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Circulating lipoprotein-carried miRNome analysis reveals novel VLDL-enriched microRNAs that strongly correlate with the HDL-microRNA profile

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ABSTRACT

Lipoproteins have been described as microRNAs (miRNAs) carriers. Unfortunately, the bibliography on this topic is scarce and shows a high variability between independent investigations. In addition, the miRNA profiles of the LDL and VLDL fractions have not been completely elucidated. Here, we profiled the human circulating lipoprotein-carried miRNome. Lipoprotein fractions (VLDL, LDL and HDL) were isolated from the serum of healthy subjects by ultracentrifugation and purified by size-exclusion chromatography. A panel of 179 miRNAs commonly expressed in circulation was evaluated in the lipoprotein fractions using quantitative real-time PCR (qPCR) assays. A total of 14, 4 and 24 miRNAs were stably detected in the VLDL, LDL and HDL fractions, respectively. VLDL- and HDL-miRNA signatures were highly correlated (rho 0.814), and miR-16–5p, miR-142–3p, miR-223–3p and miR-451a were among the top 5 expressed miRNAs in both fractions. miR-125a-5p, miR-335–3p and miR-1260a, were detected in all lipoprotein fractions. miR-107 and miR-221–3p were uniquely detected in the VLDL fraction. HDL showed the larger number of specifically detected miRNAs (n = 13). Enrichment in specific miRNA families and genomic clusters was observed for HDL-miRNAs. Two sequence motifs were also detected for this group of miRNAs. Functional enrichment analysis including the miRNA signatures from each lipoprotein fraction suggested a potential role in mechanistic pathways previously associated with cardiovascular disease: fibrosis, senescence, inflammation, immune response, angiogenesis, and cardiomyopathy. Collectively, our results not only support the role of lipoproteins as circulating miRNA carriers but also describe for the first time the role of VLDL as a miRNA transporter.

Abbreviations: ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; CAD, coronary artery disease; Cq, quantification cycle; GGE, gradient gel electrophoresis; HF, heart failure; Lp(a), Lipoprotein a; miRNAs, microRNAs; mRNA, messenger RNA; ncRNAs, non-coding RNAs; qPCR, quantitative real-time PCR; RT, reverse transcription; SEC, size-exclusion chromatography.

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1. Introduction

MicroRNAs (miRNAs) are endogenous short (18–25 nucleotides) regulatory noncoding RNAs (ncRNAs) that posttranscriptionally modulate gene expression through messenger RNA (mRNA) targeting [1,2]. In the last two decades, gain- and loss-of-function studies have demonstrated a prominent role of miRNAs in the physiology of multiple pathologies, including cardiovascular diseases, such as coronary artery disease (CAD) and heart failure (HF) [3,4]. Indeed, recent advances in therapies based on miRNAs have become an interesting approach for the development of novel treatments in the cardiovascular arena [5]. Previous studies have also highlighted the regulatory role of miRNAs in lipoprotein metabolism [6]. For instance, inhibition of miR-148a increases hepatic LDL receptor and ATP-binding cassette-A1 (ABCA1) expression, reduces LDL cholesterol (LDL-C), and increases HDL cholesterol (HDL-C) levels *in vivo* [7]. Similarly, the inhibition of miR-33a/b increases HDL-C and reduces VLDL triglyceride plasma levels [8]. In turn, a reciprocal association has also been reported since lipoproteins also modulate the expression of miRNAs. HDL stimulates miR-223–3p biogenesis and export in myeloid cells [9].

Although miRNAs act at the intracellular level, cell-free forms have been described in different body fluids, including whole blood. Since its discovery in the circulation in 2008 [10], extensive studies have been published exploring their clinical application as useful biomarkers in clinical decision-making in a variety of diseases [11,12]. Valadi et al. [13] proposed for the first time extracellular miRNAs as a novel mechanism of intercellular communication. More specifically, the authors suggested that miRNAs are released from the origin cell through exosomes and then regulate gene expression of the target cell, which could be very distant from the origin tissue [14]. This mechanism of cell cross-talk seems to be highly controlled and selective [15]. Furthermore, proteins, such as Argonaute2 [16] and Nucleophosmin 1 [17], have also been described as miRNA transporters. In 2011, Vickers et al. [18] provided the first evidence that extracellular miRNAs are also transported by lipoproteins, in particular HDL and LDL. The authors demonstrated that the miRNA signatures in HDL differ in normal and familial hypercholesterolemic subjects, suggesting their potential as biological markers. Using diverse *in vitro* and *in vivo* models, they reported that HDL delivers miRNAs to recipient cells and induces alterations in their phenotype. These results were supported by subsequent investigations [19–21].

Additional investigations are fundamental despite these promising findings. Most studies on miRNA carriers are focused on extracellular vesicles (mainly exosomes). Few data exist regarding lipoproteins as circulating miRNA transporters. This information is relevant as it is known that in addition to the quantity of lipoproteins, their qualitative properties, including size, electric charge or lipid and apolipoprotein composition, determine their atherogenicity. However, the content of miRNAs in all lipoprotein fractions is not well defined, and there is no information regarding miRNA transport by VLDL. Therefore, the aim of the current investigation was to profile the circulating lipoprotein-carried miRNome and to analyze the disparities and similarities between its different signatures.

2. Materials and methods

2.1. Lipoprotein isolation and characterization

Lipoprotein fractions (VLDL, LDL and HDL) were isolated from human pooled serum by sequential ultracentrifugation using a four-step protocol [22] (Supplemental Methods). A detailed procedure is shown in Supplemental Fig. S1. Each pool was composed of 15–30 serum samples (30–60 mL per pool) collected in the Lipids Department at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The criteria for sample selection were normolipemia and normoglycemia, according to serum values of cholesterol, triglycerides, and glucose. In addition, patients with a history of dyslipidemia, diabetes, hypertension, cardiovascular disease, severe kidney disease, hepatic disease or inflammatory pathologies were not included. The study was approved by the Ethics Committee of the Hospital de Sant Pau (IIBSP-REL-2017–27). The study conformed to the guidelines set out in the Declaration of Helsinki.

Initially, serum was treated with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The range of density for each lipoprotein was VLDL< 1.019 g/mL, LDL 1.019–1.050 g/mL and HDL 1.100–1.210 g/mL. Therefore, VLDL also contained IDL, a fraction that is present in the density fraction 1.006–1.019 g/mL. However, IDL is a very minor lipoprotein in normolipemic subjects, being only moderately abundant in patients with dysbetalipoproteinemia. The amount of IDL is almost negligible in our samples. Given that lipoprotein(a) (Lp(a)), another minor lipoprotein fraction, is isolated in the density range of 1.050–1.100 g/mL, an extra centrifugation step was performed to ensure the purity of LDL and HDL fractions. The Lp(a)-containing fraction was discarded and not included in further analysis. Density solutions were prepared using a density solution of 1.006 g/mL pH 7.4 (0.15 M NaCl, 1 mM EDTA, 0.167 mM gentamicin, 0.154 mM chloramphenicol), and potassium bromide was added according to the Radding and Steinberg equation [23]. In each step, the tubes were ultracentrifuged at 100,000 g for 21 h at 4 °C. Lipoprotein fractions were dialyzed in Buffer Tris-EDTA pH 7.4 (10 mM Tris, 1 mM EDTA) at 4 °C with SnakeSkin™ Dialysis Tubing (Thermo Scientific, MA, USA) with a molecular weight cutoff of 3.5 K.

To further increase the purity of the lipoprotein preparations, size-exclusion chromatography (SEC) was performed. Lipoprotein fractions (500 μL for each run) were repurified in an NGC™ Chromatography System (Bio-Rad, California, USA) using a Superose™ 6 Increase 5/150 GL (GE Healthcare, Chicago, IL, USA) size exclusion column at a flow rate of 0.5 mL/min, as described [24]. The protein content of the SEC fractions was monitored by measuring the absorbance at 280 nm. One hundred μL fractions were collected, and those corresponding to each specific lipoprotein peak were pooled, approximately 7–10 fractions (0.7–1.0 mL), depending on the lipoprotein. Native polyacrylamide gradient gel electrophoresis (GGE) and sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS—PAGE) were carried out to determine the purity of each fraction. GGE was performed in 2.5–16 % acrylamide gradient gels with samples prestained with Sudan Black, as described [25]. The samples were run in borate buffer (0.09 M Tris, 0.003 M EDTA, 0.08 M boric acid, pH 8.3) at 100 volts for 6 h. For SDS—PAGE [26], each sample was mixed with Laemmli sample buffer under nonreducing conditions and run using Tris-glycine buffer with SDS (5 M Tris, 0.384 M glycine, 4 mM SDS, pH 8.0). The lipid composition of each fraction (VLDL+IDL, LDL and HDL), including total and free cholesterol, triglycerides, phospholipids, apolipoprotein A-I (ApoA-I), apolipoprotein B (ApoB) and nonesterified fatty acids, was analyzed. Composition was determined in a clinical chemistry autoanalyzer Cobas 6000/c500 (Roche Diagnostics). Each lipoprotein fraction was concentrated using centrifugal filter units Amicon™ Ultra15 with a 50 kDa molecular weight cutoff (Merck, Darmstadt, Germany). Protein concentration was measured using a BCA Assay (Pierce™ Rapid Gold BCA Protein Assay, Thermo Scientific).

The presence of other miRNA carriers, such as microvesicles, in the lipoprotein fractions and in serum was evaluated using fluorescence-activated cell sorting (FACS) flow cytometry in the Cytometry Platform from the Sant Pau Biomedical Research Institute (Barcelona, Spain), as previously described [27–29].

2.2. MicroRNA isolation

Lipoprotein fractions (VLDL, LDL and HDL) from six pools and their corresponding serum samples were included in the miRNome analysis. Total RNA was isolated from 300 μg of lipoproteins or from 200 μL of serum in a final volume of 200 μL using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to the

manufacturer's instructions with an extension of 2 min in the incubation time for protein lysis and precipitation. Synthetic *Caenorhabditis elegans* cel-miR-39–3p (1.6×10^8 copies/μL) (Qiagen) was added as an external reference miRNA. To improve RNA yield, 1 μg of MS2 bacteriophage RNA (an RNA carrier not containing miRNAs) (Roche Diagnostics, Mannheim, Germany) was added to the mixture. All reagents were spiked into samples after incubation with the denaturing solution. RNA was eluted into 20 μL of nuclease-free water and stored at − 80 °C.

The RNA profile of the lipoprotein fractions obtained was evaluated using an Agilent 2100 Bioanalyzer (Total RNA 6000 Pico Kit, Agilent, CA, USA) according to the manufacturer's instructions in the Genomics Platform from the Biomedical Research Institute Sant Pau, as previously described [29].

2.3. Reverse transcription and quantitative PCR

miRNA quantification was performed according to the protocol for the miRCURY LNA Universal RT microRNA PCR System (Qiagen). The system offers optimal accuracy and reproducibility [30]. The reverse transcription (RT) of lipoprotein fractions and serum RNA was performed using the miRCURY LNA RT Kit (Qiagen). Due to the low amount of RNA in lipoprotein fractions, the volume of RNA template was 2-fold (2 μL) the volume used for serum (1 μL). The RT reaction was performed in a total volume of 10 μL using the following conditions: incubation for 60 min at 42 °C followed by heat inactivation for 5 min at 95 °C and subsequent cooling to 4 °C. Then, cDNA was stored at − 20 °C until quantitative real-time PCR (qPCR). miRNA profiling was performed using the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel (384-well plates) in a 10-μL qPCR volume. This kit is designed for profiling 179 human miRNAs stably detected in serum and plasma. qPCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, MA, USA). qPCR conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s and 56 °C for 1 min, followed by melting curve analysis.

SDS v2.3 software was used for the quantification cycle (Cq) determination. Melting curve analysis was performed to analyze the specificity of the qPCR. Synthetic UniSp3 was analyzed as an interplate calibrator and qPCR control. Homogeneous efficiencies in RNA extraction were confirmed by analyzing the Cq values of cel-miR-39–3p. A negative control without template was also included in the quality control analysis. The method described by Blondal et al. [31] was used to exclude hemolysis in the serum samples. All samples passed the test ($\Delta Cq_{\text{(miR-23a-3p - miR-451a)}} < 7$).

To identify stably expressed miRNAs in VLDL, LDL and HDL fractions, a given miRNA was considered stably expressed in a lipoprotein fraction when Cq values < 33 in the six pools (100 % of samples, excluding technical errors). Relative expression quantification was performed using the 2^{-dCq} method, where $dCq = Cq_{[miRNA]} - Cq_{[cel\text{-}miR\text{-}39\text{–}3p]}$. Cqs above 33 cycles were considered undetectable and were censored at the minimum detectable level using our technology. Expression levels were $\log_{10}$-transformed for statistical analysis.

2.4. Statistical and bioinformatic analyses

The statistical R software, version 4.0.2 (www.r-project.org), was used for statistical analyses. The Spearman correlation coefficient was used to assess the correlation between the miRNA signatures of the lipoprotein fraction and/or the serum. A p-value < 0.05 was considered significant. The web-based tool miRWalk2.0 was used to identify the predicted and miRNA-binding sites of known human genes (TargetScan filter, accessed date August 10th, 2022) [32]. Enrichment analysis was performed with the same tool by combining the results with the KEGG, GO and Reactome databases. The identification of sequence motifs was performed using MEME SUITE [33]. An E-value < 0.05 was considered significant.

3. Results

3.1. Presence of small RNA in the lipoprotein fractions

The purity of the lipoprotein fractions was evaluated using both GGE and SDS—PAGE. The SEC procedure resulted in pure lipoprotein fractions, where lipoproteins elute at different retention times based on particle size (VLDL; yellow, LDL; red and HDL; blue) (Fig. 1a). GGE (Fig. 1b) shows the band profiles of VLDL, LDL, and HDL according to their sizes (>35 nm, 25–30 nm and 9–15 nm, respectively). SDS—PAGE (Fig. 1c) shows the presence of apolipoproteins characteristic of each lipoprotein fraction. ApoB was present in both VLDL and LDL fractions but not in the HDL fraction. Moreover, ApoA-I was the most abundant apolipoprotein present in the HDL fraction and was almost absent in VLDL and LDL. As expected, microvesicles, evaluated by FACS, were detected in serum but not in lipoprotein fractions (Fig. 1d). As shown in Fig. 1e, small RNA was detected in total RNA isolated from highly purified lipoproteins.

The lipid and protein composition are indicators of the purity of the lipoprotein particles. Supplemental Table S1 shows the composition of the three lipoprotein fractions from the serum pools. The relative proportion of each component is consistent with the well-established composition of each lipoprotein.

3.2. Distinct microRNA signatures among lipoprotein fractions

To determine whether the miRNAs were differentially expressed between serum and lipoprotein fractions, a total of 179 miRNAs commonly found in the blood cell-free compartment were evaluated in VLDL, LDL and HDL fractions and their corresponding serum. Supplemental Fig. S2 displays the miRNA profile for each fraction and each pool. Hierarchical clustering based on the miRNA expression signature clearly separated the three lipoprotein profiles from the serum profile (Fig. 2a & 2b). The miRNA signature of VLDL was clustered with the HDL-miRNA signature (Fig. 2a & 2b). In contrast, the LDL-miRNA signature was grouped in a distinctive cluster of VLDL and HDL (Fig. 2a & 2b). The cluster relation was confirmed by the high correlation between VLDL and HDL-miRNA profiles (rho 0.814, p-value <0.001) (Fig. 2c & 2d). The highest correlation with serum was observed for HDL (rho 0.823, p-value <0.001) (Fig. 2c & 2d). Nevertheless, a high correlation was also observed for VLDL (rho 0.749, p-value <0.001).

According to our criteria, a total of 14 (7.8 % from total), 4 (2.2 % from total) and 24 (13.4 % from total) miRNAs were stably detected in the VLDL, LDL and HDL fractions, respectively (Fig. 3a & Supplemental Table S2). In spite of the subtle differences in miRNA expression, the data suggested that the most abundant miRNAs were miR-451a, miR-532–5p and miR-142–3p for VLDL, LDL and HDL fractions, respectively. miR-16–5p, miR-142–3p, miR-223–3p and miR-451a were among the top 5 expressed miRNAs in both VLDL and HDL fractions. Three miRNAs, namely, miR-125a-5p, miR-335–3p and miR-1260a, were detected in all lipoprotein fractions (Fig. 3b). Eight miRNAs were shared by VLDL and HDL, and one (miR-532–5p) by VLDL and LDL. No shared miRNAs were observed for LDL and HDL fractions. miR-107 and miR-221–3p were uniquely detected in VLDL. HDL showed the largest number of specifically expressed miRNAs (n = 13). No miRNAs were specifically expressed in the LDL fraction.

A group of miRNAs stably detected in HDL are members of the same miRNA family. The overrepresented families were let-7–5p/98–5p (for let-7a-5p and let-7b-5p), miR-17–5p/20–5p/93–5p/106–5p/519–3p (for miR-17–5p, miR-20a-5p and miR-106b-5p), miR-23–3p (for miR-23a-3p and miR-23b-3p) and miR-27–3p (for miR-27a-3p and miR-27b-3p) (Supplemental Table S3). In addition, two sets of HDL-miRNAs were clustered in their genomic location (inter-miRNA distance <10 kb): miR-23b-3p/miR-24–3p/miR-27b-3p on chromosome 9 and miR-23a-3p/miR-24–3p/miR-27a-3p on chromosome 19 (Supplemental Table S4).

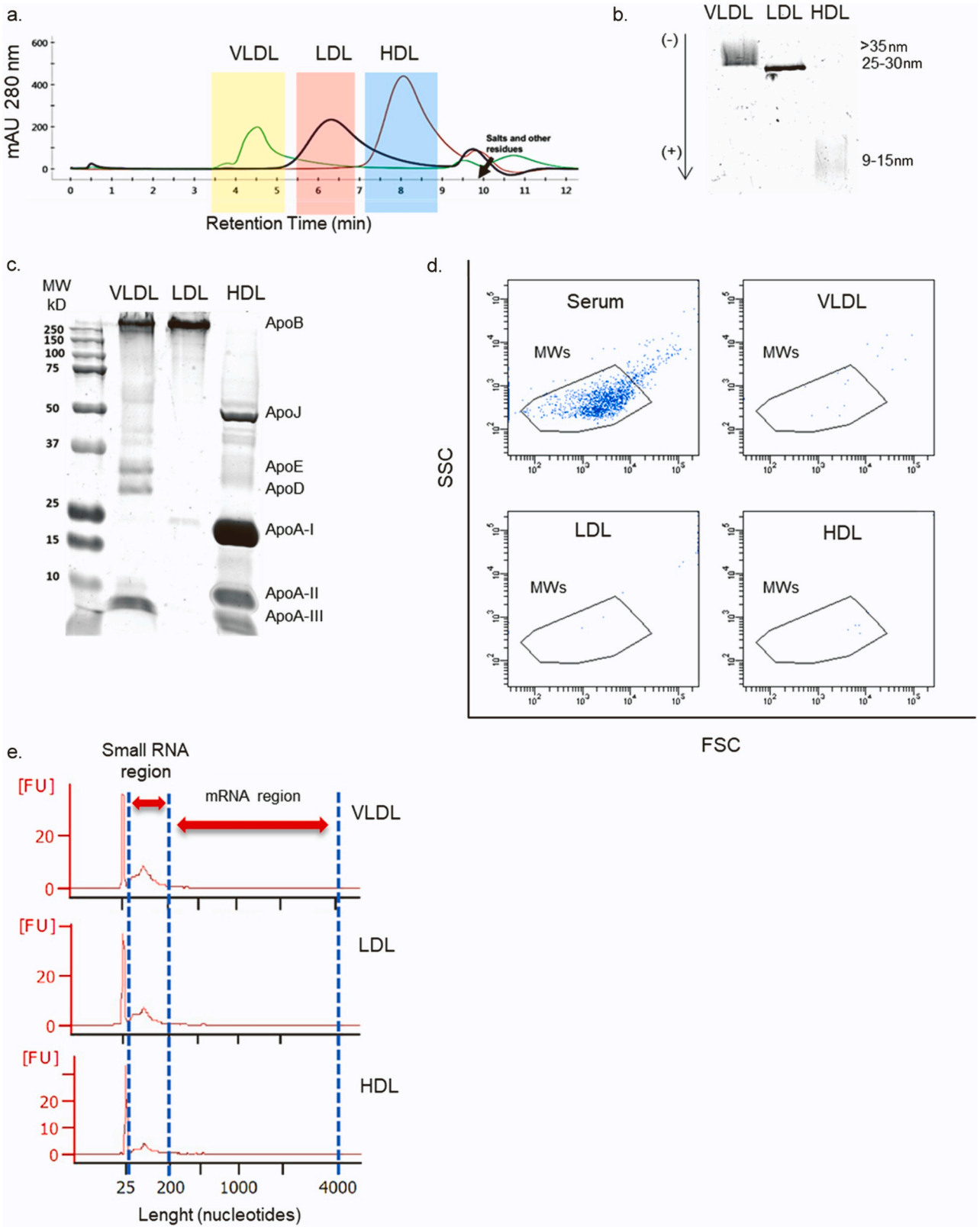

Fig. 1. Lipoprotein fraction analysis. a) Size-exclusion chromatography (SEC) distribution of three lipoprotein fractions: VLDL (yellow band), LDL (red band) and HDL (blue band). b) Native polyacrylamide gradient gel electrophoresis (GGE) of lipoprotein fractions. c) SDS—PAGE of the lipoprotein fraction with corresponding apolipoproteins. d) Fluorescence-activated cell sorting (FACS) flow cytometry of three lipoprotein fractions (VLDL, LDL, HDL) and serum. The square area indicates the area corresponding to microvesicles (MVs). FSC (forward scatter) and SSC (side scatter) characteristics; e) Bioanalyzer Pico lipoprotein analysis of total RNA: VLDL, LDL and HDL. The electropherograms of microRNA isolation show the size distribution in nucleotides and fluorescence intensity (FU) of total RNA in lipoproteins and serum samples. All figures are representative of the six pools included in the study.

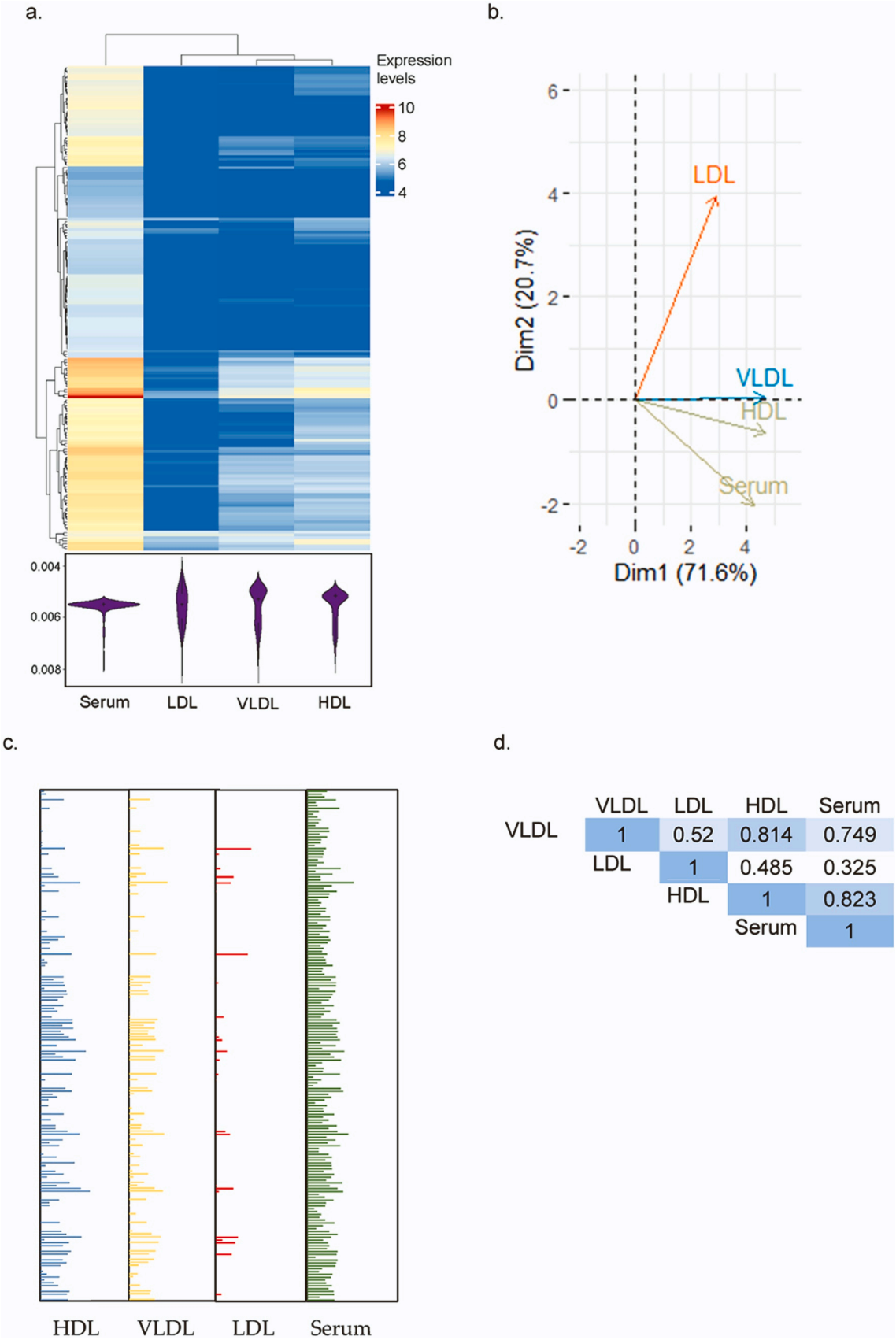

Fig. 2. microRNA profile of the lipoprotein fractions and serum. a) Heat-map (upper panel) and violin plot (bottom panel) of microRNAs (miRNAs) detected in serum and lipoprotein samples using Euclidean distance for clusters. b) Principal component analysis (PCA) plot of miRNA expression profile. c) miRNA profile of HDL, VLDL, LDL and serum. Graphic bars represent miRNA expression in each fraction. Each bar graphic reflects the relative expression of each miRNA analyzed. d) Spearman's rho correlation matrix for VLDL, LDL, HDL, and serum fractions.

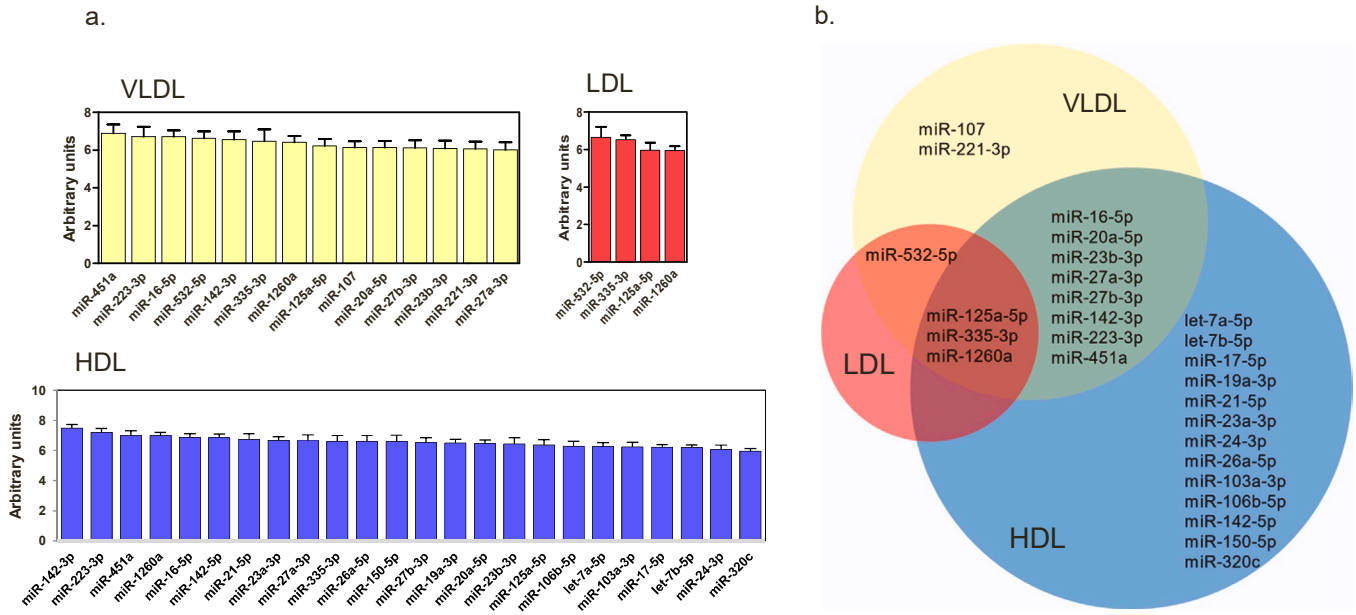

Fig. 3. microRNA profile in the lipoprotein fractions. a) miRNA expression levels in lipoprotein fractions. The expression levels are shown as $\log_{10}2^{-dCq}$ for statistical purposes. Error bars represent standard deviations. Ordered by miRNA expression level. b) Venn diagram of miRNAs in VLDL, LDL and HDL fractions. Colors represent each lipoprotein fraction. Blue indicates HDL, red indicates LDL and yellow indicates VLDL. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Multiple alignment analysis was used to determine whether the presence of specific nucleotide motifs in miRNAs could explain their different presence in lipoproteins. Two sequence motifs were detected in the HDL-miRNAs (Table 1) with *MEME E-values* of 3.8×10^{-5} and 5.0×10^{-3}. No shared sequence motifs were observed in VLDL or LDL-miRNAs.

3.3. Lipoprotein-carried microRNAs may regulate pathways implicated in cardiovascular diseases

Enrichment analysis of the detected miRNAs suggested a broad enrichment of pathways, including those related to cardiovascular pathology (Fig. 4). In brief, among the top 20 biological pathways and GO terms, we identified MAPK, Wnt and cellular senescence signaling pathways for the VLDL fraction; Arrhythmogenic right ventricular cardiomyopathy, MAPK signaling pathway, Wnt signaling pathway, Hypertrophic cardiomyopathy, Dilated cardiomyopathy, Adrenergic signaling in cardiomyocytes, and several mechanisms associated with inflammation and immune function for the LDL fraction; and MAPK, Wnt, mTOR, PI3K-Akt and VEGF signaling pathways for the HDL fraction.

4. Discussion

The current investigation simultaneously analyzed the miRNome of the circulating lipoprotein fractions VLDL, LDL and HDL. In addition to describing the VLDL, LDL and HDL-miRNA signatures, we report that: i) miRNAs can be stably detected in the VLDL fraction; ii) the miRNA profiles of VLDL and HDL show a high similarity to each other and with serum; iii) three miRNAs (miR-125a-5p, miR-335–3p and miR-1260a) are stably detected in VLDL, LDL and HDL fractions; iv) specific miRNAs are detected in HDL fractions (n = 13) and to a lesser extent in VLDL fractions (n = 2); v) two sequence motifs are present in miRNAs detected in the HDL fraction; and vi) although further experimental validation is fundamental, functional enrichment analyses suggested that the miRNAs detected in each fraction may be implicated in common mechanistic pathways associated with cardiovascular diseases.

Previous reports have demonstrated the transport of miRNAs by LDL and especially HDL [18,34,35]. Similar to other lipoproteins, the presence of phosphatidylcholine in VLDL, which has been described as crucial for nucleotide binding [36], could mediate the interaction with small RNAs and protect them from external RNases. However, whether VLDL particles act as miRNA carriers has not been previously addressed. To the best of our knowledge, we show for the first time that VLDL also transports miRNAs in the circulation. In detail, we detected 14 miRNAs in the VLDL fraction, from which two, miR-107 and miR-221–3p, were uniquely detected in isolates from this lipoprotein. Compared to other lipoproteins, the VLDL-miRNA signature was strongly correlated with the HDL-miRNA signature. A close correlation with serum was also observed. Indeed, miR-16–5p, miR-142–3p, miR-223–3p and miR-451a were among the most highly expressed miRNAs in both VLDL and HDL

Table 1
Motifs detected in the HDL-microRNA signature.

Motif	*E*-value	Sites	miRNAs
	3.8×10^{-5}	3	miR-17–5p, miR-20a-5p, miR-106b-5p.
	5.0×10^{-3}	5	miR-23a-3p, miR-23b-3p, miR-27a-3p, miR-27b-3p, miR-223–3p.

The identification of sequences motifs was performed using MEME SUITE (Bailey et al., 2009). E-value < 0.05 was considered significant. The height of each letter is proportional to the frequency of the nucleotide indicated.

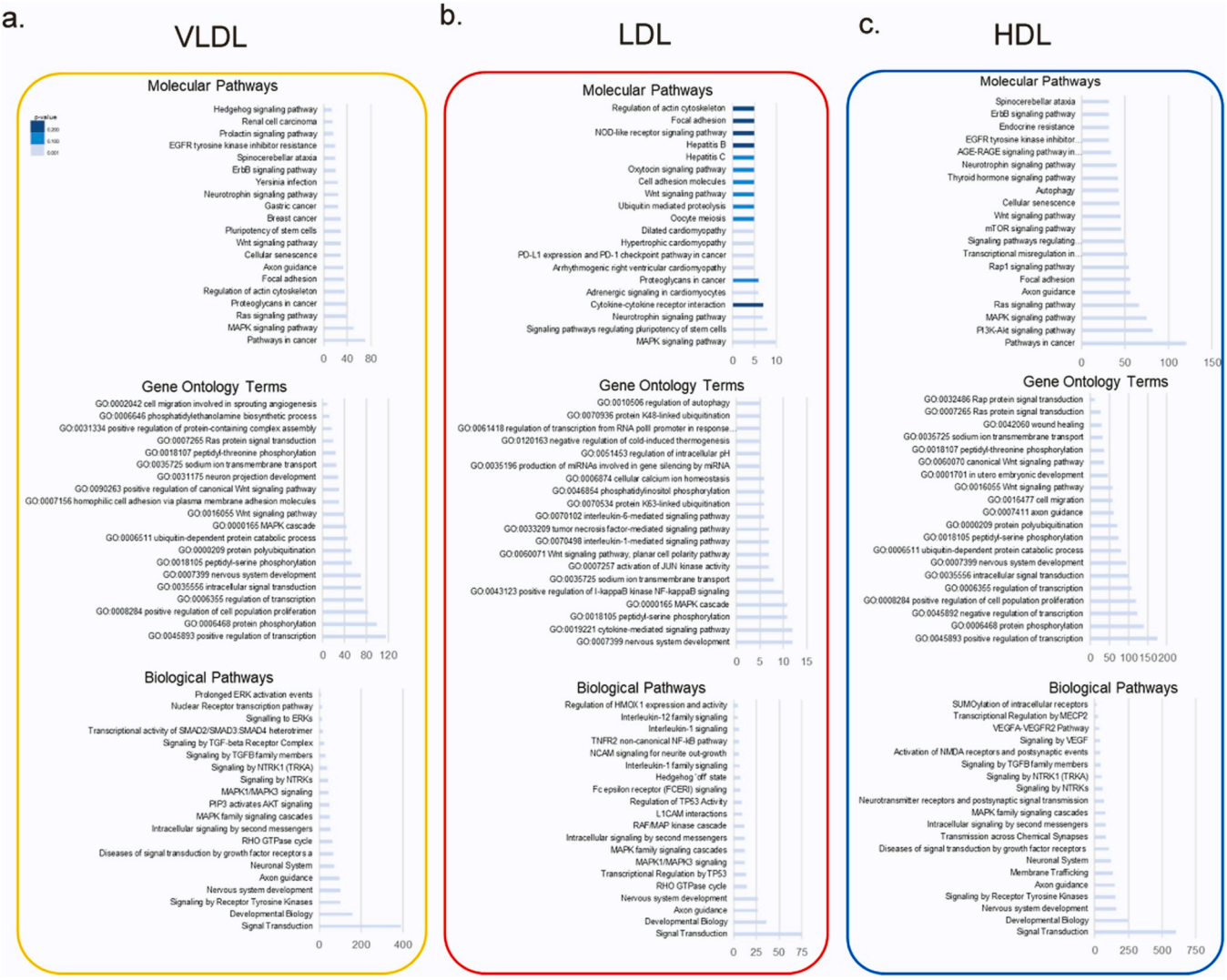

Fig. 4. Functional enrichment analysis of the lipoprotein fractions. The microRNAs (miRNAs) detected in each fraction were included in the analyses. Graph representing the number of target genes for each miRNA (the color represents the p-value) involved in the top 20 pathways as determined by KEGG (a), GO (b) and Reactome (c) analysis. miRWalk2.0 (accessed date August 10th, 2022) was used to predict the interaction of miRNAs with their targets (TargetScan filter).

fractions and serum. The number of miRNAs transported by VLDL (n = 14) was higher than that observed for LDL (n = 4) but lower than that observed in HDL (n = 24). These differences may suggest specific miRNA binding to VLDL [37]. However, we did not detect any enrichment in specific miRNA families, genomic clusters or sequence motifs, which has been observed in HDL. Overall, the identification of VLDL as a miRNA carrier may favor a better understanding of metabolic diseases related to triglyceride management and energy requirements, the identification of innovative biomarkers of cardiovascular risk and the development of new therapeutic approaches.

Another relevant finding of our study is the high correlation between VLDL and the HDL-miRNA signature (rho 0.814). The origin of miRNAs bound to HDL is unclear. However, as previously mentioned, it has been attributed to the capacity of phosphatidylcholine to bind to small RNAs [38]. In this sense, Vickers et al. [18] demonstrated that miRNAs can bind to HDL in plasma *in vivo*. Wagner et al. [35] raised the question of whether the presence of miRNAs in HDL could only be a reflection of nonspecific binding in plasma. Interestingly, the authors found that some miRNAs are specifically enriched in HDL compared with plasma, again suggesting some binding specificity [35]. More recently, Sedgeman et al. [37] reported the first evidence of a direct export of miRNAs to HDL. These authors showed that beta cells export miR-375–3p to HDL in a process regulated by insulin and perhaps mediated by K_{ATP} channels. However, this process was specific for miR-375–3p and has not been fully elucidated in other miRNAs. Thus, in parallel to a direct export of some specific miRNAs from cells to HDL, the high correlation between the VLDL and HDL-miRNA signature observed in the present study opens the possibility that at least part of the miRNAs found in HDL have a hepatic origin and have been secreted into circulation associated with VLDL. This aspect has not been studied until now, and further studies will be necessary to decipher VLDL-associated miRNA secretion.

Supporting previous findings, we observed a similar correlation between LDL and the HDL-miRNA signature (rho 0.485) as that reported by Vickers et al. [18] (R 0.54). The number of individual miRNAs was substantially increased in HDL compared with LDL (n = 24 vs. n = 4), which is consistent with the increased capacity of HDL to transport miRNAs compared to LDL, as demonstrated by independent investigators [18,34,35]. Differences in protein composition, biogenesis and metabolism have been proposed as the cause of this different affinity for miRNAs [18,34]. Our study contributes significantly to this issue, since our data indicate that the LDL-miRNA signature is distinct from VLDL, except for the presence of miR-532–5p, which is detected in both VLDL and LDL but not in HDL.

Similar to previous publications [18,35,39], we observed that miR-223–3p is strongly enriched in HDL fractions. Furthermore, both miR-125a-5p and miR-532–5p have been previously detected in LDL

fractions [18]. In contrast to our results, miR-335–3p, which was stably quantified in all lipoprotein fractions, has not been previously detected in LDL or HDL. miRNAs that have been previously described as expressed in LDL or HDL fractions, such as miR-30a-5p, miR-92a-3p, miR-105–5p, miR-106a-5p, miR-126–3p, miR-135a-3p, miR-188–5p, miR-375–3p, miR-222–3p, miR-486–5p or miR-877–5p [18,34,35,37,39–41], were not detected in any lipoprotein fraction. These findings highlight the methodological differences between the investigations. For example, the use of different qPCR platforms, ranging from RT-qPCR to Next Generation Sequencing (NGS), with different detection rates, sensitivities, and specificities [30]. The lack of standardized methods for the quantification of cell-free miRNAs could also impact on the miRNA result. Indeed, the differences in the pre-analytical and analytical phases have been recognized as crucial aspects to control the reproducibility [42], e.g., sample collection time, sample matrix, storage and centrifugation time and speed, among others. Furthermore, the comparisons may be affected by certain features of the serum donors, the lipoprotein isolation method, e.g., ultracentrifugation or immunoprecipitation, miRNA quantification, e.g., primer design, and data analysis. In order to avoid false-positives, we used very strict criteria to consider a miRNA as expressed: Cq< 33 and detected in 100 % of the samples (after excluding technical errors). In addition, we used targeted quantification of a panel of 179 miRNAs stably detected in the circulation. The impact of biological and pathological factors may also influence the results. For instance, high glucose levels inhibit miR-375–3p export to HDL in beta cells [37]. Furthermore, lipoprotein-carried miRNAs can vary with trans fatty acid intake [43], high-protein diet [40], high-fat diet [44] or estrogen-based hormonal therapy [39] and disease states, such as familial hypercholesterolemia [21,45], coronary artery disease [34] or diabetic complications [19,46].

Previous evidence suggested that lipoproteins, in particular HDL, deliver endogenous miRNAs to recipient cells with functional targeting capabilities [18,19]. Therefore, miRNAs transported by lipoproteins, at least by HDL, may participate in lipoprotein physiological functions. Interestingly, several miRNAs from the same family and sharing genomic clusters were identified in the HDL-miRNA signature. miRNAs within the same family share seed sequences and could be functionally related. Furthermore, miRNAs clustered in their genomic location (<10 kb) could be cotranscribed together and thus could share the same regulatory functions. Thus, we evaluated the predicted impact on cell phenotype attributed to the miRNA profiles of each lipoprotein fraction. Many of the mechanistic pathways associated with the miRNA signatures have roles in vascular and heart diseases: inflammation, immune response, angiogenesis, cell death, fibrosis, among others. Some mechanistic pathways were shared by the three lipoprotein fractions (e.g., MAPK and Wnt signaling pathways). Supporting the high correlation between the VLDL and HDL signatures, an overrepresentation of mechanisms associated with cell death and growth factors was observed for both lipoprotein fractions. Additionally, we observed an enrichment of mechanisms depending on the miRNA signature. An enrichment in pathways related to metabolic homeostasis and angiogenesis was observed for HDL-miRNAs. Of note, targets for LDL-miRNAs were enriched in pathways related to inflammation, immune system function and different cardiomyopathies. Further studies should explore the role of LDL-miRNAs in the onset and development of cardiomyopathies.

Given that miRNA packaging into lipoproteins and secretion mechanisms remain to be elucidated, we explored the existence of mechanisms that could control the sorting of miRNAs into lipoproteins. It has been proposed that miRNA cargo in exosomes is regulated by the presence of specific motifs in miRNAs [15]. Therefore, we analyzed the presence of common motifs among the miRNAs detected in VLDL, LDL and HDL fractions. Two motifs were described in 5 (20 % of total miRNAs detected in HDL) and 3 (12.5 % of total miRNAs detected in HDL) miRNAs from HDL fractions. The presence of these motifs may partially explain miRNA selection for HDL cargo. Nevertheless, additional mechanisms may be implicated in this process. The presence/absence of a motif could be used to decipher the functional aspects of miRNAs, its target genes and miRNA cargo.

Some limitations should be noted. First, we established strict expression criteria to ensure the presence of miRNAs stably expressed in the lipoprotein fractions, but their absence does not necessarily represent a noncarried miRNA. Second, the number of pools for lipoprotein isolation was relatively small. However, it is appropriate for a screening study, and we compared four miRNA sources (VLDL, LDL, HDL, and serum) from each pool. Third, the impact of individual sociodemographic (age, sex, or body mass index), clinical (comorbidities) and pharmacological factors (anticoagulation agents) on the lipoprotein miRNome warrants future research. Forth, additional studies should explore the relevance of the motifs in miRNA cargo and their biological role. Finally, functional enrichment analysis should be considered as a tool to guide mechanistic studies, i.e., *in vitro* and *in vivo* investigations.

5. Conclusions

The present study suggests for the first time VLDL as a miRNA transporter. In addition, the findings support the role of lipoproteins as circulating miRNA carriers. The identification of specific miRNA signatures in the different lipoprotein fractions provides the rationale for further functional studies to better understand the pathogenesis of cardiovascular disease and innovative therapeutic strategies. The potential use of lipoprotein fractions as a novel source of biomarkers should also be explored.

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Author contributions

DdGC, JLSQ, NR, GHR and **TB** conceived the experiments, **VLC, NR, SB, ARU, JC, GHR** and **TB** conducted the experiments, **DdGC, JLSQ, NR, GHR** and **TB** analyzed the results. **DdGC, JLSQ, NR, GHR** and **TB** wrote the manuscript. All authors reviewed the manuscript.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114623.

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