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TITLE: High density lipoprotein efficiently accepts surface but not internal oxidised lipids from oxidised low density lipoprotein

Running title: Transfer of oxidised lipid from LDL to HDL

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Abstract

Objective- Oxidised low density lipoprotein (oxLDL) contributes to atherosclerosis, whereas high density lipoprotein (HDL) is known to be atheroprotective due, at least in part, to its ability to remove oxidised lipids from oxLDL. The molecular details of the lipid transfer process are not fully understood. We aimed to identify major oxidised lipid species of oxLDL and investigate their transfer upon co-incubation with HDL with varying levels of oxidation.

Approach and Results- Seven major species of oxidised phosphatidylcholine and oxidised cholesteryl ester from oxLDL were identified using an untargeted mass spectrometry approach. HDL obtained from pooled plasma of normolipidemic subjects (N=5) was oxidised under mild and heavy oxidative conditions. Unoxidised (native) HDL and oxidised HDL were co-incubated with oxLDL, re-isolated and lipidomic analysis was performed. Lipoprotein surface lipids, oxidised phosphatidylcholines and oxidised cholesterols (7-ketocholesterol and 7 β -hydroxycholesterol), but not internal oxidised cholesteryl esters, were effectively transferred to native HDL. Saturated and monounsaturated lyso-phosphatidylcholines were also transferred from the oxLDL to native HDL. These processes were attenuated when HDL was oxidised under mild and strong oxidative conditions. The impaired capacities were accompanied by an increase in a ratio of sphingomyelin to phosphatidylcholine and a reduction in phosphatidylserine content in oxidised HDL, both of which are potentially important regulators of the oxidised lipid transfer capacity of HDL.

Conclusions- Our study has revealed the differential transfer efficiency of surface and internal oxidised lipids from oxLDL and their acceptance onto HDL. These capacities were modulated when HDL was itself oxidised.

Word count: 244 words

Keywords: lipidomic, cholesteryl ester, oxysterols, phosphatidylcholine, transfer, anti-oxidative

Non-standard Abbreviations and Acronyms

CE	Cholesteryl ester
LC-MS	Liquid chromatography coupled with mass spectrometry
LPC	Lyso-phosphatidylcholine
MUFA	Monounsaturated fatty acids
OxCE	Oxidised cholesteryl ester
OxHDL	Oxidised high density lipoprotein
OxLDL	Oxidised low density lipoprotein
OxPC	Oxidised phosphatidylcholine
PC	Phosphatidylcholine
PS	Phosphatidylserine
SFA	Saturated fatty acids
SM	Sphingomyelin

1 Introduction

Oxidised low density lipoprotein (oxLDL) contributes to atherosclerosis¹⁻³. Lipid oxidation products including oxidised phosphatidylcholine (oxPC), oxidised cholesteryl ester (oxCE) and lyso-phosphatidylcholine (LPC) which is produced by the action of phospholipases on oxPC, have been detected and characterised in oxLDL⁴, plasma⁵ and atherosclerotic lesions⁶. These oxidation products may represent bioactive lipids with potential pro-inflammatory capacity that affect plaque progression and stability⁴.

High density lipoprotein (HDL) is known to possess atheroprotective capacities including anti-oxidative properties^{7, 8}. Studies have characterised HDL anti-oxidative properties by its ability to delay LDL oxidation, typically monitored by the production of conjugated diene. There is also a growing body of evidence highlighting the defective anti-oxidative capacity of HDL in atherosclerosis. Decreased protection of LDL against oxidation^{9, 10}, and triglyceride and serum amyloid A enrichment of HDL¹¹ have all been associated with its impaired anti-oxidative capacity. Earlier studies on HDL subpopulations from normolipidemic individuals showed that small dense HDL3c exhibited the greatest potency in inhibiting LDL oxidation. Analysis of the lipid composition demonstrated that the small dense HDL3c was preferentially enriched in sphingosine-1-phosphate, phosphatidylserine (PS), and phosphatidic acid, and was depleted in sphingomyelin (SM)^{12, 13}.

HDL has also been reported to remove and inactivate lipid hydroperoxides from oxLDL upon co-incubation^{14, 15}. These processes are governed by the redox status of HDL-associated apolipoprotein AI (apoAI), and the surface rigidity of the phospholipid monolayer of the acceptor HDL (as determined by the ratio of sphingomyelin/phosphatidylcholine)¹⁴. This ability to transfer oxidised lipids from oxLDL to HDL may play an important part of the overall HDL anti-oxidative capacity. However, the molecular details of the oxidised lipid transfer capacity of HDL are not yet fully elucidated. Our knowledge on the identities of the oxidised lipids that are actually transferred by HDL is limited partly because studies have relied heavily on conjugated diene measurement and HPLC chemiluminescence coupled with UV detection measurement of lipid hydroperoxides. Here we aimed to oxidise lipoproteins in an artificial system using copper chloride and to identify major oxidised lipid species of oxLDL including oxPC, oxCE, and oxidised cholesterol and investigate their transfer upon co-incubation with HDL with varying levels of oxidation.

2 Materials and Methods

2.1 Isolation of human LDL and HDL

Five healthy non-diabetic normolipidemic male and female volunteers aged between 25 to 65 years were recruited for the study. All subjects provided informed written consent. They fasted overnight (≥ 12 hours) prior to the blood collection. Venous blood was collected from the antecubital vein into sterile EDTA tubes. Plasma was isolated, pooled, and sucrose was added (final concentration of 0.6 %) as a cryoprotectant for lipoproteins^{16, 17} prior to storage at -80°C . Each aliquot of plasma was thawed only once before the isolation of lipoproteins.

LDL and HDL were isolated by sequential ultracentrifugation¹⁸. EDTA was added to plasma to give a final concentration of 2 mmol/l. The samples were centrifuged in an Optima MAX-TL ultracentrifuge (Beckman Coulter, New South Wales, Australia) using TLA 120.2 rotor at 435,680 xg (100,000 rpm), 16°C for 3 hr for the isolation of each lipoprotein. LDL (density of 1.019 g/ml - 1.063 g/ml) and HDL (density of 1.063 g/ml to 1.21 g/ml) were dialysed against phosphate-buffered saline, pH 7.4 containing 5 $\mu\text{mol/l}$ of EDTA with three buffer changes (225x sample volume) over 24 hr. Sucrose was added to aliquots of lipoprotein fractions to give a final concentration of 10% (w/v); this is to preserve the lipoprotein function¹⁹ prior to storage at -80°C .

2.2 Lipid extraction

Aliquots of isolated lipoproteins (100 μl of approximately 0.1 mg protein/ml of LDL and 0.3 mg protein/ml of HDL) were lyophilised and subsequently reconstituted in 10 μl of deionised water for lipid extraction. Lipoprotein lipids were extracted as previously described²⁰. Briefly, lipoprotein samples (10 μl) were combined with 15 μl of internal standards mix (Supplemental Table I) containing 10,000 pmol cholesterol (d7), 1,000 pmol cholesteryl ester 18:0 (d6), 100 pmol 7-ketocholesterol (d7), 100 pmol lysophosphatidylcholine 13:0, 100 pmol phosphatidylcholine 13:0/13:0, 100 pmol phosphatidylserine 17:0/17:0, and 200 pmol sphingomyelin C12:0, per sample. The lipids were extracted using 200 μl chloroform:methanol (2:1). The mixtures were briefly vortexed, mixed for 10 min (on a rotary mixer), sonicated for 30 min and then allowed to stand at room temperature for 20 min before they were centrifuged at 16,000 xg, room temperature for 10 min. The supernatant containing extracted lipids were dried under a stream of nitrogen at 40°C and subsequently reconstituted in 100 μl of a mixture of water saturated butanol and methanol (1:1) containing 5 mmol/l of ammonium formate.

2.3 Identification of oxPC and oxCE by untargeted lipidomics

To identify the major oxidised lipids in oxLDL, we performed a time-course oxidation of LDL (1 mg protein/ml) with 8 $\mu\text{mol/l}$ copper sulphate for 1, 2, 4, 8, and 24 hr at 37°C . The lipids were subsequently extracted and analysed using an Agilent 1200 liquid chromatography system with an Agilent 6520 quadrupole-time of flight mass spectrometer (LC-MS). Lipid extracts (5 μl) were injected on a 2.1 x 100 mm C18 Zorbax-Eclipse column (Agilent, USA) at 250 $\mu\text{l/min}$. The following gradient conditions were used: 0 % B to 40 % B over 4 min, 40 % B to 63.6 % B over 13 min, 63.6 % B to 100 % B over 12 min, 100% B over 3 min, and a return to 0 % B over 1 min, followed by 0 % B over 7 min. Solvents A and B consisted of water:terahydrofuran:methanol in the ratio of 60:20:20 and 5:75:20 respectively, both containing 10 mmol/l ammonium formate. Analysis was conducted using MZmine 2.10, developed by Orešič *et al.*^{21, 22} and Pluskal *et al.*²³.

2.4 Assessment of the transfer of oxidised lipids from oxLDL to HDL

The ability of HDL to act as an acceptor of oxidised lipids from oxLDL was assessed by incubating HDL with oxLDL at a protein ratio of 3:1 (HDL:LDL). This ratio provides approximately equal amounts of HDL and LDL lipids in the assay system. The lipoproteins were then re-isolated and the lipid composition was analysed by liquid chromatography electrospray ionisation tandem mass spectrometry.

Prior to co-incubation with HDL, LDL (0.6 mg protein/ml) was oxidised with 6 μ mol/l copper chloride for 2 hr. Oxidised LDL and HDL with final protein concentrations of 0.1 mg/ml and 0.3 mg/ml respectively were co-incubated in the presence of 120 μ mol/l EDTA at 37°C for 2 hr. To assess the effect of oxidation on the ability of HDL as an acceptor of oxidised lipids, HDL (1.8 mg protein/ml) was oxidised with 6 μ mol/l copper chloride for 60 and 200 min to obtain mildly and heavily oxidised forms of HDL, respectively. The mildly- and heavily-oxHDL were co-incubated with oxLDL as described above. Following co-incubation, the lipoproteins were re-isolated using a similar density ultracentrifugation procedure method as described above. Briefly, aliquots of 400 μ l mixture of LDL and HDL were adjusted to a final density of 1.063 g/ml using NaBr solution and then overlaid with the same NaBr solution to a total volume of 1.0 ml. The samples were centrifuged (435,680 xg, 16°C, 3 hr). The LDL (density of 1.019 g/ml - 1.063 g/ml) was aspirated with the top 400 μ l of the density gradient. The HDL (density of 1.063 g/ml to 1.21 g/ml) was isolated in the lower 400 μ l of the density gradient. The intermediate layer (200 μ l) in between the LDL and HDL was analysed and found to contain only 7-10 % of lipids, demonstrating clear separation of the LDL and HDL fractions. Further, SDS-PAGE analysis of the reisolated oxLDL and HDL showed no cross contamination of ApoA1 and ApoB (data not shown). The re-isolated lipoproteins were dialysed against phosphate-buffered saline (100 x total sample volume) containing 5 μ M EDTA overnight. Aliquots of 100 μ l of the samples were frozen at -80°C and then lyophilised. They were reconstituted in 10 μ l of deionised water prior to lipid extraction as described above.

2.5 Lipid analysis by liquid chromatography electrospray ionisation tandem mass spectrometry

Lipids were quantified using multiple reaction monitoring on an Agilent 1200 HPLC system coupled to a QTrap 4000 triple quadrupole mass spectrometer (Applied Biosystems) using methodology similar to that described previously ²⁰ (Supplemental Table I). Liquid chromatography separation was performed on a 2.1 x 100 mm C18 Poroshell column (Agilent, USA) at 300 μ l/min. The following gradient conditions were used: 10 % B to 100 % B over 13 min, 100 % B over 3 min, and a return 10 % B over 1 min, followed by 10 % B over 3 min. Solvents A and B consisted of water:tetrahydrofuran:methanol in the ratio of 60:20:20 and 5:75:20 respectively, both containing 10 mmol/l ammonium formate.

The oxPC and oxCE species identified using the untargeted LC-MS approach described above (Table 1) were added to the multiple reaction monitoring list for quantification using Q3 product ion values of 184.1 and 369.4 for oxPCs and oxCEs respectively; Declustering, entrance, and cell exit potential were set at 100, 10, and 11 V, respectively for oxPC species, and 30, 10, and 12 respectively for oxCE species (Supplemental Table I). The collision energy was set at 45 V for oxPC species, and 20 V for oxCE species. Retention times were established by comparing oxLDL with native LDL (Table 1). Additionally, 7-ketocholesterol and 7 β -hydroxycholesterol, known products of cholesterol oxidation ⁵, were included in the targeted analysis. Multiple reaction monitoring transitions and tandem mass spectrometry conditions for these lipids were established by comparison against authentic standards and quantification was achieved by comparison against corresponding deuterium labelled standard of 7-ketocholesterol ⁵ (Supplemental Table I). Tandem mass spectrometry conditions for other lipids we analysed including cholesteryl ester (CE), cholesterol, phosphatidylcholine (PC), LPC, SM and PS were listed in Supplemental Table I.

Peak integration was carried out using MultiQuant software v.2.1.1. Relative lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding internal standard (Supplemental Table I). Total lipids of each class were calculated as the sum of the relative concentration of individual lipid species within the class

²⁰.

2.6 Data analysis and statistics

To correct for differences in sample recovery following re-isolation of the lipoprotein fractions, the concentration of oxidised lipids, oxPC, oxCE, oxysterols (7-ketocholesterol and 7 β -hydroxycholesterol) as well as LPC were expressed relative to total level of saturated and/or monounsaturated fatty acid-containing PC (SFA+MUFA PC). These species were found to be resistant to oxidation and so represent a stable factor for normalisation. OxCE and oxysterols were also normalised to SFA- and MUFA-containing CE and cholesterol, respectively to confirm what we observed with the earlier normalisation method (ie. relative to SFA+MUFA PC). Other lipids such as SM and PS were expressed relative to levels of total PC to reflect the relative contribution these lipid classes made to the surface lipids of the lipoprotein particles. Normalisation of lipid concentration to protein content was not possible in this case as the re-isolation of lipoproteins and subsequent dialysis resulted in the dilution of samples and thus low and inaccurate protein estimates. Statistical significance between sample groups was determined using Student t-tests.

3 Results

3.1 Identification of the major oxPC and oxCE species in oxLDL

In order to measure the transfer of oxidised lipids from oxLDL to HDL, we first characterised the major oxidised lipid species present in the oxLDL using untargeted LC-MS. Untargeted LC-MS analysis was performed on LDL which had been subjected to oxidation for 0, 1, 2, 4, 8 and 24 hr. Upon oxidation multiple new features were evident in the lipidomic analysis of the HDL (Figure 1). While complete characterisation of these new species was beyond the scope of the current work, we observed several features which correspond to the addition of one or two oxygen atoms to major species of PC (PC (34:3), PC (34:2), PC (36:3), PC (36:2)), and CE (CE (16:1), CE (16:0), CE (18:3), CE (18:2), CE (18:1)) present in LDL (Figure 1, Table 1). The absence of significant oxidation products arising from PC containing SFA- and MUFA indicates their resistance to oxidation and this supports our normalisation of the levels of oxidised lipids and LPC to these lipids.

The experimentally determined mass of these features was within 10 mDa of the mass of the proposed oxidised lipid, and they were consistent with the previously reported masses of the oxidised lipid species (Table 1). The retention times of these species were earlier than their corresponding non-oxidised counterparts consistent with an increase in polarity upon oxidation. Product ion analysis of these newly identified species showed major product ions of m/z 184.1 and m/z 369.4 corresponding to the phosphocholine head group and cholesterol respectively.

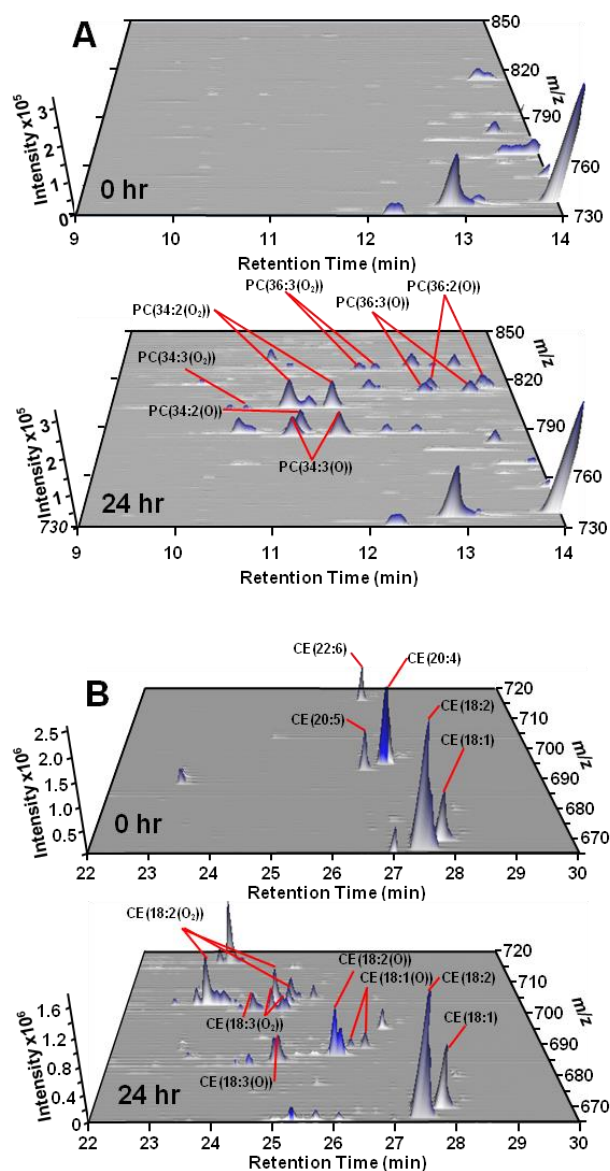


Figure 1. Lipidomic analysis of native and oxidised LDL. LDL was oxidised for 24 hr with copper sulphate as described in Materials and Methods. Lipids were extracted and untargeted lipidomic analysis was performed. Panel A shows the region of the analysis corresponding to oxPC while Panel B shows the region corresponding to oxCE.

Table 1. Comparison of the observed and exact masses for the oxidised lipid species identified in copper-catalysed oxidation of LDL via untargeted LC-MS analysis

Oxidised species*	Parent ion	Observed mass	Exact mass	Error (mDa)	Retention time (min)†	Previous reports
PC (34:3(O))	[M+H] ⁺	772.551	772.549	2	9.8	4
PC (34:2(O))	[M+H] ⁺	774.561	774.564	-3	9.0	4
PC (34:3(O ₂))	[M+H] ⁺	788.537	788.544	-7	8.8	4
PC (34:2(O ₂))	[M+H] ⁺	790.551	790.559	-8	8.8	4
PC (36:3(O))	[M+H] ⁺	800.577	800.58	-3	10.2	4
PC (36:2(O))	[M+H] ⁺	802.590	802.596	-6	10.7	4
PC (36:3(O ₂))	[M+H] ⁺	816.570	816.575	-5	8.9	4
CE (16:1(O))	[M+NH ₄] ⁺	656.598	656.598	0	13.0	-
CE (16:0(O))	[M+NH ₄] ⁺	658.611	658.613	-2	13.1	-
CE (18:3(O))	[M+NH ₄] ⁺	680.598	680.598	0	12.8	6
CE (18:2(O))	[M+NH ₄] ⁺	682.605	682.613	-8	13.6	6
CE (18:1(O))	[M+NH ₄] ⁺	684.628	684.629	-1	13.6	-
CE (18:3(O ₂))	[M+NH ₄] ⁺	696.591	696.593	-2	12.9	6
CE (18:2(O ₂))	[M+NH ₄] ⁺	698.605	698.608	-3	12.8	6

*PC – phosphatidylcholine ; CE – cholesteryl ester

†Retention time was based on LC run on a 2.1 x 100 mm C18 Poroshell column at 300 µl/min as described in Materials and Methods.

Extracted ion chromatograms of these features (Figure 2, Supplemental Figure I) demonstrate that the signal intensity increased with oxidation time. The chromatograms represent a mixture of isotopologues and in many instances, the oxidation has led to multiple chromatographic features consistent with multiple isomeric products presumably differing in the location of the oxygen(s). We have not further characterised these isomeric species but represent them with a O or O₂ in parenthesis following the sum composition of the fatty acid

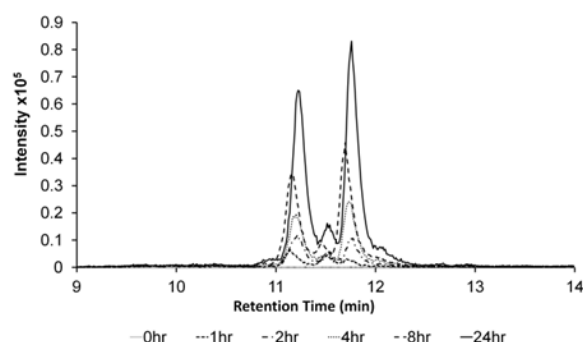


Figure 2. Extracted ion chromatogram ($m/z = 772.5440 - 772.5540$) of the oxidised lipid corresponding to PC(34:3(O)) in LDL. LDL was oxidised for 0, 1, 2, 4, 8, and 24 hr as described in the Materials and Methods. Lipids were extracted and untargeted lipidomic analysis was performed.

Following the initial identification of oxPC and oxCE species, we undertook to include these species in our previously established triple quadrupole based targeted lipidomics approach. MRM transitions for the oxidised lipids were established using product ions of 184.1 and 369.4 for the oxPC and oxCE respectively. Retention times were then established using the oxLDL samples.

3.2 HDL acceptance of oxidised lipids from oxLDL

To assess the ability of HDL to transfer and accept oxidised lipids from oxLDL, native, mildly-oxidised and heavily-oxidised HDL were co-incubated with oxLDL. The HDL and oxLDL were also incubated with buffer only as controls. The lipoproteins were re-isolated and the amount of the newly identified oxidised lipids species was measured as a percentage relative to the total level of SFA and MUFA containing PC (SFA+MUFA PC) by multiple reaction monitoring in tandem mass spectrometry.

3.2.1 Oxidised phosphatidylcholine (oxPC)

There was a significant net transfer of oxPC (sum of oxPC species measured) from the oxLDL to native (non-oxidised) HDL (Table 2). Relative to total level of SFA+MUFA PC, the concentration of oxPC in the re-isolated oxLDL decreased significantly (-67%, $P < 0.05$) compared to the oxLDL which was not incubated with HDL (i.e. oxLDL control) (Figure 3A, Table 2). In parallel, oxPC in the re-isolated HDL increased from 2.6% to 11% ($P < 0.001$) post-incubation with oxLDL (Figure 3B, Table 3). This process was modulated when pre-oxidised HDL under mild and heavy oxidative conditions were used. There was a 43% ($P = 0.09$) decrease and 27% ($P = 0.33$) increase of oxPC in the re-isolated oxLDL following incubation with mildly and heavily oxidised HDL, respectively (Figure 3A, Table 2). Correspondingly, there was an increase from 19% to 23% of oxPC ($P = 0.09$) and a decrease from 47% to 42% of oxPC ($P = 0.33$) in the mildly and heavily oxidised HDL, respectively (Figure 3B, Table 3).

3.2.2 Oxidised cholesteryl ester (oxCE)

As compared to the oxLDL control, there was no significant difference in the concentration of total oxCE (sum of all oxCE species measured) in the re-isolated oxLDL relative to SFA+MUFA PC, post-incubation with native HDL, mildly-, or heavily-oxHDL (Figure 3C, Table 2). However, an increase in oxCE (relative to SFA+MUFA PC) was observed in the re-isolated native HDL from 10% to 26% ($P < 0.05$); the increase in oxCE was attenuated in the mildly- and heavily-oxHDL (73% to 77%, $P = 0.78$ and 159% to 160%, $P = 0.91$ respectively) (Figure 3D, Table 3). Similarly, relative to SFA and MUFA containing CE (SFA+MUFA CE), analysis of oxCE showed no significant difference in the concentration of total oxCE in the re-isolated oxLDL post-incubation with native HDL, mildly-, or heavily-oxHDL (Supplemental Figure IIA). In addition, a 2.5-fold increase (3.6% to 8.6%, $P < 0.01$) in oxCE was observed in native HDL; whereas no significant change was observed in both mildly- and heavily-oxHDL (25% to 26%, $P = 0.90$ and 53% to 54%, $P = 0.90$, respectively) (Supplemental Figure IIB).

3.2.3 7-ketocholesterol and 7 β -hydroxycholesterol

Native HDL effectively reduced the concentration of LDL-derived oxidised cholesterol (7-ketocholesterol and 7 β -hydroxycholesterol) relative to SFA+MUFA PC by 48% and 66%, respectively ($P < 0.05$ for both) as compared with the oxLDL control (Table 2). In parallel, the concentration of total oxidised cholesterol (sum of 7-ketocholesterol and 7 β -hydroxycholesterol, relative to SFA+MUFA PC) in the re-isolated HDL increased from 0.004% to 2% ($P = 0.001$) (Table 3). However, this process was attenuated when HDL was oxidised under mild and heavy oxidative conditions; changes in oxLDL 7-ketocholesterol of -41% ($P = 0.08$) and 49% ($P = 0.43$) and of 7 β -hydroxycholesterol of -55% ($P = 0.06$) and -25% ($P = 0.34$) post-incubation with mildly- and heavily-oxHDL respectively were observed (Table 2). In addition, a smaller increase from 0.4% to 2% ($P < 0.05$) and a decrease from 6.3% to 4.7% ($P = 0.62$) in the total oxidised cholesterol levels (relative to total SFA- and MUFA-PC) in the mildly- and heavily-oxHDL, respectively was observed (Table 3). Consistent with these findings, analysis of the oxidised cholesterols relative to non-oxidised cholesterol revealed a reduction in the concentration of LDL-derived 7-ketocholesterol and 7 β -hydroxycholesterol by -21% ($P = 0.66$) and -44% ($P = 0.36$), respectively post incubation with native HDL; Furthermore, these levels were attenuated in oxLDL post incubation with mildly- and heavily-oxHDL (-8%, $P = 0.86$ and -19%, $P = 0.74$, of 7-ketocholesterol; and 28%, $P = 0.66$ and -11%, $P = 0.87$ of 7 β -hydroxycholesterol), respectively (Supplemental Table II). Correspondingly,

the total concentration of oxidised cholesterol in the re-isolated native HDL increased from 0.002% to 0.6% ($P<0.001$), but this was attenuated in mildly- and heavily-oxHDL (0.2% to 0.7%, $P<0.05$ and 3% to 2%, $P=0.57$) (Supplemental Table III).

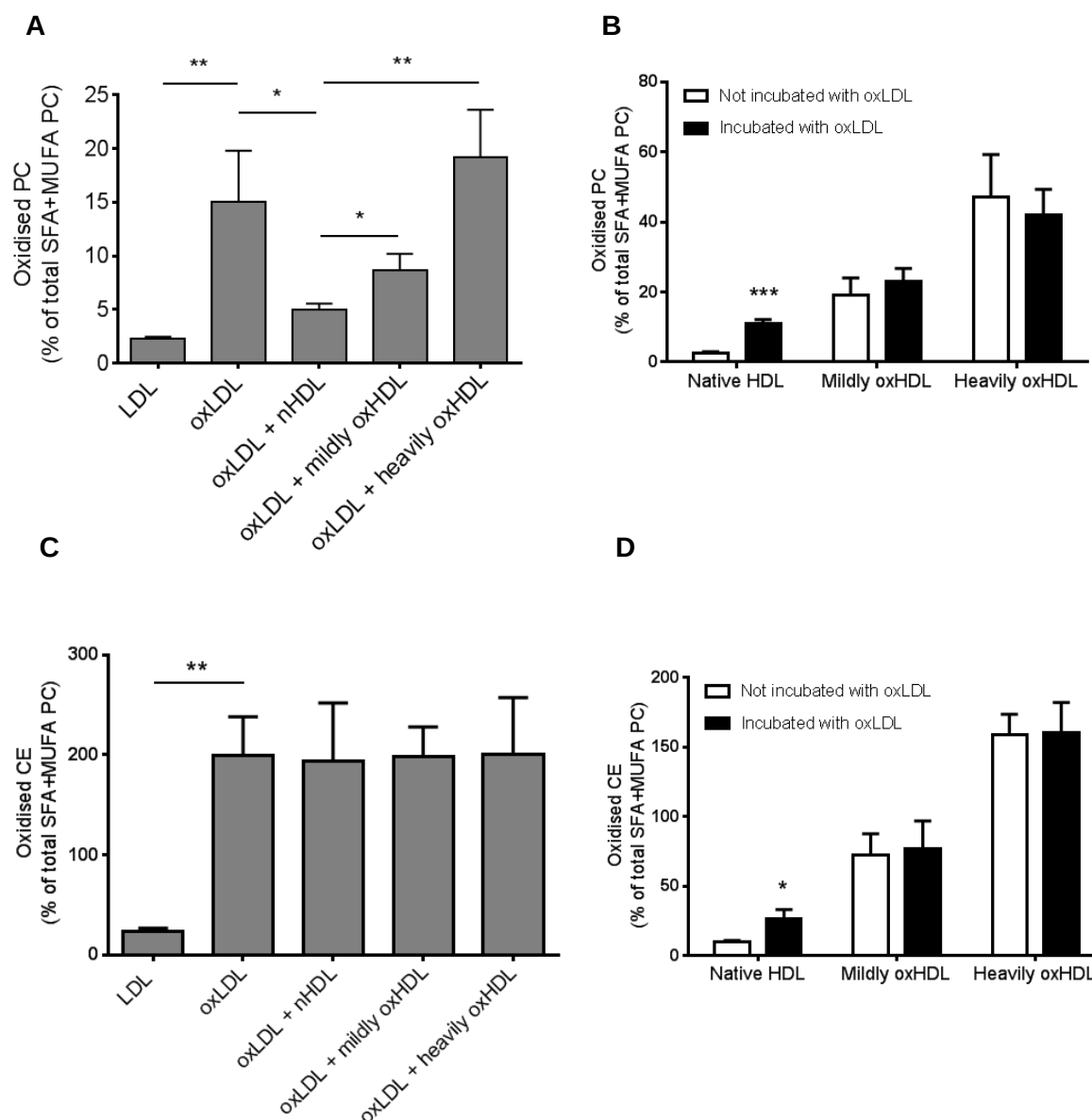


Figure 3. Effective transfer of oxidised PC, but not oxidised CE from oxidised LDL by HDL. Oxidised PC content in the re-isolated LDL (A); and re-isolated HDL (B), and oxidised CE content in the re-isolated LDL (C); and re-isolated HDL (D), expressed as mean $\pm$ SD of % of total PC containing saturated and monounsaturated fatty acids (SFA+MUFA PC). LDL denotes non-oxidised LDL; oxLDL denotes oxidised LDL which was not incubated with HDL (i.e. oxLDL control); oxLDL + nHDL denotes oxidised LDL which was co-incubated with native HDL; oxLDL + mildly oxHDL denotes oxidised LDL which was co-incubated with mildly oxidised HDL; and oxLDL + heavily oxHDL denotes oxidised LDL which was co-incubated with heavily oxidised HDL. Data on the re-isolated LDL (A) and (C) were compared to oxLDL unless indicated otherwise whereas data on the re-isolated HDL (B) and (D) were compared to their corresponding HDL samples which were not incubated with oxLDL. Data were analysed using Student t-test; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

1 **Table 2. Changes in the level of oxidised lipids in the re-isolated LDL after co-incubation.**

2

Assigned lipid name	LDL + nHDL*		LDL + mildly oxHDL [†]			LDL + heavily oxHDL [‡]		
	% change ^s	P-value	% change ^s	P-value	P-value (relative to nHDL) [#]	% change ^s	P-value	P-value (relative to nHDL) [#]
Oxidised PC								
PC (34:3(O))	-69	0.028	-46	0.134	0.165	22	0.381	0.000
PC (34:2(O))	-65	0.020	-38	0.102	0.007	24	0.384	0.007
PC (34:3(O ₂))	-84	0.025	-63	0.072	0.121	158	0.215	0.082
PC (34:2(O ₂))	-72	0.018	-44	0.079	0.005	3	0.871	0.000
PC (36:3(O))	-63	0.030	-45	0.086	0.062	7	0.745	0.000
PC (36:2(O))	-48	0.026	-26	0.116	0.021	13	0.567	0.024
PC (36:3(O ₂))	-70	0.028	-48	0.085	0.024	52	0.238	0.018
Total oxPC	-67	0.022	-42	0.090	0.017	27	0.334	0.005
Oxidised CE								
CE (16:0(O))	-5	0.890	-7	0.807	0.930	14	0.785	0.711
CE (16:1(O))	-14	0.648	-11	0.688	0.917	2	0.964	0.714
CE (18:1(O))	3	0.924	3	0.916	0.996	19	0.691	0.752
CE (18:2(O))	10	0.719	5	0.710	0.897	13	0.680	0.922
CE (18:2(O ₂))	-15	0.501	-3	0.879	0.298	-20	0.374	0.709
CE (18:3(O))	-2	0.905	-3	0.863	0.992	2	0.908	0.828
CE (18:3(O ₂))	-22	0.462	-18	0.481	0.871	-12	0.735	0.762
Total oxCE	-2	0.910	0	0.989	0.912	1	0.979	0.905
Oxidised cholesterol								
7-ketocholesterol	-48	0.030	-41	0.077	0.561	49	0.434	0.153
7-β hydroxycholesterol	-66	0.015	-55	0.066	0.536	-25	0.344	0.078
Total oxidised cholesterol	-49	0.029	-41	0.073	0.545	47	0.444	0.151

3

4 * LDL + nHDL - oxidised LDL which was co-incubated with native HDL.

- 1 [†] LDL + mildly oxHDL - oxidised LDL which was co-incubated with mildly oxidised HDL.
2 [‡] LDL + heavily oxHDL- oxidised LDL which was co-incubated with heavily oxidised HDL.
3 [§] Change (%) with reference to oxLDL (oxLDL which was not incubated with HDL).
4 ^{||} The sample groups (LDL with and without co-incubation with native, mildly oxidised and heavily oxidised HDL) were analysed using Student t-
5 test; values in bold indicate statistical significance ($P<0.05$).
6 [#] Indicates the significance of the difference between the level of oxidized lipid remaining in the oxLDL compared to the level remaining
7 following treatment with nHDL.

1

2 **Table 3. Changes in the levels of oxidised lipids in re-isolated HDL with or without co-incubation with oxLDL.**

Assigned lipid name	Native HDL			Mildly oxHDL			Heavily oxHDL		
	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]
Oxidised PC									
PC (34:3(O))	0.0600 ± 0.003	1.31 ± 0.157	0.000	1.61 ± 1.020	2.46 ± 0.781	0.319	5.50 ± 1.130	5.07 ± 0.686	0.604
PC (34:2(O))	0.950 ± 0.191	4.57 ± 0.320	0.000	6.64 ± 0.952	8.25 ± 0.865	0.096	10.8 ± 1.720	10.5 ± 1.10	0.778
PC (34:3(O ₂))	0.0200 ± 0.015	0.430 ± 0.083	0.001	0.550 ± 0.380	0.930 ± 0.296	0.251	5.73 ± 4.030	4.59 ± 2.33	0.695
PC (34:2(O ₂))	0.360 ± 0.068	1.52 ± 0.320	0.003	4.50 ± 1.210	7.49 ± 0.760	0.005	12.7 ± 4.270	10.8 ± 3.55	0.023
PC (36:3(O))	0.511 ± 0.084	1.29 ± 0.432	0.013	1.91 ± 0.899	2.48 ± 0.863	0.434	4.17 ± 0.659	3.58 ± 1.230	0.302
PC (36:2(O))	0.641 ± 0.120	1.44 ± 0.102	0.001	2.66 ± 0.324	2.95 ± 0.343	0.355	4.16 ± 0.556	4.02 ± 0.476	0.764
PC (36:3(O ₂))	0.112 ± 0.032	0.473 ± 0.080	0.002	1.16 ± 0.266	1.27 ± 0.169	0.566	4.07 ± 1.99	3.44 ± 1.250	0.656
Total oxPC	2.65 ± 0.325	11.0 ± 1.160	0.000	19.0 ± 5.000	22.9 ± 3.840	0.345	47.1 ± 12.200	41.9 ± 7.440	0.562
Oxidised CE									
CE (16:0(O))	0.00900 ± 0.015	0.153 ± 0.046	0.006	0.229 ± 0.091	0.264 ± 0.100	0.616	0.822 ± 0.181	0.856 ± 0.210	0.837
CE (16:1(O))	0.0230 ± 0.020	0.214 ± 0.090	0.023	0.340 ± 0.128	0.487 ± 0.150	0.265	1.34 ± 0.334	1.39 ± 0.360	0.866
CE (18:1(O))	0.524 ± 0.0340	2.67 ± 0.976	0.018	5.58 ± 1.83	6.69 ± 1.960	0.510	18.3 ± 4.540	19.0 ± 5.420	0.887
CE (18:2(O))	8.94 ± 1.000	16.0 ± 3.440	0.018	45.9 ± 5.38	45.7 ± 7.240	0.982	57.3 ± 22.000	59.3 ± 18.600	0.772
CE (18:2(O ₂))	0.508 ± 0.113	3.53 ± 1.69	0.032	11.3 ± 3.33	12.6 ± 4.610	0.699	37.6 ± 6.000	36.6 ± 9.100	0.868
CE (18:3(O))	0.181 ± 0.045	1.54 ± 0.507	0.010	6.52 ± 3.26	6.72 ± 3.340	0.945	12.1 ± 3.170	12.6 ± 1.880	0.837
CE (18:3(O ₂))	0.0460 ± 0.041	1.97 ± 0.945	0.024	2.63 ± 2.10	4.22 ± 3.230	0.511	31.2 ± 12.400	30.7 ± 13.200	0.964
Total oxCE	10.2 ± 0.679	26.1 ± 6.700	0.017	72.5 ± 15.0	76.8 ± 20.100	0.783	159 ± 14.900	160 ± 21.500	0.915
Oxidised cholesterol									
7-ketocholesterol	0.00 ± 0.000	2.00 ± 0.439	0.001	0.392 ± 0.382	1.93 ± 0.809	0.041	6.24 ± 4.740	4.62 ± 2.070	0.616
7-β hydroxycholesterol	0.00400 ± 0.001	0.0310 ± 0.014	0.033	0.0200 ± 0.013	0.0660 ± 0.061	0.266	0.0740 ± 0.049	0.0760 ± 0.051	0.951
Total oxidised cholesterol	0.00400 ± 0.001	2.03 ± 0.435	0.001	0.412 ± 0.390	1.99 ± 0.867	0.045	6.32 ± 4.790	4.70 ± 2.030	0.619

1

2 * Percentage level of oxidised lipids relative to total SFA+MUFA containing PC in re-isolated HDL which was incubated alone. Data is
3 expressed as mean $\pm$ SD.

4 † Percentage level of oxidised lipids relative to total SFA+MUFA containing PC in re-isolated HDL which was incubated with oxLDL. Data is
5 expressed as mean $\pm$ SD.

6 ‡ The sample groups (HDL with and without co-incubation with oxLDL) were analysed using Student t-test; values in bold indicate statistical
7 significance ($P<0.05$).

3.3 Transfer of lyso-phosphatidylcholine from oxLDL to HDL

We examined the profile of LPC containing SFA and MUFA which has previously been shown to be implicated in inflammation and LDL oxidation⁴. Upon oxidation of LDL there was a significant increase in the relative level of LPC species containing SFA and MUFA (Figure 4A). The same was observed in HDL with increasing oxidation levels (Figure 4B). Upon incubation of the oxLDL with HDL these LPC were effectively transferred to the HDL particles. We observed a 57% reduction of LPC 16:0 ($P<0.05$) in oxLDL when co-incubated with native HDL (Figure 4A, Supplemental Table IV) and this correlated with an increase of LPC 16:0 (relative to total SFA- and MUFA-PC) in the HDL from 5.8% to 18% ($P<0.001$) (Figure 4B, Supplemental Table V). This effect was diminished with the oxidised forms of HDL; we observed a 31% reduction ($P=0.21$) and a 33% increase ($P=0.30$) of LPC 16:0 in the re-isolated oxLDL following the co-incubation with mildly- and heavily-oxHDL, respectively (Figure 4A, Supplemental Table IV). This also correlated with less significant changes in the amount of LPC 16:0 in mildly- and heavily-oxHDL; An increase from 17% to 26% ($P=0.10$) and a decrease from 53% to 50% ($P=0.81$), respectively (Figure 4B, Supplemental Table V). These effects were also observed in other species of LPC with SFA and MUFA (Supplemental Table IV and V).

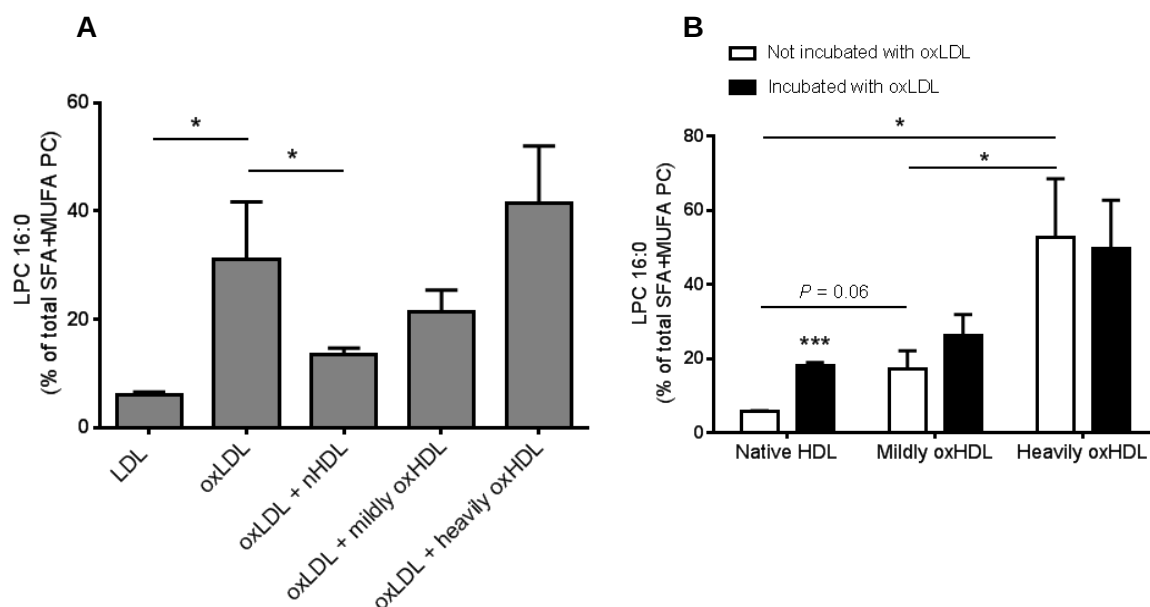


Figure 4. Transfer of lyso-phosphatidylcholine from oxLDL to native and oxHDL. Content of LPC 16:0 in (A) re-isolated LDL; and (B) re-isolated HDL. Data is expressed as mean $\pm$ SD of % of total PC containing saturated and monounsaturated fatty acids (SFA+MUFA PC). LDL denotes non-oxidised LDL; oxLDL denotes oxidised LDL which was not incubated with HDL (i.e. oxLDL control); OxLDL + nHDL denotes oxidised LDL which was co-incubated with native HDL; oxLDL + mildly oxHDL denotes oxidised LDL which was co-incubated with mildly oxidised HDL; and oxLDL + heavily oxHDL denotes oxidised LDL which was co-incubated with heavily oxidised HDL. Data on the re-isolated LDL (A) were compared to oxLDL whereas data on the re-isolated HDL (B) were compared to their corresponding HDL samples which were not incubated with oxLDL, unless indicated otherwise. Data were analysed using Student t-test; * $P<0.05$ and *** $P<0.001$.

3.4 Sphingomyelin to phosphatidylcholine ratio in native and oxidised HDL

The ability to transfer lipid hydroperoxides has been shown to be dependent on the ratio of SM to PC that contributes to the surface rigidity of phospholipid monolayer of the acceptor particle ¹⁴. Therefore, we examined the total level of SM relative to total PC of native and oxidised HDL to investigate whether the difference in the ratio could have affected the oxidised lipid and LPC transfer activity of HDL. The level of SM increased with increasing oxidative conditions of the HDL ($P < 0.05$ for all comparisons among the HDL samples which were not incubated with oxLDL) (Figure 5) and with decreasing ability to accept oxidised lipids (Figure 3, Table 3). The level of SM was further elevated in the re-isolated native HDL post-incubation with oxLDL, but there was no significant difference in the level of SM in the mildly- and heavily-oxHDL as compared to the corresponding samples which were incubated without oxLDL (Figure 5).

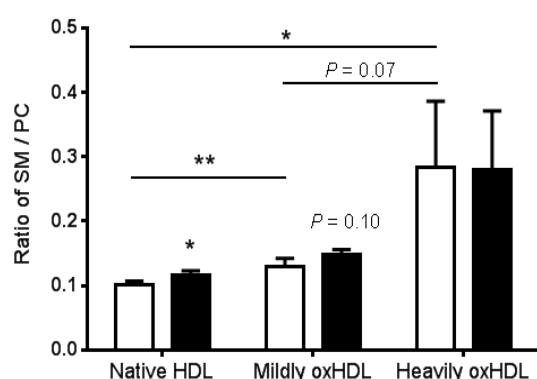


Figure 5. Level of sphingomyelin relative to total phosphatidylcholine as a contributing factor to the ability of HDL to transfer oxidised lipids. Open bars indicate HDL which was incubated without oxLDL. Closed bars indicate HDL which was incubated with oxLDL. Data is expressed as the ratio of total SM to total PC in native, and mildly- and heavily oxidised HDL. Data were compared to their corresponding HDL samples which were not incubated with oxLDL, unless indicated otherwise, and analysed using Student t-test; * $P < 0.05$ and ** $P < 0.01$.

3.5 Phosphatidylserine in the native and oxidised HDL

PS is a negatively charged lipid which has been shown to induce a conformational change of apoAI ²⁴. Thus, we analysed the concentration of total PS to investigate whether the change in the PS could have affected the oxidised lipid and LPC transfer capacity of HDL.

In HDL, the amount of PS was progressively decreased upon more severe oxidative conditions. There was no significant change in the level of PS in the samples which were incubated alone and incubated with oxLDL (Figure 6).

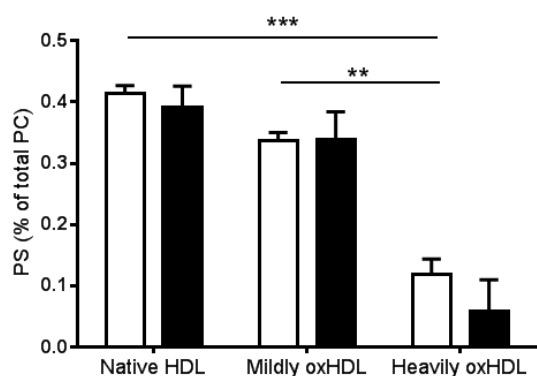


Figure 6. Phosphatidylserine in the native and oxidised HDL. Re-isolated HDL contents of total PS, expressed as mean $\pm$ SD of % of total PC. . Open bars indicate HDL which was incubated without oxLDL. Closed bars indicate HDL which was incubated with oxLDL. PS contents of the HDL samples which were not incubated with oxLDL were compared to each other and were analysed using Student t-test; ** $P < 0.01$, and *** $P < 0.001$.

4 Discussion

Using an untargeted lipidomic approach, our study has identified major oxidised lipid species in oxLDL, representing the internal and surface lipids of the lipoprotein. We expect oxPC and oxidised cholesterol to be found at the surface of lipoprotein particles while oxCE is expected to be more closely associated with the hydrophobic core. We find here that oxPC and oxidised cholesterol but not oxCE are effectively transferred from LDL to HDL. Oxidation of HDL attenuated these capacities.

While the exact structure of the oxidised lipids identified has not been fully elucidated and in most instances may represent isomeric mixtures of molecular species, the observed mass and chromatographic properties are consistent with these assignments. Additionally the proposed oxPC and oxCE species are consistent with oxidised lipids previously detected in human atherosclerotic plaques^{6, 25} and plasma of a rabbit model of atherosclerosis⁵, thus highlighting their physiological relevance. Further investigation is required to determine whether the products of lipid oxidation by copper chloride overlap with those produced by myeloperoxidase, an enzyme which catalyses lipoprotein oxidation²⁶ and has been shown to be associated with CAD²⁷.

Using these oxidised lipids as markers, we were able to assess the ability of native HDL and oxHDL to accept oxidised lipids from oxLDL particles. Upon co-incubation of the lipoproteins, we observed transfer of oxPC and oxidised cholesterol (7-ketocholesterol and 7 β -hydroxycholesterol) from oxLDL to HDL. This was clearly demonstrated by the marked reduction in the oxPC and oxidised cholesterol in oxLDL post-incubation with native HDL, and the corresponding increase in the oxPC and oxidised cholesterol in the HDL particles. This ability of HDL to act as an acceptor of oxPC and oxidised cholesterol is not limited to oxLDL particles; previous studies by Vila *et al*^{28, 29} demonstrated that phospholipid and cholesterol-derived hydroperoxides could be transferred spontaneously between cell membranes and LDL, while Terasaka *et al*³⁰ showed that 7-ketocholesterol, but not cholesterol was exported to HDL from ABCG1-transfected 293 cells.

Greenberg *et al*³¹ demonstrated that oxidative truncation to the *sn*-2 fatty acyl chain of phospholipids resulted in the re-orientation of the lipid in the membrane and its protrusion into the aqueous phase. It would be expected that this would effectively lower the free energy of activation for transfer between lipid surfaces thereby increasing the rate of transfer. Whilst the oxPC species measured in this study were not truncated, the addition of the oxygen would increase polarity potentially leading to a re-orientation within the surface lipid layer. This re-orientation may modulate lipid phase rigidity in the oxLDL particles resulting in a decrease in the free energy of activation and so facilitate the removal of the lipids by HDL when the lipoproteins interact. Lund-Katz and Phillips³² showed that the half-time of cholesterol exchange from human HDL to LDL at 37°C was 2.9 min whereas the half-time for dipalmitoyl-phosphatidylcholine was 5 hr. The large difference between these two molecules presumably relates to their free energy of activation, a function of size and polarity. Nonetheless the time scale is comparable to our experimental scale, particularly if the half-time of the oxPC exchange is decreased relative to non-oxidised PC as expected.

In contrast to the efficient transfer of oxPC and oxidised cholesterol from oxLDL to HDL we observed that oxCE species were transferred inefficiently with no significant reduction of oxCE in the oxLDL and only a small increase of oxCE in the HDL following co incubation. The same findings were obtained when we analysed the levels of oxCE and oxidised cholesterol relative to SFA- and MUFA- CE (Supplemental Figure II) and non-oxidised free cholesterol (Supplemental Table II and III), respectively, demonstrating that the amount of oxCE transferred is low relative to other major lipid constituents of the lipoprotein particles.

The larger size and lower polarity of the CE compared to the phospholipids and cholesterol, result in the CE being primarily located within the hydrophobic core of the lipoprotein particles^{33, 34}. Oxidation of the CE increases polarity, potentially driving the oxCE into the

surface lipid layer, and lowers the free energy of activation, as previously demonstrated by the preferential transfer of CE hydroperoxides from HDL to Hep G2 cells relative to non-oxidised CE³⁵. However, in this system oxidation of CE was insufficient to cause bulk movement of the oxCE from the LDL to the HDL particles.

Oxidation of both LDL and HDL leads to an increase in LPC species containing SFA and MUFA. In contrast, no significant changes were observed in the level of LPC containing polyunsaturated fatty acids in HDL and oxHDL, with or without co-incubation with oxLDL (data not shown). Similarly, in oxLDL, no changes were observed following co-incubation with HDL or oxHDL. Therefore, we focused our analyses on LPC species containing SFA and MUFA. LPC 16:0 is the most abundant species in LDL and following co-incubation with HDL showed similar trends with other SFA and MUFA containing LPC species (Supplementary Table IV and V). Therefore, we used LPC 16:0 as a representative species to demonstrate the changes of LPC in the lipoproteins following co-incubation. Oxidised PC are known to be preferred substrates for Lipoprotein-associated Phospholipase A₂ which cleaves the *sn*-2 fatty acid, typically the site of polyunsaturated fatty acids to yield the corresponding LPC species. In addition to the oxidised lipids, we observed a significant decrease in LPC 16:0 in LDL following incubation with HDL. In parallel, LPC 16:0 was elevated in the native HDL post-incubation with oxLDL. These suggest the net transfer of LPC from oxLDL to HDL.

In this study we observed a smaller reduction or a non-significant increase in oxidised lipids and LPC in oxLDL upon co-incubation with more heavily oxidised HDL. This can be attributed to the higher initial concentration of the oxidised lipids and LPC in the oxHDL particles, thus limiting their capacity to uptake more oxidised lipids. It is possible that the transfer of oxidised lipids and LPC can also proceed from the HDL to the LDL in situations where the oxidation of HDL is greater than the oxidation of LDL. However, further studies using stable isotope labelled lipid species will be required to confirm this. The mechanistic details of the transfer of oxidised lipids from oxLDL to HDL are yet to be elucidated. We speculate that lipidomic components of HDL play a role in the transfer of the oxidised lipids and LPC. A previous study using HPLC chemiluminescence and UV detection to measure lipid hydroperoxides have demonstrated that the transfer of a few molecular species of PC hydroperoxides can be influenced by the availability of the lipid-transfer protein (cholesteryl ester transfer protein) and the surface rigidity of the acceptor particle; a low ratio of SM/PC reduced surface rigidity and aided in the transfer efficiency of oxidised lipids, and in the delay of LDL oxidation^{14, 36}. Once transferred the lipid hydroperoxides were subsequently reduced to their respective hydroxides by HDL-associated ApoAI as first described by Garner *et al*³⁷. This process was governed by the total HDL content of apoAI and the redox status of the methionine residues of apoAI¹⁴. In light of this earlier study, we examined the level of SM relative to PC in our HDL acceptor particles. We demonstrated that the SM/PC ratio was increased in oxHDL due to a reduction in the content of the polyunsaturated PC (data not shown), thus suggesting a possible overall increase in the surface rigidity of the HDL upon oxidation. Consistent with our finding, native HDL with the lower ratio of SM/PC most effectively accepted oxidised lipids and LPC from oxLDL as compared to mildly- and heavily-oxHDL.

PS is a negatively charged lipid which has been reported to be enriched in HDL3c particles¹³. It induces a conformational change of apoAI²⁴, and facilitates the electrostatic interaction of the protein with polar phospholipids, allowing the penetration of the protein into the phospholipid monolayer^{13, 38}, of other lipoproteins. We observed a decrease in the PS content in HDL with increasing oxidative conditions. The high level of polyunsaturated fatty acids in PS relative to PC makes it more susceptible to oxidation and thus is likely to contribute to the observed decrease in the PS/PC ratio. In light of the previous studies, our findings suggest that a decrease in the PS content may lead to a modulation of the capacity of HDL-associated apoAI to penetrate and interact with the polar oxidised lipids in oxLDL, as

well as a general shift away from features of HDL3c which was associated with the greatest potency of anti-oxidative capacity. This notion is supported by our findings where the capacities of oxHDL to transfer and uptake oxidised lipids and LPC were impaired.

In conclusion, our study has demonstrated that while surface lipids (oxPC and oxidised cholesterol) are readily transferred from oxLDL to HDL, lipids located in the hydrophobic core of oxLDL (oxCE) are less readily transferred. This has important implication for the role of HDL in the prevention and/or reversal of atherosclerosis where oxCE makes up a major component of the lipid core of the atherosclerotic plaque. The composition of the HDL particle (SM/PC and PS content) appears to influence the antioxidant capacity opening the way for lipidomic assessment of HDL to quantify atheroprotective functionality which is currently not reflected in routine clinical measurement of circulating HDL-cholesterol levels. Our findings may also have relevance for the development of new HDL therapeutics where the lipid composition of such formulations may influence functionality and thereby efficacy.

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7 Disclosures

None.

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High density lipoprotein efficiently accepts surface but not internal oxidised lipids from oxidised low density lipoprotein

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Supplementary materials

Supplemental Table I. Conditions for liquid chromatography electrospray ionisation tandem mass spectrometry analysis of different lipid species

Lipid class	No. of species	Internal standard	Parent ion	Fragmentation*	Voltage settings (V) [†]			
					DP	EP	CollE	CXP
Cholesteryl ester	25	Cholesterol ester 18:0 (d6)	[M+NH ₄] ⁺	PI, m/z 369.3	30	10	20	12
Cholesterol	1	Cholesterol (d7)	[M+NH ₄] ⁺	PI, m/z 369.3	55	10	17	12
Lyso-phosphatidylcholine	22	Lysophosphatidylcholine 13:0	[M+H] ⁺	PI, m/z 184.1	100	10	45	11
Oxidised cholesteryl ester	7	Cholesterol ester 18:0 (d6)	[M+NH ₄] ⁺	PI, m/z 369.3	30	10	20	12
Oxidised phosphatidylcholine	7	Phosphatidylcholine 13:0/13:0	[M+H] ⁺	PI, m/z 184.1	100	10	45	11
Oxysterol (7-ketocholesterol)	1	7-ketocholesterol (d7)	[M+H] ⁺	PI, m/z 175.1	60	10	37	8
Oxysterol (7 β -hydroxycholesterol)	1	7-ketocholesterol (d7)	[M+H] ⁺	PI, m/z 159	80	10	35	24
Phosphatidylcholine	51	Phosphatidylcholine 13:0/13:0	[M+H] ⁺	PI, m/z 184.1	100	10	45	11
Phosphatidylserine	5	Phosphatidylserine 17:0/17:0	[M+H] ⁺	NL, 185 Da	86	10	29	16
Sphingomyelin	20	Sphingomyelin C12:0	[M+H] ⁺	PI, m/z 184.1	65	10	35	12

*Abbreviations for voltage settings: DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – cell exit potential.

[†] PI – product ion; NL – neutral loss.

Supplemental Table II. Changes in oxidised cholesterol levels relative to non-oxidised cholesterol in the re-isolated LDL

Assigned lipid name	LDL + nHDL*		LDL + mildly oxHDL [†]		LDL + heavily oxHDL [‡]	
	% change [§]	<i>P</i> -value	% change [§]	<i>P</i> -value	% change [§]	<i>P</i> -value
7-ketocholesterol	-21	0.662	-8	0.858	28	0.658
7- β hydroxycholesterol	-44	0.361	-19	0.738	-11	0.871
Total oxidised cholesterol	-42	0.061	-31	0.179	91	0.367

* LDL + nHDL - oxidised LDL which was co-incubated with native HDL.

[†] LDL + mildly oxHDL - oxidised LDL which was co-incubated with mildly oxidised HDL.

[‡] LDL + heavily oxHDL - oxidised LDL which was co-incubated with heavily oxidised HDL.

[§] Change (%) with reference to oxLDL (oxLDL which was not incubated with HDL).

^{||} The sample groups (LDL with and without co-incubation with native, mildly oxidised and heavily oxidised HDL) were analysed using Student t-test.

Supplemental Table III. Changes in oxidised cholesterol levels relative to non-oxidised cholesterol in the re-isolated HDL

Assigned lipid name	Native HDL		<i>P</i> -value [‡]	Mildly oxHDL		<i>P</i> -value [‡]	Heavily oxHDL		<i>P</i> -value [‡]
	without incubation (%) [*]	With incubation (%) [†]		without incubation (%) [*]	with incubation (%) [†]		without incubation (%) [*]	with incubation (%) [†]	
7-ketocholesterol	0.00 ± 0.00	16.5 ± 2.06	0.000	2.87 ± 2.69	15.1 ± 5.39	0.024	33.8 ± 30.1	25.1 ± 18.7	0.693
7-β hydroxycholesterol	0.0300 ± 0.00	0.262 ± 0.138	0.044	0.154 ± 0.100	0.509 ± 0.444	0.248	0.399 ± 0.319	0.465 ± 0.417	0.839
Total oxidised cholesterol	0.00200 ± 0.00	0.609 ± 0.100	0.000	0.196 ± 0.178	0.695 ± 0.178	0.027	2.63 ± 1.94	1.91 ± 0.562	0.573

* Percentage level of oxidised lipids relative to total non-oxidised cholesterol in re-isolated HDL which was incubated alone. Data is expressed as mean ±SD.

† Percentage level of oxidised lipids relative to total non-oxidised cholesterol in re-isolated HDL which was incubated with oxLDL. Data is expressed as mean ±SD.

‡ The sample groups (HDL with and without co-incubation with oxLDL) were analysed using Student t-test; values in bold indicate statistical significance (*P*<0.05).

Supplemental Table IV. Changes in the level of saturated and monounsaturated lyso-phosphatidylcholine in the re-isolated LDL after co-incubation.

Lipid species	% of change*		
	oxLDL + nHDL [†]	oxLDL + mildly oxHDL [‡]	oxLDL + heavily oxHDL [§]
LPC 14:0	-51	-33	40
LPC 15:0	-55	-34	45
LPC 16:0	-57	-31	33
LPC 16:1	-70	-40	-29
LPC 17:0	-58	-43	27
LPC 17:1	-100	-9	59
LPC 18:0	-41	-25	40
LPC 18:1	-41	-32	5
LPC 20:0	-49	-46	-3
LPC 20:1	-18	-12	-5

* Percentage of change of oxLDL with reference to oxLDL (oxLDL which was not incubated with HDL); Values in bold indicate significant difference (at least $P < 0.05$) between the sample and the corresponding reference.

[†] oxLDL + nHDL - oxidised LDL which was co-incubated with native HDL.

[‡] oxLDL + mildly oxHDL - oxidised LDL which was co-incubated with mildly oxidised HDL.

[§] oxLDL + heavily oxHDL - oxidised LDL which was co-incubated with heavily oxidised HDL.

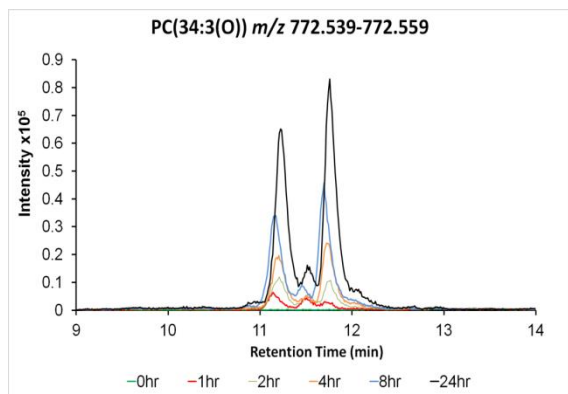
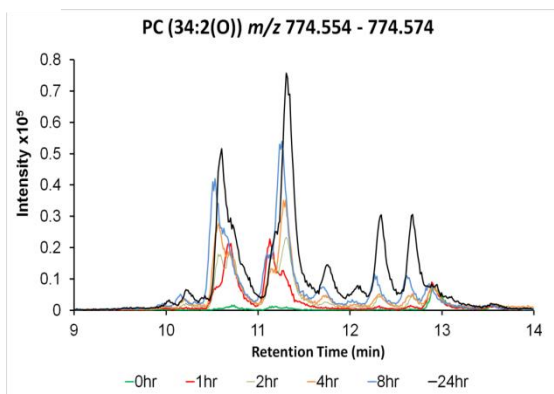
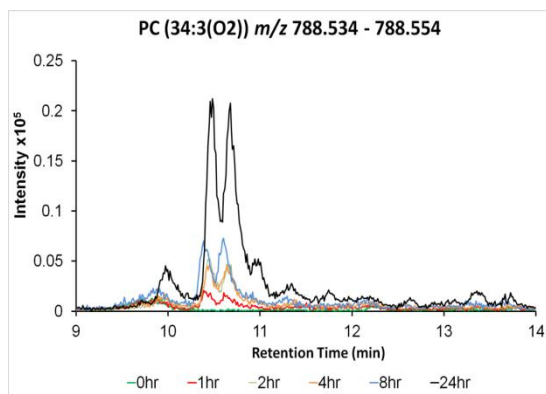
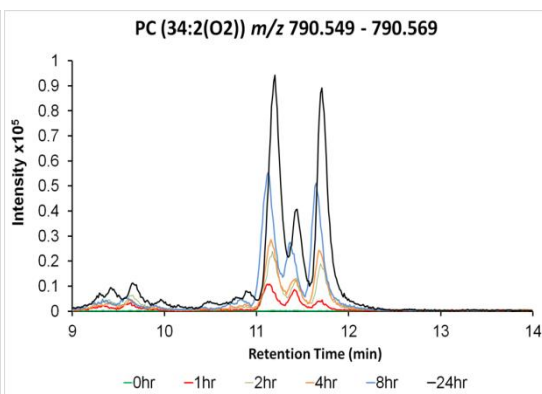
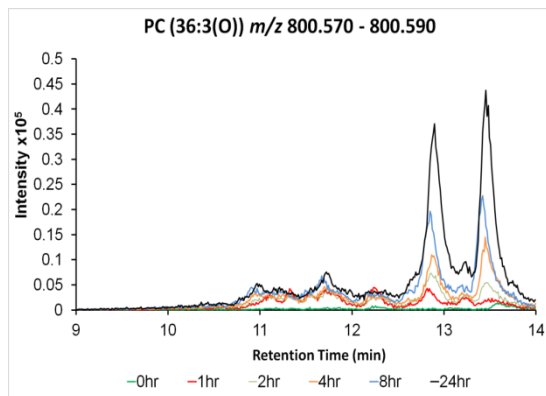
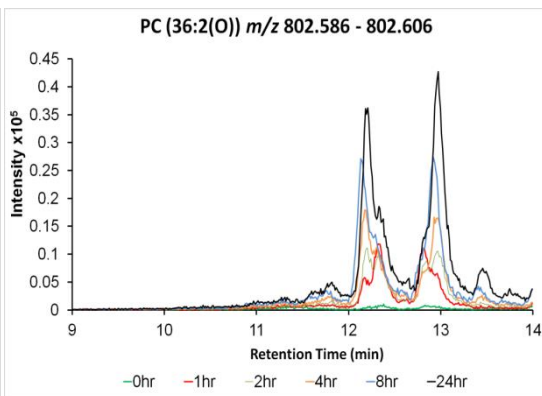
Supplemental Table V. Changes in the level of saturated and monounsaturated lyso-phosphatidylcholine in the re-isolated HDL after co-incubation.

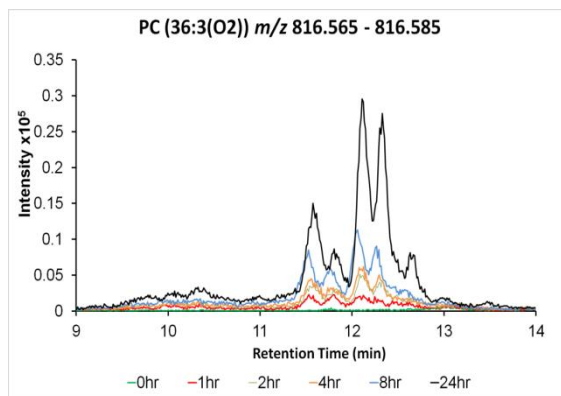
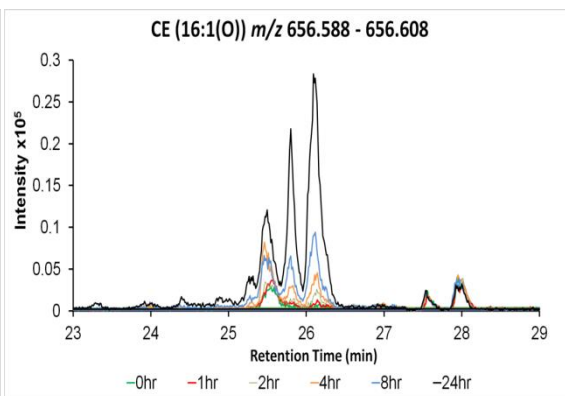
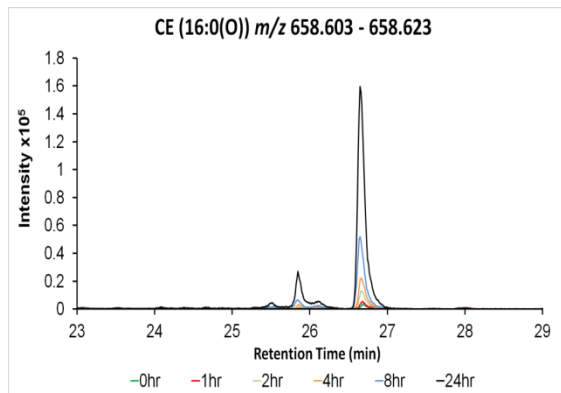
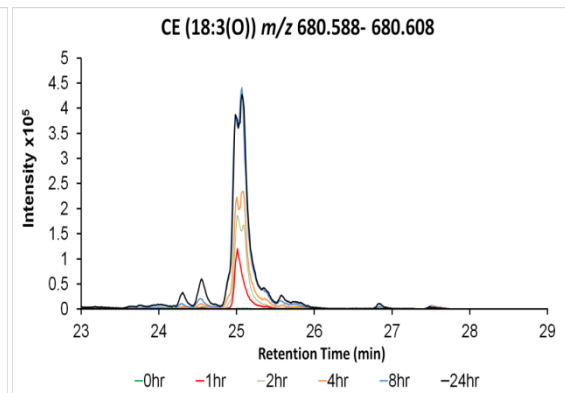
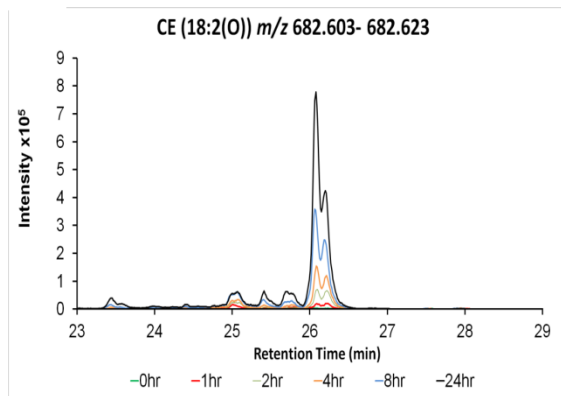
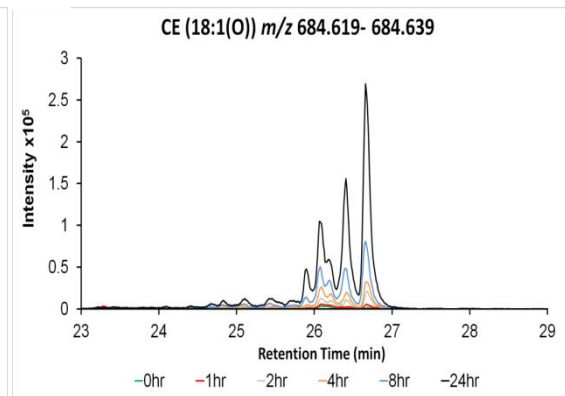
Lipid species	Native HDL			Mildly oxHDL			Heavily oxHDL		
	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]	without incubation (%) [*]	with incubation (%) [†]	<i>P</i> -value [‡]
LPC 14:0	0.0500 ± 0.00	0.154 ± 0.0140	0.000	0.121 ± 0.0220	0.224 ± 0.0380	0.015	0.401 ± 0.0910	0.402 ± 0.0990	0.997
LPC 15:0	0.0510 ± 0.00	0.135 ± 0.0100	0.000	0.152 ± 0.0260	0.222 ± 0.0390	0.061	0.485 ± 0.143	0.428 ± 0.0950	0.597
LPC 16:0	5.81 ± 0.0930	18.0 ± 0.969	0.000	17.3 ± 4.81	26.3 ± 5.59	0.101	52.6 ± 15.9	49.6 ± 13.1	0.812
LPC 16:1	0.00 ± 0.00	0.358 ± 0.0320	0.000	0.327 ± 0.0430	0.514 ± 0.0640	0.014	0.565 ± 0.490	0.921 ± 0.242	0.321
LPC 17:0	0.242 ± 0.0390	0.420 ± 0.0240	0.002	0.492 ± 0.0690	0.615 ± 0.104	0.165	1.28 ± 0.316	1.18 ± 0.250	0.685
LPC 17:1	0.0160 ± 0.0140	0.0420 ± 0.005	0.033	0.0360 ± 0.00	0.0520 ± 0.00	0.043	0.0970 ± 0.013	0.0920 ± 0.0160	0.692
LPC 18:0	3.21 ± 0.135	6.77 ± 0.475	0.000	6.44 ± 0.506	9.08 ± 1.59	0.051	16.5 ± 4.78	15.5 ± 3.33	0.778
LPC 18:1	2.68 ± 0.051	3.93 ± 0.250	0.001	3.51 ± 0.275	4.71 ± 0.312	0.007	6.94 ± 0.804	7.24 ± 1.43	0.766
LPC 20:0	0.0280 ± 0.00	0.0380 ± 0.00	0.018	0.0340 ± 0.00	0.0380 ± 0.00	0.059	0.0550 ± 0.00	0.0520 ± 0.00	0.683
LPC 20:1	0.0560 ± 0.00	0.0830 ± 0.0120	0.021	0.0770 ± 0.00	0.101 ± 0.00	0.009	0.136 ± 0.0110	0.145 ± 0.0210	0.539

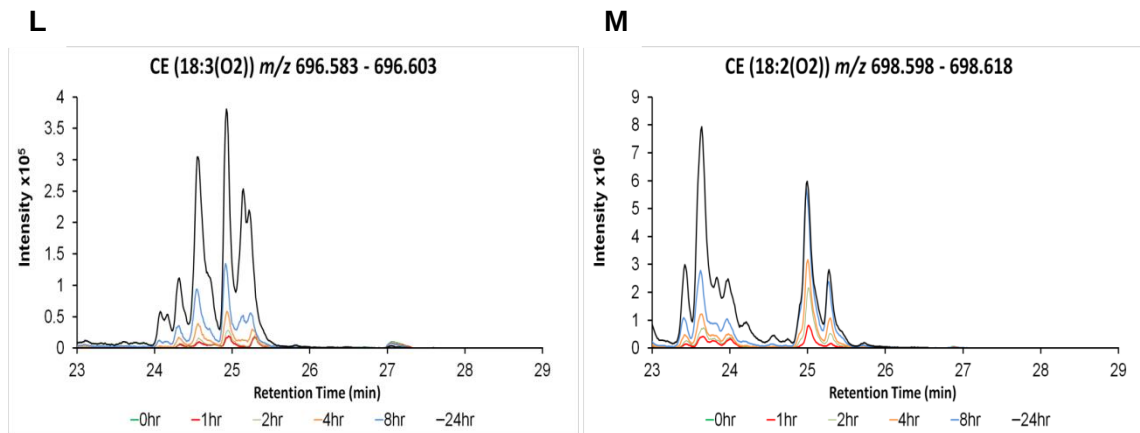
* Percentage level of LPC relative to total SFA+MUFA containing PC in re-isolated HDL which was incubated alone. Data is expressed as mean ± SD.

† Percentage level of LPC relative to total SFA+MUFA containing PC in re-isolated HDL which was incubated with oxLDL. Data is expressed as mean ± SD.

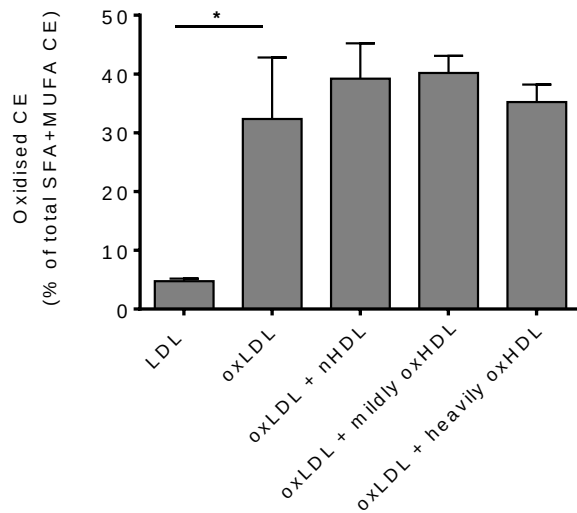
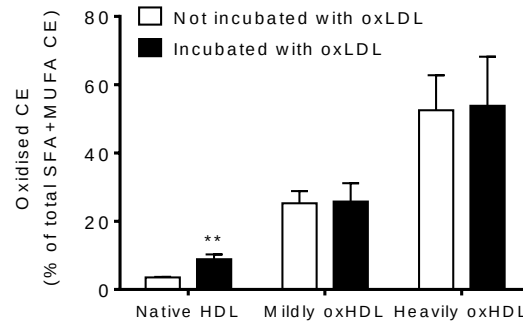
‡ The sample groups (HDL with and without co-incubation with oxLDL) were analysed using Student t-test; values in bold indicate statistical significance (*P*<0.05).

A**B****C****D****E****F**

G**H****H****I****J****K**



Supplemental Figure I. Extracted ion chromatograms of oxidised lipids from oxidised LDL, demonstrating increasing intensity with increasing oxidation time. (A) to (G) correspond to oxidised phosphatidylcholine and (H) to (M) correspond to oxidised cholesteryl ester.

A**B**

Supplemental Figure II. Transfer of oxidised cholesteryl ester from oxidised LDL to HDL of different oxidative stress levels. Oxidised CE content in the re-isolated LDL (A); and re-isolated HDL (B), expressed as mean $\pm$ SD of % of total CE containing saturated and monounsaturated fatty acids (SFA+MUFA CE). LDL denotes non-oxidised LDL; oxLDL denotes oxidised LDL which was not incubated with HDL (i.e. oxLDL control); oxLDL + nHDL denotes oxidised LDL which was co-incubated with native HDL; oxLDL + mildly oxHDL denotes oxidised LDL which was co-incubated with mildly oxidised HDL; and oxLDL + heavily oxHDL denotes oxidised LDL which was co-incubated with heavily oxidised HDL. Data on the re-isolated LDL (A) were compared to oxLDL whereas data on the re-isolated HDL (B) were compared to their corresponding HDL samples which were not incubated with oxLDL. Data were analysed using Student t-test; * $P < 0.05$, and ** $P < 0.01$.