, namely 9,10,13-triHOME and 9,12,13-triHOME, whose levels were increased ~25 fold in 12h-OxLDL (Fig. 2C and Supplemental Table 2), and further increased by ~5 fold in 24h-OxLDL compared to baseline. These trihydroxyoctadecenoic acids (triHOMEs) are the end products of oxidation of linoleic acid. Though certain studies have reported a pro-inflammatory role for these oxylipins, little is known about the physiological significance of these triHOMEs. Recently, Brash et al. [37] had shown that triHOMEs play an important physiological role in the skin barrier function of the human and porcine epidermis. Our previous work reports significantly higher concentrations of these two triHOMEs in older individuals (45–64 years) compared to younger individuals (19–28 years) [38]. This finding is important as people in the older age group of (45–64 years) are all atherosclerotic although the symptoms for this disease are not noticeable. Moreover, the levels of these triHOMEs were reported to be significantly elevated in inflammatory diseases such as Chronic Obstructive Pulmonary Disease (COPD) [39] and asthma [40,41]. Though the observed higher concentrations of these pro-inflammatory triHOMEs may provide mechanistic insights to explain the ongoing inflammatory response associated with atherosclerosis, their role as inflammatory messengers in atherothrombotic events demands further investigations.

The distribution of oxylipins in LDL by category revealed a major role for alcohols alongside with prostaglandins and triols (Fig. 3A). The most abundant alcohols in OxLDL were the linoleate derived 13-hydroxy-9,11-octadecadienoic acid (13-HODE) and 9-hydroxy-10,12-octadecadienoic acid (9-HODE) (Fig. 3C). In 1991, Belkner and coworkers [42] had reported the presence of 9-HODE and 13-HODE cholesterol esters in lipid-laden plaques in humans who had died from atherosclerosis. Moreover, they have reported a positive correlation between the concentration of these two metabolites and the severity of the atherosclerotic lesions. In our study, from baseline to 12h-OxLDL, the concentrations of 13-HODE increased from 1.00 ± 0.35 ng/mg to 26.19 ± 11.53 ng/mg and 9-HODE from 1.46 ± 0.35 ng/mg to 51.56 ± 18.87 ng/mg (Supplemental Table 7). These findings are also consistent with earlier investigations which reported that esterified linoleic acid in LDL can be oxidized to its 9- and 13-hydroperoxy metabolites and the amount of these products is increased in cholesterol-fed rabbits [43]. Among the alcohols, the concentrations of three AA-derived hydroxy eicosatetraenoic acids (HETEs) (5-HETE, 8-HETE and 11-HETE) were also found to be significantly increased at 12h-OxLDL compared to n-LDL. Though these mono-HETEs have previously been reported to positively correlate with atherosclerosis and the incidence of coronary heart disease in females [44,45], our data, for the first time, support the relationship between these mono-HETEs and the extent of LDL oxidation.

PLS-DA score plot (Fig. 4A) has revealed that ten oxylipins, primarily prostaglandins, could be used to clearly differentiate non-oxidized human LDL particle from oxidized LDL particle. This suggests that there exist considerable differences in oxylipins between non-oxidized and oxidized LDL, and these molecules could also serve as markers of oxidative stress. Considering the fact that these oxylipins are not entirely independent of each other and are all part of the same cascade (Fig. 6), valuable information can be gained by observing the patterns of these oxylipins. Among these, all metabolites other than 11β-PGE2 displayed a similar pattern (Fig. 4B); gradual increase in their concentration up to 24 h of LDL oxidation before eventually declining. Whatever the precise mechanism involved may be, these findings implicate these molecules in the generation of oxidatively modified LDL. If we assume that a similar process happens *in vivo,* also at a rate significant enough to contribute to atherosclerosis, then evaluating the level of these oxylipins would provides a means to measure the degree of atherosclerosis *in vivo*. Among these, PGE2 is known to exert both pro-inflammatory and anti-inflammatory responses [46,47] and can act as a mediator to maintain local homeostasis in the body, while PGE1 is a potent vasodilator with known anti-inflammatory properties. Previous studies [48] have reported an induction of PGE1 by OxLDL, suggesting an anti-inflammatory response to atherosclerotic injury caused by oxidation of LDL. This shows that oxidized LDL is a complex mixture of several bioactive oxylipins with complementary and opposing effects. This also accounts for the reported dual role exhibited by OxLDL [4,49] as pro- and anti-inflammatory, as well as pro- and anti-atherogenic at the same time.

4.1. Conclusions

To the best of our knowledge, this is the first attempt to look at the effect of oxidation on LDL oxylipin profile. The analysis of 162 oxylipins in LDL particle unveiled apparent differences between non-oxidized and oxidized LDL extracts. Here we describe the finding that higher degree

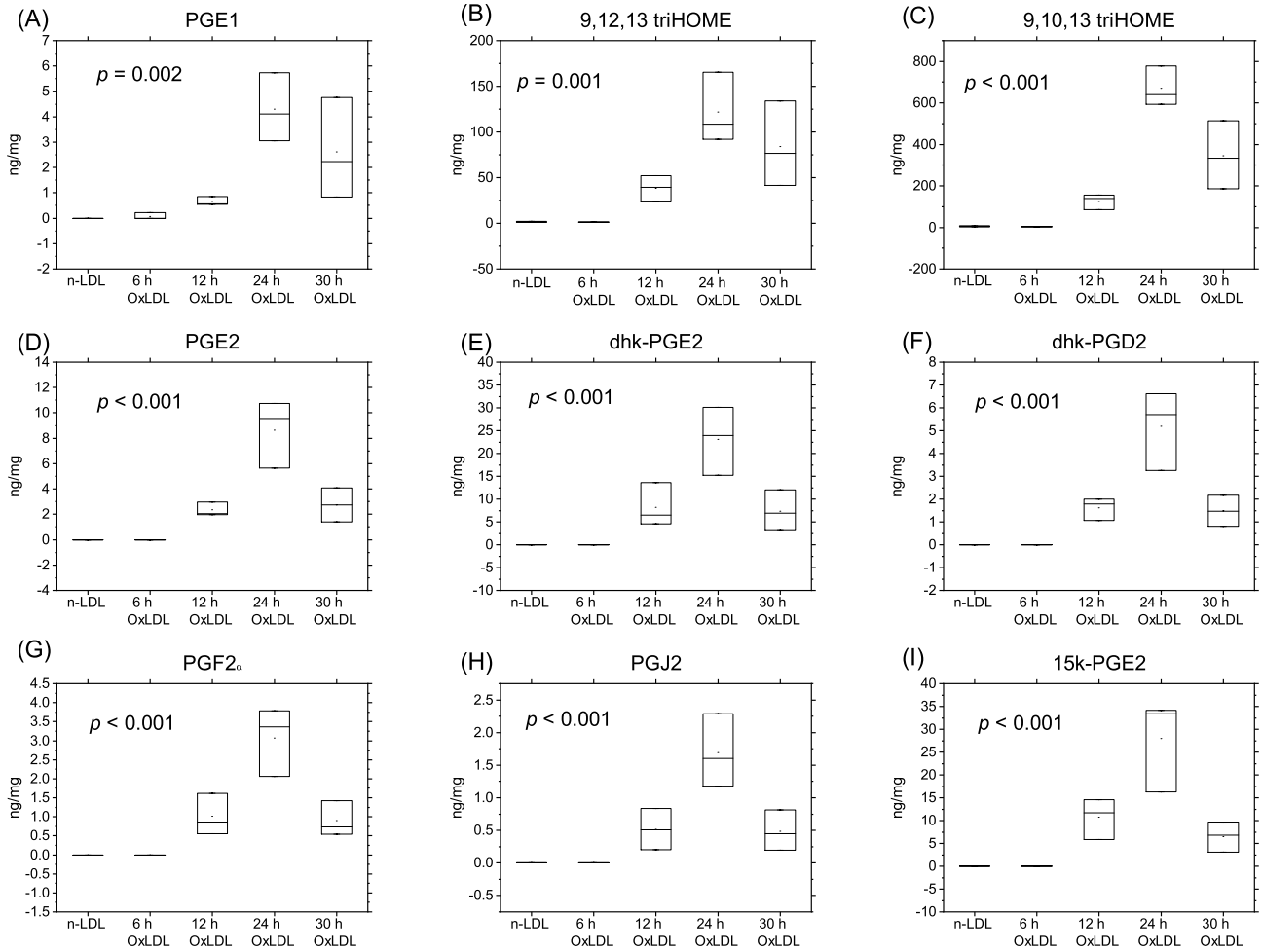

Fig. 5. Box plot of oxylipins exhibiting a similar pattern.
Boxplots are shown for concentrations of (A) PGE1, (B) 9,12,13 triHOME, (C) 9,10,13 triHOME, (D) PGE2, (E) dhk-PGE2, (F) dhk-PGD2, (G) PGF2α, (H) PGJ2 and (I) 15k-PGE2 across various time intervals expressed as ng mg^{-1} of LDL. Statistical significance was determined using one-way analysis of variance ($p < 0.05$).

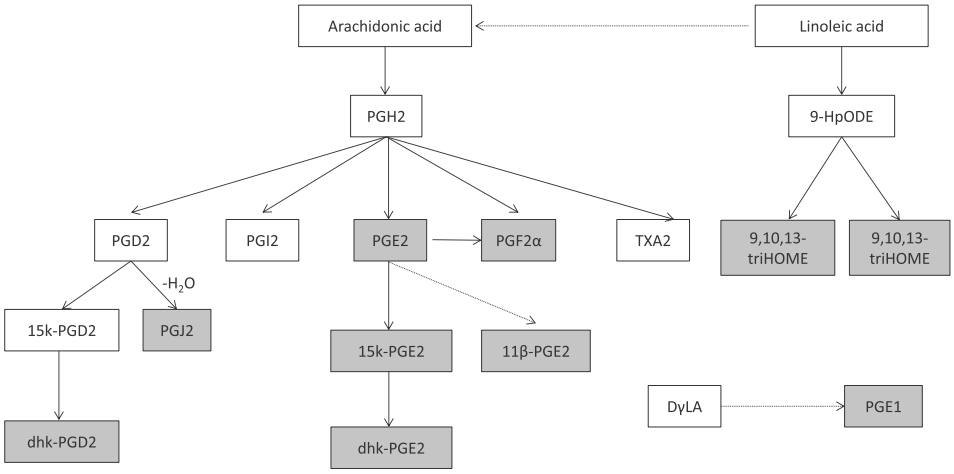

Fig. 6. Overview of the oxylipin biosynthesis pathway.
The metabolic pathways of oxylipins, which could be used to differentiate non-oxidized human LDL particle from oxidized LDL particle. Shaded boxes represent the oxylipin revealed by PLS-DA analysis and non-shaded boxes represent other oxylipins which are part of the pathway. PG, prostaglandin; TX, thromboxane; HpODE, hydroperoxy-octadecadienoic acid; triHOME, trihydroxy-octadecenoic acid; 15k, 15 keto; dhk, dihydro-keto; DγLA, dihomo-γ-linolenic acid.

of oxidation induced profound changes in the LDL oxylipin profile. Between oxidized LDL and native LDL, the oxylipin composition is different, which depends upon the parent PUFA and varies with the extent of oxidation. In addition, oxidative modification of LDL generates numerous new oxylipins, with both pro- and anti-inflammatory properties. Our findings contribute to our understanding on how oxidation impacts the oxylipin content of low-density lipoprotein.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

AR conceived and designed the experiments and helped draft the

manuscript. AS carried out the data analyses, statistical analyses and helped draft the manuscript. HZ, AE and TW performed the experiments. HA provided intellectual insight with data analyses and helped draft the manuscript.

Acknowledgements

This work was supported by Research Manitoba, Canada for Arun Surendran, Research Manitoba, Canada for Dr. Amir Ravandi and Heart and Stroke Foundation of Canada for Dr. Amir Ravandi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.07.018.

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