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Article

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An obesity model and corona multiomics analysis reveal high-density lipoprotein effects on lipid nanoparticle function

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Abstract

Lipid nanoparticles (LNPs) are currently of great interest for therapeutic nucleic acid delivery. Lipid-based nanoparticles are, however, difficult to study analytically and so our understanding of the interaction between LNPs and biological systems remains obscure, particularly in terms of biomolecular corona formation and the impact this has on therapeutic efficacy and targeting. Ideally, we would like to engineer particles to acquire corona components that facilitate targeting, cargo delivery and improved safety. This requires studying the relationship between LNPs, biofluids and the resulting coronas. The particle-corona complexes are, however, fragile and biofluids also contain particles (such as lipoproteins) with sizes and biochemical characteristics similar to lipid nanoparticles, so contamination with biological components is a challenge. Here we describe a rapid, automated, and unbiased isolation method for LNP biomolecular coronas, coupled with proteomic and lipidomic analysis. Using these analytical methods, we systematically studied, in lean and obese rats, the variation in LNP-mediated mRNA delivery caused by individual physiology. A comprehensive multivariate model links LNP corona content to efficacy, identifying and validating high-density lipoproteins as a previously unidentified factor affecting particle efficacy.

Keywords: LNPs, nanoparticles, biomolecular corona, mRNA delivery, proteomics, lipidomics, lipoprotein, obesity

1 **Main**

2 Lipid nanoparticles (LNPs) are the lead non-viral delivery system for clinical nucleic acid
3 therapeutics, widely used for the encapsulation of nucleic acids, including antisense
4 oligonucleotides (ASOs), small interfering RNA (siRNA), single guide RNA (sgRNA), or
5 messenger RNA (mRNA)¹. LNPs are designed to deliver these therapeutic agents and cure
6 diseases by silencing infarcted genes, expressing functional proteins, or using targeted genome
7 editing². Compared to liposomes that were often used previously, LNPs usually contain ionizable
8 cationic lipids, helper phospholipids, cholesterol, and polyethylene glycol lipid (PEG-lipid)³.
9 Microfluidic, rapid mixing techniques can formulate sub-100 nm, highly-potent, reproducible LNP
10 batches at large scale⁴, allowing LNPs to progress quickly from concept to clinical applications⁵.
11 ⁶. In 2018, the FDA and EMA approved Onpattro® for the treatment of hereditary amyloidogenic
12 transthyretin, which was the first clinical milestone for a therapeutic, nucleic acid-based, LNP
13 formulation⁷. The SARS-CoV-2 vaccine development has recently highlighted the remarkable
14 impact of LNPs as mRNA delivery vehicles, saving billions of lives⁸.

15 So far, the advance of LNP-based therapies is predominantly due to improved formulation
16 component and composition designs. For example, incorporating cationic ionizable lipids has
17 successfully increased LNP potency by facilitating cargo release from endosomes following local
18 pH reduction⁹. In contrast, the addition of targeting ligands at the nanoparticle surface, which was
19 intended to optimize targeting efficacy and, distribution tropism by utilizing surface receptors
20 expressed by target tissues¹⁰, has been less successful^{11, 12}. One reason for this is that these
21 interactions can be adversely affected by the biomolecules, collectively termed the biomolecular
22 corona, adsorbed onto nanoparticle surfaces. This is exemplified by nanoparticles that have been

surface-functionalized with transferrin motifs. The ability of this ligand to augment nanoparticle function is strongly suppressed following corona formation within serum *in-vitro*¹³. Thus, in earlier trials, introducing modifications such as pegylation became a gold standard for reducing protein binding and preventing corona formation, thereby prolonging circulation and improving delivery efficacy¹⁴.

Recently, the modulation of biomolecular corona formation has become another promising approach to improve LNP potency^{15, 16}. The biomolecular corona formed by hundreds of biomolecules (primarily proteins and lipids) creates a new biological identity for nanoparticles¹⁷. Growing evidence has suggested that this new biological identity can affect the pharmacokinetics and pharmacodynamics of LNPs and is essential for effective delivery [Citation: Gallud et al, publication under review]. A recent study also indicated that the prolonged circulation was not due to the complete elimination of corona formation. In contrast, the adsorption of a specific corona protein, Clusterin, on the PEGylated surface, allows nanoparticles to evade clearance by the immune system¹⁸.

Among all LNP corona biomolecules, modulation of corona Apolipoprotein E (ApoE) content has been most intensively explored as a targeting tactic to improve LNP delivery to the liver^{19, 20}. Onpattro®, the FDA-approved first-in-class LNP-enabled therapy, has been shown to target hepatocytes mainly by recruiting ApoE into the corona¹⁹. The interaction between corona ApoE and cell surface low-density lipoprotein receptor (LDLR) drives the internalization of LNP into hepatocytes¹⁹ and other cell types in the liver²¹. Interestingly, another study using ApoE (*apoE*^{-/-}) and LDLR (*LDLR*^{-/-}) knockout mice did, however, indicate the LNP potency was only

dependent on ApoE²², suggesting the possibility of alternative ligand-receptor pairs involving ApoE.

Though most mechanistic studies have focused primarily on unbound free ApoE, a large proportion of ApoE in blood plasma associates with various endogenous lipoprotein particles, creating a greater diversity of biomolecules and higher complexity²³. In addition, ApoE can actively incorporate into LNPs, affecting both their structure and composition²⁴. How and why biomolecules accumulate in LNP coronas remains poorly understood, making it difficult to modulate corona composition effectively. The examples above highlight the need to understand corona-centric LNP potency modulation mechanistically, ideally in various physiological or pathological settings.

A major obstacle for studying LNP coronas is the technical challenges associated with the isolation of lipid particles and their associated corona components. For metallic, colloidal, and polymeric nanoparticles, the corona composition and the factors determining composition have been extensively investigated and relatively well-understood^{17, 25}. In contrast, current methods for LNP corona isolation are generally laborious, low-throughput, material-consuming, and often require additional particle modifications or methods that can perturb the corona composition and integrity²⁶. Most unfavorably, they cannot guarantee a distinct separation of LNPs and endogenous lipoproteins or extracellular vesicles^{26, 27, 28, 29}.

There is also an increased demand for multi-omics phenotyping of the biomolecular corona surrounding LNPs. Besides protein components, many lipid species also have high affinities for the surface of nanoparticles³⁰. Lipid metabolism also likely influences the efficacy of lipid-based

nanoparticle therapeutics³¹. Therefore, evaluating the LNP biomolecular corona composition changes in an easy, fast and reliable way enables multi-omics interrogation, becoming another challenge.

Finally, the corona composition is highly variable from person to person and is strongly influenced by individual pathophysiology, underlying disease, and co-medication³² [Citation: Liu et al, publication under review]. This greater pathological complexity affects nanomedicine targeting, clearance, distribution, and cargo release through altered corona formation³² [Citation: Liu et al, publication under review]. For example, obesity can strongly affect protein and lipid homeostasis³³. As a result, the biodistribution of nanoparticles in obese animals deviates significantly from their healthy counterparts³⁴, so this is important to know when engineering nanomedicines for human patients with similarly variable physiologies. This individual diversity also complicates LNP corona analysis. Conversely, deciphering corona composition on an individual basis will offer more insight than standardized conditions and contribute mechanistic knowledge regarding corona modulation of LNP potency.

This study address the impact of individual physiology on corona formation and LNP potency to reveal the mechanistic relationships between the two. We established a rapid, affinity-based, automated, high-throughput corona isolation method coupled with downstream omics analysis. A clinically relevant LNP formulation was incubated with plasma obtained from individual lean or obese rats. The protein and lipid corona, analyzed by quantitative multi-omics, revealed an apparent correlation between the corona components (high-density lipoprotein (HDL) in particular) and the LNP efficacy, measured as the functional delivery of mRNA and protein expression levels. Our results reveal a novel mechanistic explanation for how corona components

affect LNP efficacy, with implications for future mRNA vaccine development, personalized nanomedicine design, and human clinical translation.

Lean and obese animals, ApoE and the effects on LNP potency

To investigate whether individual pathology influences the LNP-mediated mRNA expression, we compared eGFP mRNA expression in hepatocytes supplemented with plasma obtained from obese and lean rats. Blood plasma was withdrawn from eight lean and eight obese Zucker rats (Crl:ZUC-*Lep^{fa}*) with matching genetic backgrounds. The obese Zucker rat is a well-characterized genetic obesity model³⁵ that exhibits spontaneous hyperlipoproteinemia and a lower level of hepatic low-density lipoprotein receptor (LDLR), resulting in an elevated number of very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL) particles in blood plasma³⁵ (Supplementary Fig. 1). After blood plasma withdrawal, a thorough characterization of individual biological plasma samples demonstrated significantly elevated levels of obesity biomarkers in obese rats, such as cholesterol, triglycerides, natural phospholipids (Supplementary Data 1), and lipid binding apolipoproteins, including ApoE, as shown in the proteomics cluster analysis (Supplementary Fig. 2). Fig. 1a illustrates the screening procedure to evaluate the LNP mRNA delivery potency regulated by individual lean and obese plasmas. After plasma withdrawal, an equal portion of obese or lean individual plasma was pooled, termed as obese pool (OP) and lean pool (LP), and this was used for initial experiments to evaluate LNP functional dose-response relationships and identify key corona components (lead generation). This was followed by an evaluation of selected LNP doses in combination with individual plasma samples.

The LNPs used throughout this work were formulated using the clinically approved ionizable cationic lipid DLin-MC3-DMA, encapsulating eGFP-encoding mRNA. To enable the evaluation

of LNP efficacy, in the context of lean and obese plasmas and, further biomolecular corona profiling, a small portion of helper lipid and mRNA cargo was substituted with rhodamine (Rhod) and cyanine 5 (Cy5) labeled counterparts according to the composition described in Supplementary Fig. 3a. The LNPs, characterized using Dynamic Light Scattering (DLS) and Electron Microscopy (EM) as previously described³, had a diameter of approx. 80 nm and a spherical morphology, with an electron-dense core, as expected (Supplementary Fig. 3b, 3c, and Table 1).

To explore the effects of lean and obese plasmas on LNP function, we employed high throughput confocal live-cell imaging for real-time quantitative measurement of cellular LNP uptake and productive cargo delivery, as eGFP mRNA expression, using rat hepatocellular cultures H4-II-E-C3 and McA-Rh-7777, human hepatocytes Huh-7 and, rat kidney fibroblasts NRK-49F for comparison. Shortly before imaging, the growth media were replaced with either plasma-free media or growth media supplemented with 1-10% lean pool or obese pool rat plasma. The cells were then treated with LNPs using a dose range from 25 ng to 400 ng mRNA/per well (or 0.5-8 µg/mL), and cellular LNP uptake and GFP production (Fig. 1b, Supplementary figures 4 and 5) was evaluated continuously over the next 10 hours.

The presence of biomolecules and corona formation is indispensable for LNP-mediated uptake and mRNA expression. Without plasma supplementation prior to LNP dosing, there was minimal LNP cellular uptake and negligible eGFP expression in the hepatocyte cell lines across all tested mRNA doses (Fig. 1b). Conversely, when the media was supplemented with 10% lean or obese plasma, the eGFP expression increased. Paradoxically, reduction of the plasma proportion to 5% resulted in enhanced eGFP expression than in the 10% plasma condition.

Comparing LP and OP samples, we observed the most noticeable difference in eGFP expression when recipient cells were supplemented with 1% lean or obese plasma prior to LNP dosing. As shown in Fig 1b, the overall eGFP expression in the 1% lean condition was reduced, compared to higher plasma concentrations, although all hepatocyte cell lines expressed an increasing amount of eGFP with higher LNP doses and longer times and, the differences between lean and obese plasma became more apparent. The most significant difference between lean and obese conditions appeared at the combination of 1% plasma and the 200 ng mRNA/well dose (Fig. 1c), where LNP efficacy was 5-10 fold improved by obese plasma supplementation. At lower LNP doses, such as the 50 ng mRNA/well dose, the contrast between lean and obese plasma effects on LNP efficacy was less apparent (Fig. 1d). The significant difference at 200 ng mRNA/well emerged as early as 4 h post-dosing and persisted until the endpoint at 10 h (Supplementary Fig. 6). The expression difference between obese and lean was less pronounced in kidney fibroblasts than in hepatocytes (Fig 1c). The relationship between the LNP and plasma dosing is crucial because it reveals that higher protein production is not simply because of the obese plasma's general anabolic effect (this is explored in more detail below).

To further explore how individual physiological variations can affect LNP efficacy, we then dosed cells with 50 ng and 200 ng eGFP mRNA containing LNPs, supplemented with individual plasmas at a 1% ratio, as the 1% plasma supplementation seemed to drive higher efficacy. The difference between lean and obese conditions remained when cell cultures were supplemented with individual lean or obese plasmas. In contrast, individual variation now emerged (Fig. 1e and Supplementary Fig. 7), although the intragroup variation (among lean or obese individuals) was less significant than the intergroup variation (lean v.s. obese).

Overall, the intracellular mRNA expression difference between lean and obese conditions is likely not due to the absolute plasma component abundance (for example, ApoE) or plasma's impact on cellular metabolism alone. For example, the higher ApoE content (i.e., obese v.s. lean, or 10% v.s. 5% plasma) did not introduce significantly higher eGFP expression levels in recipient hepatocytes at the tested dose range. In contrast, the 1% obese supplementation resulted in the highest eGFP expression. This suggests that ApoE is not the sole determinant of LNP hepatic potency. The imaging results (Fig. 1b) also implied that the difference in eGFP expression did not directly correlate to the overall amount of ApoE or other nutrients provided by plasma.

Based on these findings, we hypothesized that the altered mRNA cargo expression is, at least partially, a result of different biomolecular corona formations. The binding of the corona components to LNPs can cause structural and compositional rearrangement²⁴, and this can affect potency. Thus, in the 1% lean condition, the attenuation in the eGFP expression was likely due to the limited availability of biomolecules necessary to form the biomolecular corona, especially at doses higher than 50 ng mRNA/well. The obese plasma condition preserved the eGFP expression and enhanced the expression compared to high plasma ratios because, in this case, a corona formed that was more compatible with hepatocytes. Alternatively, lowering the plasma ratio may also unmask corona interactions with recipient cells, as previously reported³⁶. Therefore, isolating LNP coronas and identifying the corona determinant driving altered eGFP mRNA expression is necessary to understand how to modulate corona composition and adapt to individual physiology and pathology variations.

Ultrafast affinity isolation enables unbiased biomolecular corona phenotyping

Given the great variation in abundance and complexity of plasma biomolecules, the LNP-corona complex (LNPCor) must be separated from unbound biomolecules and endogenous nanosized structures in the plasma, as illustrated in Fig 2a. Preservation of corona function and integrity is crucial for studying the corona and revealing the identity of its components. So aggressive chemical and mechanical separation procedures are not suitable for LNPCor isolation. We assessed various methods to retrieve the LNP-corona complexes from plasma prior to corona analysis. Retrieving these complexes is technically challenging as plasma components overlap in size, composition, and physicochemical characteristics. The commonly used size exclusion chromatography (SEC) is prone to co-isolate unbound protein and endogenous particles together with nanoparticles³⁷. Latest techniques, such as asymmetric flow field-flow fractionation (AF4), is an improved size-based separation technique that utilizes a liquid cross-flow to generate a parabolic flow profile in a narrow channel³⁸. It has previously been utilized in the characterization of lipid-based nanoparticles³⁹. The size and density of LNPs do, however, overlap with endogenous lipoprotein particles, especially very-low-density lipoproteins (VLDLs). And this inevitably led to contamination of LNP-corona populations with endogenous lipoprotein particles and potentially extracellular vesicles (Supplementary Fig. 9). In agreement with the literature, we confirmed that conventional isolation approaches for LNPs, utilizing size or density fractionation, were insufficient for retrieving LNPs from plasma because of the unfavorable overlap with other plasma components.

Therefore, we developed an affinity-based, magnetic isolation workflow employing antibodies against polyethylene glycol (PEG) on the surface of LNPs. The assay is a miniaturized high

throughput 96-well format. As shown in Fig 2a, antibodies against PEG were conjugated to the surface of magnetic microbeads to pull down the LNPcor complex. The isolation procedure contains three essential steps: LNPcor capture, wash, and elution. The optimization of these three steps is illustrated in the design of the experiment (DoE) in Fig 2b. As one of the LNPs' structural components, PEG existing on the LNPs surface was employed here as a primary "endogenous" affinity tag. The introduction of an additional surface affinity tag is a common approach for affinity-based isolation. This may, however, further modify the LNPs' surface properties and affect the biomolecular corona formation²⁶. Washing steps are also necessary to remove unbound small molecules and endogenous particles. The inclusion of detergent typically included in bead-based assays posed a threat to the colloidal stability of LNPs. During the elution, strong detergents such as sodium dodecyl sulfate can disrupt the binding of antibodies to their ligand. Again, it may cause a destructive effect on the LNPcor's integrity. PBS, in this case, served as an alternative wash buffer. A moderate change of pH, on the other hand, is a relatively milder way to disassociate LNPcor from antibodies. Nevertheless, a pH lower than the pKa of ionizable cationic included in the LNPs (i.e., DLin-MC3-DMA) usually results in LNPs disassembly⁴⁰. The DoE optimization revealed that a combination of antibody against PEG backbone, PBS wash and basic pH elution resulted in robust LNPcor recovery in the final elution.

We then tested this miniaturized, high-throughput setting to identify optimal incubation and elution conditions to retrieve maximal LNPs without introducing impurities by prolonged incubation and elution period (Supplementary Fig. 10). Then, the optimized approach is used to retrieve LNPcor incubated in 1% lean pool and obese pool plasmas. As shown in Fig. 2c, this method successfully recovered the majority of the LNPcor complex. We also observed a similar recovery profile

between LNPcor formed in the two plasma conditions. The number and recovery rate of nanosized particles within the elution was examined by nanoparticle tracking analysis (NTA) to confirm that LNPcor was harvested in a particle-like format. The recovery rate of lipid and mRNA content was calculated by Rhod and Cy5 fluorescent intensity associated with LNPs. Overall, the recovery rate of nanosized particles matched lipid and mRNA recovery, indicating that there was very little lipoprotein contamination. Controls with antibody-conjugated microbeads incubated within 1% pooled lean or obese plasma without the presence of LNPs confirmed negligible unspecific binding of endogenous nanosized particles. This affinity-based magnetic isolation workflow had superior specificity compared to conventional density or size exclusion-based methods.

To verify the LNP integrity and functionality following the LNPs pull-down procedure, we retrieved the LNPs from PBS (LNP_{PD}), lean pool (Lean_{PD}), or obese pool (Obese_{PD}) plasmas following 4 h incubation at 37°C. Compared to LNPs without the above incubations, the mRNA encapsulation was not reduced during the pull-down procedure or corona formation. It demonstrated that the LNP integrity was preserved during the isolation procedure (Supplementary Fig. 11a). The original LNP and LNP_{PD} (LNP isolated from PBS without corona) were dosed to the recipient cells. The two types of LNPs provoked similar eGFP expression in recipient cells supplemented with lean pool or obese plasma without significant difference. It indicated the LNP functionality is not affected by the isolation procedure. (Supplementary Fig. 11b). When comparing the original LNP to the LNPcor, the original LNP induced negligible cellular uptake in the plasma-free condition. In contrast, LNPs with fully formed biomolecular corona was taken up by cells in the absence of plasma. It highlighted that the isolation procedure can harvest LNPcor with functional corona (Supplementary Fig. 11c).

Cryogenic transmission electron microscopy (Cryo-EM) was also employed to reveal the macroscopic effects that the corona exerts on LNPs (Supplementary Fig. 12). Cryo-EM showed that the lean LNPcor remained mostly as electron-dense spheres as described earlier³. Intriguingly, the obese LNPcor demonstrated multi-laminar structures, likely resulting from the incorporation of biomolecules with detergent-like properties, such as apolipoproteins⁴¹.

Overall, the result confirmed that the ultrafast affinity isolation can harvest intact LNPcor complex with functionality preserved, minimizing impurities that biased conventional isolation methods.

Individual lean or obese conditions altered LNP biomolecular corona formation and content

We then analyzed the LNPcor complex formed and isolated from each individual lean or obese rat plasma using the optimized harvest procedure. Characterized by dynamic light scattering (DLS), the obese-plasma-derived LNPcor demonstrated, on average, a significantly larger hydrodynamic diameter than the lean-plasma-derived particles, while the polydispersity was lower (Supplementary Fig. 13a and 13b.). The LNPcor size and polydispersity matched the LNPcor morphology obtained by Cryo-EM (Supplementary Fig. 12).

The individual coronas were also evaluated using quantitative proteomics, revealing significant differences between lean and obese coronas. On average, obese-plasma-derived biomolecular coronas harbored 1.8-fold more protein than those derived from lean plasma (Fig. 3a). Control experiments were conducted without LNPs to test for unspecific protein and lipid adsorption onto the magnetic microbeads and plastic surfaces. The amount of unspecifically bound proteins (Fig. 3a, BG) and endogenous plasma particles (Supplementary videos) were substantially lower

compared to the LNPcor samples. The relative protein abundance (RPA) of identified proteins was also calculated by comparing to the total number of detected protein sequences. The group average RPA of corona apolipoproteins increased from 76.04% to 94.40%, when moving from lean to obese plasmas (Fig. 3b). This increase of lipid-binding apolipoproteins posed a potential explanation for the Cryo-EM morphology change of obese derived LNPcor due to their amphipathic properties (Supplementary Fig. 12). Other consistently detected corona proteins were involved in the acute-phase response, redox homeostasis, iron transfer, complement and coagulation processes, and immune response-related proteins.

In general, the LNP protein corona did not mimic the plasma protein composition, implying selective absorption of corona proteins. Fig. 3c illustrates the breakdown of major corona proteins (relative protein abundance, RPA>0.1%, summarized from individual coronas) and their abundance in original blood plasma. We found that in obese-plasma-derived coronas, apolipoprotein A-II (Apo A-II), apolipoprotein C-II (Apo C-II), apolipoprotein C-III (Apo C-III), and apolipoprotein M (Apo M) were elevated. In contrast, the ApoE abundance was moderately reduced in all obese-plasma-derived coronas compared to corona samples derived from lean plasmas, despite the improved cargo eGFP mRNA expression following LNP exposure to obese plasma (Fig. 1b). Antibodies against apolipoproteins were employed to confirm the presence of Apo A-II, C-II, C-III, and E in lean and obese-plasma-derived coronas (Supplementary Fig. 15). Fig. 3d illustrates in more detail the major corona proteins in individual coronas. In Fig. 3e, the protein corona fingerprints were used as individual protein corona fingerprints classifiers for similarity hierarchical clustering analysis (HCA). Obese and lean-derived coronas self-organized into two clear and distinct groups, highlighting that while there is variation within the corona

protein content introduced by individual plasma samples, the lean and obese corona phenotypes are very distinct.

Whereas the protein components of the corona on various nanoparticles have been investigated in more detail⁴², the effect of corona lipids on delivery efficacy remains obscure, which is surprising given that lipids are a prominent component of blood plasma. They are also usually bound with apolipoproteins to form lipoprotein particles, and as demonstrated above, apolipoproteins are enriched in LNPs protein coronas. Therefore, we examined the content of naturally occurring lipids in the LNPs corona.

The lipid components within each individual plasma-derived LNPcor complex were extracted as previously described⁴³, revealing elevated lipid content in obese plasma-derived coronas (Supplementary data 2). Four lipid families, phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), and sphingomyelin (SM), were measured for individual plasmas and coronas, and the results are summarized in Fig. 4. While comparing the obese plasmas to the lean counterparts, we observed a universal elevation of all examined lipid species. Although the obese plasma-derived coronas showed higher PC, LPC, and PE content, but not SM. Similarly, the individual lipid corona fingerprints were used for hierarchical clustering analysis. Again, obese and lean plasma-derived coronas formed two distinct clusters, highlighting the variation within the corona lipid contents introduced by lean and obese plasmas.

HDL, rather than ApoE, determined hepatic LNP mRNA delivery

Multi-omics analysis generated comprehensive data on the coronas formed following exposure to the individual lean and obese plasmas. Corona components function cooperatively, so it is

problematic to consider them in isolation. Multivariate regression models have been shown to outperform single-component models when predicting the effects of corona composition on nanoparticle performance⁴⁴. Therefore, we utilized an orthogonal partial least squares (OPLS) analysis to reveal the latent multivariant correlation between the individual plasma-derived biomolecular coronas and eGFP expression. In OPLS plots (Fig. 5a), the distance on the Y-axis indicates orthogonality to the OPLS model. Omics hits that localize closer to 0 on the y-axis have lower orthogonality and are more relevant. While the distance along the x-axis reveals the relative correlation. A hit with a positive x value, for example, is positively correlated to the cellular eGFP expression. Overall, the OPLS correlation model can reveal the potential correlation between the corona content resulting from lean and obese plasma exposure and LNP performance.

Hits derived from the -omics analysis (see the proteomic and lipidomic methods for lead identification methods) were normalized using z-score normalization for unbiased correlation (Supplementary data 3). In Fig. 5a, the correlation between corona contents (protein and lipid contents, X variables) and the eGFP expression obtained by quantitative imaging (AUC_{10h}, Y variables) for the OPLS model was illustrated as cumulative R²X, R²Y, and Q² (R²X_{cum}, R²Y_{cum}, Q²_{cum}, range 0-1). The correlation model explained 74.1% (R²X_{cum}=0.741) of the X variable behavior and 93.9% (R²Y_{cum}=0.939) of the Y variable behavior. An R² value greater than 0.66 is considered indicative of a good model fitness⁴⁵. Furthermore, the overall model predictive power (X variables' impact on Y variables) of the OPLS is 68.6% (Q²_{cum}=68.6). Overall, the OPLS correlation model can reveal the potential correlation, between the corona content resulting from lean and obese plasmas and, LNP performance.

The OPLS analysis revealed two groups of corona proteins that were of particular interest concerning LNP efficacy: immune response proteins (exemplified by complement proteins) and proteins related to lipid physiology (apolipoproteins) (Fig. 5a). The immune response proteins are not considered here and are the focus of separate, ongoing experiments. The presence of many apolipoproteins in LNP coronas correlated with LNP efficacy ($-0.1 < \text{Orthogonality} < 0.1$). In particular, Apo A-II, B, C-II, and C-IV, the structural proteins found in HDL, VLDL, and chylomicron (CM) lipoproteins, were positively correlated with LNPs performance. Apo M, which is found primarily on HDL⁴⁶, also exhibited a high positive correlation to LNP performance. In contrast, Apo E and Apo A-IV⁴⁷, which are primarily found in VLDL and CM demonstrated a moderate negative correlation to LNPs performance (Fig. 5a). While Apo E is often considered highly correlative to LNP hepatic delivery efficacy, the amount of corona Apo E in obese-plasma-derived coronas declined modestly compared to lean-derived counterparts (Supplementary Fig. 14). Remarkably, quantitative imaging revealed that LNPs stimulated higher cellular eGFP mRNA expression when supplemented with obese plasmas that resulted in coronas with less ApoE (Fig. 1e). As will be shown later, this is also not simply because the obese serum stimulates protein production, but rather that specific plasma components accumulate in LNP coronas and this leads to enhanced LNP function.

While not all protein components of LNP coronas are strongly correlated with LNP function, the majority of lipids associated with LNPs did correlate with enhanced LNP performance. A lipid-rich corona, in general, is favorable for LNP mediated mRNA delivery. However, the orthogonality of lipid hits was higher than most of the protein hits ($\text{Orthogonality} > 0.1$ or < -0.1), suggesting that the corona proteins are more relevant for explaining the differences between LNP

function in lean and obese contexts. The plasma lipids became more relevant (orthogonality dropped below 0.1 in most cases) when considering particle uptake as opposed to function. While particle uptake was not our primary readout, it is interesting that PC, for example, has previously been shown to be important for particle uptake in an SR-BI-dependent manner⁴⁸.

In addition to the intergroup correlation between X and Y variables, OPLS also revealed the intragroup correlation within X variables and Y variables separately. Within the Y variables group (eGFP expression), the human and rat hepatocytes were located in close proximity to each other, indicating the LNP performance with the three hepatocyte lines were similar when supplemented with either lean or obese plasmas. On the contrary, the non-hepatocytes were located further away from the hepatocytes cluster. This indicates that the LNPs efficacy is likely cell-type and tissue-origin specific. Within the X variables, the apolipoprotein clusters (Apo A-II, C-II, C-III, C-IV, and ApoM) are adjacent to the corona lipids, suggesting the apolipoproteins and lipids, which are commonly co-assembled into lipoprotein particles, formed LNP coronas collectively. Additionally, the OPLS revealed acute phase, complement cascade, and coagulation function associated proteins are less likely to be present in an apolipoprotein-abundant LNP corona. Overall, the above intragroup correlations show that OPLS is a valuable method for elucidating the multivariant relationship between corona content and LNP mRNA expression efficacy.

Our focus then turned to identify the mechanism that triggered different corona formation and associated efficacy variation in lean and obese conditions. While OPLS analysis revealed complex multivariant correlations, linear classifiers are a more intuitive way to explore the impact of corona components on LNP function. There was a moderate correlation between individual corona ApoE and cellular eGFP expression and, according to our data, both corona and plasma ApoE were not

the optimal indicators for hepatic mRNA expression following LNP delivery (Fig 5b and 5c). In contrast, the correlation between individual ApoM and cellular eGFP expression was much stronger. The corona ApoM also correlated more with LNP function than plasma ApoM (Fig 5b and 5c). In addition, other major HDL-associated apolipoproteins, such as ApoA-II, were also highly correlated with cellular eGFP expression (Supplementary Fig. 16). Lipoprotein fragments, even intact lipoproteins, have previously been identified in nanoparticle coronas^{49, 50}, so we hypothesized that LNP corona formation involved interactions with various lipoprotein particles. In terms of composition, lipoproteins particles are, however, complex and dynamic. While our analysis revealed that certain lipoproteins correlated more with increased LNP efficacy, it is challenging to map this proteomic fingerprint onto a naturally occurring lipoprotein particle population, although our data suggested that HDL particles were a likely source of the efficacious corona components.

To explore LNP interactions with lipoprotein particles experimentally, Huh7 hepatocytes were exposed to LNPs together with 1% lean plasma spiked with purified HDL, VLDL, and CM to determine how these components affected cellular mRNA expression (recall that LNPs supplemented with 1% lean plasma are not very functional in Fig. 1). The lipoprotein candidates were spiked into lean plasma using a wide concentration range, either separately or combined, at the 200 ng/well eGFP mRNA dose, and the resulting eGFP production was measured (Fig. 5d, and supplementary Fig. 17).

We found that, unlike other lipoproteins, only corona HDL could potentiate cellular responses to LNP exposure, and even at the lowest spike-in concentration (5×10^5 particles/well), HDL alone could stimulate the expression of mRNA delivered by LNPs, with a maximum response at 1.6×10^7

particle/well. At higher HDL concentrations, a decline in eGFP mRNA expression was observed. In comparison, even at high concentrations, the addition of VLDL and CM did not affect LNP performance compared to lean plasma alone (dotted line, Fig 5d). When HDL, VLDL, and CM were spiked in an equal concentration together, the LNP performance enhancement was also not different from HDL alone. In contrast, at higher spike-in doses, the lipoprotein combination mitigated somewhat the eGFP expression reduction seen when using higher doses of HDL.

Next, we investigated whether the HDL-potentiated cellular mRNA expression was LNP/HDL ratio-dependent. We dosed the LNPs to recipient cells at various combinations of LNP doses and HDL concentrations. At lower LNP doses, the maximum mRNA expression occurred at lower HDL concentrations, while high LNP doses required more HDL but resulted in higher mRNA expression (Fig. 5e), suggesting a constant optimal ratio between corona HDL and the LNPs in terms of productive cargo delivery.

We also verified the HDL-enhanced cellular mRNA expression in human lipoprotein deficient serum (Supplementary Fig. 18). When human Huh7 hepatocytes were exposed in the human serum with lipoprotein removed, LNPs did not significantly evoke eGFP expression at 25, 50, and 200 ng/well mRNA doses. When HDL was added, the same relationship between HDL and LNP numbers was seen, with lower plateaus at lower doses and higher plateaus at higher doses. Again, further increasing the amount of HDL added inhibited cellular eGFP expression. In comparison, VLDL and CM did not stimulate effective cellular eGFP expression. The decline of cellular eGFP expression at high HDL doses is likely a result of increased competition, between corona components and the unbound counterparts in plasma, for cell surface receptors, such as the previously mentioned SR-BI⁵¹ (Supplementary Fig. 19). This is interesting, as it suggests that

LNPs interact with HDL at lower doses and it is only at relatively higher doses that cell surface scavenger receptor blockade negatively impacts LNP performance.

Finally, thanks to our efficient method for isolating LNPs and their associated coronas, we could allow the coronas to form, retrieve the LNPs and then dose them to Huh7 hepatocytes (Supplementary Fig. 20). The LNPcor was formed and retrieved from 1% lean pool (Lean_{PD}) and 1% obese (Obese_{PD}) as described above. The retrieved LNPcor were then dosed together with 1% plasma from lean or obese animals. In agreement with our previous data, the obese plasma containing higher HDL levels clearly inhibited the uptake of LNPs with pre-formed HDL-enriched coronas.

Conclusions

Much effort has been devoted to the development of highly effective LNPs through sophisticated chemical composition designs. However, most of these efforts proceed in a “trial and error” manner. How these designs can affect delivery efficacy and tissue tropism is largely unpredictable, particularly due to the tremendous diversity of pathophysiological conditions. This severely hinders the development of personalized precision nanomedicine. Understanding corona formation is, on the other hand, an emerging approach for optimizing nanomedicine design towards desirable biological outcomes.

Our study demonstrated that individual physiological states can affect LNP function through corona formation. The heterogeneity amongst patients/diseases can hinder the success of clinical nanomedicines, whereas most nanomedicine studies are carried out in unstratified patient populations⁵² and, our data suggest that corona composition is more predictive than plasma

biomarkers. For example, although animal 12 was classified as obese using relevant biomarkers, its plasma resulted in an LNP corona composition closer to lean plasma-derived corona and eGFP expression similar to the lean group. Our study is the first to show new LNP designs, informed by corona composition generated in a stratified patient populations or individuals, can improve the delivery of and response to, nanomedicine therapies.

Therefore, unbiased corona analysis is essential for decoding the relationships between LNP composition, corona composition, and LNP function. Using a streamlined process for corona isolation, multi-omics corona assessment, and multivariate analysis, we successfully pinpointed HDL as a determinant of LNP mRNA delivery and expression efficacy and, we confirmed this by adding exogenous HDL. Interestingly, we uncovered a relationship between how HDL is associated with the LNP coronas, the amount of free HDL and the cellular capacity for HDL-mediated uptake. At higher plasma concentrations, free HDL functioned antagonistically, whereas reduced plasma concentrations resulted in lower free HDL, greater particle uptake and, subsequently, improved mRNA expression. At the lowest lean plasma concentrations, there was not enough HDL to populate LNP coronas, especially when using high doses of LNPs and this degraded LNP performance. The number of HDL particles is, however, elevated in obese rat plasma, so the LNPs remained functional even at low obese plasma concentrations, and under these conditions, there was also less competition from unbound HDL. Taken together, these results are first to indicate that LNP designs that encourage association with HDL or HDL components will improve LNP performance by tipping the balance between LNP uptake and, free-HDL blockade of cellular uptake mechanisms (such as SR-B1⁵³).

This study highlights the importance of HDL for LNP corona formation and mRNA expression efficacy. Most early studies accredited LNP delivery, especially hepatic delivery, to ApoE-mediated machinery. However, the lipid-bound state of Apo E⁵⁴ and competition with non-corona ApoE⁵¹ may further obscure the correlation of corona Apo E-induced uptake, and we found that corona ApoE content was not the best predictor for LNP efficacy. The current study illustrated that among ApoE containing lipoproteins, only HDL modulated LNP mRNA expression efficacy. Thus, for LNP corona content, HDL lipoproteins ApoM and/or ApoAII may serve as better predictors of LNP efficacy. This raises the question of how co-medication might affect the therapeutic efficacy of nanomedicines. While commonly used cholesterol-lowering drugs such as statins have a moderate stimulatory effect on HDL, Fibrate and prescription-strength Niacin (which can also be obtained in the everyday diet) can effectively increase HDL levels⁵⁵.

The OPLS analysis also indicated that lipids (rather than lipoproteins) were contributive but less decisive for LNPs' performance. Although one lipid hit LPC 20:1 did display a negative correlation to LNP performance, and LNPs formulated with LPC demonstrated increased tropism towards liver endothelial cells⁵⁶, making LPC 20:1 a potential candidate for delivery to tissues other than liver hepatocytes.

In summary, we have created an efficient method for isolating LNPs, with intact coronas from plasma followed by an automated and unbiased mass spectrometric analysis of corona protein and lipid content. We used this method to evaluate how corona composition affects LNP function (in terms of effective mRNA delivery) by forming coronas in plasma from individual lean and obese animals. Our study identified a lipoprotein fingerprint that promotes LNP function, leading us to examine the role of HDL-LNP interactions as an essential factor for LNP efficacy. With these

476 methods, it will now be possible to explore a greater variety of particles, biofluids, tissues, and
477 physiological states in order to more fully explore the relationships between lipid nanoparticle
478 corona content and delivery efficacy. While designing LNPs to promote particular corona
479 compositions is a significant engineering challenge, the complexity of these interactions creates
480 many opportunities for improving safety, reducing cost, targeting tissues and, tuning therapeutic
481 particles for specific biological and pathobiological contexts.

Methods

Materials

Cholesterol (C8667), DSPC (LP-R4-076) was purchased from Merck. DOPE-Rhod (810150) was from Avanti Polar Lipids. DMPE-PEG2k (PM-020CN) was obtained from Nof America Corporation. Cationic lipids DLin-MC3-DMA were chemically synthesized in-house. Other chemicals, unless specified, were purchased from Merck and used as received.

Formation of LNPs

Commonly used and clinically relevant reference LNPs containing DLin-MC3-DMA were formulated using a NanoAssemblr microfluidic mixer (Precision NanoSystems). As described in Supplementary Fig.2, to achieve a 10:1 (w/w) lipid:mRNA ratio (N:P ratio = ~3:1), lipids were prepared in ethanol at a ratio of 50:38.5:9.9:1.5:0.1 (MC3: Cholesterol: DSPC: DMPE-PEG2000: DOPE-Rhod). Unlabelled and Cy5 labelled eGFP mRNA were prepared at a 4:1 ratio (TriLink: L7201/7701) in 50 mM citrate buffer (pH 3, TekNova: Q2445). Lipid and mRNA-containing solutions were mixed 1:3 (ethanol: citrate) at a constant flow rate of 12 ml/min to form LNPs. Formulated LNPs were dialyzed overnight in PBS (pH 7.4) at 4 °C.

Physical particle characterization

Particle size (Z-average diameter) and polydispersity index (PDI) were determined by dynamic light scattering utilizing a Malvern Zetasizer ZS for newly formulated LNPs by NanoAssemblr or a Malvern Zetasizer APS for LNPs and harvested LNPcor. Following formulation, the LNPs surface zeta-potential was measured by a Malvern Zetasizer ZS. In both cases, viscosity and

refractive index values, 0.8872 mPa and 1.33 respectively, were used for data analysis (Zetasizer software, v7.12).

The mRNA concentration and encapsulation of LNPs were evaluated by Ribogreen dye (Thermo Fisher Scientific) according to the manufacturer's guidelines. $\pm 1\%$ Triton was used to ascertain the fraction of encapsulated mRNA by comparison to a relevant free mRNA standard curve.

Particle number and size distribution of LNPs, coronated LNPs and, endogenous particles in blood plasmas were determined by nanoparticle tracking analysis (NTA). The analysis was performed at RT using a Nanosight LM14 model (Nanosight, UK) equipped with a blue laser (405 nm, 70 mW) and SCMOS camera. The samples were prepared by diluting stocks from 1:2 000 to 1:10 000 in PBS before analysis to obtain an appropriate concentration for the NTA measurements. Three 90-second videos were recorded. The data analysis was performed using the NTA software v3.0.

Cell culture

H4-II-E-C3 (CRL-1600), McA-RH-7777 (CRL-1601), and NRK-49F (CRL-1570) cells were purchased from ATCC. Huh7 (Riken - RCB1366) cells were a kind gift from Samir El-Andaloussi (KI, Stockholm). All cell lines were authenticated by STR profiling and tested negative for mycoplasma contamination. Cells were maintained in an incubator at 37 °C with humidity in the complete media (DMEM + Glutamax supplemented with 10% fetal bovine serum).

Animals

Ten week-old male lean and obese Zucker rats (CrI:ZUC-*Lep^{fa}*) were purchased from Charles River Laboratories (Maryland, USA) and group-housed ($n=4/\text{cage}$) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility at 20-

22°C and relative humidity of 40-60 % with a 12-h day/night cycle. The rats had free access to water and a standard rodent chow diet (R70, Lactamin AB). At 20 weeks of age, the rats fasted for 4 hours, and a tail vein blood sample was obtained for glucose (Accu-Chek® Mobile, Roche Diagnostics) and glycosylated hemoglobin (HbA1c, PTS Diagnostics) analyses. Thereafter, the rats were euthanized using isoflurane anesthesia (Forene®, Abbott) and blood was collected from the heart and EDTA plasma was isolated and stored at –20 °C. The experimental procedures were approved by the local Ethics Committee for Animal Experimentation (Gothenburg region, Sweden).

Blood plasma analysis

Plasma insulin levels were measured using a mouse/rat insulin kit (#K152BZC-1, Meso Scale Discovery). Plasma triglyceride (#11877771, Roche Diagnostics), cholesterol (#A11A01634, Horiba Medical) and alanine aminotransferase (ALT, #A11A01627, Horiba Medical) levels were analyzed using an ABX Pentra 400 instrument (Horiba Medical).

Imaging experiments and quantification

Cells were seeded at appropriate densities into CellCarrier-384 Ultra plates (#6007558, PerkinElmer) in complete media a minimum of 16 h prior to treatment. At the experimental start, the media on cells was removed and replaced with media containing the experimental treatment as denoted in the relevant figures. The lean and obese rat plasma used were withdrawn as described above and characterized as shown in Supplementary Fig.2 and Data 1. The human lipoprotein deficient serum (#LP4, Merck), HDL (#LP3, Merck), VLDL (#LP1, Merck), and CM (#SPR6304, Merck) were commercially available.

Following a 1 h incubation at 37 °C with 100% humidity, the cell plate was then imaged. Live-cell imaging was carried out with a CV7000 (Yokogawa) spinning disk confocal microscope utilizing a 20x objective (NA 0.75) in a humidified chamber maintained at 5% CO₂. Images were obtained using a bright-field lamp (for digital phase contrast, DPC) and the following fluorescence excitation (emission) wavelengths: 488 nm laser (BP522/35), 561 nm laser (BP600/37) and 640 nm laser (BP676/29). For time-resolved measurements, the same fields of view were imaged over time with three optical planes (slicing interval: 3µm). Final fluorescent image stacks were constructed using maximum intensity projections. Images were processed and analyzed for relevant features and parameters indicated in figures utilizing Columbus image-analysis software (Perkin Elmer, v2.9.0). Briefly, cells were identified using DPC using a ‘find cells’ building block (Method M) within Columbus software that identifies individual cell boundaries. Within individual cell regions of interest, fluorescent intensities were quantified for each relevant fluorophore. Data were exported and plotted with JMP (v15.0.0) and Graphpad Prism (v9.0.0) with appropriate statistical analysis. The overall eGFP measured over the 10 h time course were summarized as a new parameter termed 10 h area under the curve (AUC_{10h}) for OPLS correlation analysis.

LNPs stability in culture medium

0.8 µg mRNA containing LNPs were resuspended in a final volume of 200 µL using different rat plasmas or PBS. 50 µL of these suspensions were pipetted in a 384 well plate (Greiner, #781209) in triplicates, and the plate was incubated for 0h, 4h and 8h at 37°C. At these timepoints, the emission spectra were obtained using a Safire II plate reader (Tecan) and 560 nm excitation with emission scanning from 570 to 750 nm. FRET efficiency is calculated as the ratio between acceptor

molecule (Cy5) emission peak (FA) and the ratio of Rhod (FD) and Cy5 emission in total (equation 1).

$$(Eq. 1) FRET = \frac{FA*}{FD**+FA}$$

*The emission peak for Cy5 (FA) was the maximum RFU measured for 668-678 nm.

**The emission peak for Rhodamine (FD) was the maximum RFU measured for 586-594 nm.

Asymmetric flow field-flow fractionation (AF4) separation

AF4 separation was performed using an Agilent 1260 Infinity HPLC (Agilent) connected to an Eclipse AF4 separation system (Wyatt Technology) followed by a Dawn Heleos-II 18-angle MALS detector (Wyatt Technology). Separations were performed using a 10-kDa molecular-mass cutoff polyether sulfone membrane with an S-350 μ m spacer in a 153 mm separation channel. Samples were introduced to the channel at an inlet flow of 0.2 mL/min and subsequently focused at the head of the channel at a focus flow rate of 1.5 mL/min. Samples were eluted over 25 min with a channel flow rate of 1 mL/min and a cross-flow gradient of 3.0–0 mL/min.

LNP corona isolation

LNPs were incubated in cell culture media supplemented with 1% individual plasmas at 200ng mRNA LNPs dose (4 μ g/mL of mRNA). M-270 Epoxy Dynabeads (#14321D, Thermo Fisher Scientific) were cross linked with monoclonal anti-PEG [PEG-2-128] (Abcam) according to the manufacturer's instructions. A KingFisher Flex magnetic purifier (Thermo Fisher Scientific) with 96 magnetic rod heads was used to separate the free-proteins, endogenous nanosized particles from the LNPcor. Briefly, the incubation, wash, and elution procedures were performed using the

optimized conditions indicated in Supplementary Fig. 10. The antibody-conjugated Dynabeads were incubated with LNPs within media for 20 min at an Antibody: mRNA (wt:wt) ratio of 1 with gentle mixing. At the end of incubation, the Dynabeads were extracted using magnet rods and washed four times with PBS. A basic pH elution buffer containing 0.5 M NH₄OH and 0.5 mM EDTA was utilized to release LNPcor from Dynabeads. LNP pull-down quantification was performed by Cy5 fluorescence readout.

Cryo-electron microscopy

For cryo-electron microscopy experiments, lean and obese LNP samples at a concentration of ~10¹³ particles/ml were incubated with glow discharged carbon-coated copper grids (SPI supplies), following vitrification at 10 °C and 99 % humidity by using a Leica EM GP automatic plunge freezer (Leica Microsystems Company). The excess sample was removed by blotting dry the grid for 2.5 sec with filter paper and plunging it into liquid ethane at -180 °C. Following vitrification, grids were stored immersed in liquid nitrogen until use. Before imaging, the grids were mounted in a Gatan 626 cryo-holder (Gatan Company) and analyzed using an FEI Tecnai G2 Spirit BioTwin transmission electron microscope (Thermo Fisher Scientific). Pictures were taken with a Morada digital camera (Olympus Soft Image Solutions) and iTEM image capture software.

Proteomics analysis

Corona protein digestion was performed on recovered LNPs from individual plasmas containing an equal amount of mRNA. Briefly, sample denaturation and reduction were performed using a 30 min one-step 8M urea (#U1250, Merck) and TCEP bond-breaker solution (#77720, Thermo Fisher Scientific), followed by a 30 min alkylation step using a 2-chloroacetamide reagent (#22790,

607 Merck). Protein digestion was done overnight in trypsin (#EMS0004, Merck) and ceased by the
608 addition of formic acid. Digestions were measured using a Q-Exactive HF mass spectrometer
609 (Thermo Fisher Scientific) coupled with an Evosep One (Evosep) automatic sample loader equipped
610 with Evotip disposable C18 trap columns (Evosep) for in-line peptide desalting and purification
611 immediately prior to analytical column separation with a preset, 30 samples per day (30-SPD),
612 loading sequence. Briefly, purified peptides were separated on an 8 cm analytical reverse phase
613 column (Evosep) with gradient off-set focusing to achieve a 3%-40% acetonitrile within a 44 min
614 loop at a 0.5 μ L/min flow rate.

615 MS raw files were analyzed by MaxQuant software (v1.6.6.0). Proteins were identified using the
616 Uniprot FASTA database (*Rattus norvegicus*, June 2019) with N-terminal acetylation and
617 methionine oxidations as variable modifications, and cysteine carbamidomethylation as a fixed
618 modification. The false discovery rate (FDR) was set to 1% by reverse database search for both
619 proteins and peptides with a minimum length of 7 amino acids. Enzyme specificity was set as
620 trypsin (cleavage at C-terminal to arginine and lysine). A maximum of 2 missed cleavages was
621 allowed in the database search. Peptide identification was performed with an initial precursor mass
622 deviation tolerance up to 6 ppm and a main mass deviation tolerance to 20 ppm. Matching between
623 runs was performed among samples. Proteins matching to the reversed database were filtered out.
624 For protein quantification, MaxQuant computes raw protein intensities as the sum of all identified
625 peptide intensities. Label-free quantification (LFQ) and intensity-based absolute quantification
626 (iBAQ) were calibrated from raw protein intensities with a minimum peptide ratio count of 1.

627 To compute protein relative abundance (PRA), intensity-based absolute quantification (iBAQ)
628 obtained from raw protein intensities were divided by the number of theoretically observable

peptides calculated using in silico protein digestion. Then, iBAQ intensities of identified protein were used to calculate PRA.

To compare each identified protein between samples, statistical analyses were first performed with the Perseus software (v1.6.2.3) using LFQ intensities. A valid value threshold was first applied to identify significantly altered corona proteins among all identified proteins. Only proteins identified within all conditions and replicates, followed by a multi-sample ANOVA test, are considered valid hits (significance cutoff: permutation-based FDR <5%). Further hierarchical clustering analysis was performed with JMP (v15.0.0). OPLS analysis was performed with SIMCA (v16.0.1), using z-score normalized LFQ values. The Pearson correlation between single corona component and cellular eGFP was carried out with Prism GraphPad (v9.0.0). The correlation coefficient r was controlled by nonparametric (Spearman) correlation P value.

Lipidomics analysis

The molecular species of corona phospholipids was extracted with the BuMe method⁴³. Briefly, the samples were combined with a BuMe mixture (butanol: methanol ratio=3:1). SPLASH I S(#330707, Avanti polar lipids) was included in the BuMe as an internal standard. Following a series of centrifugations, the extracted lipids were then evaporated and redissolved in MeOH and analyzed by HILIC-UPLC-ESI-MS/MS. The UPLC-MS/MS system was an Acquity I class coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer with a BEH amide analytical column (#186004801, Waters).

The mobile phase A was composed with 95% Acetonitrile and 5 mM Ammonium Formate. The mobile phase B was 10 mM Ammonium Formate (#17843, honeywell). The mass spectrometer

was operated in positive ESI mode. 29 transitions were monitored for PE and 11 for LPE, all transitions having a neutral loss of 141 Da. 29 transitions were monitored for PC, 13 for SM, and 20 for LPC all transitions having a common 184 Da product ion. The Cone voltage was kept at 30 V for all transitions and the collision energy was kept at 25 V for all transitions.

Immunoblotting

2µL solution containing LNPhor or corresponding control samples was spotted to Nitrocellulose membranes (#LC2001, Thermo Fisher Scientific). Membranes were blocked with Intercept (TBS) blocking buffer (LI-COR) for 30 min at RT and incubated with primary antibodies Cy5 (#ab52061, Abcam), PEG (#ab51257, Abcam), Apo AII (#ab92478, Abcam), Apo CII (#ab230447, Abcam), Apo CIII (#ab76305, Abcam) and ApoE (#ab183597, Abcam) where appropriate, diluted 1:1000 in blocking buffer at for 30 min at RT. Membranes were washed three times with 0.1% TBS-Tween and incubated for 30 min at RT with IRDye 680RD Goat anti-Mouse IgG (#926-68070, LI-COR) and 800CW goat anti-rabbit IgG (#926-32211, LI-COR) diluted 1:2000 in 0.1% TBS-Tween. Following three washes, membranes were visualized with the Odyssey CLx imaging system and processed in Image Studio (v4.0, LI-COR).

Statistics and reproducibility

Statistical testing was carried out with relevant multiple comparisons and post-testing where appropriate as indicated in the respective methods. For all experiments, no data were excluded from the analysis and all reported results were replicable. For imaging experiments, statistical testing was carried out upon three independent experimental replicates. For omics experiments, a minimum number of two independent experimental replicates were used for data statistics. See

671 figure legends for full details of replicates, statistical testing, and significance. For further
672 multivariate analysis information, please see the proteomics analysis section and the main text.

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Competing financial interests

Kai Liu, Ralf Nilsson, Elisa Lázaro-Ibáñez, Tasso Miliotis, Michael Lerche, Rita Salgado Ribeiro, Marie Strimfors, Hanna Duàn, Johan Ulander, Daniel Lindén and Alan Sabirsh are or were at the time of work employees of AstraZeneca.

Figures, Legends, and supplementary material

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Figures

Figure 1

The efficacy of LNPs mRNA delivery is individual plasma and dose dependent. a, The experimental design for evaluating LNP potency under lean and obese conditions. The candidate mRNA doses and the lean (LP) and obese pooled (OP) plasma concentrations resulting in the largest difference between lean

and obese states were identified prior to exploring individual plasma samples. b, A series of mRNA doses (25-400 ng/well) were tested in the presence of LP or OP plasma (0, 1, 5 and 10%) using H4-II-E-C3, McA-RH-7777, Huh7 and NRK-49F cell lines over a 10 h time course. The cellular eGFP mean fluorescent intensity (MFI) was quantified using image analysis and visualized as contour maps (each black dot represents the mean of n=3). In general, the eGFP expression increased over time. The obese plasma induced higher eGFP expression at the 1% plasma concentration in hepatocytes. c,d, The fold change of mRNA cargo expression (eGFP) and LNP uptake (Rhodamine label for lipids; and Cy5 label for mRNA) at the 10 h endpoint was calculated using the data from panel b as indicated by triangles (solid triangle: 50ng/well dose; hollow triangle: 200ng/well dose). At the 50 ng/well mRNA dose, the lean and obese plasma-complexed LNPs resulted in comparable cellular uptake and eGFP expression. The uptake of LNPs, following lean and obese plasma supplementation, was mildly improved at the 200 ng mRNA/well, whereas the eGFP expression was significantly elevated, particularly in rat hepatocytes. e, The difference in LNP efficacy was assessed using a 200ng/well mRNA dose in vitro. An apparent variation was observed between lean and obese plasmas, and among every individual. The error bars represent standard deviation of the mean (n=3).

Figure 2

The development of affinity based magnetic isolation of LNP-corona complexes. a, Schematic illustration of the ultrafast affinity-based, 96-well isolation method. Anti-PEG antibody conjugated magnetic beads capture LNPcor from plasma containing free protein, extracellular vesicles and lipoprotein particles. b, The design of experiment (DoE) space of LNPcor capture (epitope), wash and elution. The circles indicate where the majority of LNPs were detected. Only when combining an antibody against PEG backbone with PBS washing and basic elution conditions, were the majority of LNPs was identified in final elution. c, The recovery ratio of LNPcor in terms of particle number, rhodamine labelled lipids (lipid) and Cy5 labelled mRNA (mRNA). Fig. 2a is created with BioRender.

Figure 3

Proteomics profiling of individual biomolecular coronas. **a**, The intensity of individual plasma derived LNPcor entities. The intensity was summarized from all identified corona proteins from each individual-plasma-derived LNPcor ($n=9$). The uncomplexed LNPs and non-specifically bound lipid binding were evaluated, respectively. For box and whisker plots, each data point represents an individual LNPcor, averaged from duplicate measurements. The box represents the interquartile range (first quartile, median and third quartile). BG is the intensity summarized from background impurity controls. **b**, The average ratio of major proteomic functional clusters summarized from lean and obese-individual-derived LNPcor. **c**, The most abundant coronal proteins (RPA> 0.1%) in lean and plasma-derived LNPcor and the corresponding RPA in original plasmas ($n=2$). RPA: relative protein abundance. CV: coefficient of variation. **d**, An overview of major corona proteins in individual LNPcor ($n=2$). UP, uncharacterized protein.

e, Hierarchical clustering of individual plasma derived LNPcor using proteomic fingerprints. Lean- and obese-plasma-derived LNPcor formed distinct clusters.

Figure 4

The lipidomics profile of corona lipids. a, The summary of natural lipid species intensity identified within individual plasma (n=9). b, The summary of natural lipid species intensity identified within individual-plasma-derived LNPcor (n=9). Uncomplexed LNPs (LNP, dotted lines) and nonspecific lipid binding (BG, dotted lines) were also evaluated. For box and whisker plots, each data point represents an individual LNPcor averaged from duplicate measurements. The box represents the interquartile range (first quartile, median and third quartile). c, Hierarchical clustering of individual plasma-derived LNPcor using lipidomics fingerprints. Lean and obese plasmas derived LNPcor formed distinct clusters.

Figure 5

High-density lipoprotein modulated LNPs performance. a, OPLS analysis to illustrate the correlation between corona contents and cellular eGFP expression. The orthogonal axis (orthogonal loading vector po of the X-part and the projection onto Y (so), poso) indicates the corona contents' orthogonality to eGFP expression; The predictive axis (X loading weight p and Y loading weight q combined to one vector, pq) implies the corona content's impact on eGFP expression. b, The correlation between corona ApoM or

ApoE (z-score normalized) and eGFP expression in a variety of cell lines with Pearson correlation coefficient r . c, The correlation coefficient r between plasma ApoM or ApoE (z-score normalized) and eGFP expression in a variety of cell lines. d, The spike-in of HDL, but not VLDL and CM stimulated LNP-mediated eGFP expression at 200 ng/well mRNA dose in Huh7 hepatocytes. e, HDL spike-in with different doses of LNPs and HDL particles in Huh7 hepatocytes. The curves reveal the relationship between LNP and HDL particle numbers. The error bars represent standard deviation of the mean ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

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