ÉCOLE NORMALE SUPÉRIEURE DE LYON

15 parvis René-Descartes
BP 7000, 69342 Lyon cedex 07
Tél. +33 (0)4 37 37 60 00
www.ens-lyon.fr

Biologie Master BioSciences

Maciel Emmanuelle

Isolation of HDL from mouse plasma in the prospect of analysing their RNA content

M2 Internship report

Internship dates : 10th January- 31st May
Laboratory : Laboratory of Neuroepigenetics, HIFO, Zürich
Supervisor : Isabelle Mansuy
Nb of words : 3200

Previous Interships :

	Year	Laboratory	Supervisor	Title or Subject
L3	2017	LBMC	F. Palladino	The impact of SET2/SET1 and SIN3/HDAC complexes on histone acetylation and their interactions in C. Elegans embryos.
M1	2018	European Neuroscience Institute	J. Clemens	Influence of auditory cues and social context on male-male courtship behaviour in Drosophila melanogaster

UNIVERSITÉ DE LYON | ENS DE LYON | Lyon 1

Early life trauma can have effects on the behaviour in adulthood, such as depression or risk-taking behaviour. These effects can be passed to the next generations. In order to study how environmental alterations during early life can affect the offspring, a mouse model of epigenetic inheritance, where pups undergo maternal separation combined with unpredictable maternal stress (MSUS) was established. In this model, pups are separated from their mothers three hours a day between post-natal day 1 and 14. At adult age, they display behavioural, molecular and metabolic alterations. Some of these changes involve alterations of the high density lipoproteins (HDLs) metabolism and of HDL-associated miRNAs, such as miR-375-3p. For example, miR-375-3p is upregulated in sperm in the first generation after maternal separation. Besides, upregulation of miR-375-3p was also observed in germ cells after they have been incubated with MSUS mouse serum. To investigate the role of HDL and their RNA cargo in the transmission of these effects through generations, we aim at analysing circulating HDL RNA content in the MSUS model. In this study, we isolated HDLs from mouse plasma in the prospect of sequencing their RNAs. For this, we tested different methods, such as size-exclusion chromatography and density gradient ultracentrifugation. After characterising the fractions obtained with the different methods by western blots against HDL and exosomal markers, we opted for density gradient ultracentrifugation to isolate HDLs.

Introduction :

MicroRNAs (miRNAs) are a type of small non-coding RNAs that can silence the expression of genes by RNA interference (1), or occasionally by causing histone modifications or DNA methylation (2). They are involved in the regulation of many biological processes including cell differentiation, proliferation and apoptosis during development and adulthood (1). Their biogenesis starts with the transcription of a primary double-stranded miRNA (pri-miRNA) by RNA polymerase II or III (3), which adopts a hairpin secondary structure. Pri-miRNAs are then cleaved in pre-miRNAs by the RNase Drosha (3), which acts in a microprocessor complex (4). Pre-miRNAs are then exported out of the nucleus by Exportin-5 (5) and further processed in the cytoplasm by Dicer (6), which cleaves the hairpin enabling the two strands to dissociate. Mature miRNAs can regulate gene expression (7) by interfering with target messenger RNAs (mRNA) in two ways. If they are in a minority compared to their target mRNAs, they associate with RISC (RNA-induced silencing complex) through proteins of the Argonaute family, such as AGO2, that recognise miRNAs, then bind to the mRNAs (8). RISC cleaves the mRNAs and the resulting fragments can themselves become interfering RNAs, thereby amplifying silencing (9). In mammals, miRNAs can circulate out of the cell of origin either freely (10), bound to AGO2 (11), in extracellular vesicles (EVs) (12) or in high density and low density lipoproteins (HDLs and LDLs) (13).

HDLs play an important role in reverse cholesterol transport, carrying cholesterol from peripheral organs to liver (14). First, their major structural protein Apolipoprotein A1 (APO-A1) is synthesized in the liver (15) or in the intestine (16). These proteins acquire cholesterol and phospholipids that are effluxed from hepatocytes and enterocytes, involving the ABC1 (ATP-binding cassette A1) receptor (17). The newly formed HDL are then released into the circulation to reach peripheral organs and other lipoproteins from which they acquire more lipids (14).

Then, they are carried to the liver, where they deliver these lipids through receptor SR-B1 (Scavenger receptor class B type 1) (18).

HDL contain proteins (14), lipids such as phospholipids, triglycerides and cholesterol, and metabolites such as steroids and other hormones (19). It was also shown recently that they carry small RNAs, and in particular miRNAs such as miR-375 and miR-223 in mice (20). In mice, miR-375 is involved in pancreatic islet cells genesis (21) and in the regulation of insulin secretion (22). It is also expressed in the pituitary and in hypothalamic cells (23). miR-223 is involved in haematopoiesis (24).

Some studies suggest that circulating miRNAs encapsulated in EVs might play a role in epigenetic inheritance by conveying information from the soma to the germline (25). It can also be investigated whether miRNAs associated with HDLs participate in epigenetic inheritance.

The mechanisms underlying epigenetic inheritance can be studied in mouse models, such as the MSUS model (26). Early life trauma is known to have consequences at adult age, such as depression (27), which were shown to be transmitted across generations (26). In the MSUS (unpredictable maternal separation combined with unpredictable maternal stress) model, female mice are chronically and unpredictably separated from their pups and stressed three hours a day between post-natal days 1 and 14 (26). This is associated with depressive-like behaviour in their male offspring at adult age, and this alteration of the phenotype is transmitted through four generations at least (28). MSUS mice do not only display behavioural but also molecular alterations: in the first generation after maternal separation, the sperm, serum, hypothalamus and hippocampus miRNA profiles differ from the control. For example, miR-375-3p, which is a specific form of miR-375, is upregulated in MSUS sperm (29). MSUS mice also display metabolic disruptions (28), such as alterations of their cholesterol metabolism. In the first generation after maternal separation, HDL levels were shown to be significantly increased in the liver of pups, and significantly decreased in adult plasma (unpublished data).

Recent results suggest that HDL might be involved in epigenetic inheritance in the MSUS model: first, germ cells (GC1) were incubated with serum from MSUS mice, and a significant increase in miR-375-3p expression was observed in those cells compared to cells incubated with serum from wild type mice (unpublished data). Next, HDL receptor SR-B1 was knocked-down in GC1 cells, and it was observed that when these cells were incubated with MSUS plasma, there was no miR-375-3p increase. This suggests that HDLs deliver miR-375-3p or factors regulating its expression to these cells. In order to confirm this hypothesis, the miRNA content of HDLs isolated from MSUS and control mice blood has to be analysed.

The aim of this study was to optimise a method to isolate HDLs from mouse plasma in the prospect of extracting the miRNAs they contain to compare their profiles between MSUS and control mice.

For this, we used size-exclusion chromatography (SEC) and density gradient ultracentrifugation (DGUC) and we characterised the fractions obtained by Western blot. We observed that the overlap between exosomal and HDL fractions was smaller when using DGUC, so we opted for this method.

Results :

In the bloodstream, miRNAs can circulate freely, bound to AGO2, encapsulated in EVs or in HDLs (10–13). Thus, HDLs have to

be separated from exosomes and AGO2 proteins to ensure that the source of miRNAs analysed is HDL only. There are several ways to isolate vesicles and protein complexes: they can be separated chemically by precipitation (30), by size or by density. We used the two latest methods: we first performed size-exclusion chromatography, and then density gradient ultracentrifugation.

HDL isolation by size-exclusion chromatography.

We first aimed at isolating HDL by size-exclusion chromatography. We used commercially available columns which are usually employed to isolate exosomes (Fig. 1A). They are filled with porous beads. When the sample travels through the column, the small molecules are trapped into the pores, while the bigger molecules are able to migrate quicker during elution (31). Thus, exosomes, which are bigger than HDL and other proteins, are expected to be eluted in the first two millilitres after elution of the column void volume, while proteins, including HDLs, should be eluted in the later fractions (Fig. 1B). Previous experiments aiming at isolating exosomes with this method had already been conducted at the lab, and the fractions obtained had been characterised by Western Blots against exosomal markers HSP70 (Fig. 1C) and flotillin (Fig. 1D). The molecular weight of HSP70 is 70 kDa, but we can see bands at around 50 kDa, because this protein has a variant weighing 54kDa (32). HSP70 is present in fractions 7 to 14, and flotillin in fractions 9 to 12, indicating that exosomes are present in these fractions. To investigate whether this method is suitable for HDL isolation, we performed this experiment again to characterise the HDL content of the fractions and to compare it with these results. We collected 26 fractions of 500 µL and we analysed the presence of HDL marker APO-A1 by Western blot (Fig. 1E). APO-A1 is detected in fractions 9 to 26, indicating the presence of HDLs in these fractions. Taken together, these results show an overlap between exosome and HDL fractions. We concluded that this method was not efficient to separate HDLs from exosomes.

HDL isolation by density gradient ultracentrifugation (DGUC).

Next, we aimed at isolating HDLs by DGUC. In DGUC, tubes are filled with layers of iodixanol solutions of decreasing densities from the top to the bottom, and the plasma sample is added on top of these layers. When the tubes are centrifuged, the particles present in the sample sedimentate if they are in a medium of lower density. Therefore, they migrate down the tube until they reach the layer where their density is equal to the density of the medium, and they are thus separated by density (35).

Previous studies established that some populations of HDLs and exosomes had the same density (30), thus they cannot be separated completely by DGUC. However, results obtained by Onódi et al. (36) suggested that the overlap was negligible, because some populations of exosomes are removed during pre-processing of plasma prior to density gradient. Therefore, we isolated HDL using a modified version of their protocol (Fig. 2A). After having performed DGUC using an iodixanol gradient, we collected 14 fractions of increasing densities (Fig. 2B). We then analysed the presence of HDL marker APO-A1 and exosome marker TGS101 by Western blot (Fig 2C, D). We observed the presence of APO-A1 in fractions 2 to 6, and of TSG101 in fractions 4 to 8, so there is a small overlap between

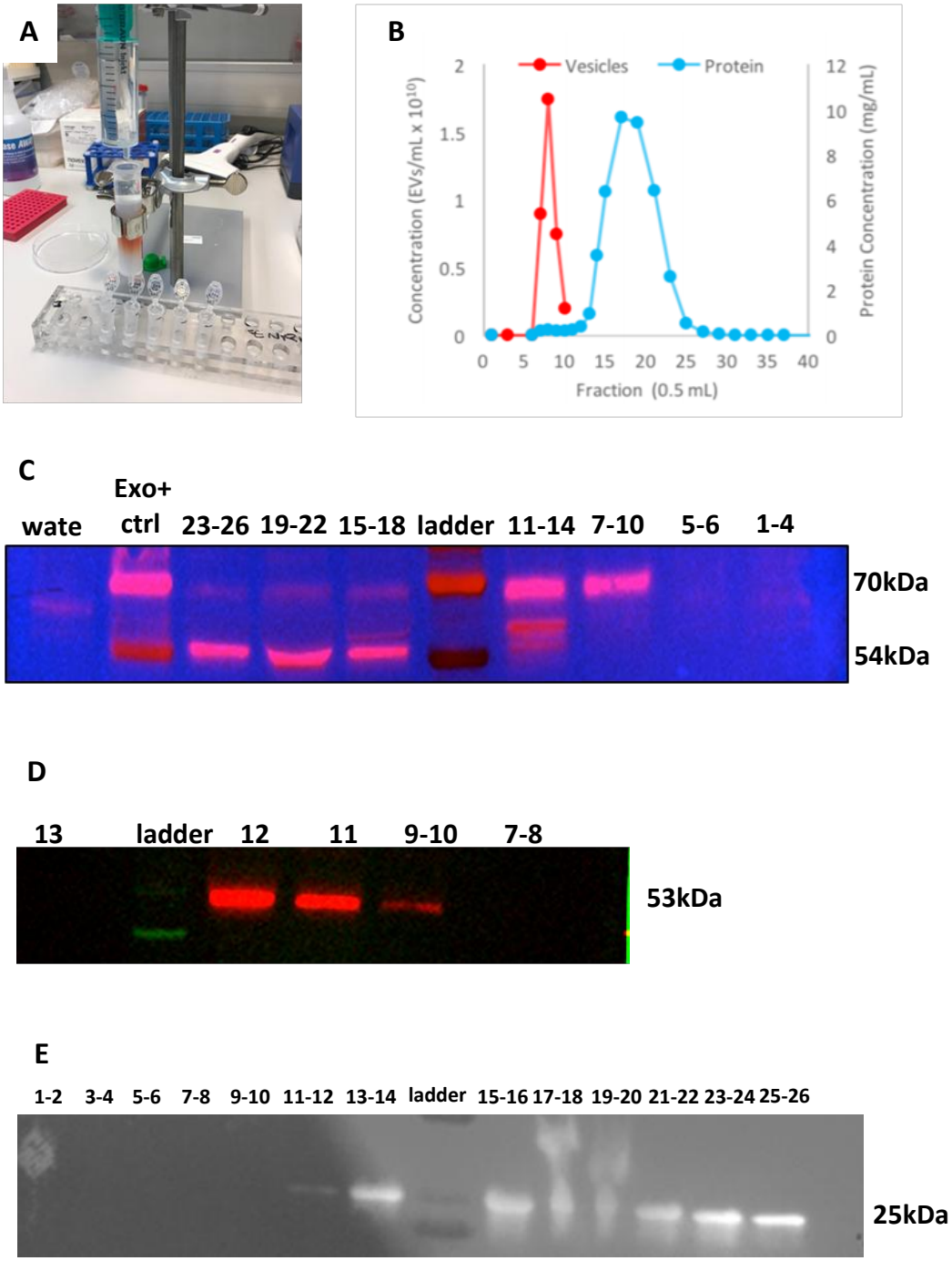

Fig1. HDL isolation by size-exclusion chromatography. A. *Experimental set-up.* ***B.*** *Expected elution profile, provided by manufacturer technical note* (33) ***C.*** *Western blot against HSP70. Each lane corresponds to several fractions obtained by size-exclusion chromatography and pooled. Exo+ ctrl is a positive control* (34) ***D.*** *Western blot against flotillin.* ***E.*** *Western blot against APO-A1. Each lane corresponds to two fractions obtained by size-exclusion chromatography.*

HDL and exosomal fractions. However, we considered that this overlap was negligible. We also performed a Western blot against AGO2 (Fig. 2E), and we saw that it was present in fractions 3 to 6, so there is an overlap between AGO2 and HDL fractions. Therefore, HDLs must also be separated from AGO2.

Simultaneously, we performed another DGUC and collected 10 fractions. This experiment was performed using the same initial amount of plasma as in the first one. We performed Western blots against APO-A1 (Fig. 2F) and exosomal marker CD9 (Fig. 2G). We observed that APO-A1 was present in fractions 2 to 6, and CD9 in fractions 6 to 8, so there is also a negligible overlap between HDL and exosomal fractions. Provided that the overlap was negligible compared to SEC where the major exosomal fractions overlapped with HDL fractions, we chose to use this method of density gradient ultracentrifugation to isolate HDLs from exosomes.

Next, we aimed at separating HDLs from AGO2. For this, we performed a filtering centrifugation of the DGUC fractions containing HDLs. We performed a Western blot against APO-A1 and we observed that it was only detected in the concentrate (Fig. 2H). However, we did not have time to perform a Western blot against AGO2, to check if the protein is present in the flowthrough.

Then, we aimed at characterising the RNA content of the 10 DGUC fractions obtained. For this, we extracted the RNAs from each DGUC fraction and we analysed their profiles by RNA electrophoresis (Fig. 3B). For HDL fractions, the expected profile is shown in Fig. 3A: HDL contain a variety of small RNAs (between 30 and 200 nucleotides), and no mRNAs. We obtained the same profile for fractions 2, 5 and 6, however, for fractions 3 and 4, the profile obtained is different from the expected one. This might be due to an experimental mistake during RNA extraction of these fractions.

Discussion

We aimed at isolating HDLs from plasma in order to extract their RNAs to compare their profiles in MSUS and control mouse. For this, we used density gradient ultracentrifugation. As the densities of exosomes and HDLs overlap, this method is usually performed in addition to another separation method, such as chemical exosome precipitation (30) or fast protein liquid chromatography (37). The HDL fractions obtained with these methods are more pure, but the HDL yield is lower. Therefore, in order to have enough HDL to perform RNA extraction and then prepare libraries for sequencing, the initial amount of plasma required is higher. As we had a limited amount of plasma at our disposal, we opted for a method with a lower purity and higher yield. However, this method needs to be further optimised in order to increase the purity of the HDL obtained. Besides, a method to separate AGO2 from HDL has to be established.

To further characterise the fractions obtained by DGUC, qPCRs detecting miR-375 and miR-223, which are HDL markers, should be performed on the RNAs extracted from each fraction (Fig. 3). The presence of exosomal marker miR-155-5p (38) and hemolysis marker miR-451a (39) should also be analysed in each fractions. For this, a miRNA specific qPCR protocol will be followed (40). The DGUC fractions do not contain any RNA the quantity of which is constant, and thus, that could be

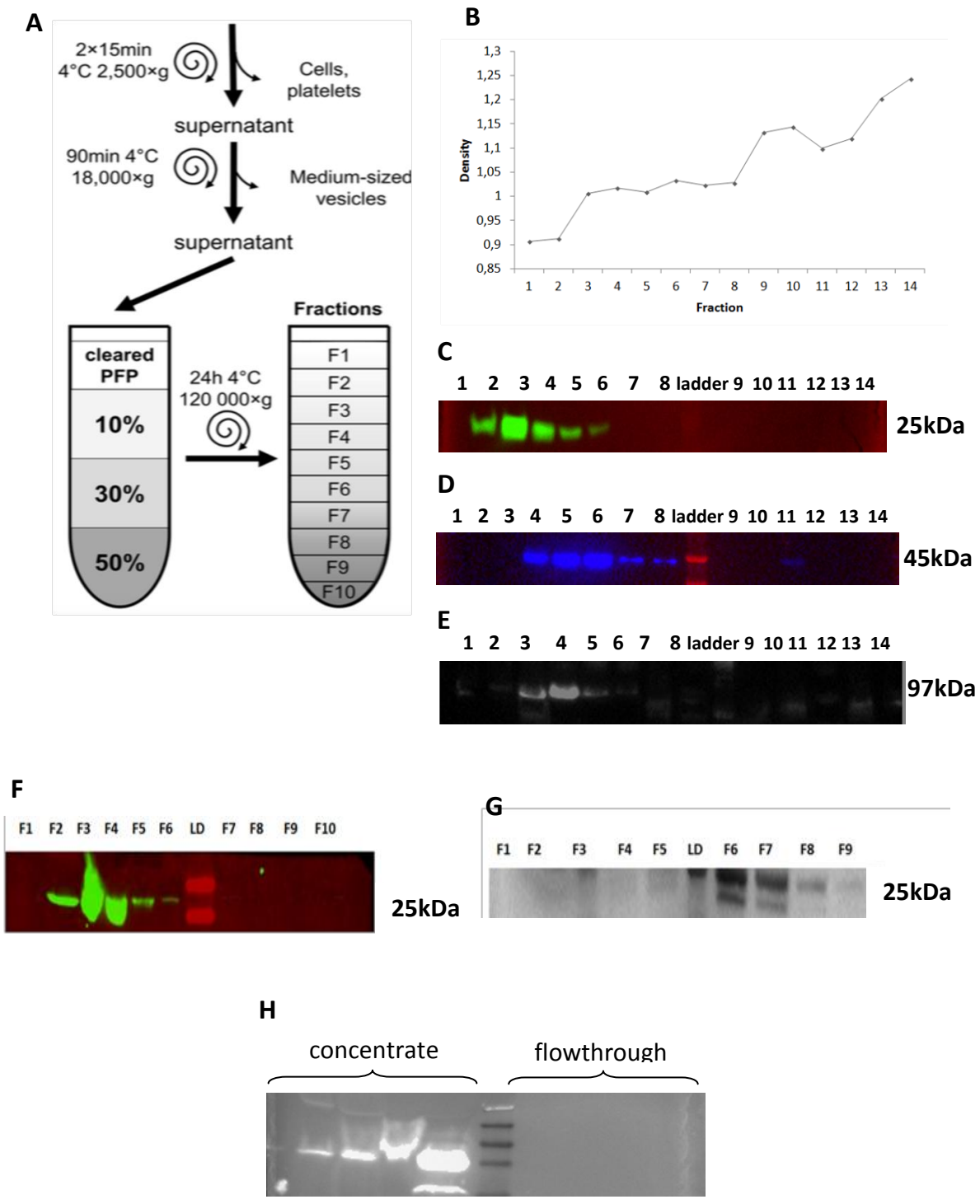

Fig2. HDL isolation by density gradient ultracentrifugation. A. *Experimental design, from the paper of Onodi et al.* (36). *F: fraction, PFP: platelet-free plasma.* ***B.*** *Density plot of the 14 fractions obtained by DGUC.* ***C,D,E.*** *Western blot against C. APO-A1, D.TSG101, E. AGO2 in the 14 fractions obtained in the first DGUC.* ***F,G.*** *Western blot against E. APO-A1, F. CD9 in the 10 fractions obtained in the second DGUC. Each lane corresponds to one fraction.* ***H.*** *Western blot against APO-A1 in the HDL fractions after filtering centrifugation.*

used as a standard; therefore, a spike-in miRNA has to be added in each fraction.
After isolating HDLs and extracting their RNAs, the next step is to prepare libraries for sequencing. As the amount of RNAs extracted is small, the protocol followed will be adapted from a single cell sequencing protocol (41). A test run following this protocol was already performed. When the results obtained are analysed, the next step consists in sequencing the RNAs extracted from MSUS and control HDL samples.

The question underlying this study is to know whether HDLs play a role in epigenetic inheritance. Several results suggest that HDL might be involved in the modification of the miRNA content of germ cells. If the results of HDL RNA cargo sequencing show a difference between MSUS and control HDL RNA profiles, it will reinforce this hypothesis. To provide direct evidence that HDL are able to modify germ cells miRNA content, GC1 cells could be incubated with HDLs isolated from MSUS and control mice plasma, and their miRNA content could be compared.

It was also shown recently that cholesterol metabolism was altered in MSUS pups at post-natal day 28 (unpublished data). For example, ELISA assays showed a significant increase of HDLs in their liver compared to control. We can investigate whether the uptake or the release pathway is affected, by performing qPCRs to assess the amount of SR-B1 and ABCA1 RNAs. We already extracted RNAs from post-natal day 28 MSUS and wild type livers and performed cDNA conversion (data not shown).

Material and methods :

DGUC. The protocol of Onódi et al.(36) was used. Solutions of 10%, 30% and 50% iodixanol were prepared: iodixanol was diluted into a solution of 25 mM sucrose and 1 mM Tris-HCl (pH 7.4). These solutions were layered in 10 mL ultracentrifuge tubes, and 1 mL of plasma was added in the tube. Tube weights were equilibrated using PBS and the samples were ultracentrifugated at 4°C at 120,000xg for 24h (Sorvall discovery 90SE, rotor: TH 64.1). Then, the tube content was separated into 10 or 14 fractions. Each fraction was diluted in 10 mL of PBS and ultracentrifugated for 3 hours at 4°C at 100,000xg using the same rotor and centrifuge. The supernatant was removed using a vacuum pump and the pellet was resuspended in 70 µL of PBS.

Size-exclusion chromatography. 500 µL of plasma was added on top of the column (IZON qEV 70 original Size Exclusion Column), and PBS was used for elution. Fractions of 1 mL were collected in each tube. In total, 14 fractions were collected. Then, the column was flushed with 10 mL PBS. Fractions were concentrated (Concentrator 5301, Eppendorf) and the volume of each fraction was equilibrated.

Gradient gel. The protocol of Walker (42) was used. Solutions of 5% and 20% acrylamide (named respectively solutions A and B) were prepared and placed in two beakers. TEMED was added into each beaker. A dual channel peristaltic pump was used to add progressively solution A into solution B (thus decreasing progressively the acrylamide concentration of solution B), while carrying solution B to the gel plates at the same rate. Then, water was added on top of the gel and it was left for 30 minutes to

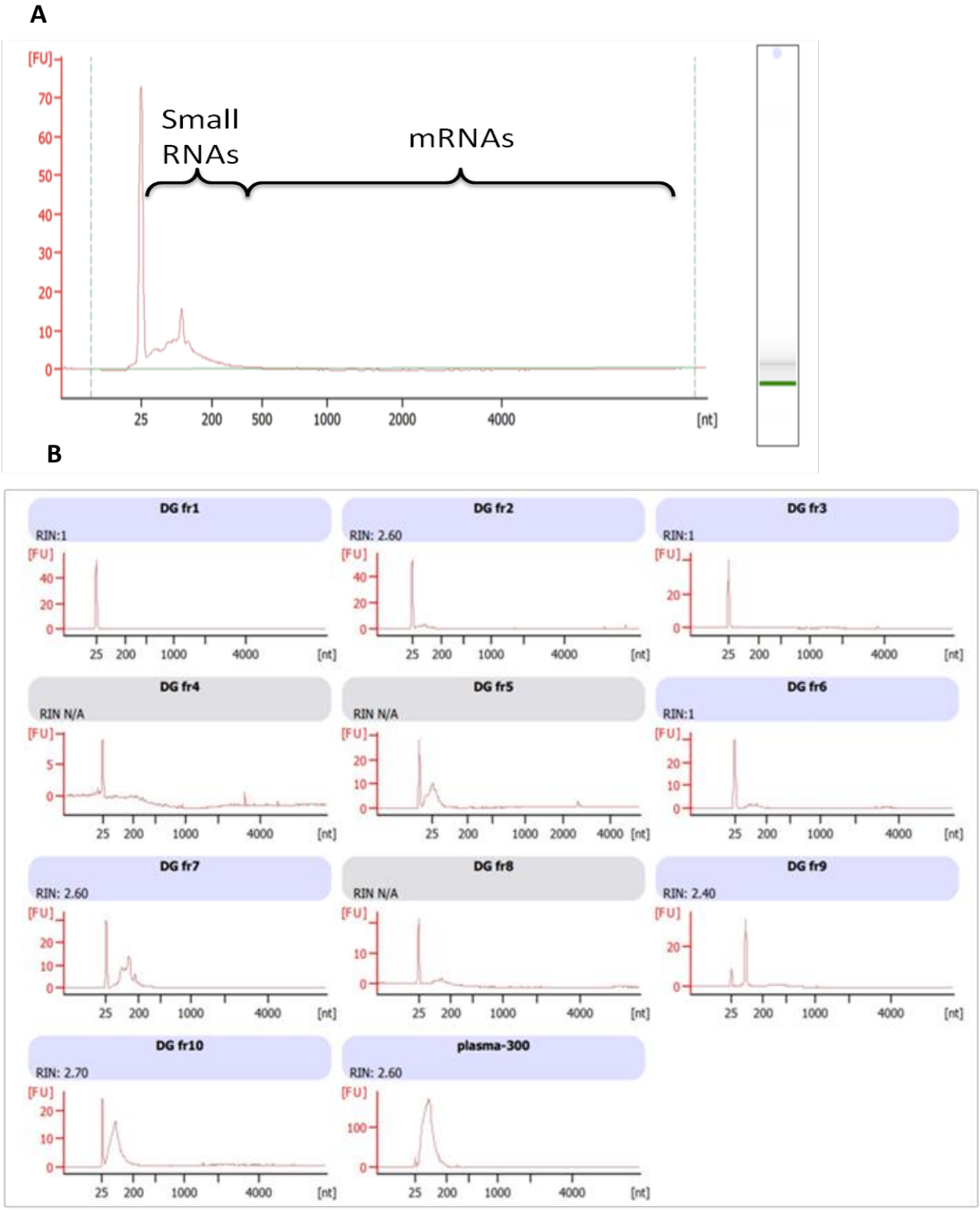

Fig3. RNA content of the DGUC fraction. A. *Expected RNA profile in HDL fractions.* ***B.*** *RNA profiles of the 10 fractions obtained by DGUC. Fractions 2 to 5 correspond to HDL fractions, fractions 6 to 8 correspond to exosomal fractions, "plasma-300" corresponds to the total RNAs extracted from 300 µL of plasma.*

polymerise. Then, water was removed and the gel plates were filled with a stacking solution (4% acrylamide). The comb (10 or 15 wells) was placed between the two plates, and the gel was stored at 4°C.

Western blots. In each fraction, protease inhibitor cocktail (Thermofisher) 100X and RIPA 10X were added. The volume added was calculated to obtain a final solution of 1X of protease inhibitor cocktail and 1X of RIPA. The protein concentration of each fraction was assessed using Qubit. Then, Laemmli buffer and beta-mercaptoethanol were added to aliquots of each fractions (volumes calculated according to manufacturer's instructions), and these samples were incubated at 95°C for 5 minutes. Samples ran at 100-125V for about 2 hours in 4-20% or 5-20% gradient gels. Proteins were transferred on a PVDF membrane (Transblot Turbo Mini PVDF Transfer pack) using Biorad TransBlot Turbo machine. The membrane was incubated for 2 hours at room temperature under agitation in a blocking solution (5% Sureblock (Lubioscience) in TBS-T). Then, the membrane was incubated with primary antibodies overnight at 4°C (for APO-A1: Genetex, rabbit, 1:2000 or 1:3000,for TSG101: Santa Cruz Biotechnology, mouse monoclonal, 1:2000). The next day, the membrane was washed three times for 15 minutes in TBS-T and then incubated for one hour with secondary antibodies in the dark at room temperature (goat anti-mouse HRP conjugated, Upstate; goat anti-rabbit HRP conjugated, Upstate). The membranes were imaged using ChemiDoc™ XRS+ (Biorad).

RNA extraction. For RNA extraction in the liver: Tissues were first lysed in TCEP and RLT plus buffer (Tissue Lyser II, Qiagen). For all RNA extractions: 150 µL of lysed tissue/ 200µL of DGUC fractions were transferred into a new tube and 1 mL of Trizol was added. The tubes were vortexed and incubated at room temperature for 3 minutes. 300 µL of chloroform was added. The tubes were vortexed and incubated at room temperature for 5 minutes. Then, they were centrifuged at 12,000 g at 4°C for 15 minutes. The aqueous phase was transferred into new tubes and 500 µL of chloroform were added. The tubes were vortexed and centrifuged as previously. The aqueous phase was transferred into new tubes containing 10 µL of glycogen. 500 µL of isopropanol were added, and then the tubes were stored at -20°C overnight. The next day, the samples were centrifuged at 12,000 g at 4°C for 30 minutes. The supernatants were removed using a vacuum pump and 1 mL of ethanol 75% was added into each tube. The pellets were resuspended and the tubes were centrifuged at 7,500 g at 4°C for 5 minutes. The samples were washed two more times with ethanol. Then after drying the samples for 5 minutes by leaving the tubes open on ice, the pellets were resuspended in 30 µL of nuclease free water.

cDNA conversion of liver RNAs. RNA samples were first diluted to obtain a concentration of 50ng/µL. 2 µL of oligodT were added to 13 µL of each sample. The tubes were incubated at 70°C for 5 minutes. Then, 5µL of M-MLV (Moloney Murine Leukemia Virus Reverse Transcriptase) buffer, 3.5 µL of dNTP (2.5 nM), 0.5 µL of RNAsin and 1 µL of M-MLV were added to the samples and they were incubated for 90 minutes at 37°C, and then 10 minutes at 65°C. 75 µL of water were then added to each tube.

Protein, RNA and DNA concentration measurements. Protein, RNA and DNA

concentrations were measured with a Qubit machine, following manufacturer's instructions.

RNA profile analysis. RNA profiles of the DGUC fractions were established using Agilent RNA 6000 Pico kit with a Bioanalyzer machine, following manufacturer's instructions.

Acknowledgements.

I performed the density gradients with the help of Anara Alshanbayeva, the size-exclusion chromatography and the Western blots of Figure 1E, 2C, 2E. I implemented the gradient gel protocol at the lab and I poured the gradient gels. I performed the RNA extractions. We performed the Bioanalyser assay and the qPCR together. Anara Alshanbayeva made the Western blots of Figures 1C, 1D, 2D, 2E, 2F.

I thank Isabelle Mansuy for giving me the opportunity of working in her group.
I thank Anara Alshanbayeva for her supervision, and all the other members of the group for their warm welcome and support.

REFERENCES

1. Ambros V (2004) The functions of animal microRNAs. *Nature* 431:350–355.
2. Hawkins P, Morris KV (2008) RNA and transcriptional modulation of gene expression. *Cell Cycle* 7(5):602–607.
3. Lee Y, et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425(6956):415–419.
4. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* 432(7014):231–235.
5. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U (2004) Nuclear export of microRNA precursors. *Science* 303(5654):95–98.
6. Lund E, Dahlberg JE (2006) Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol* 71:59–66.
7. Carrington JC, Ambros V (2003) Role of microRNAs in plant and animal development. *Science* 301(5631):336–338.
8. Khraiwesh B, et al. (2010) Transcriptional Control of Gene Expression by MicroRNAs. *Cell* 140(1):111–122.
9. Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics* 9(2):102–114.
10. O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne)* 9:402.
11. Arroyo JD, et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A* 108(12):5003–5008.
12. Valadi H, et al. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature Cell Biology* 9(6):654–659.
13. Bayraktar R, Van Roosbroeck K, Calin GA (2017) Cell-to-cell communication: microRNAs as hormones. *Mol Oncol* 11(12):1673–1686.
14. Feingold KR, Grunfeld C (2000) Introduction to Lipids and Lipoproteins. *Endotext*, eds De Groot LJ, et al. (MDText.com, Inc., South Dartmouth (MA)). Available at: http://www.ncbi.nlm.nih.gov/books/NBK305896/ [Accessed January 15, 2019].
15. Hamilton RL, Williams MC, Fielding CJ, Havel RJ (1976) Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J Clin Invest* 58(3):667–680.
16. Glickman RM, Green PH (1977) The intestine as a source of apolipoprotein A1. *Proc Natl Acad Sci U S A* 74(6):2569–2573.
17. Brooks-Wilson A, et al. (1999) Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nature Genetics* 22(4):336.
18. Acton S, et al. (1996) Identification of Scavenger Receptor SR-BI as a High Density Lipoprotein Receptor. *Science* 271(5248):518–520.
19. Vickers KC, Remaley AT (2013) Functional Diversity of HDL Cargo. *J Lipid Res*:jlr.R035964.
20. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT (2011) MicroRNAs are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat Cell Biol* 13(4):423–433.

21. Lynn FC, et al. (2007) MicroRNA Expression Is Required for Pancreatic Islet Cell Genesis in the Mouse. *Diabetes* 56(12):2938–2945.
22. Poy MN, et al. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432(7014):226–230.
23. Kapsimali M, et al. (2007) MicroRNAs show a wide diversity of expression profiles in the developing and mature central nervous system. *Genome Biol* 8(8):R173.
24. Johnnidis JB, et al. (2008) Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* 451(7182):1125–1129.
25. Sharma A (2014) Bioinformatic analysis revealing association of exosomal mRNAs and proteins in epigenetic inheritance. *J Theor Biol* 357:143–149.
26. Franklin TB, et al. (2010) Epigenetic Transmission of the Impact of Early Stress Across Generations. *Biological Psychiatry* 68(5):408–415.
27. Mandelli L, Petrelli C, Serretti A (2015) The role of specific early trauma in adult depression: A meta-analysis of published literature. Childhood trauma and adult depression. *European Psychiatry* 30(6):665–680.
28. van Steenwyk G, Roszkowski M, Manuella F, Franklin TB, Mansuy IM (2018) Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation. *Environ Epigenet* 4(2). doi:10.1093/eep/dvy023.
29. Gapp K, et al. (2014) Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature Neuroscience* 17(5):667–669.
30. Seneshaw M, et al. (2016) Fast and Simplified Method for High Through-put Isolation of miRNA from Highly Purified High Density Lipoprotein. *J Vis Exp* (113). doi:10.3791/54257.
31. Sun T, Chance RR, Graessley WW, Lohse DJ (2004) A Study of the Separation Principle in Size Exclusion Chromatography. *Macromolecules* 37(11):4304–4312.
32. Tsukahara F, Yoshioka T, Muraki T (2000) Molecular and Functional Characterization of HSC54, a Novel Variant of Human Heat-Shock Cognate Protein 70. *Mol Pharmacol* 58(6):1257–1263.
33. qEVoriginal Technical Note 2018.pdf Available at: https://www.schaefer-tec.it/sites/default/files/qEVoriginal%20Technical%20Note%202018.pdf [Accessed May 28, 2019].
34. Human Exosome Lysate Positive Protein Control *System Biosciences*. Available at: https://www.systembio.com/products/exosome-research/exosome-detection/human-exosome-lysate-positive-protein-control/ [Accessed May 31, 2019].
35. Density Gradient Ultracentrifugation | Protocol Available at: https://www.jove.com/science-education/5685/density-gradient-ultracentrifugation [Accessed May 31, 2019].
36. Onódi Z, et al. (2018) Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma. *Front Physiol* 9. doi:10.3389/fphys.2018.01479.
37. Michell DL, et al. (2016) Isolation of High-density Lipoproteins for Non-coding Small RNA Quantification. *J Vis Exp* (117). doi:10.3791/54488.
38. Alexander M, et al. (2015) Exosome-delivered microRNAs modulate the inflammatory response to endotoxin. *Nature Communications* 6:7321.
39. Shkurnikov MY, et al. (2016) Analysis of Plasma microRNA Associated with Hemolysis. *Bull Exp Biol Med* 160(6):748–750.

40. Goldrick M (2013) Reduce microRNA RT-qPCR Assay Costs by More Than 10-fold Without Compromising Results.
41. Hagemann-Jensen M, Abdullayev I, Sandberg R, Faridani OR (2018) Small-seq for single-cell small-RNA sequencing. *Nature Protocols* 13(10):2407.
42. Walker JM (1994) Gradient SDS polyacrylamide gel electrophoresis of proteins. *Methods Mol Biol* 32:35–38.