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SHORT COMMUNICATION

# Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation

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Extracellular vesicles (EVs) facilitate intercellular communication by carrying bioactive molecules such as proteins, messenger RNA, and micro (mi)RNAs. Recently, high-density lipoproteins (HDL) isolated from human plasma were also reported to transport miRNA to other cells. HDL, when isolated from human plasma, ranges in density between 1.063 and 1.21 g/mL, which grossly overlap with the reported density of EVs. Consequently, HDL and EV will be co-isolated when using density gradient ultracentrifugation. Thus, more stringent isolation/separation procedures of EV and HDL are essential to know their relative contribution to the pool of circulating bioactive molecules.

Keywords: *Isolation*; *RNA*; *lipoproteins*; *extracellular vesicles*

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Recently, the presence of micro(mi)RNAs in high-density lipoprotein (HDL) fractions from the plasma of patients with stable coronary artery disease or acute coronary syndromes has been reported by Wagner et al. (1). An earlier study by Vickers et al. also demonstrated that HDL contains miRNA-223, which can be transferred to recipient cells (2). Also, extracellular vesicles (EVs) in human body fluids have been reported to contain signalling molecules such as bioactive lipids, mRNA, and micro (mi)RNA (3). Thus, the findings that both HDL and EV may contain genetic information further highlight the relevance of intercellular communication by vesicles and/or lipoprotein particles.

To date, lipoproteins are isolated from plasma by density gradient ultracentrifugation, which separates fractions of lipoproteins such as very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and HDL on differences in density (4). A common method is density gradient ultracentrifugation using potassium bromide (KBr) (1,2,4). Also, Wagner et al. isolated HDL using KBr-density gradient ultracentrifugation (1). Because plasma also contains EVs in concentrations up to $10^{11}$–$10^{12}$/mL with a density (d) between 1.13 and 1.19 g/mL, which is essentially similar to HDL (d = 1.063–1.21 g/mL) (2,5–7), EVs will be present in isolated HDL when no further purifications steps are applied such as size-exclusion chromatography or HDL immunoprecipitation (2). Consequently, the contribution of HDL as carriers of plasma miRNAs may be overestimated (1).

In our study, we isolated lipoprotein fractions (VLDL, LDL, and HDL) using KBr-density gradient ultracentrifugation. We investigated the expected presence of EVs in HDL fraction with a density of 1.063–1.21 g/mL. In this study, we emphasize that careful monitoring is needed upon the isolation and analysis procedures in order to interpret the results and conclusions between different research groups correctly.

## Materials and methods

### *Plasma isolation*

Blood was collected in a sodium citrate plastic tube (0.109 mol/L; Becton Dickinson, CA) from 2 fasting healthy donors. Plasma was prepared by centrifugation within 15 minutes after collection at 1,550 × g for 20 minutes at 20°C (twice), and used directly for lipoprotein isolation (Fig. 1a).





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### Lipoprotein isolation

VLDL ($0.94 < d < 1.006$ g/mL), LDL ($1.006 < d < 1.063$ g/mL), HDL ($1.063 < d < 1.21$ g/mL), and lipoprotein-deficient plasma (LPDP; $d > 1.21$ g/mL) were isolated from 3 mL plasma by KBr-density gradient ultracentrifugation according to Redgrave et al. (4) (Fig. 1b) with some minor modifications (omission of sucrose in the salt gradient, and use of a 29,000 rpm instead of a 41,000 rpm). Briefly, plasma density was adjusted to $d = 1.25$ g/mL with solid KBr (0.398 g/mL plasma) and a 3 mL aliquot was pipetted into $14 \times 89$ mm polyallomer ultracentrifuge tubes (Beckman Coulter Inc., CA). A discontinuous gradient was formed by carefully layering 2 mL of $d = 1.225$ g/mL KBr solution on top of the plasma, followed by 4 mL of $d = 1.100$ g/mL KBr solution. Finally, a top layer of 3 mL of $d = 1.006$ g/mL KBr solution was added. The samples were centrifuged for 19 hours at 10°C at 29,000 rpm in a SW 41 Ti rotor (Beckman Coulter Inc., CA). The fractions of interest were sliced out of the ultracentrifuge tube and applied to Amicon® Ultra 3K centrifugal filter device (Merck Millipore, MA) for KBr removal. Each fraction was washed 3 times by addition of phosphate-buffered saline (PBS, pH 7.45), containing 154 mmol/L NaCl, 1.24 mmol/L $Na_2HPO_4.2H_2O$, and 0.21 mmol/L $NaH_2PO_4.2H_2O$. All chemicals are from Merck, Darmstadt, Germany. In addition, the KBr density gradient was measured in the fractions after ultracentrifugation by conductivity determination using a Jenway 4,200 conductivity meter (Jenway, Gransmore Green Felsted, Essex, UK).

### Transmission electron microscopy

VLDL, LDL, HDL, and LPDP fractions were fixed at room temperature for at least 18 hours by using 0.1% (weight (w)/volume (v)) paraformaldehyde (Electron Microscopy Sciences, PA). Next, fractions (10 μL) were

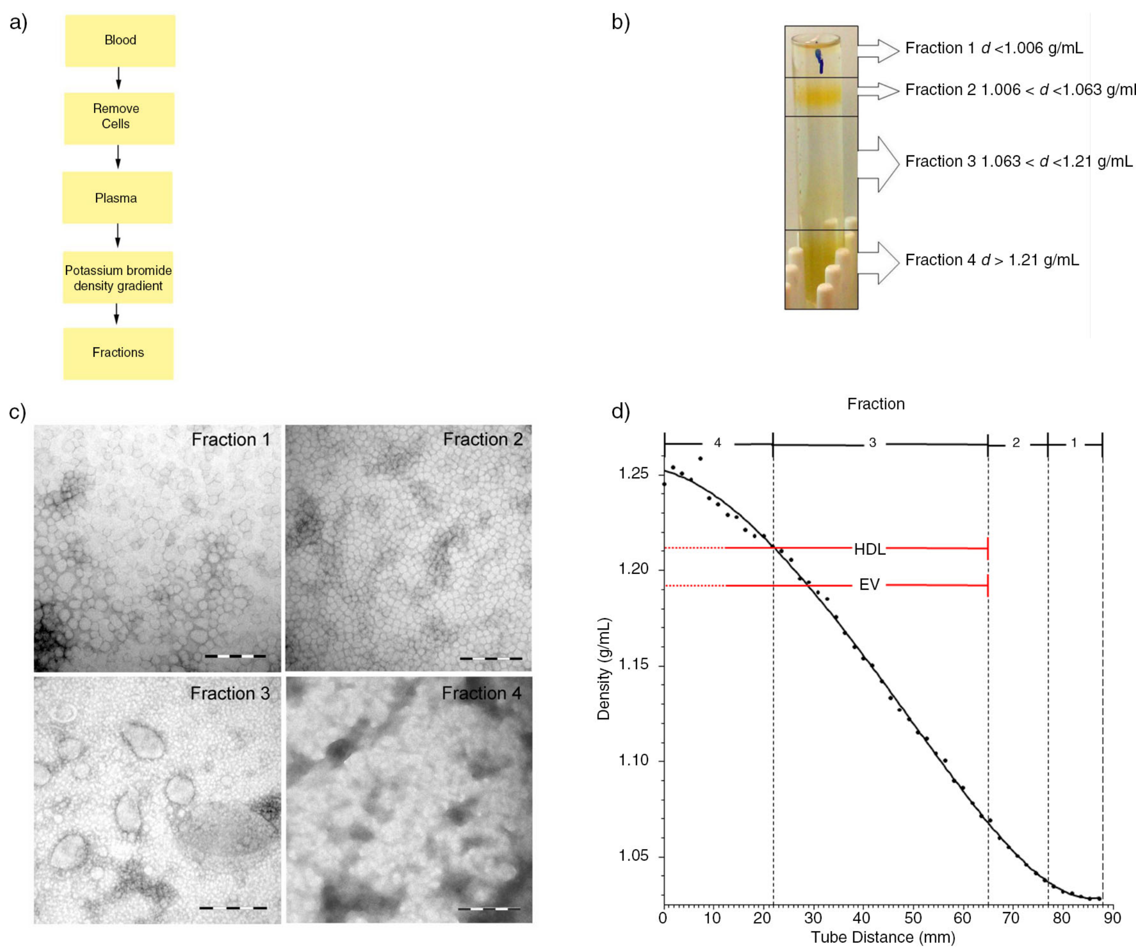


*Fig. 1.* Isolation and morphological characterization of the lipoprotein fractions. Plasma was subjected to KBr-density gradient ultracentrifugation (a). After centrifugation, lipoprotein-containing fractions (Fraction 1–3) and lipoprotein-deficient fraction (Fraction 4) were collected (b). Each fraction was imaged by transmission electron microscopy and negative staining using uranyl acetate (c). From upper left to upper right and lower left to lower right, VLDL (Fraction 1), LDL (Fraction 2), HDL and EV (Fraction 3), and LPDP (Fraction 4) are shown. The scale bars in the individual pictures represent a total length of 200 nm. (d) Density of fractions isolated by KBr-density gradient ultracentrifugation. The overlap between the density of HDL and EV is shown.


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applied on a 200-mesh EM copper grid with formvar coating (Electron Microscopy Sciences), and incubated for 7 minutes at room temperature. The grids were transferred to 1.75% uranyl acetate (w/v) for negative staining. The grid was imaged under a Tecnai 12 electron microscope (FEI Company, Eindhoven, The Netherlands), operated at 80 keV. Two-dimensional data was collected and images were recorded at 60,000x magnification.

### Apolipoprotein A-I assay

Concentrations of apolipoprotein A-I were measured in fractions after KBr-density gradient ultracentrifugation by turbidimetry using an Architect ci8200 (Abbott Laboratories, IL). Reagents (apolipoprotein A-I and apolipoprotein calibrator) were from the same manufacturer, and measurements were performed according the manufacturer's instructions.

### Flow cytometry

Five microlitres of VLDL, LDL, HDL, or LPDP fraction were stained by the addition of either 5 μL fluorescein isothiocyanate (FITC)-labelled lactadherin (100-fold diluted; Haematologic Technologies Inc., VT) or 5 μL phycoerythrin (PE)-labelled mouse anti-human CD61 clone VI-PL2 (50-fold diluted; BD Biosciences, CA) and 5 μL FITC-labelled mouse anti-human CD63 clone CLBGran/12 (25-fold diluted; Beckman Coulter, CA) in PBS. Unlabelled sample was used as a negative control for lactadherin-labelled sample. As negative controls of antibody staining, mouse IgG1 PE clone X40 and mouse IgG1 FITC clone X40 (BD Biosciences) were used at the same concentrations as the anti-CD41 and anti-CD63 antibodies. Samples were incubated for 15 minutes at room temperature and diluted to 350 μL with PBS before being measured on Apogee A50-Micro (Apogee Flow Systems, Hemel Hempstead, UK) for 5 minutes.

## Results and discussions

Based on transmission electron microscopy (TEM) images, fractions isolated at density ranges 0.94–1.006 (fraction 1) and 1.006–1.063 g/mL (fraction 2) contain VLDL and LDL particles, respectively (Fig. 1c). The diameter of

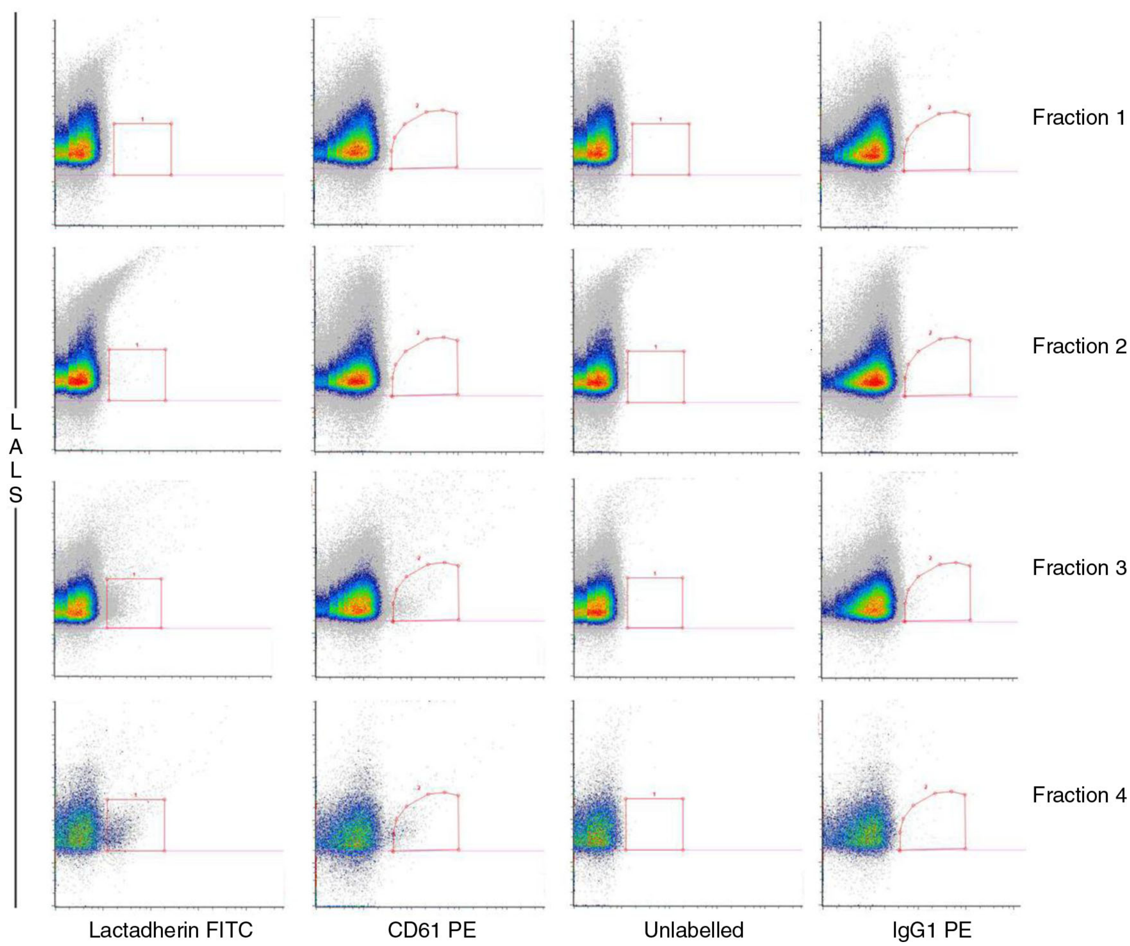


*Fig. 2.* Flow cytometry analysis of fractions isolated from KBr-density gradient ultracentrifugation using Apogee A50-Micro. Fractions 1–4 were stained using lactadherin FITC or anti-CD61 PE. Unlabelled or IgG1 PE stained-fractions were used as negative controls to define the positive events for lactadherin and CD61 in all fractions, respectively. LALS (Y-axis): large angle light scattering.

VLDL particles is approximately 60 nm, whereas the diameter of LDL particles is of 25 nm. The fraction isolated at density range 1.063–1.21 g/mL (fraction 3) contains HDL particles with diameter around 10 nm, as well as much larger vesicular structures with a diameter of about 100 nm, most likely EV based on the typical cup-shaped morphology (7). After counting vesicles in representative TEM images ($n = 6$), we estimate the ratio of EV:HDL particles about 1:100. Fraction 4 ($d > 1.21$ g/mL) contains protein complexes/aggregates.

To confirm the presence of HDL, we measured apolipoprotein A-I (Apo A-I) in all fractions. Fractions 1 and 2 contained no detectable levels of Apo A-I, fraction 3 contained 1.44 g/mL Apo A-I, and fraction 4 contained 0.65 g/mL Apo A-I. These results confirm the presence of HDL particles in fraction 3.

Flow cytometry also confirmed the presence of vesicles in fraction 3 (Fig. 2). Vesicles binding lactadherin were mainly present in fractions 3 ($4.06 \times 10^6$ events/mL) and 4 ($2.22 \times 10^7$ events/mL), whereas vesicles exposing CD61 were only detectable in fractions 3 ($1.17 \times 10^6$ events/mL) and 4 ($4.67 \times 10^6$ events/mL). Vesicles exposing CD63 were below the detection limit (data not shown). Taken together, flow cytometry confirms the presence of vesicles in fractions 3 and 4. Although the high-density fraction 4 ($d > 1.21$ g/mL) is supposed to contain protein aggregates, our results suggest that this fraction contains vesicles. Our results confirm earlier findings of other investigators, who demonstrated the presence of vesicles in the high-density fraction ($d > 1.23$ g/mL) obtained by sucrose-density gradient centrifugation (8,9).

Thus, within a density range of 1.063–1.21 g/mL, both EVs and HDLs will be isolated when KBr-density gradient centrifugation is applied (Fig. 1d). Similarly, also EVs isolated from plasma by sucrose-density gradient ultracentrifugation will include HDL particles (6). Consequently, results from studies using density gradient ultracentrifugation to isolate either EV or HDL, and attribute specific roles to those entities, should be interpreted with caution.

Despite the work of Vickers et al. (2), Wagner et al. (1) reported the presence of miRNA in HDL (1.063–1.21 g/mL) isolated by KBr-density gradient ultracentrifugation without further downstream steps. Likely, their HDL isolation contained EVs. In density gradient HDL preparation, the amount of EV is approximately 1% on a particle-to-particle ratio. Although this may look insignificant, one single vesicle has a much larger diameter than a single HDL particle, for example 100 nm vs. 10 nm in diameter. This implicates that the total volume of EVs would be 10-fold more than the total volume of HDL particles.

EVs in numerous studies have been reported to carry miRNA (3). Thus, the contribution of EVs to the pool of miRNA in the study of Wagner et al. (1) should be verified. Another consideration is that the total surface area of EVs is comparable to the total surface area of HDL particles in fraction 3. An earlier study by Vaisar et al. (10), which shows proteomics of HDL isolated by using KBr-density gradient centrifugation, showed the presence of multiple proteins playing a role in complement activation. As EVs will be present in such fractions, and because EVs are known to expose proteins involve in complement activation (11,12), the contribution of EVs in such proteomics studies may be significant.

Taken together, robust procedures for isolation and purification of EV and lipoprotein particles are essential to gain better insight into their contribution as potential carriers of genetic information and their protein antigens.

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## Conflict of interest and funding

The authors declare no conflict of interest.

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