HDL surface lipids mediate CETP binding as revealed by electron microscopy and molecular dynamics simulation

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Cholesteryl ester transfer protein (CETP) mediates the transfer of cholesterol esters (CE) from atheroprotective high-density lipoproteins (HDL) to atherogenic low-density lipoproteins (LDL). CETP inhibition has been regarded as a promising strategy for increasing HDL levels and subsequently reducing the risk of cardiovascular diseases (CVD). Although the crystal structure of CETP is known, little is known regarding how CETP binds to HDL. Here, we investigated how various HDL-like particles interact with CETP by electron microscopy and molecular dynamics simulations. Results showed that CETP binds to HDL via hydrophobic interactions rather than protein-protein interactions. The HDL surface lipid curvature generates a hydrophobic environment, leading to CETP hydrophobic distal end interaction. This interaction is independent of other HDL components, such as apolipoproteins, cholesteryl esters and triglycerides. Thus, disrupting these hydrophobic interactions could be a new therapeutic strategy for attenuating the interaction of CETP with HDL.

A n elevated level of low-density lipoprotein-cholesterol (LDL-C) and/or a low level of high-density lipoprotein cholesterol (HDL-C) in human plasma are major risk factors for cardiovascular disease (CVD). Cholesteryl ester transfer protein (CETP), with a molecular mass of 53 kDa (composed of 476 amino acids) before post-translational modification1, mediates the cholesteryl ester (CE) transfer from high-density lipoprotein (HDL) to low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), and in exchange for triglyceride (TG). CETP deficiency has been shown to be associated with elevated HDL cholesterol levels2,3. Loss-of-function mutations in the CETP gene were negatively associated with premature atherosclerosis, and have been implicated in longevity in some studies4. On the other hand, there is also evidence that CETP mutations are associated with an increased incidence of coronary heart diseases4. Despite these inconsistencies and because of the urgent public desire to expand treatment options beyond statins, the most financially successful drug to reduce LDL-C levels to date, CETP has been used as a promising drug target for designing inhibitors in order to treat heart disease5–10. Four large clinical trials of CETP inhibitors5,8–10 have been undertaken to date. The first two CETP inhibitors failed in phase III clinical trials due to increased mortality related to off-target effects and lack of efficacy. Two other CETP inhibitors9,10 are currently being investigated in large clinical outcome trials. As such, an improved understanding of CETP’s molecular interactions could eventually provide beneficial and definitive descriptions of CETP function, thereby leading to new CETP-related drug design.

The structure of CETP, revealed by X-ray crystallography, resembles a banana shape with dimensions of roughly 3 × 3 × 13 nm and contains four structural components: an N-terminal β-barrel domain, a C-terminal β-barrel domain, a central β-sheet, and a C-terminal extension (a distorted amphipathic helix, helix X, Glu465-Ser476 at the C terminus)11,12. Electron microscopy (EM) has shown that CETP forms a bridge between HDL and LDL, with its N-terminal β-barrel domain penetrating the HDL surface and its C-terminal β-barrel domain penetrating the LDL surface13. A molecular dynamics (MD) simulation has revealed that the distal portions of the N- and C-terminal β-barrel domains of CETP remain highly flexible in solution14. This flexibility may be necessary for conformational changes to occur at the distal ends, a necessary step for the formation of a tunnel through the entire molecule13,15.

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Although CETP has been intensively studied, a detailed understanding of how CETP senses and binds to HDL remains unknown due to the heterogeneity and dynamics of HDL. HDLs vary in size, shape, and composition. Considering that most plasma CETPs are naturally bound to HDLs, an investigation of how different components of HDL affect the interaction of spherical HDL with CETP is essential for a complete understanding of CETP function.

Here, we studied CETP interactions with various HDLs and liposome vesicles using optimized negative-staining (OpNS), cryo-electron tomography (Cryo-ET), and molecular dynamics simulations in order to understand how the different HDL components affect CETP binding.

Results

EM images of CETP bound to plasma HDL2. Based on previous reports, the spherical HDL2 in plasma varies in diameter (~9 to ~15 nm) and in density (from 1.063 to 1.125 g/ml)\(^{19,20}\). Earlier studies\(^{18,19,21}\) showed the average molecular weight of HDL2 is 360 kDa and that the particles contain three major surface components: apolipoprotein A-I and A-II (apoA-I and apoA-II) (total ~40.2% of MW) and phospholipids (~31.3% of MW), as well as core lipids: cholesterol esters (~17.6% of MW) and triglycerides (~4.2% of MW). A small amount of free cholesterol (~5.8% of MW) is distributed between the particle surface and the core\(^{21}\).

In the present study, CETP was incubated with isolated HDL2 at a molar ratio of 1:4 (HDL2: CETP), then prepared as described for optimized negative-staining (OpNS) EM method\(^{22,23}\) (a method that minimizes rouleaux formation), and examined by electron microscopy (EM). A large field EM micrograph and representative particles showed that CETP-HDL2 complexes had the appearance of rod shaped CETP penetrating spherical shaped HDL2 (Fig. 1a). No CETP was found to bridge two HDL2 particles or to adhere to the convex surface of HDL2 via its concave surface as hypothesized by...
40% of the HDL3 particles were bound to CETP (compared to Fig. 1f). The micrographs and statistical analyses showed more than two CETP molecules bound to more than two HDL2 particles (Fig. 1b). Among these CETP molecules (Fig. 1b), considering that HDL3 and HDL2 have a molar ratio of 4:1 (CETP: HDL3), prepared for OpNS and examined by EM. The EM micrographs showed that the CETP-HDL3 complexes were similar to CETP-HDL2 complexes, with the rod-shaped CETP adhering to the spherical shaped surface of HDL3 (Fig. 1b). As before, no CETP bridged two HDL2 particles or adhered to the convex surface of HDL3 via its concave surface. The diameter of HDL3 that was bound to CETP (Fig. 1e) was ~11.4 ± 1.7 nm, similar to that of HDL3 alone (~10.9 ± 1.0 nm) (Supplementary Fig. 1e). The rod shaped CETP in the complexes had a similar width to CETP itself (Supplementary Fig. 1e), but were shorter in length (~8.6 ± 1.2 nm, Fig. 1f). The micrographs and statistical analyses showed more than 40% of the HDL3 particles were bound to CETP (compared to ~50% for HDL2), in which 32.8% ± 7.0% (mean ± sd) of the HDL3 particles bound to one CETP molecule, while 8.2% ± 1.4% (mean ± sd) of the HDL3 particles bound to two and more CETP molecules. Among these bound HDL3, ~20.2% was also bound to two or more CETP molecules (Fig. 1b). Considering that HDL3 and HDL2 have the same surface components (apoA-I, apoA-II, and phospholipids), the lower percentages (~41.1% vs. ~50.7%) of CETP bound to HDL that have a lower percentage (22.7% vs. 31.3%) of surface phospholipids suggest that the surface phospholipids/protein ratio may have contributed to some variation in CETP binding.

TEM images of CETP bound to plasma HDL3. To investigate how three major HDL surface components: phospholipids, apoA-I and apoA-II affect the CETP binding, the above experiment was repeated with HDL3 that contain a different surface protein and lipid ratio than HDL2. Earlier studies showed that HDL3 vary in size, but have an average molecular weight of 175 kDa, a density of ~1.125 to ~1.210 g/ml, and contain apoA-I and apoA-II (~55.5% of MW), phospholipids (~22.7% of MW), free cholesterol (~2.8% of MW), cholesterol esters (~14.7% of MW) and triglycerides (~3.4% of MW). Thus, HDL3 has a lower surface percentage of lipids, but a higher surface percentage of proteins than HDL2.

In this study, the HDL3 sample was incubated with CETP at a molar ratio of 4:1 (CETP: HDL3), prepared for OpNS and examined by EM. The EM micrographs showed that the CETP-HDL3 complexes were similar to CETP-HDL2 complexes, with the rod-shaped CETP adhering to the spherical shaped surface of HDL3 (Fig. 1b). As before, no CETP bridged two HDL2 particles or adhered to the convex surface of HDL3 via its concave surface. The diameter of HDL3 that was bound to CETP (Fig. 1e) was ~11.4 ± 1.7 nm, similar to that of HDL3 alone (~10.9 ± 1.0 nm) (Supplementary Fig. 1e). The rod shaped CETP in the complexes had a similar width to CETP itself (Supplementary Fig. 1e), but were shorter in length (~8.6 ± 1.2 nm, Fig. 1f). The micrographs and statistical analyses showed more than 40% of the HDL3 particles were bound to CETP (compared to ~50% for HDL2), in which 32.8% ± 7.0% (mean ± sd) of the HDL3 particles bound to one CETP molecule, while 8.2% ± 1.4% (mean ± sd) of the HDL3 particles bound to two and more CETP molecules. Among these bound HDL3, ~20.2% was also bound to two or more CETP molecules (Fig. 1b). Considering that HDL3 and HDL2 have the same surface components (apoA-I, apoA-II, and phospholipids), the lower percentages (~41.1% vs. ~50.7%) of CETP bound to HDL that have a lower percentage (22.7% vs. 31.3%) of surface phospholipids suggest that the surface phospholipids/protein ratio may have contributed to some variation in CETP binding.

TEM images of CETP bound to spherical recombined HDL (rHDL). To investigate which HDL surface component dominates CETP binding, one of three major HDL surface components, apoA-II, was excluded by repeating the above experiment using reconstituted HDL (rHDL). The rHDL contain apoA-I as the only protein (~43% in MW) and POPC as the only phospholipid (~32.9% in MW). Cholesteryl esters were the only core lipid components (43% in MW) and POPC as the only phospholipid (~32.9% in MW). Cholesteryl esters were the only core lipid components (~21.7% in MW) and a small amount free cholesterol (~2.4%) was distributed between the surface and the core.

In this study, the rHDL sample was incubated with CETP (molar ratio of 4:1 for CETP: rHDL) prepared for OpNS and examined by EM. The EM micrographs showed ~42.9% of rHDL particles were attached to CETP. No CETP was found to connect two rHDL particles or bind to the rHDL surface via its concave surface. The average diameter of rHDL particles in rHDL-CETP complexes was 10.2 ± 1.2, similar to rHDL (10.3 ± 0.7 nm) alone (Supplementary Fig. 1e). The CETP protrusion length of ~9.2 ± 1.3 nm was shorter than that of CETP alone, but slightly longer than the CETP protrusions on the surfaces of HDL2 and HDL3 (~9.2 nm vs. ~8.6 nm, Fig. 1f). The micrographs and statistical analyses showed ~34.6% ± 5.3% (mean ± sd) of the rHDL particles bound to one CETP molecule, and ~7.6% ± 3.8% and ~0.7% ± 1.3% (mean ± sd) of the rHDL particles bound to two and more CETP molecules. Approximately 19.4% of the CETP-rHDL complexes were bound to two or more protruding CETP molecules. Notably, most multi-CETP binding complexes (~75%) showed the protruding CETP sharing the same hemisphere of rHDL (Fig. 1c). Moreover, the total number of CETP molecules bound to rHDL can exceed the total number of apoA-I molecules in
EM images of CETP bound to liposomes. To investigate which one of the remaining two HDL surface components, apoA-I or phospholipids, dominates the CETP binding, a lipid vesicle without apoA-I was further used to repeat the above experiments. The lipid vesicle (a POPC-only liposome) was incubated with CETP, and the sample was prepared by OpNS and examined by EM. The EM micrographs and representative liposomes showed the liposomes had a distribution of ~7.7 to ~116.2 nm in diameter (Fig. 1d), which is within the diameter range of all types of lipoproteins (from small HDL to large VLDL). Zoomed in views of a micrograph (Supplementary Fig. 3) and representative particle images (Fig. 1d, top right panel) showed spherical liposomes with surface CETP protrusions with an average length of ~8.5 ± 0.6 nm (Fig. 1f). The liposome particles retained their shape and structure after CETP insertion. Incubation with CETP did not cause particle disruption. Additionally, CETP did not form a bridge between two liposome particles, or bind to a liposome so that its concave surface was adjacent to the convex surface of the liposome. The micrographs and statistical analyses showed ~19.1% ± 3.2% of the liposome particles bound to 1–2 CETP molecules, ~6.0% ± 2.7% of the liposome particles bound to 3–4 CETP molecules, and ~5.1% ± 3.6% and 2.7% ± 1.7% bound to 5–6, or 7 and more CETP molecules respectively. In total, ~32% of the liposomes contained at least one surface CETP protrusion. Among these CETP bound liposomes, ~66% were bound to two or more CETP molecules (Fig. 1d). Notably, smaller liposomes had more CETP molecules bound (Fig. 2). For instance, 5–7 CETP molecules were bound to liposomes ~15 nm in diameter, while liposomes over ~40 nm were rarely bound to CETP (Fig. 2b and Supplementary Fig.4). This relationship was analyzed based on a linear fitting the number of bound CETP against the liposome diameters (Fig. 2a). This result suggests that the smaller liposomes had a higher binding affinity to CETP. Moreover, it confirmed that apoA-I in HDL do not dominate the CETP binding.

Three dimensional structure of a complex of CETP bound to liposome by individual-particle cryo-electron tomography. To confirm that the conformation of liposome-CETP complex was not due to negative-stain artifact, the sample was also flash-frozen in vitreous buffer without any negative-staining. The frozen sample was then examined by cryo-electron microscopy (cryo-EM) at ~170°C and under a low-dose imaging condition (Fig. 3a). The micrographs and selected particles showed the CETP liposome surfaces adhering with rod-shape CETP (arrows in Fig. 3b), similar to that from the OpNS (Fig. 1d). To reveal the detailed structure in three dimensions (3D), the sample was also imaged by cryo-electron tomography (cryo-ET) through a series of tilting angles (angle range of ~57° to 60° in steps of 1.5°). A 3D structure of a representative complex was reconstructed by individual-particle electron tomography (IPET)27. IPET was designed for 3D reconstruction of an asymmetric individual particle rather than averaging different particles like in conventional single-particle reconstruction. The naturally varying particle diameter of liposomes made it impossible to achieve any 3D reconstruction by conventional cryo-EM single-particle reconstruction methods.

A total of 79 tilting images of a representative liposome-CETP complex were windowed from CTF-corrected (by TOMOCTF28) cryo-ET micrographs (Fig. 3c, left panel). Although the tilt images (but CTF corrected) were noisy, a protrusion of CETP was marginally visible (arrows in Fig. 3c, left panel). To confirm that the
protrusion was from a bound CETP instead of noise, three images from consecutively tilted angles (28.5°, 30° and 31.5°) were averaged together to enhance the signal contrast and to reduce the noise level. The averaged image showed the protrusion was more clearly visible (arrow in Supplementary Fig. 5a) than in any individual tilt image, suggesting that the protrusion is indeed from CETP instead of noise. During IPET reconstruction, the tilt images were gradually aligned precisely to their "global center" via an iteration process. The noise during IPET reconstruction, the tilt images were gradually aligned precisely to their "global center" via an iteration process. The noise during IPET reconstruction, the tilt images were gradually aligned precisely to their "global center" via an iteration process.

The averaged image showed the protrusion was more clearly visible (arrow in Supplementary Fig. 5a) than in any individual tilt image, suggesting that the protrusion is indeed from CETP instead of noise. During IPET reconstruction, the tilt images were gradually aligned precisely to their "global center" via an iteration process. The noise during IPET reconstruction, the tilt images were gradually aligned precisely to their "global center" via an iteration process.

To test this hypothesis, we employed MD simulation to study the liposome surface hydrophobicity against the liposome size by generating a series of liposome vesicles with diameters of ~12 nm, ~20 nm, ~27 nm, ~35 nm and ~42 nm (Fig. 4, and supporting Table 1). After energy minimization (Supplementary Fig. 8), the analyses on the surface hydrophobicity of each liposome showed that the percentages of surface hydrophobic area (measured by the solvent accessible surface area, SASA) were ~12.9%, ~9.2%, ~8.0%, ~7.4% and ~7.3% respectively (Fig. 4b and d), suggesting that smaller liposomes had a higher hydrophobicity than the larger liposomes (Fig. 4e).

Discussion
Our experiments showed that, i) the similar morphology in CETP binding between plasma HDL and rHDL implied that the absence of Apo-A-II and TG in rHDL did not affect CETP binding to HDL (Fig. 1); ii) the total number of CETP molecules bound to rHDL can exceed the total number of apoA-I molecules in each rHDL particle; iii) non-apoA-I containing liposomes showed similar CETP protrusions to apoA-I containing HDL (Fig. 1d); iv) that there is a significant correlation between liposome size and number of bound CETP molecules; and v) a significant correlation between liposome size and the percentage of surface hydrophobic area. Those results suggest that apoA-I and apoA-II may not be involved in CETP binding; however, the surface phospholipids, and surface curvature likely dominate the interaction of CETP with the surface of HDL.

A larger surface lipid area provides a higher opportunity for CETP binding. We noticed that HDL2, which has has a larger diameter than HDL3 (~13 vs. ~11), also has a higher percentage of bound CETP particles than HDL3 (~50% vs. ~42%). This may be due to the HDL3 surface lipids occupying a larger area than the surface lipids of HDL3 (~31.3% vs. ~22.7%). This is consistent with liposomes which are...
convex with the N-terminal end to open up); ii) how CE molecules can be transferred through a ~10 nm channel; iii) how TGs can be transferred back to HDL from LDL; iv) how CE and TG exchange between LDL and VLDL; v) how CETP homo-exchanged the radiolabeled lipid transfer among HDL particles. Our results highlight the CETP N-terminal β-barrel domain hydrophobic distal end as a potential drug target, which may lead to a next-generation drug to treat CVDs.

**Methods**

**Protein and liposome isolation.** Recombinant human CETP (~53 kDa with no post-translational modifications) was expressed and purified from the Chinese hamster ovary cell line DG44 [1, 2]. The CETP construct was previously described [3]. The CETP concentration was determined by absorbance at 280 nm. Native plasma HDL2 and HDL3, in phosphate buffered saline (PBS) were isolated from fresh, pooled samples of human plasma by ultracentrifugation as reported [4]. Pooled samples from multiple donors were used to make sure that the sample was representative and that the results could not be attributed to something that specifically related to a single individual. Spherical, reconstricted HDL (rHDL) in PBS were prepared [5, 6]. Liposome vesicle samples were produced by Encapsula NanoSciences (Brentwood, TN). The sample contained 1 mg/ml 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC, from Avanti Polar lipids) with a peak vesicle size of ~50 nm in a buffer containing 20 mM Tris-Cl, 154 mM NaCl, pH 7.4.

**Specimen preparation for negative-staining EM.** Specimens were prepared for EM using the optimized negative-staining (OpNS) protocol [7, 8], which minimizes the rouleaux artifact that is observed with lipoproteins [9–11]. In brief, CETP (0.28 mg/ml) was incubated with HDL2, HDL3, VLDL and LDL at a molar ratio of ~4:1 (CETP: lipid macromolecular particle). CETP-lipid macromolecular complexes were diluted to 1.5 μg/ml with Dulbecco’s phosphate buffered saline (DPBS). A 4 μl aliquot was placed on a thin-carbon-coated copper grid (300 mesh TEM grid, Cu-300CN, Pacific Grid-Tech, San Francisco, CA) that had been glow-discharged. After one minute, excess solution was blotted with filter paper, followed by washing and negative staining with 1% (w/v) uranyl formate (UR) [12]. After air drying, the grids were further dried for an hour at 40°C.

**Electron microscopy data acquisition and image pre-processing.** The OpNS micrographs were acquired under defocus between ~0.6 μm to ~2.2 μm on a Gatan UltraScan 4 K X 4 K CCD equipped on a Zeiss Libra 120 Plus transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). The TEM was operated under a high-tension of 120 kV, energy filtering of 20 eV and magnification range of 31.5 K to 80 K, in which each pixel of the micrographs corresponded to 3.68 to 1.4 Å respectively. A total of ~15–72 micrographs were imaged from each sample. The contrast transfer function (CTF) of each micrograph was determined and then corrected by the phase-flip option using cifit (EMAN software) [13]. ~300–3000 particles from each sample were selected and windowed by the boxer software in the EMAN software package [14] and submitted for Gaussian low-pass filtering before size measurement.

**Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) data acquisition.** Cryo-EM specimens were prepared on lacy carbon film coated copper grid (Cu-200LC, Pacific Grid-Tech, San Francisco, CA). Cryo-EM data of liposome binding to CETP specimens were acquired under a less than ~2.3 μm defocus with a high-sensitivity 4 K X 4 K CCD Camera on a Zeiss Libra 120 Plus transmission electron microscope. A 4 μl aliquot was placed on a thin-carbon-coated copper grid (300 mesh TEM grid, Cu-300CN, Pacific Grid-Tech, San Francisco, CA) that had been glow-discharged. After one minute, excess solution was blotted with filter paper, followed by washing and negative staining with 1% (w/v) uranyl formate (UR) [12]. After air drying, the grids were further dried for an hour at 40°C.

**Correction of contrast transfer function (CTF) for cryo-ET data.** Tilting series of micrographs were initially aligned together with the IMOD software package [15]. The defocus near the tilt-axis area of each tilt micrograph was examined by fitting CTF parameters to its power spectrum by cffind3 in the FREALIGN software package [16] and then examined by cifit (EMAN software package) [17]. The CTF was then corrected by TOMOCIF [18]. The tilt series of each CETP-liposome image in windows of 200 × 200 pixels was tracked and selected by IPET software.

**Individual-particle electron tomography (IPET) 3D reconstruction.** Ab-initio 3D reconstruction of an individual-liposome-CETP complex was conducted by the IPET method [19]. In IPET, a small image containing only a targeted liposome-CETP complex was windowed from each tilted whole-micrograph (CTF corrected). An ab-initio model was generated by these single-particle reconstructions into a 3D map. The map was then refined via three rounds of refinement loops (including more than a hundred iterations) by the focused electron tomography reconstruction (PETR) algorithm [20]. In FETR, an automatically-generated dynamic Gaussian low-

larger than HDL2 or HDL3 having ~6 bound CETP molecules (Fig. 1d and 2b), compared to the average of 1–2 CETP molecules that are bound to HDL2 and HDL3 (Fig. 1a and b). This suggests that the binding affinity of CETP is regulated by surface lipid area, as well as surface curvature.

Our results favor the tunnel mechanism rather than the shuttle mechanism for the transfer of CE from HDL to LDL via CETP. Both of these mechanisms were hypothesized to be involved in the CETP-mediated transfer of CE between HDL and LDL two decades ago [21, 22]. In the shuttle mechanism, CETP interacts with HDL and acquires CEs, after which the “CE-enriched” CETP is released from the HDL for subsequent deposition into LDL. The “CE-poor” CETP is then released from the LDL surface for subsequent interaction with HDL in another cycle of CE transfer [23]. In the tunnel mechanism, CETP binds to HDL and LDL simultaneously to form a ternary complex. CETP then undergoes a conformational change to form a hydrophobic tunnel whereby CEs transfer from HDL to LDL.

Our results showed that CETP bound to only one HDL particle at a time, and did not form a bridge between two HDL2 HDL3 or rHDL particles. This suggests that the binding of CETP to HDL is directional. When the fact that i) we were unable to demonstrate CETP bridging two HDL particles (Fig. 1), ii) small HDL/liposome particles with a higher surface curvature and hydrophobicity are more likely to bind to more CETP molecules (Fig. 1 and 2), iii) the N-terminal β-barrel domain of CETP binds to HDL while the C-terminal β-barrel domain binds to LDL, and iv) the distal end of the N-terminal β-barrel domain of CETP is relatively hydrophobic [24], are considered in light of the current results which show that CETP binds to only one liposome particle at a time, and does not bridge two liposomes (Fig. 1, 2 and 3), it follows that the distal end of the CETP N-terminal β-barrel domain is most likely inserted into the liposome particle surface via a protein-lipid interaction. This interaction is different for LDL particles, where the distal end of the CETP hydrophobic tunnel whereby CEs transfer from HDL to LDL.

Individual-particle electron tomography (IPET) 3D reconstruction. Ab-initio 3D reconstruction of an individual-liposome-CETP complex was conducted by the IPET method. In IPET, a small image containing only a targeted liposome-CETP complex was windowed from each tilted whole-micrograph (CTF corrected). An ab-initio model was generated by these single-particle reconstructions into a 3D map. The map was then refined via three rounds of refinement loops (including more than a hundred iterations) by the focused electron tomography reconstruction (PETR) algorithm. In FETR, an automatically-generated dynamic Gaussian low-
pass filter and an automatically generated soft-mask were applied to both the references and tilted images to achieve the final 3D reconstruction. Since the specimen holder has a limitation to tilt to ±90° angle, a wedge shaped data was missing in final 3D reconstruction, which resulted in a certain level of artifact, especially along the Z direction. The missing wedge data was estimated via our newly developed interactive algorithm (related manuscript in preparation). As an implementation of this algorithm, we computed the missing wedge data and contributed to the final reconstruction. The crystal structure (PDB entry 2OBD) was fitted into the final IPET 3D reconstruction by using a rigid-body fitting option in UCSF Chimera.

Fourier shell correlation (FSC) analysis. To analyze tomographic 3D reconstruction results, the in-plane resolved raw ET images were split into two groups based on having an odd- or even-numbered index in the order of tilt angles. Each group was used to generate a 3D reconstruction image; the two 3D reconstructions from both groups were then used to compute the FSC curve over their corresponding spatial frequency shells in four space (using the "RF 3° command in SPIDER (Supplementary fig. 3)). The frequency at which the FSC curve declined to a value of 0.5 and 0.143 (gold standard) was used to represent the resolution of the final reconstruction.

Statistical analyses of CETP binding to HDL particles and liposomes. Particle size was determined by measuring the diameter in two orthogonal directions, as described before. In brief, the geometric mean of the perpendicular diameters was used to represent the particle diameter. The aspect ratio of the long and perpendicular diameters was used to represent particle shape. Histograms of the particle diameters were generated with 2.17 nm sampling steps. Each histogram was fitted with a 9th degree polynomial function in R for data analysis. Since the CETP bound to the specimen holder has a limitation to tilt to 90° angle, a wedge shaped data was missing in final 3D reconstruction, which resulted in a certain level of artifact, especially along the Z direction. The missing wedge data was estimated via our newly developed interactive algorithm (related manuscript in preparation). As an implementation of this algorithm, we computed the missing wedge data and contributed to the final reconstruction. The crystal structure (PDB entry 2OBD) was fitted into the final IPET 3D reconstruction by using a rigid-body fitting option in UCSF Chimera.

Liposome structures by molecular dynamics (MD) simulation and hydrophobicity analysis. Based on the size range of liposomes in our experiment, five initial liposome models with diameters of 12 nm, 20 nm, 27 nm, 35 nm and 42 nm were simulated. The initial models to simulate the liposomes were generated as below, the lipid bilayer vesicles (~65 Å/Lipids and the average thickness ~36.8 Å) with an open pore (with 13% of surface area) were surrounded by a solution containing water molecules and NaCl at 0.1 M. These pores on the vesicles would allow the lipid flipping and transferring between inner and outer membranes during energy minimization and MD simulations.

As the liposomes contained a large number of molecules, we used the Residue-based Coarse Graining (RBGG) MD method to simplify our initial models in order to enable a long time-scale simulation. In RBGG, about every 10 atoms were grouped together and assigned as a bead model based on their chemical functionality. One POPC molecule was assigned 13 beads (one bead for choline, one for phosphate, two beads for glycero and nine for two fatty acid chains) based on Marrink’s CG lipid model. The interaction potentials between beads and the bead masses were used as previously reported.

Energy minimization was conducted by Nanoscale Molecular Dynamics (NAMD)The criteria to determine whether the liposomes were stabilized as follows: (i) the surface pores were completely closed; (ii) liposomes should have a spherical shape determined by Radial Distribution Function (RDF), which is computed with respect to the liposome’s center of mass (supplementary fig. 8); and (iii) stabilized surface hydrophobicity. The area of the hydrophobic surface on each stabilized liposome was measured by the Solvent Accessible Surface Area (SASA) function in Visual Molecular Dynamics (VMD). The percentages of surface hydrophobic area on each liposome were computed by taking their hydrophobic surface area and dividing by the whole liposome surface area. The average of the last 300 frames (12 ns) was used to calculate the hydrophobicity.

1. Drayna, D. et al. Cloning and sequencing of human cholesteryl ester transfer protein cDNA. Nature 327, 632–6 (1987).
2. Inazu, A. et al. Increased high-density lipoprotein levels caused by a common mutation in the cholesteryl ester transfer protein gene despite increased HDL: Excimer probe study. J Biol Chem 263, 238–40 (1990).
3. Ihm, J., Quinn, D. M., Busch, S. J., Chataing, B. & Hannay, J. A. Kinetics of plasma protein-catalyzed exchange of phosphatidylcholine and cholesteryl ester between plasma lipoproteins. J Biol Chem 270, 1238–42 (1995).
4. Miller, T. J., Li, X., Catherwood, C. & Rinaldi, R. A. Cyt and determination and correction in electron cryomicrography. Ultramicroscopy 106, 587–96 (2006).
5. Derks, M., Anzures-Cabrera, J., Turnbull, L. & Phelan, M. Safety, tolerability and pharmacokinetics of dalceparib following single and multiple ascending doses in healthy subjects: a randomized, double-blind, placebo-controlled, phase I study. Clin Drug Invest 31, 325–35 (2011).
6. Gotto, A. M., Jr. et al. Evaluation of lipids, drug concentration, and safety parameters following cessation of treatment with the cholesteryl ester transfer protein inhibitor anacetrapib in patients with or at high risk for coronary heart disease. Am J Cardiol 113, 78–84 (2013).
7. Friedrich, S. et al. The pharmacokinetics and pharmacokinetic/pharmacodynamic relationships of evacetrapib administered as monotherapy or in combination with statins. CPT Pharmacometrics Syst Pharmacol 3, e94 (2014).
8. Derks, M. et al. Crystal structure of cholesteryl ester transfer protein reveals a long tunnel and four bound lipid molecules. Nat Struct Mol Biol 14, 106–13 (2007).
9. Liu, S. et al. Crystal structures of cholesteryl ester transfer protein in complex with inhibitors. J Biol Chem 287, 37321–9 (2012).
10. Zhang, L. et al. Structural basis of transfer between lipopolysaccharides by cholesteryl ester transfer protein. Nat Chem Biol 8, 342–9 (2012).
11. Lei, D. P. Structural features of cholesteryl ester transfer protein: a molecular dynamics simulation study. Proteins 81, 415–25 (2013).
12. Pulskamp, P. N. & Zibolomeye, M. A. Atomicistic molecular dynamics simulation reveals the mechanism by which CETP penetrates into HDL enabling lipid transfer from HDL to CETP. J Lipid Res (2014).
13. Koivuniemi, A., Vuorela, T., Kovanen, P. T., Vattulainen, I. & Hyvonen, M. T. Molecular basis of lipid transfer protein deficiency in a family with Fabry disease. J Biol Chem 275, 27824–9 (2010).
14. Jeyarajah, E. J., Cromwell, W. C. Measurement issues related to CETP and CETP-mediated lipoprotein exchange. Clin Drug Investig 31, 325–35 (2011).
15. Rye, K. A., Hime, N. J. & Barter, P. J. Evidence that cholesteryl ester transfer protein-mediated reductions in reconstituted high density lipoprotein size involve particle fusion. J Biol Chem 272, 3935–60 (1997).
40. Rye, K. A. & Barter, P. J. The influence of apolipoproteins on the structure and function of spheroidal, reconstituted high density lipoproteins. J Biol Chem 269, 10298–303 (1994).
41. Ludtke, S. J., Baldwin, P. R. & Chiu, W. EMAN: semiautomated software for high-resolution single-particle reconstructions. J Struct Biol 128, 82–97 (1999).
42. Kremer, J. R., Mastronarde, D. N. & McIntosh, J. R. Computer visualization of three-dimensional image data using IMOD. J Struct Biol 116, 71–6 (1996).
43. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. J Struct Biol 142, 334–47 (2003).
44. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem 25, 1605–12 (2004).
45. Frank, J. et al. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. J Struct Biol 116, 190–9 (1996).
46. Scheres, S. H. & Chen, S. Prevention of overfitting in cryo-EM structure determination. Nat Methods 9, 853–4 (2012).
47. Kucerka, N., Nieuw, M. P. & Katsaras, J. Fluid phase lipid areas and bilayer thicknesses of commonly used phosphatidyldicholines as a function of temperature. Biochim Biophys Acta 1808, 2761–71 (2011).
48. Tahara, Y. & Fujiyoshi, Y. A new method to measure bilayer thickness: cryo-electron microscopy of frozen hydrated liposomes and image simulation. Micron 25, 141–9 (1994).
49. Risselada, H. J. & Marrink, S. J. Curvature effects on lipid packing and dynamics in liposomes revealed by coarse grained molecular dynamics simulations. Phys Chem Chem Phys 11, 2056–67 (2009).
50. de Vries, A. H., Mark, A. E. & Marrink, S. J. Molecular dynamics simulation of the spontaneous formation of a small DPPC vesicle in water in atomistic detail. J Am Chem Soc 126, 4488–9 (2004).
51. Marrink, S. J. & Mark, A. E. Molecular dynamics simulation of the formation, structure, and dynamics of small phospholipid vesicles. J Am Chem Soc 125, 15233–42 (2003).
52. Marrink, S. J., de Vries, A. H. & Mark, A. E. Coarse grained model for semiquantitative lipid simulations. Journal of Physical Chemistry B 108, 750–760 (2004).
53. Marrink, S. J., Risselada, J. & Mark, A. E. Simulation of gel phase formation and melting in lipid bilayers using a coarse grained model. Chem Phys Lipids 135, 223–44 (2005).
54. Shih, A. Y., Freddolino, P. L., Arkhipov, A. & Schulten, K. Assembly of lipoprotein particles revealed by coarse-grained molecular dynamics simulations. J Struct Biol 157, 579–92 (2007).
55. Phillips, J. C. et al. Scalable molecular dynamics with NAMD. J Comput Chem 26, 1781–802 (2005).
56. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. J Mol Graph 14, 33–8, 27–8 (1996).

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Author contributions
This project was initiated and designed by R.C., L.Z., K.A.R., X.Q., D.G.J. and G.R.; and refined by M.Z. and M.A.C.; X.Q., D.G.J., M.P. and K.A.R. isolated the CETP and HDL samples; M.Z., L.Z. and R.C. conducted the experiments and acquired the OpNS data; H.T. and L.Z. acquired the cryo-EM data; M.Z. processed the NS data, computed the statistics and performed the molecular dynamics simulation; L.Z., M.Z. and F.W. processed the cryo-ET data; A.R. analyzed the projections and averages of 3D reconstruction; M.Z., L.Z., R.C., M.A.C., M.I.R. and G.R. interpreted and manipulated the data; M.Z., R.C., M.A.C. and G.R. drafted the initial manuscript, which was revised by L.Z., K.A.R., A.R., M.P., M.J.R., X.Q. and D.G.J.

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