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The lipidomic profile of the nanoparticle-biomolecule corona reflects the diversity of plasma lipids†

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The spontaneous self-assembly of biomolecules around the surface of nanoparticles (NPs) once exposed to plasma and other biofluids, has been termed the 'biomolecule corona'. While the protein composition of the biomolecule corona has been widely characterised, the interaction of NPs with the plasma lipidome has not been fully investigated. Here, we use targeted and untargeted lipidomics to analyse a wide spectrum of bioactive lipids adsorbed onto the surface of liposome NPs post-incubation with human plasma. Our data indicate that the biomolecule corona contains a diverse mixture of simple and complex lipid species, including sphingolipids such as ceramides and sphingomyelins, glycerolipids, glycerophospholipids, cholesteryl esters, as well as oxylipin and *N*-acyl ethanolamine derivatives of fatty acids. Although the corona lipidomic profiles reflected the overall composition of the plasma lipidome, mono-hydroxy- and oxo-fatty acid oxylipins, mono-, di- and tri- acylglycerols, sphingomyelins and ceramides showed a preferential binding for liposome NP surface. Interestingly, the biomolecule corona lipid profiles appeared to mirror those of the lipoprotein lipid cargo, suggesting that lipid species may be carried within the lipoprotein complexes attached to the corona. Proteomic analysis of corona-associated proteins showed the presence of several apolipoproteins (A-I, A-II, A-IV, B, C-I, C-III, C-IV, C2-C4, D, E, L, M and lipoprotein Lp(A)), supporting this notion. Our findings reveal the wide lipid diversity of the biomolecule corona and indicate a potential lipoprotein-mediated adsorption mechanism of lipids onto liposome NPs, highlighting the importance of bridging proteomics with lipidomics to fully comprehend the interactions at the bio-nano interface.

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Introduction

Once nanoparticles (NPs) are exposed to biological fluids, they are immediately covered by a shell of biomolecules. This spontaneous self-assembly of biomolecules onto the surface of NPs is termed the 'biomolecule corona'.^{1–3} The formation of the biomolecule corona alters the pristine physicochemical properties of NPs, confers them with a new identity and becomes their interface with biological systems.^{4,5} As the clinical devel-

opment and use of NP-based formulations is rising,⁶ it has become imperative to determine the molecular composition of the biomolecule corona and understand its impact on the bio-nano interactions. To date, the majority of studies focus on the protein composition of the NP-biomolecule corona, with only a few investigating its lipid^{7–17} and/or other components.^{18–28}

Lipids are a diverse group of biomolecules with distinct physicochemical properties allowing them to exhibit a range of bioactivities. They are key components of the cellular membrane, having both structural and signaling roles, and are known to be involved in physiological and pathophysiological processes, including inflammation, neuropathy, metabolic disorders, cardiovascular diseases, tumorigenesis and cancer.^{29–34} Human plasma contains an array of lipid species produced by the endothelium, platelets and other blood cells, adipose tissue, liver and various organs. These plasma lipids are transported *via* lipoproteins, albumin and other protein complexes.^{35,36} Recent plasma lipidomic studies have identified an array of glycerophospholipids, glycerolipids, sphingolipids including ceramides, sterol lipids, prenol lipids, as well

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as fatty acids (FA) and, their oxylipin and *N*-acyl ethanolamine (NAE) derivatives.^{34,37–43} As plasma lipid profiles can be affected by disease, they have been targeted as potential biomarkers.^{30,33,40–43} Particularly attractive for biomarker discovery are bioactive lipid mediators such as ceramides and oxylipins. Ceramides are potent sphingolipids involved in apoptotic pathways, and currently considered markers of cardiovascular disease with potential heritable characteristics, whilst oxylipins are oxygenated derivatives of polyunsaturated fatty acids (PUFA) with diverse properties and critical biological roles in a wide array of homeostatic and pathophysiological events, including systemic inflammation.^{31,40,44–46}

The large number and structural diversity of lipids found in biofluids necessitates the lipidomic analysis of the NP-biomolecule corona, in order to decipher the role and potential impact of corona-associated lipids on critical biological events occurring at the bio-nano interface, including NP cellular internalisation, active targeting, clearance and cytotoxicity. Hellstrand *et al.* were the first to report that copolymer NPs interact with cholesterol, triacylglycerols and phospholipids, as well as with high-density lipoprotein (HDL) complexes from human plasma.⁷ This work triggered more investigations into the lipid composition of the biomolecule corona formed in various biofluids (*e.g.* plasma, serum, pulmonary surfactant), using several experimental methods (*e.g.* cholesterol assays, glycerol assays, metabolomic- and lipidomic-based liquid chromatography mass spectrometry) and different NP types (*e.g.* titanium dioxide, iron oxide, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) liposomes, magnetic, gold, single-walled carbon nanotubes).^{8–23} These studies demonstrated the presence of glycerophospholipids (*e.g.* phosphatidylcholines (PC), phosphatidylinositols (PI), lysophosphatidylcholines (LPC), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (LPE)), glycerolipids (*e.g.* mono

(MAG)-, di (DAG)-, tri (TAG)-acylglycerols), sphingolipids (*e.g.* sphingomyelins (SM)), cholesterol and free fatty acids (FFA), as well as lipoprotein complexes. The lipid cargo of lipoproteins comprises simple lipids, such as ceramides, oxylipins and cholesterol, as well as complex glycerophospholipids, sphingolipids and acylglycerols,^{47,48} that may contribute to the lipid diversity of the NP-biomolecule corona.^{10,19} However, more work to fully characterise the lipidome of the biomolecule corona is needed to support this notion. Studies combining proteomics and lipidomics have also provided a better understanding of the interaction mechanism between the plasma components and NPs, as well as its impact at the bio-nano interface,^{9,11,12} suggesting that the complete characterisation of the biomolecule corona may be key to improving our knowledge on its formation and evolution.

To date, most studies examining the lipidome of the NP-biomolecule corona have reported the presence of glycerophospholipids, glycerolipids, sphingomyelins, cholesterol and some FFA species, while there is a notable lack of information on the presence of bioactive lipid mediators including ceramides and oxylipins.^{7–17} Liposome NPs are considered the most clinically established nanoplatform for drug delivery, yet their interaction with plasma lipids has been scarcely explored, with only one published study to date.¹⁰ La Barbera *et al.* demonstrated the presence of glycerophospholipids (PC, PE and PI), sphingolipids and a few FFA on positively-charged DOTAP liposomes using a metabolomics approach. Nevertheless, more work is required to assess the interactions of clinically used liposomal formulations with plasma lipids.

In this work, we present an in-depth lipidomic analysis of the plasma-derived liposome-biomolecule corona using both targeted and untargeted lipidomics (Table 1 and ESI Fig. S1A†). Our findings show that the liposome NP-corona is rich in simple and complex bioactive lipids, including an array of previously unexplored oxylipins, NAEs and ceramides, reflecting the diversity of the human plasma lipidome. Interestingly, liposome NPs show a preference towards mono-hydroxy- and oxo-FA oxylipins, 2-MAG, DAG, TAG, SM and ceramides. In addition, proteomic analysis of the biomolecule corona was carried out to examine potential links between the corona identified lipids and proteins. Our data reveal the presence of lipid-binding proteins in the corona, including lipoproteins and albumin, suggesting a potential protein-mediated interaction of liposome NPs with plasma lipids. Our results demonstrate that the biomolecule corona composition mimics the lipidomic fingerprints of the biofluid of origin and highlight the importance of integrating lipidomics with proteomics in future investigations of the bio-nano interface.

Results & discussion

Although there has been a strong interest and intense effort to comprehensively characterise the protein content of the NP-biomolecule corona, to date only a few studies have interrogated the spontaneous interaction of NPs with plasma



Marilena Hadjidemetriou

Marilena obtained a BSc in Pharmacy at the University of Athens. In 2013, she completed the MSc in Drug Delivery at the UCL School of Pharmacy and she then joined the Nanomedicine Lab at The University of Manchester as a Marie Curie Early-Stage Fellow. After her PhD, Marilena was awarded a Medical Research Council post-doctoral fellowship and the CRUK Pioneer Award to work on the nanoparticle-enabled discovery of blood biomarkers.

In 2018, Marilena was appointed as a Research Fellow and Group Leader in Nano-Omics and in 2020, she was appointed as Lecturer. Her group aims to develop multi-omics nanotechnology platforms to explore disease pathways and uncover molecular biomarkers.



Table 1 Lipid classes investigated in human plasma NP-biomolecule corona samples. Lipids were analysed by targeted (UPLC/ESI-MS/MS) or untargeted (UHPSFC/ESI-QTOF-MS^E) lipidomics. Lipid class profiles are presented in this manuscript in the following order: oxylipins, *N*-acyl ethanolamines (NAE), 2-monoacyl glycerols (2-MAG) and free fatty acids (FFA) (shown in Fig. 1); ceramides (shown in Fig. 2); glycerophospholipids, sphingomyelins, glycerolipids and cholesteryl esters (shown in Fig. 3)

Lipid category	Lipid class	Lipidomic analysis approach	Data shown in
Fatty acyls and derivatives	Oxylipins	Targeted (UPLC/ESI-MS/MS)	Fig. 1
	<i>N</i> -Acyl-ethanolamines (NAE)	Targeted (UPLC/ESI-MS/MS)	Fig. 1
	Free fatty acids (FFA)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 1
Sphingolipids	Ceramides	Targeted (UPLC/ESI-MS/MS)	Fig. 2
	Sphingomyelins (SM)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
Glycerolipids	Mono-acylglycerols (2-MAG)	Targeted (UPLC/ESI-MS/MS)	Fig. 1
	Di-acylglycerols (DAG)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
	Tri-acylglycerols (TAG)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
Glycerophospholipids	Phosphatidylcholines (PC)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
	LysoPC (LPC)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
	Phosphatidylethanolamines (PE)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
	Phosphatidylinositol (PI)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3
Sterol lipids	Cholesteryl esters (CE)	Untargeted (UHPSFC/ESI-QTOF-MS ^E)	Fig. 3

lipids.^{7–12,28} Blood contains a wide range of lipid species,^{34,37–43} yet our current knowledge of the lipidomic content of the NP-biomolecule corona is limited to the analysis of glycerophospholipids and glycerolipids.^{7–12,18} In this study we report an extensive lipidomic profile of the human plasma liposome NP-biomolecule corona that includes a range of glycerophospholipids (PC, LPC, PE, PI), sphingolipids (ceramides, SM), glycerolipids (DAG, TAG), FFA and fatty acyl derivatives of the oxylipin, NAE and 2-MAG classes (Table 1). We also perform a lipidomic analysis of whole plasma, plasma processed by the two-step purification protocol used to isolate the corona-coated NP (referred to as ‘purified plasma’) and of bare liposomes, as controls (see experimental section for details). Our results provide the first experimental evidence that the liposome NP-biomolecule corona contains bioactive lipid mediators such as ceramides and oxylipins, known to be involved in systemic inflammatory disease and also explored for biomarker discovery.^{30,32,40,43}

Overall, only very small amounts of some lipid species were detected in the purified plasma and/or bare liposomes controls (marked with # in Fig. 1–3), indicating that the corona lipid profiles mainly resulted from the adsorption of plasma lipids onto liposome NPs and were not artefacts of incomplete purification or components from the employed liposomal formulation. The liposome NP-corona purification protocol employed ensures retention of the tightly adsorbed molecules onto the NP surface, also referred to as the ‘hard corona’.

It should be mentioned that any lipids found in the NP-corona that were also identified at equal concentrations in bare liposomes were excluded from Fig. 1–3. These were either components identified by the supplier or impurities of the HSPC lipid used (ESI Table S2†) and include the NAE stearyl ethanolamine (STEA), the CER[NP] species N(26)P(17) and N(26)P(18), PC 34:0, PC 36:0, LPC 18:0, LPC 20:0, LPC 20:4, the FFA stearic acid (C18:0) and palmitic acid (C16:0), as well as the linoleic and alpha-linolenic acid oxylipin derivatives 9-HOTrE, 9(10)-EpOME, 9,10-DiHOME, 12,13-DiHOME (ESI Tables S3–S7†).

Oxylipin composition of the plasma liposome NP-biomolecule corona

A total of 38 oxylipins, derivatives of the PUFAs linoleic acid (LA; C18:2n-6), alpha-linolenic acid (ALA; C18:3n-3), dihomo-gamma-linolenic acid (DGLA; C20:3n-6), arachidonic acid (AA; C20:4n-6), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) were quantitated in human plasma samples using targeted lipidomics (UPLC/ESI-MS/MS)⁴⁹ (Fig. 1A and ESI Table S3†). These species were mainly derivatives of lipoxygenase (LOX) and cytochrome P450 monooxygenase/soluble epoxide hydrolase (CYP/sEH) enzymatic metabolism of PUFA. Although a number of cyclooxygenase (COX)-derived mediators were also detected, they were not consistently found to be above the limit of quantitation of the assay (data not shown).

Of these 38 plasma oxylipins, 19 species were found in the liposome NP-corona (Fig. 1A). A number of CYP/sEH epoxy- and dihydroxy-fatty acids (*i.e.* EpOME, EpDPE, DiHOME, DHET, DiHETE, DiHDPA) were found mainly in whole plasma, while monohydroxy- and oxo-fatty acids (*i.e.* oxo-ODE, HODE, *Trans*-EKODE, HOTrE, oxo-ETE, HETE, HEPE and HDHA) were also identified in the liposome NP-corona. These differences are reflected in the relative abundance of oxylipins, when grouped according to their enzymatic origin. Although 35% of the whole plasma oxylipins were LOX-derived monohydroxy-FA, their proportion in the liposome NP-corona increased to 81%, with no CYP/sEH-derived dihydro-FA species being identified in the corona (Fig. 1B). When grouped according to their precursor PUFA, 87% of the oxylipins found in whole plasma were LA-derivatives with only 5% derived from AA. However, the liposome NP-corona profiles differed, with 48% LA-derivatives and 32% AA-derivatives (Fig. 1C). Furthermore, the liposome NP-corona had no PUFA dihydroxy- and epoxy-FA compared with whole plasma (32% and 29% of these found in whole plasma, respectively), but had a higher abundance of oxo-FA derivatives (19% compared with 3% in whole plasma).

Oxylipins are a group of low abundance oxygenated derivatives of PUFA, better known for their involvement in



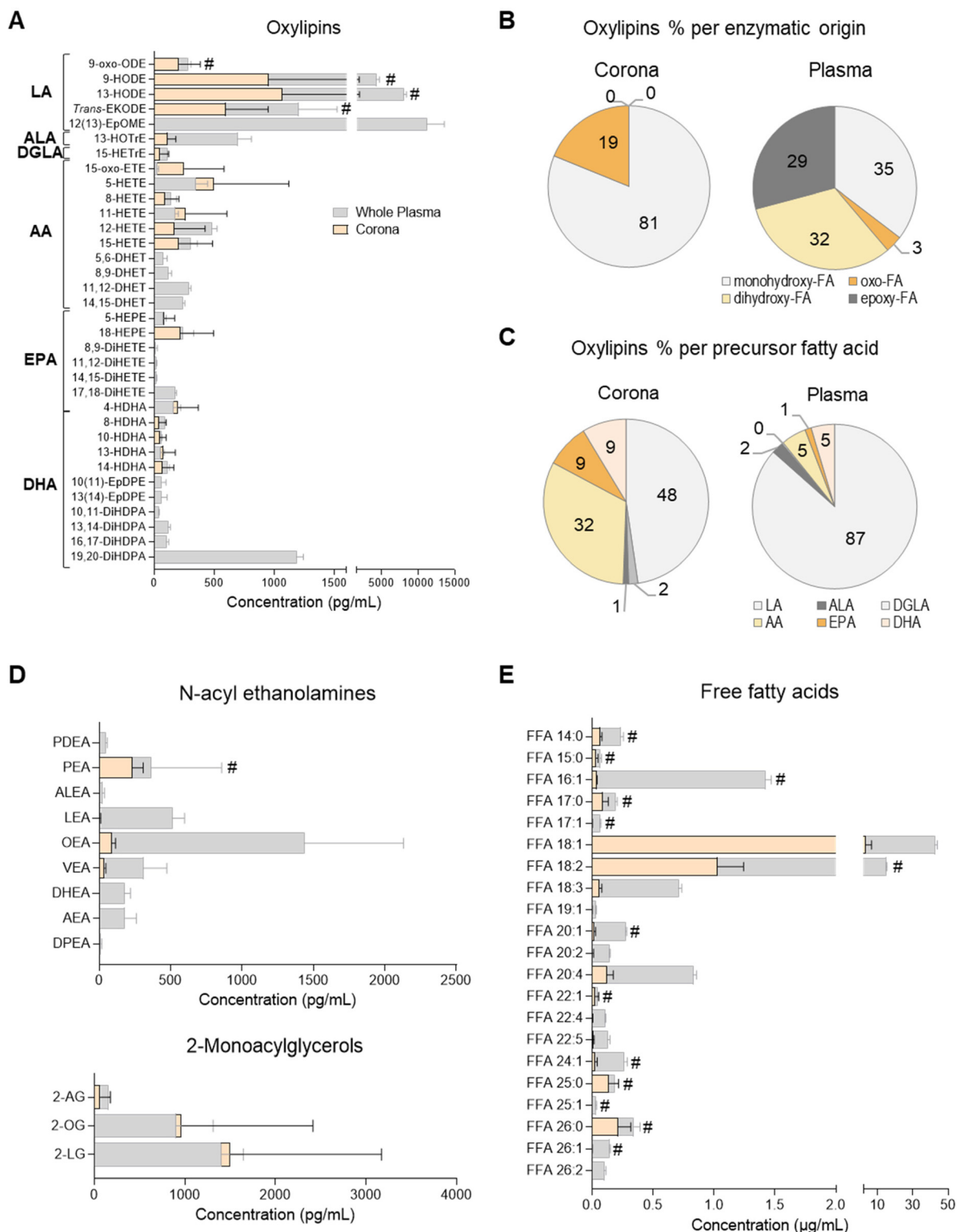


Fig. 1 Profiles of oxylipin, *N*-acyl ethanolamine (NAE), 2-monoacyl glycerol (2-MAG) and free fatty acid (FFA) species in human plasma and the liposome nanoparticle (NP)-biomolecule corona. (A) Concentration of oxylipin derivatives of the polyunsaturated fatty acids (PUFA) linoleic acid (LA; C18:2), alpha linolenic acid (ALA; C18:3), dihomo gamma linolenic acid (DGLA; C20:3), arachidonic acid (AA; C20:4), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6). Relative abundance of oxylipins grouped per (B) enzymatic origin or (C) precursor PUFA. Concentration of (D) NAE, 2-MAG and (E) FFA species. Lipid mediators were analysed by UPLC/ESI-MS/MS and FFAs by UHPSFC/ESI-QTOF-MS^E. Data shown as the mean \pm SD of $n = 5$ analyses; # denotes lipid species also found in bare liposomes.



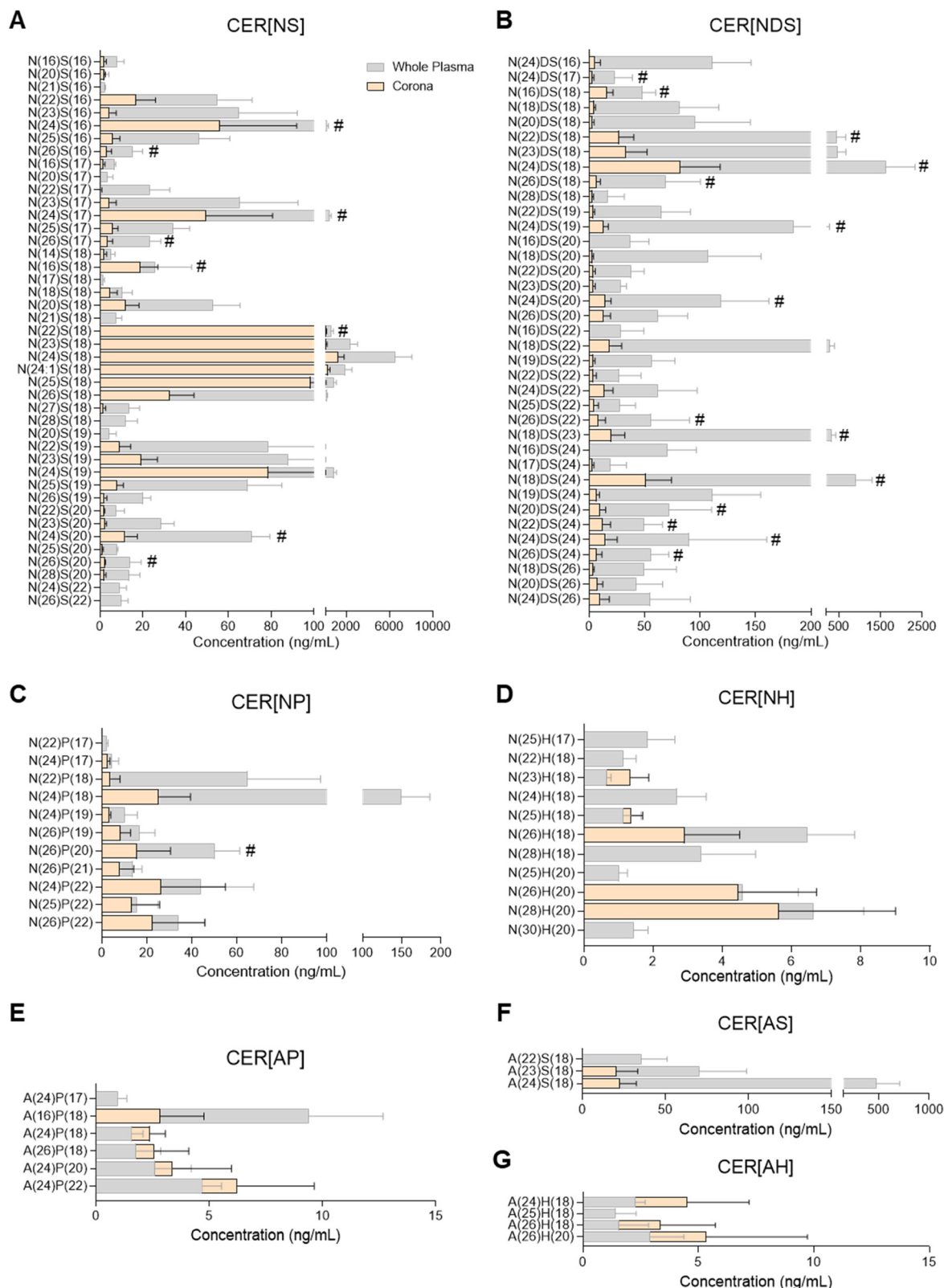


Fig. 2 Profiles of ceramide species in human plasma and the liposome nanoparticle (NP)-biomolecule corona. (A) CER[NS], non-hydroxy fatty acid/sphingosine base ceramide; (B) CER[NDS], non-hydroxy fatty acid /dihydro-sphingosine base ceramide; (C) CER[NP], non-hydroxy fatty acid/phyto-sphingosine base ceramide; (D) CER[NH], non-hydroxy fatty acid /6-hydroxy-sphingosine base ceramide; (E) CER[AP], alpha-hydroxy fatty acid /phyto-sphingosine base ceramide; (F) CER[AS], alpha-hydroxy fatty acid/sphingosine base ceramide; (G) CER[AH], alpha-hydroxy fatty acid /6-hydroxy-sphingosine base ceramide. Ceramides were analysed by UPLC/ESI-MS/MS. Data shown as the mean \pm SD of $n = 5$ analyses; # denotes lipid species also found in bare liposomes.



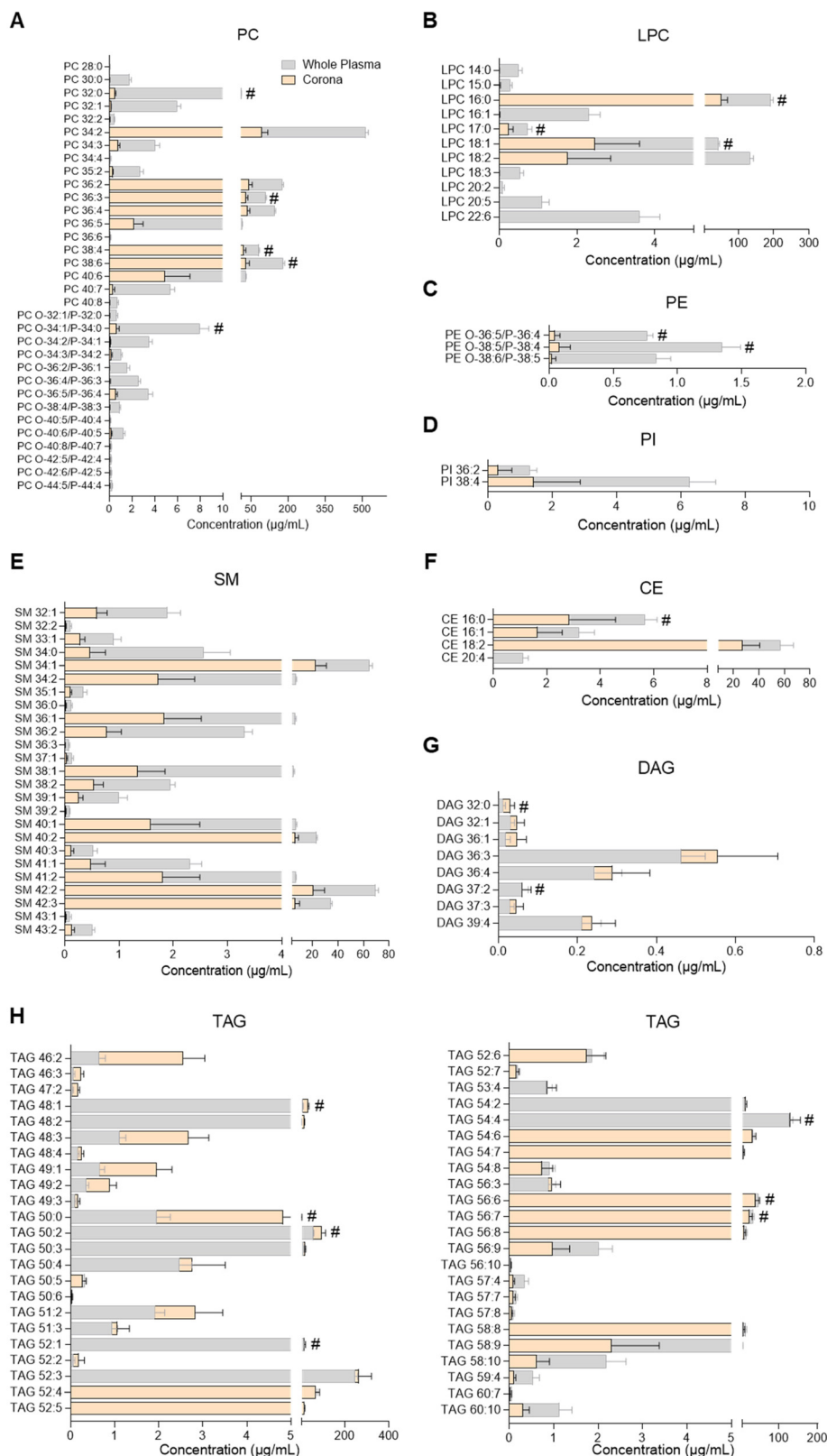


Fig. 3 Profiles of glycerophospholipids, sphingomyelins, glycerolipids and cholesteryl esters in human plasma and the liposome nanoparticle (NP)-biomolecule corona. (A) Phosphatidylcholines (PC); (B) lysophosphatidylcholines (LPC); (C) phosphatidylethanolamines (PE); (D) phosphatidylinositols (PI); (E) sphingomyelins (SM); (F) cholesteryl esters (CE); (G) diacylglycerols (DAG); (H) triacylglycerols (TAG). Lipids were analysed by UHPSFC/ESI-QTOF-MS^E. Data shown as the mean \pm SD of $n = 5$ analyses; # denotes lipid species also found in bare liposomes. Constituent lipids of the liposomes have been excluded from the figures but can be seen in ESI Table S2.†



It is now well established that the composition of the biomolecule corona varies in the presence of a disease,^{24,56–58} and this has led to the exploitation of the biomolecule protein corona as a novel protein biomarker discovery tool to meet pressing clinical needs.^{56–62} The emerging Nano-omics paradigm seeks to apply the knowledge garnered at the bio-nano interface in order to unveil novel disease specific fingerprints

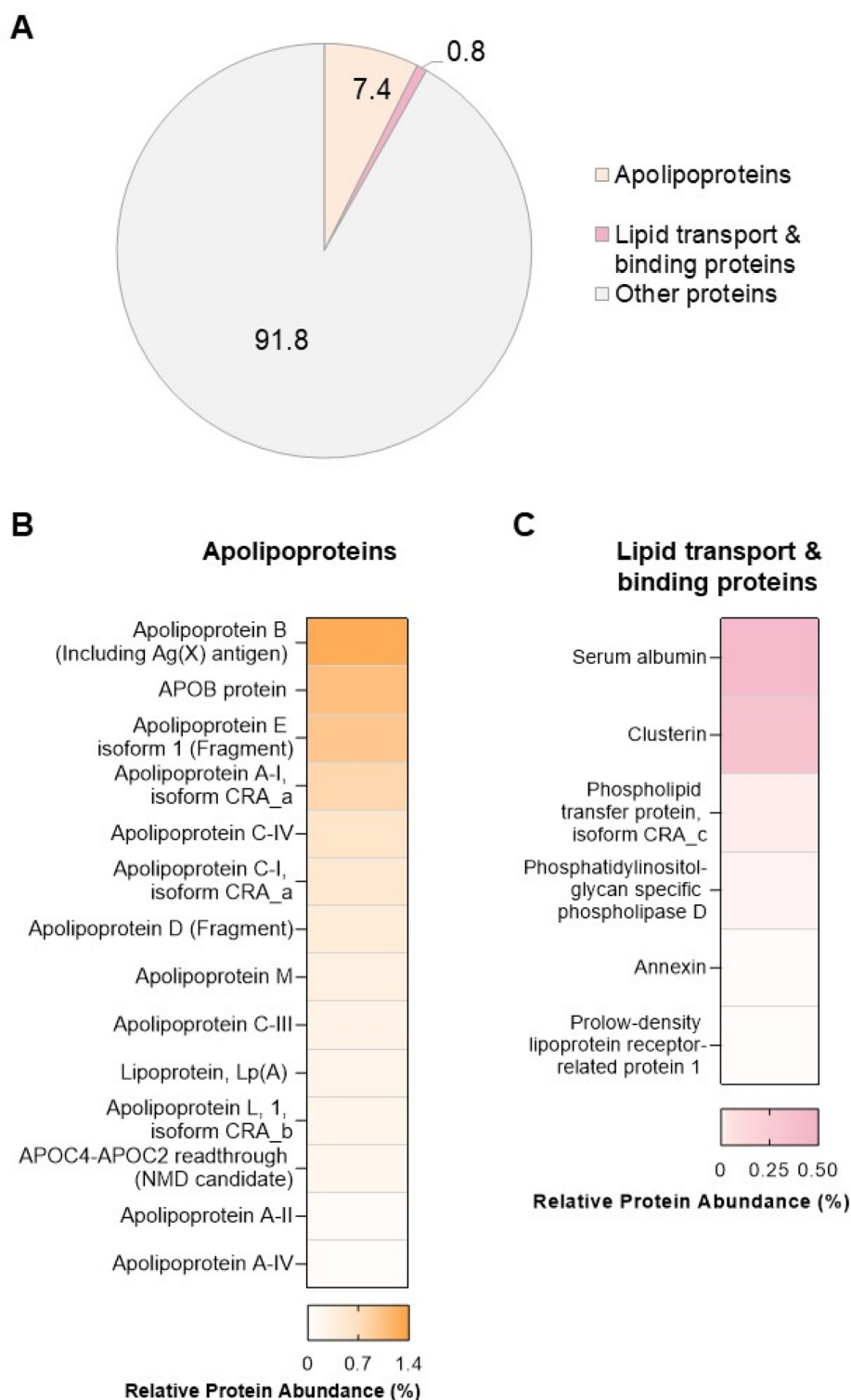


Fig. 4 Proteomic analysis of the liposome nanoparticle (NP)-biomolecule corona. (A) Contribution of apolipoproteins and lipid-transport/binding proteins to the total biomolecule corona. Percentages shown in the pie chart represent the sum relative protein abundance (RPA%) calculated for each protein category. Heatmaps of the RPA% of each (B) apolipoprotein and (C) lipid transport/binding protein identified in the NP-protein corona. Relative abundance of apolipoproteins and lipid transport/binding proteins was assessed by LC-MS/MS. Data shown as % of total proteins measured (mean of $n = 3$ analyses).



in blood and other biological fluids.⁵² Proteogenomic analysis of the liposome NP biomolecule corona has recently revealed a protein-mediated interaction of liposomes with cfDNA and highlighted the potential exploitation of the biomolecule corona as a novel blood-analysis nanoscale tool for cancer diagnostics.²⁴ Considering the diversity of the NP-biomolecule corona lipids identified in this study, we anticipate that this work will provide impetus for further studies aiming to explore the use of NPs as a scavenging tool for the enrichment and analysis of lipid biomarkers. Overall, the complex multi-omics fingerprint of the biomolecule corona offers an exciting opportunity to develop nanoscale multi-omics platforms for biofluid based biomarker discovery.

Conclusion

In this study, we describe the lipidomic profile of the biomolecule corona formed around human plasma-incubated liposome NPs, using targeted and untargeted approaches. Our findings reveal a complex lipidomic corona fingerprint that reflects to a great extent the composition of the plasma lipidome. We found that the corona lipidome comprises a range of free fatty acids, glycerolipids, glycerophospholipids and lysoglycerolipids, sphingomyelins, cholesteryl esters, ceramides, oxylipins and *N*-acyl ethanolamines. Compared to plasma, the NP-biomolecule corona demonstrated preferential binding for monohydroxy-fatty acids, mono-, di- and tri- acylglycerols, sphingomyelins and an array of ceramides. Proteomic analysis of the NP-biomolecule corona revealed the presence of lipoproteins and other lipid-transport proteins suggesting a protein-mediated mechanism of lipid adsorption onto the NP surface. Our work highlights the importance of integrative multi-omics to fully comprehend the multi-molecular composition of the biomolecule corona and opens up new possibilities for the exploitation of the bio-nano interactions as a novel multi-omic blood analysis nanoscale tool.

Experimental

Human plasma samples

K2 EDTA plasma samples from healthy female donors were supplied by Cambridge Bioscience, UK (Batches: 385365, 385429, 385446, 386259 and 386260; ESI Table S1†). Frozen plasma samples were received on dry ice and stored at -80°C . Samples were thawed, centrifuged for 10 min at 13 000 rpm, 4°C , pooled together, aliquoted in 1 mL aliquots and stored at -80°C . These pooled samples were used for all experiments ($n = 5$ aliquots per assay).

Preparation and physicochemical characterisation of liposome NPs (HSPC : Cholesterol 59.6 : 40.4 w/w%)

Liposome NPs (12.5 mM) were prepared using phosphatidylcholine from hydrogenated soy bean (HSPC; Lipoid, Germany; ESI Table S2†) and cholesterol (Sigma Aldrich, UK) by the thin

lipid film method followed by hydration and extrusion, as previously described.⁵³ This liposome composition was chosen as it forms the basis of the clinically-used liposomal doxorubicin formulation (Doxil®). The size and surface charge of liposome NPs were measured by Dynamic Light Scattering and Electrophoretic Light Scattering, respectively, using a Zetasizer Nano ZS (Malvern Panalytical, Instruments, UK) (ESI Fig. S3A†).

Ex vivo liposome NP-biomolecule corona formation

The biomolecule corona was allowed to form by adding liposome NPs (180 μL , 12.5 mM) to human plasma samples (820 μL) and incubating for 10 min at 37°C in an orbital shaker (ThermoFisher, MaxQ™ 4450 Benchtop Orbital Shaker) at 250 rpm. Samples were then subjected to a two-step purification protocol, comprising size exclusion chromatography and membrane ultrafiltration, as previously described, to isolate the corona-coated liposome NPs from unbound plasma biomolecules.^{24,53,54,56–59,63} Corona lipids were then extracted using organic solvents and analysed by targeted (UPLC/ESI-MS/MS) and untargeted lipidomics (UHPLC/ESI-QTOF-MS^E) (Table 1). Plasma samples from the same healthy donors were also analysed (whole plasma) and compared with the resulting liposome-corona profiles. To confirm that lipids identified as part of the corona lipidome were indeed liposome-NP adsorbed lipids and not unbound, co-eluting species, or artefacts of the purification process, plasma samples without liposome NPs added, were subjected to the same two-step purification protocol (purified plasma) and analysed as a control. As the NPs used in this study were lipid-based, lipidomic analysis of liposome NPs without any interaction with plasma (bare liposomes) was performed, as another control. It should be noted that for all sample preparations described here (liposome NP-corona, whole and purified plasma), we started with the same volume (820 μL) of plasma.

Quantitation of liposome NPs

The concentration of phospholipids (mM) in the liposome preparations was determined by the Stewart assay (ESI Fig. S3B†), as previously described.^{24,53,54,56–59,63} In brief, liposome NPs were treated with ammonium ferrothiocyanate $[(\text{NH}_4)_4\text{Fe}(\text{SCN})_6]$ and the absorbance of the resulting solution was measured at 485 nm using a Cary 50 Bio Spectrophotometer (Agilent Technologies, USA).

Quantitation of liposome NP-corona proteins

The total amount of proteins adsorbed onto the surface of liposome NPs was quantitated by the BCA Protein assay kit (ThermoFisher Scientific), as previously described.^{24,53,54,56–59,63} Protein binding (Pb) values, defined as amount of protein per mole of liposome NP, were then calculated per sample. Data shown as the mean \pm SD (μg of protein/ μmole of liposome NP) $n = 25$ technical repeats per treatment group (ESI Fig. S3B†).



Analysis of oxylipins by ultra-performance liquid chromatography tandem mass spectrometry with an electrospray ionisation source (UPLC/ESI-MS/MS)

Extraction and analysis of oxylipins was performed, as previously described.^{39,49} Briefly, plasma samples (1 mL), corona-coated liposome NPs (100 μ L) and controls (purified plasma 100 μ L; bare liposome NPs 100 μ L) ($n = 5$ technical repeats per group) were spiked with deuterated internal standards: 20 ng each of prostaglandin PGB₂-*d*4, 12-HETE-*d*8, 15-HETE-*d*8, 8,9-EET-*d*11, 8,9-DHET-*d*11 and 12,13-DiHOME-*d*4; Cayman Chemicals, Ann Arbor, MI, (USA). Oxylipins were extracted using ice-cold methanol (15% v/v) followed by solid phase extraction (SPE). The extracted oxylipins were separated on a C18 column (Acquity UPLC \otimes BEH C18, 2.1 \times 50 mm, 1.7 μ m; Waters, UK) and analysed by UPLC/ESI-MS/MS (UPLC Acquity and Xevo TQ-S, Waters, USA). Detection was performed by multiple reaction monitoring (MRM) in the negative ionisation mode (ESI⁻). Quantitation was performed using calibration lines constructed with commercially available standards. Instrument and data analysis were carried out by MassLynx and TargetLynx software (Waters, UK).

Analysis of NAE and 2-MAG by UPLC/ESI-MS/MS

Extraction and analysis of NAE and 2-MAG were performed, as previously described.^{39,49} Briefly, samples (500 μ L each) of plasma, corona-coated liposome NPs and controls (purified plasma and bare liposome NPs) ($n = 5$ technical repeats per group) were spiked with 20 ng of AEA-*d*8 and 40 ng of 2-AG-*d*8; Cayman Chemicals, Ann Arbor, MI, (USA) and extracted using ice-cold chloroform:methanol (2:1 v/v). NAE and 2-MAG were recovered in the organic layer and analysed by UPLC/ESI-MS/MS (UPLC Acquity and Xevo TQ-S, Waters, USA) using a C18 column (Acquity UPLC \otimes BEH C18, 2.1 \times 50 mm, 1.7 μ m; Waters, UK) and gradient elution. Detection was performed in the positive ionisation mode (ESI⁺) using MRM. Lipid species were quantitated using calibration lines constructed using commercially available standards. MassLynx and TargetLynx software (Waters, UK) were used for instrument control and data analysis.

Analysis of ceramides by UPLC/ESI-MS/MS

Extraction and analysis of ceramides was performed, as previously described.^{39,49} Briefly, 50 μ L of plasma samples, corona-coated liposome NPs and controls (purified plasma and bare liposome NPs) ($n = 5$ technical repeats per group) were spiked with 4 ng each of CER[N(16)S(18)]-*d*9, CER[N(16)DS(18)]-*d*9, CER[N(16)H(18)]-*d*9, CER[N(16)P(18)]-*d*9, CER[A(16)S(18)]-*d*9, CER[A(16)DS(18)]-*d*9, CER[A(16)H(18)]-*d*9 and CER[A(16)P(18)]-*d*9, Avanti Polar Lipids, Alabaster, AL, (USA) and extracted using ice-cold chloroform:methanol (2:1 v/v) before clean-up using silica SPE cartridges (100 mg Strata SI-1 Silica, Phenomenex, UK). The analysis of ceramides was performed by UPLC/ESI-MS/MS. Ceramides were separated on a C8 column (UPLC BEH C8, 2.1 \times 100 mm, 1.7 μ m; Waters, UK). Detection was performed by MRM using ESI⁺. Ceramides

were identified by retention time and characteristic fragmentation patterns. Relative quantitation of ceramide species was carried out using class-specific deuterated internal standards. Instrument and data analysis were carried out by MassLynx and TargetLynx software (Waters, UK).

Analysis of complex lipids (glycerophospholipids, sphingomyelins, glycerolipids, cholesteryl esters) and FFA by ultra-high performance supercritical fluid chromatography coupled to quadrupole time of flight mass spectrometry with electrospray ionisation (UHPSFC/ESI-QTOF-MS^E)

Extraction and analysis of complex lipids was performed, as previously described.^{46,64} Briefly, 50 μ L of plasma samples, corona-coated liposome NPs and controls (purified plasma and bare liposome NPs) ($n = 5$ technical repeats per group) were spiked with 4 μ g each of CE 15:0-*d*7, SM 16:0-*d*31, PC 16:0-*d*31-18:1, LPC 26:0-*d*4, PE 16:0-*d*31-18:1, TAG 17:0-17:117:0-*d*5, DAG 18:1-*d*5, lyso-PE (LPE) 18:1-*d*7, 12 μ g of phosphatidylglycerol (PG 16:0-*d*31-18:1) (Avanti Polar Lipids, AL, USA); and 4 μ g of palmitic acid-*d*31 (Sigma Aldrich, UK) and extracted using ice-cold chloroform:methanol (2:1 v/v). After the addition of ice-cold water, the organic layer was retrieved by centrifugation, evaporated to dryness and reconstituted in chloroform/2-propanol (1:1, v/v). The complex lipids and FFA were analysed by untargeted UHPSFC/ESI-QTOF-MS^E (UHPSFC, Acquity UPC2, Waters; Synapt G2 High Definition QTOF, Waters, Milford, MA, USA) in both ESI⁺ and ESI⁻. Chromatographic separation was carried out using an Acquity UPC2 Torus 2-PIC column (100 mm \times 3.0 mm \times 1.7 μ m; Waters, UK) and a gradient elution with CO₂ and methanol with 1% ultrapure water and 10 mM ammonium acetate. Data acquisition was done by MS^E in full scan mode over the range m/z 150–1200 Da. Mass data were corrected during acquisition using leucine enkephalin (m/z 556.2771 for ESI⁺ and 554.2615 for ESI⁻). MassLynx software (Waters, UK) and Progenesis QI (Nonlinear Dynamics, Newcastle, UK) were used for data acquisition and processing, respectively. Relative quantitation was based on the concentration of class-specific deuterated internal standards.

Analysis of proteins by LC-MS/MS

Digestion of corona proteins was performed prior to LC-MS/MS analysis by suspension trapping (S-trap). Briefly, proteins (10 μ g) were mixed overnight with lysis buffer (5% SDS, 50 mM triethylammonium bicarbonate TEAB pH 7.5), reduced with 5 mM dithiothreitol (DTT, Fisher) and alkylated with 15 mM iodoacetamide (IAM, Sigma Aldrich). Samples were then acidified by aqueous phosphoric acid (12% w/w) and S-trap binding buffer (90% aqueous methanol containing 100 mM TEAB pH 7.1) was added to the protein lysates. Subsequently, samples were loaded onto S-trap columns (ProtiFi, LLC, USA) with trypsin (0.1 μ g μ L⁻¹) at 47 $^{\circ}$ C for 1 hour and digested peptides were collected. Peptide samples were finally desalted using oligo R3 beads in 50% acetonitrile and dried in a vacuum centrifuge (Heto Speedvac). Following digestion, samples were analysed by LC-MS/MS using an UltiMate \otimes 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA)



coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ (ThermoFisher Scientific, Waltham, MA) mass spectrometer. LC-MS/MS data processing was carried out using Scaffold software (version 4.8.5, Proteome Software Inc., Portland, OR), as previously described.^{53,63}

Author contributions

All authors have given approval to the final version of the manuscript. L. P. designed and performed the experiments, analysed all data and took responsibility for planning and writing the manuscript. A. K. performed lipidomics experiments and contributed to data analysis. D. C.-M. performed lipidomics experiments and data analysis. A. N. and M. H. initiated, designed, directed, supervised and provided intellectual input throughout the study and contributed to the writing of the manuscript. L. P., A. N. and M. H. wrote the manuscript.

Abbreviations

2-AG	2-Arachidonoyl-glycerol
2-LG	2-Linoleoyl-glycerol
2-MAG	2-Monoacylglycerol
2-OG	2-Oleoyl-glycerol
AA	Arachidonic acid
AEA	Arachidonoyl ethanolamine /anandamide
ALA	α -Linolenic acid
ALEA	Alpha-linolenoyl ethanolamine
CE	Cholesteryl ester
CER[AH]	Alpha-hydroxy fatty acid/6-hydroxy-sphingosine base ceramide
CER[AP]	Alpha-hydroxy fatty acid/phyto-sphingosine base ceramide
CER[AS]	Alpha-hydroxy fatty acid/sphingosine base ceramide
CER[NDS]	Non-hydroxy fatty acid/dihydro-sphingosine base ceramide
CER[NH]	Non-hydroxy fatty acid/6-hydroxy-sphingosine base ceramide
CER[NP]	Non-hydroxy fatty acid/phyto-sphingosine base ceramide
CER[NS]	Non-hydroxy fatty acid/sphingosine base ceramide
COX	Cyclooxygenase
CYP/SEH	Cytochrome P450 monooxygenase/soluble epoxide hydrolase
DAG	Diacylglycerol
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DHET	Dihydroxyeicosatrienoic acid
DiHDPA	Dihydroxydocosapentaenoic acid
DiHETE	Dihydroxyeicosatetraenoic acid
DiHOME	Dihydroxyoctadecenoic acid

DOTAP

DHEA

DPEA

EPA

EpDPE

EpOME

FFA

HDHA

HDL

HEPE

HETE

HETrE

HODE

HOTrE

HSPC

LA

LDL

LEA

LOX

LPC

LPE

MAG

NAE

NPs

OEA

oxo-ETE

oxo-ODE

PC

PC-O

PDEA

PE

PEA

PI

PUFA

RPA

SEA

S-trap

SM

TAG

TEAB

trans-EKODE

UHPSFC/

ESI-QTOF-MS^E

UPLC/ESI-MS/

MS

VEA

VLDL

1,2-Dioleoyl-3-trimethylammonium-propane

Docosahexaenoyl ethanolamine

Docosapentaenoyl ethanolamine

Eicosapentaenoic acid

Epoxydocosapentaenoic acid

Epoxyoctadecenoic acid

Free fatty acids

Hydroxydocosaheptaenoic acid

High-density lipoprotein

Hydroxyeicosapentaenoic acid

Hydroxyeicosatetraenoic acid

Hydroxyeicosatrienoic acid

Hydroxyoctadecadienoic acid

Hydroxyoctadecatrienoic acid

Hydrogenated soy bean

Linoleic acid

Low-density lipoprotein

Linoleoyl ethanolamine

Lipoxygenase

Lysophosphatidylcholine

Lysophosphatidylethanolamine

Monoacylglycerol

N-Acyl-ethanolamine

Nanoparticles

Oleoyl ethanolamine

Oxo-eicosatetraenoic acid

Oxo-octadecadienoic acid

Phosphatidylcholine

Ether-phosphatidyl glycerol

Pentadecenoyl ethanolamine

Phosphatidylethanolamine

Palmitoyl ethanolamine

Phosphatidylinositol

Polyunsaturated fatty acids

Relative protein abundance

Stearoyl ethanolamine

Suspension-trapping

Sphingomyelin

Triacylglycerol

Triethylammonium bicarbonate

trans-Epoxyketooctadecenoic acid

Ultra-high performance supercritical fluid chromatography coupled to quadrupole time of flight mass spectrometry with electrospray ionisation

Ultra-performance liquid chromatography tandem mass spectrometry with electrospray ionisation

Vaccenoyl ethanolamine

Very low-density lipoprotein.

Conflicts of interest

There are no conflicts to declare.



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