European Journal of Nutrition (2021) 60:1999–2011
https://doi.org/10.1007/s00394-020-02390-2

ORIGINAL CONTRIBUTION

One-year dietary supplementation with walnuts modifies exosomal miRNA in elderly subjects

María-Carmen López de las Hazas[1] · Judit Gil-Zamorano[1] · Montserrat Cofán[2,3] · Diana C. Mantilla-Escalante[1] · Almudena Garcia-Ruiz[1] · Lorena del Pozo-Acebo[1] · Oscar Pastor[3,4] · María Yañez-Mo[5,6] · Carla Mazzeo[5,6] · Mercè Serra-Mir[2] · Monica Doménech[2] · Cinta Valls-Pedret[2] · Sujatha Rajaram[7] · Joan Sabaté[7] · Emilio Ros[2,3] · Aleix Sala-Vila[8,9] · Alberto Dávalos[1]

Received: 5 May 2020 / Accepted: 14 September 2020 / Published online: 26 September 2020

Abstract

Purpose Epidemiological studies and clinical trials support the association of nut consumption with a lower risk of prevalent non-communicable diseases, particularly cardiovascular disease. However, the molecular mechanisms underlying nut benefits remain to be fully described. MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression and play a pivotal role in health and disease. Exosomes are extracellular vesicles released from cells and mediate intercellular communication. Whether nut consumption modulates circulating miRNAs (c-miRNAs) transported in exosomes is poorly described.

Methods Cognitively healthy elderly subjects were randomized to either control ($n=110$, abstaining from walnuts) or daily supplementation with walnuts (15% of their total energy, ≈30–60 g/day, $n=101$) for 1-year. C-miRNAs were screened in exosomes isolated from 10 samples, before and after supplementation, and identified c-miRNA candidates were validated in the whole cohort. In addition, nanoparticle tracking analysis and lipidomics were assessed in pooled exosomes from the whole cohort.

Results Exosomal hsa-miR-32-5p and hsa-miR-29b-3p were consistently induced by walnut consumption. No major changes in exosomal lipids, nanoparticle concentration or size were found.

Conclusion Our results provide novel evidence that certain c-miRNAs transported in exosomes are modulated by walnut consumption. The extent to which this finding contributes to the benefits of walnuts deserves further research.

Keywords c-miRNA · Dietary intervention · Exosomes · Lipidomics · Walnuts

Abbreviations

c-miRNAs	Circulating-miRNAs
EVs	Extracellular vesicles
miRNAs	MicroRNAs
NTA	Nanoparticle tracking analysis
qRT-PCR	Quantitative real-time PCR

María-Carmen López de las Hazas, Judit Gil-Zamorano and Montserrat Cofán have contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00394-020-02390-2) contains supplementary material, which is available to authorized users.

✉ Aleix Sala-Vila
asala3@imim.es

✉ Alberto Dávalos
alberto.davalos@imdea.org

Extended author information available on the last page of the article

Introduction

Nuts are an energy-dense food composed by unsaturated fatty acids [1–3] and other phytochemicals such as vitamin E, phytosterols and polyphenols [1]. A robust body of evidence from prospective studies suggests that sustained nut consumption (30–42.5 g/day [4, 5]) inversely relates to the risk of cardiovascular disease (CVD) and associated mortality [6]. Also produces effects on hypertension [7], neurodegenerative disorders [8], and all-cause mortality [9]. A consistent cholesterol-lowering effect of nut-enriched diets has also been observed in many feeding studies [10]. However, the molecular mechanisms underlying the benefits of nut consumption remain to be fully uncovered.

Extracellular vesicles (EVs) are stable nanovesicles released from cells and present in all biological fluids. Exosomes (a type of EVs with size between 50 and 150 nm) display an important role in intercellular communication

by carrying functional/relevant information to target cells [11, 12]. Exosomes are normally enriched in cholesterol, sphingomyelin, glycosphingolipids and phosphatidylserine [13], and its cargo contains genetic material (including microRNAs [miRNAs]), proteins and lipids, which are released into the intercellular space by exocytosis upon body requirements.

miRNAs are small RNAs (18–22 nucleotides) involved in post-transcriptional gene regulation. miRNAs circulate in plasma associated with lipoproteins, Argonaute protein family or exosomes [14, 15], which increases their resistance to degradation by ribonucleases (RNases). Recently, the modulation of circulating-miRNAs (c-miRNAs) by different epigenetic factors has been described [16, 17] including diet and dietary components [18, 19].

Previous data suggest that dietary supplementation with a mixture of nuts (almonds and walnuts) influences circulating levels of miRNAs, some of which are correlated to lipid levels [20]. However, their transport in exosomes has not been assessed. Moreover, previous studies have also suggested that certain dietary polyphenols could promote exosome secretion [21] or interfere with the biogenesis of EVs [22]. Indeed, walnuts are a source of the dietary polyphenols ellagitannins [23]. The ability of exosomes to cross different barriers, i.e., blood–brain barrier could have implications on neurological health [12]. For that reason, the study of c-miRNAs after a dietary intervention could be a valuable tool to handle the progression of different diseases [19, 24].

The high stability of exosomal circulating miRNAs in biological samples highlight their validity as biomarkers [12]. Circulating miRNAs have been previously described to be modulated by dietary intervention [19]. Whether long-term walnut consumption influences the secretion of exosomes in humans is unknown. To address this issue, we explored long-term (1 year) changes in plasma exosome composition (content of miRNA, morphology, and lipidomic profile) in elders who added walnuts to their daily diet, compared with those following a diet without walnuts.

Material and methods

Study population

The current study was conducted within the frame of Walnuts And Healthy Aging (WAHA) study (https://clinicaltrials.gov/show/NCT01634841). WAHA is a dual-center (Hospital Clínic, Barcelona, Spain; and Loma Linda University, CA), randomized, parallel-group, observer-blinded, controlled clinical trial aimed to assess whether a walnut-enriched diet for 2 years would prevent or slow down age-related cognitive decline and macular degeneration compared with a control diet (abstention from walnuts) in cognitively healthy individuals aged 63–79 years. Detailed information on the project can be found in [25, 26]. Briefly, all participants were randomized to follow their usual diet, abstaining from walnuts (control group), or to add to their usual diet a daily amount of walnuts providing roughly 15% of their total energy intake (ranging from 30 to 60 g/day of walnuts depending on energy requirements; walnut group). Once randomized, we scheduled participants for a visit with the dietitians every 2 months, aimed at assessing compliance, increasing retention, collecting data on tolerance, and delivering walnuts when appropriate. At baseline, in a face-to-face visit, a study dietitian measured height, weight, and waist circumference by standard methods. In the same visit, participants also completed a validated short version of the Minnesota physical activity questionnaire [27] and a 3-day food record. At each visit, for participants in the walnut group, dietitians provided 8-week allotments of pieced walnuts (in sachets for daily consumption), noted any side effects, and collected used walnut sachets as a measure of compliance. In addition, they monitored food consumption through 3-day food records at the 0, 6-, and 12-month visits. We estimated energy requirements of each participant by using the World Health Organization formula for energy needs for adults > 60 years [28]. Dietitians obtained follow-up data on adiposity, physical activity and food consumption through 3-day food records from all participants at the 12-month visit. We calculated the nutrient composition of the diets with Food Processor Plus software (ESHA Research, Salem, Oregon, USA) adapted to nutrient databases of local foods when appropriate.

We collected overnight fasting blood samples at baseline and end of years 1 and 2, and we stored them at − 80 °C until analysis. The present sub-study was conducted only in statin-naïve participants completing the first year of intervention in the Barcelona site ($n = 211$, Table 1).

Pool of samples

Previous data suggest that statins might influence the transport of c-miRNAs and certain exosomal miRNAs [29], as well as exosomal release in certain cell types [30]. To avoid the possible modulatory effects of statins on exosomes and their c-miRNAs, participants remaining statin-naïve for the period of interest were selected. This resulted in $n = 211$ participants ($n = 110$ control group, $n = 101$ walnut group). Plasma samples were pooled to identify robust differences between intervention arms and because of the sensitivity of lipidomic analysis, which was not sufficient to analyze single samples lipids within individual isolated exosomes. Pools of 1.8 mL of plasma were prepared according to intervention group, gender and age range. Samples were pooled from about 10 participants (Fig. 1S-Online Resource). 40 pools were obtained: 7 pools of women and 3 pools of men × each

Table 1 Baseline characteristics of the studied population by intervention group

Variables	Control diet ($n=165$)	Walnut diet ($n=166$)
Women—no. (%)	110 (67.1)	114 (68.7)
Age—year	68.7 (68.2–69.2)	69.0 (68.5–69.5)
Ever smoking, yes—no. (%)	47 (28.7)	46 (27.7)
Weight—kg	71.1 (69.1–73.1)	69.9 (68.1–71.7)
Body mass index—kg/m^2	27.4 (26.8–28.1)	26.8 (26.2–27.3)
Waist circumference—cm	99 (97–101)	97 (96–99)
Hypertension—no. (%)	94 (51.4)	89 (53.6)
Type-2 diabetes—no. (%)	16 (9.8)	20 (12.0)
Dyslipidemia—no. (%)	87 (53.0)	89 (53.6)
Physical activity*	2412 [1562–3834]	2629 [1770–3909]

Data are *n* (%) or mean (95% confidence interval), except for physical activity, expressed as medians [interquartile ranges]

*Physical activity is expressed in MET-min/day, minutes/day at a given metabolic equivalent level (units of energy expenditure in physical activity, 1 MET-min roughly equivalent to 1 kcal)

intervention (control group and walnut group) × each time-point (baseline and 1 year of intervention).

Nanoparticle tracking analysis of exosomes

500 µL of 16 pooled samples (2 pools × each intervention × each time-point × each gender) was employed for nanoparticle tracking analysis (NTA) (Fig. 1S-Online Resource). In brief, plasma was diluted 1.5 times with PBS and was sequentially centrifuged s at 300*g* twice for 4 min, 2000 g for 4 min, and 10,000 g for 1 min at 4 °C, to remove large particles, dead cells and cellular debris. Then, the supernatants were centrifuged at 100,000*g* for 120 min at 4 °C. After discarding the supernatant, pellets were rinsed with PBS and resuspended into 100 µL of filtered PBS. Then, samples were diluted 500 times for the analysis of exosome concentration and size by using DS500 nanoparticle characterization system (NanoSight, Salisbury, United Kingdom) and NanoSight NTA 3.1. program and ZetaView® NTA system (Particle Metrix, Germany), respectively.

Screening of c-miRNAs transported in exosomes

Screening of c-miRNAs was performed in 500 µL of the 20 pools of walnut group (10 from each time-point) (Fig. 1S-Online Resource). Briefly, exosomes were isolated with miRCURY® Exosome Serum/plasma kit (Exiqon, Denmark) following the manufacturer's instructions. Immediately, total exosome RNA was isolated using miRCURY™ RNA isolation kit—Biofluids (Exiqon)—adding RNA spike-in kit (UniSp2, UniSp4, UniSp5, UniSp6 and cel-miR-39-3p) (Exiqon) following the manufacturer's instructions. After that, cDNA was synthesized using miScript II RT kit (Qiagen, Denmark) following the manufacturer's instructions. Then, miRNAs transported in exosomes were screened using miRCURY LNA miRNA miRNome PCR Human panel I + II, V4 using ExiLENT SYBR green master mix (Exiqon) by quantitative real-time PCR (qRT-PCR) on a 7900HT fast Real-Time PCR System (Applied Biosystems, CA, USA). Ct values were normalized using GenEx software (MultiD Analyses AB, Sweden).

Validation of c-miRNAs candidates transported in exosomes

Top miRNAs candidates obtained from the screening were then validated in the whole population ($n=333$), including participants who did not remain statin-naïve during the first year of intervention. Exosomal miRNA and cDNA from baseline and 1-year plasma samples were obtained as previously described. Validation of miRNAs candidates was performed by quantitative real-time PCR (qRT-PCR) using the ExiLENT SYBR green master mix (Exiqon) and LNA™ Oligonucleotides (Exiqon). The exosomal miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [31].

Lipidomic analysis

In a random sub-sample of 81 participants ($n=42$ in the control group and $n=39$ in the walnut group), we objectively assessed compliance by measuring changes in the red blood cell (RBC) status of alpha-linolenic acid (C18:3n3, ALA), an integral compound of walnuts, as described [25]. Exosomes isolated by miRCURY® exosome Serum/plasma kit (Exiqon) from 500 µL of each pool of plasma samples were used for lipidomic characterization. Lipids from exosomes were extracted following the method of Folch et al. with minor modifications [32]. The relative quantification of individual lipid species was performed using an internal standard mixture composed by phosphatidyl choline (PC) 28:2 (14:1/14:1), phosphatidylethanolamine (PE) (16:1/16:1), lysophosphatidylcholine (LPC) 17:0, dihydroceramide (dhCer) 35:0 (d18:0/17:0), ceramide (Cer) 37:1 (d181/19:0), hexosylceramide (HexCer) 33:1 (d18:1/15:0), sphingomyelin (SM) 30:1(d18:1/12:0), triglyceride (TG) 46:2(18:1/10:0/18:1), cholesteryl ester (CE): d7-CE 18:1 and free cholesterol (FC): d7-FC (Solna, Sweden). The exosome lipid extract was reconstituted in 200 µl of acetonitrile /isopropanol (1:1). Then 10 µL were injected on the LC–MS/MS system (QTrap 4000, AB SCIEX) equipped with a Kinetex C18 column (100 × 2.1 mm, 1.7 µm; Phenomenex) at 55 °C as previously described by [33]. Phospholipids (PC, PE and LPC), sphingolipids (Cer, dhCer, SM, HexCer and

dhHexCer) and TG lipid species were analyzed by ESI (electrospray ionization) following specific multiple reaction monitoring transition for each class. The analysis of FC and CE species was done on a second injection of the same lipid extract, on the same system, employing APCI (atmospheric-pressure chemical ionization) mode [34]. The annotation of lipid species followed published recommendations [35].

Bioinformatic studies

Validated target genes of differentially expressed miRNAs in exosomes supported by strong experimental evidence (reporter assay and Western blot) were obtained from the miRWalk 3.0 database [36]. Functional enrichment of target genes was performed with GeneCodis3 algorithm using Gene Ontology (GO) Biological Process annotation [37–39]. Prediction of possible sources of those miRNAs previously validated circulating in exosomes was performed using miRNA tissue expression data from the first version of the Human miRNA Tissue Atlas database [40].

Statistical analyses

This study was initially conceived as an opportunistic WAHA sub-study to be conducted in participants of the Barcelona node. The power calculation of the parent study can be found in Sala-Vila et al. [26] No run power calculation was specifically conducted for this sub-study; we validated the miRNAs candidates in the whole cohort because sample size/power calculations should also consider other size effects (i.e., elderly, sex, other dietary habits, etc.) and estimate of an association obtained in the discovery phase may be inflated because of a "winner's curse" phenomenon [41, 42].

Descriptive values are presented as means (interquartile range). We assessed between-group differences in ALA proportion of RBC membranes by one-way ANOVA. Regarding the screening for c-miRNA in exosomes, paired ANOVA was used to compare baseline and end of intervention. Significant levels were adjusted for multiple comparisons by Bonferroni's correction ($P < 0.00014$) and false discovery rate (FDR q-value < 0.00286533). The GenEx Pro qPCR data analysis software (Exiqon) was used for all data processing. One-way ANOVA followed by Tukey's comparison post hoc test was used for miRNA validation in all four groups and to evaluate gender effects and establish lipidomic differences between groups, Differences at the $p < 0.05$ level were considered statistically significant. In addition, paired ANOVA was used to determine statistical significance of differences in miRNA expression from baseline to 1 year of intervention in the overall sample and by gender. All analyses were performed with the GraphPad Prism software V.5 (La Jolla, CA, USA).

Results

Dietary compliance and evaluation of the nutritional changes

First, to determine compliance, we determined changes in ALA levels in RBC in a randomly selected sub-sample of participants. Whereas we found no between-group differences at baseline (Fig. 1a), significant differences were observed at the end of the intervention, with participants allocated to walnut group showing higher increase than those observed in the control group (Fig. 1b), indicating good compliance.

Baseline and 1-year changes in energy and nutrient intake and nut consumption by intervention group are presented in Table 2. No significant in-trial differences in walnut consumption were observed in the control group, while the walnut group increased consumption, as planned. At the end of the trial, participants in the walnut diet arm increased dietary energy and total fat and reciprocally decreased carbohydrate, translating into significant differences with changes observed in the control diet. 1-year increases in fiber, ALA, and polyunsaturated fatty acid intakes in the walnut group were also significantly higher than those observed in the control group, reflecting the nutrient composition of walnuts.

Walnut supplementation does not influence size and concentration of plasma exosomes

Exosomes were isolated from pooled samples before and after supplementation and subjected to NTA (Fig. 1S-Online Resource). Exosome size distribution and median size analyses demonstrated successful isolation of circulating exosomes, but no between-group differences in size or plasma concentration were observed (Fig. 2S-Online Resource and Table 3). Regarding the particle quantification, NanoSight NS300 and ZetaView NTA devices showed different particle quantification and nanosphere concentration (Fig. 2S).

Exosomal circulating miRNAs screening

Walnut-modulated exosomal c-miRNAs were screened in a subset of the statin-naïve participants of the walnut group before and after dietary supplementation ($n = 20$). The miRNAs analyzed were the most abundant 179 miRNAs present in human plasma. From the 179 miRNAs analyzed, 165 were detected in the exosomal samples of walnut group participants (Table 1S-Online Resource). 20 miRNAs were significantly modulated by walnut consumption (Fig. 1c; Table 4).

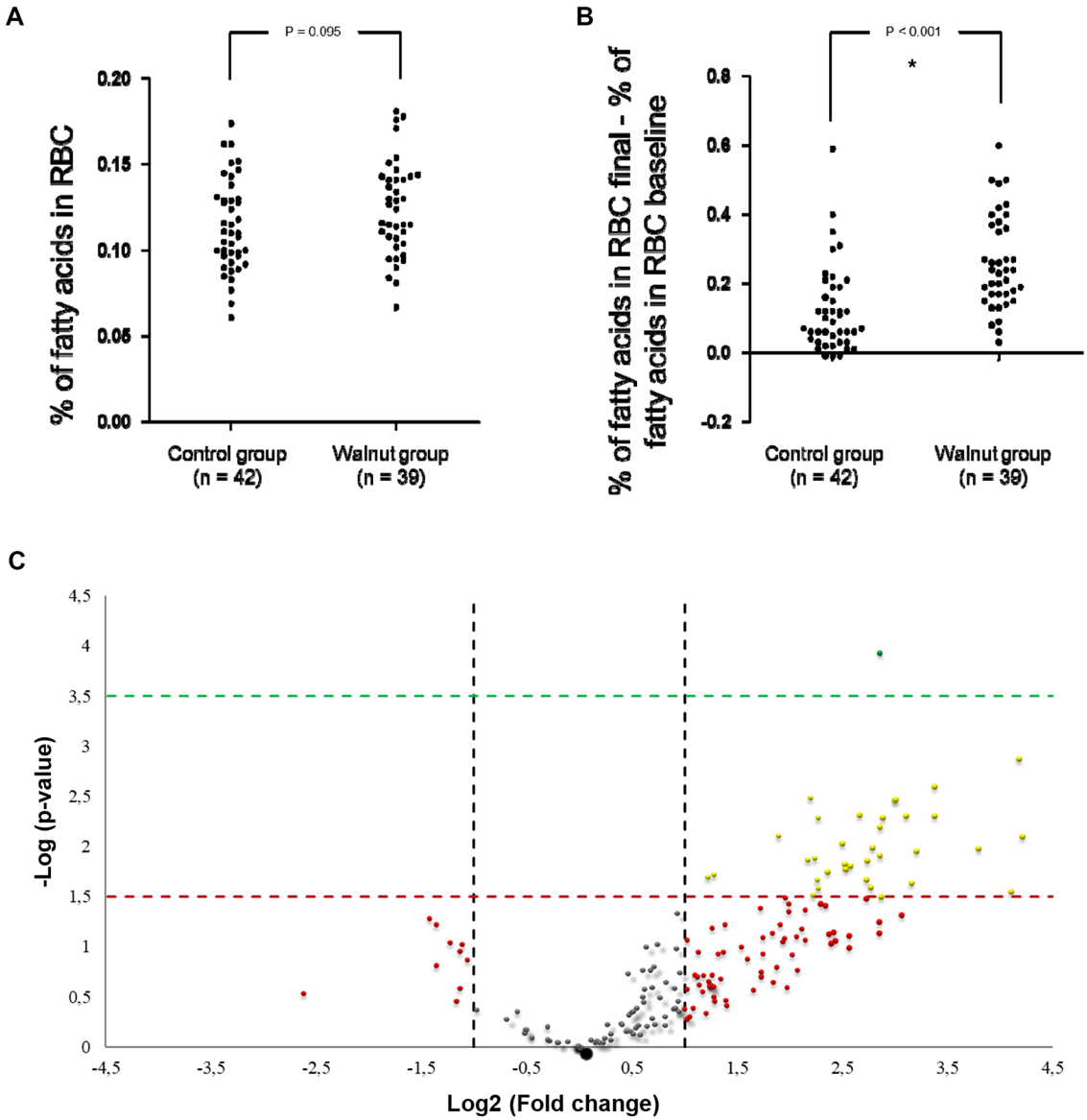

Fig. 1 Adherence to walnut supplementation and circulating miRNA screening. **a** Percentage of fatty acids, α-linolenic acid (ALA), in red blood cells (RBC) at baseline for the walnut and control groups. **b** Percentage of change of total fatty acids ALA in RBC after 1 year. *, significant different at $P < 0.05$ by one-way ANOVA. **c** Circulating exosomal miRNA screening of plasma samples assayed by qRT-PCR ($n = 20$ pools) after normalization using exogenous spike-in was performed and 20 miRNAs were differentially expressed (FDR < 0.05). Volcano plots were constructed using fold-change and P values. Dashed vertical lines indicate fold-change in miRNA expression threshold is 1.0-fold up and down, respectively. The horizontal red line represents a P value of 0.05, and the horizontal green line a P value of 0.01. Green points in the plot are significantly differentially expressed miRNAs ($P < 0.01$); meanwhile yellow points are significant ($P < 0.05$)

Walnut supplementation modulates exosomal miR-32-5p and miR-29b-3p

To determine if candidate c-miRNAs (Table 4) are also differentially modulated in a larger number of subjects, the top 10 significantly modulated exosomal c-miRNAs were validated by qRT-PCR in the whole cohort ($n = 333$). miR-194-5p was excluded from the validation set because of its absence in the first version of Vesiclepedia database [43], which lists miRNA transported in exosomes. Statistical significance was observed for only two c-miRNAs: hsa-miR-32-5p and hsa-miR-29b-3p (Fig. 2). As we found a trend for certain c-miRNAs in the walnut group after 1-year intervention, we searched for a possible effect of gender. We found some differences between males and females when miRNAs analysis was separated by sex.

Table 2 Baseline and 1-year changes in energy and nutrient intake, and nut consumption by intervention group

Variable	Visit	Control diet ($n=165$)	Walnut diet ($n=166$)	P value*
Energy—kcal/day	Baseline	1643 (1596 to 1691)	1718 (1659 to 1777)	0.053
	Change	12 (− 36 to 59)	140 (84 to 195)	0.001
Protein—% energy	Baseline	18.5 (18.1 to 19.0)	18.1 (17.6 to 18.6)	0.199
	Change	0.1 (− 0.5 to 0.7)	− 1.1 (− 1.6 to − 0.5)	0.005
Carbohydrate—% energy	Baseline	41.9 (40.9 to 42.9)	41.9 (40.8 to 43.0)	0.958
	Change	1.3 (0.1 to 2.5)	− 3.7 (− 4.8 to − 2.7)	< 0.001
Total fat—% energy	Baseline	39.2 (38.4 to 40.1)	39.3 (38.3 to 40.2)	0.990
	Change	− 1.2 (− 2.4 to − 0.1)	6.1 (5.0 to 7.2)	< 0.001
SFA—% energy	Baseline	9.8 (9.4 to 10.1)	9.9 (9.5 to 10.2)	0.656
	Change	− 0.3 (− 0.7 to 0.2)	− 0.8 (− 1.1 to − 0.4)	0.073
MUFA—% energy	Baseline	21.0 (20.4 to 21.6)	20.7 (20.0 to 21.3)	0.419
	Change	− 0.9 (− 1.8 to − 0.1)	− 1.6 (− 2.4 to − 0.9)	0.228
PUFA—% energy	Baseline	5.3 (5.0 to 5.6)	5.1 (4.9 to 5.4)	0.390
	Change	− 0.4 (− 0.7 to − 0.1)	8.6 (8.0 to 9.1)	< 0.001
ALA—% energy	Baseline	0.44 (0.42 to 0.46)	0.42 (0.40 to 0.44)	0.362
	Change	− 0.01 (− 0.04 to 0.02)	1.78 (1.68 to 1.88)	< 0.001
Fiber—g/day	Baseline	17.5 (16.6 to 18.3)	18.4 (17.3 to 19.5)	0.182
	Change	0.4 (− 0.5 to 1.4)	2.1 (1.1 to 3.1)	0.025
Alcohol—g/day	Baseline	5.4 (4.2 to 6.7)	6.4 (5.0 to 7.9)	0.218
	Change	0.0 (− 1.0 to 1.0)	− 0.5 (− 1.7 to 0.6)	0.522
Cholesterol—mg/day	Baseline	247 (229 to 266)	251 (237 to 265)	0.764
	Change	− 21.5 (− 43.6 to 0.6)	− 17.2 (− 34.9 to 0.6)	0.761
Walnuts—g/day	Baseline	0.7 (0.4 to 1.0)	0.4 (0.2 to 0.7)	0.221
	Change	− 0.3 (− 0.8 to 0.1)	39.9 (37.9 to 41.9)	< 0.001
Total nuts—g/day	Baseline	3.2 (1.9 to 4.5)	2.4 (1.5 to 3.4)	0.327
	Change	− 1.2 (− 2.7 to 0.4)	38.9 (36.8 to 41.1)	< 0.001

Data are means (95% CIs). SFA, saturated fatty acids

MUFA monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *ALA* alpha-linolenic acid

*Obtained by 1-way ANOVA

Table 3 Characterization of exosomes: size distribution of exosomes from walnut and control groups before and after intervention and gender effects measured with NanoSight NS 300

			Walnuts		Control	
			Baseline	1-Year	Baseline	1-Year
63–66	♀	Mean (nm)	201.4 ± 11.9	196 ± 0.4	212.3 ± 1	197.5 ± 6.2
		Mode (nm)	130.3 ± 6.9	135.5 ± 8.6	143.5 ± 9.6	140.9 ± 9.8
		s.d.	87.1 ± 3.1	88.2 ± 1.5	98.6 ± 1.9	94.8 ± 3.2
		Exosome	$3.85*10^8$	$3.15*10^8$	$1.08*10^9$	$7.50*10^8$
	♂	Mean (nm)	226.1 ± 3	228.2 ± 4.2	193 ± 3.8	214.4 ± 4.1
		Mode (nm)	138.6 ± 4.2	142.4 ± 11.1	137.5 ± 8.7	128.7 ± 13
		s.d.	109.7 ± 2.3	116.5 ± 6.8	90.5 ± 5.4	108.7 ± 4.4
		Exosome	$7.23*10^8$	$6.54*10^8$	$7.41*10^8$	$4.39*10^8$
67–69	♀	Mean (nm)	193.4 ± 6.1	172 ± 15.8	238.8 ± 7.1	154.9 ± 2.5
		Mode (nm)	119.1 ± 7.9	132.4 ± 19.7	152.4 ± 7.4	102.8 ± 5.1
		s.d.	81.8 ± 2.7	96 ± 23.4	111.7 ± 3.3	76.9 ± 3.9
		Exosome	$4.16*10^8$	$1.34*10^7$	$1.77*10^9$	$6.94*10^8$
	♂	Mean (nm)	203.6 ± 2.6	189.4 ± 3.9	165.8 ± 0.6	187.8 ± 2.9
		Mode (nm)	132.6 ± 14.4	126.8 ± 11.6	109.1 ± 2.8	124 ± 5.2
		s.d.	94.6 ± 3.8	100.2 ± 4.3	77.1 ± 1.5	90 ± 0.4
		Exosome	$6.17*10^8$	$9.1*10^8$	$7.23*10^8$	$7.32*10^8$

♀ Female, ♂ male, *s.d.* standard deviation

Table 4 List of circulating exosomal microRNAs differentially expressed (FDR < 0.05) after 1 year of walnut consumption comparing with basal levels in the screening phase ($n = 20$ pools)

	(Group-2) vs (Group-1)	Fold change	*P* value	FDR
1	**hsa-miR-15b-5p**	6.77858	0.000106	0.00065
2	**hsa-miR-106b-5p**	13.73081	0.001089	0.01469
3	**hsa-miR-151a-3p**	3.74096	0.002266	0.0229
4	**hsa-miR-424-5p**	9.07264	0.002291	0.0229
5	**hsa-miR-32-5p**	6.99093	0.002917	0.02628
6	**hsa-miR-107**	6.00084	0.003703	0.02957
7	**hsa-miR-148a-3p**	5.93548	0.004716	0.02957
8	hsa-miR-194-5p	3.93065	0.004987	0.02957
9	**hsa-miR-15a-5p**	8.61543	0.005017	0.02957
10	**hsa-miR-29b-3p**	7.37336	0.005088	0.02957
11	**hsa-miR-144-3p**	16.17018	0.006645	0.03932
12	hsa-miR-331-3p	4.96418	0.007268	0.0409
13	hsa-miR-451a	3.71696	0.007935	0.04269
14	hsa-miR-130a-3p	4.30391	0.008836	0.04459
15	hsa-miR-425-5p	10.53997	0.009584	0.04459
16	hsa-miR-660-5p	5.5079	0.010022	0.04459
17	hsa-miR-145-5p	5.49845	0.010312	0.04459
18	hsa-miR-142-5p	5.61277	0.010389	0.04459
19	hsa-miR-484	3.66003	0.010773	0.04459
20	hsa-miR-590-5p	6.51234	0.011368	0.0456

Candidates in bold were selected for downstream validation

Indeed, a greater effect of walnuts in women for hsa-miR-32-5p, hsa-miR-29b-3p and miR-144-3p (Fig. 3S-Online Resource) was observed. Compared to the whole population, miR-144-3p was only induced in females after walnut supplementation.

Functional analysis and possible origin of walnut-modulated miRNAs

We next performed a bioinformatic pathway analysis using validated target genes obtained from the miRWalk database [36]. Gene Ontology analysis suggested the involvement of targets genes in biological process of regulation of transcription (GO: 0006355), nervous system development (GO: 0007399), positive regulation of transcription (GO: 0045944), and positive regulation of cell proliferation (GO: 0008284) (Fig. 3a and Online Resource Table S2) for hsa-miR-32-5p. Panther pathway analysis of miR-29b-3p targets suggested their involvement on the integrin signaling pathway (P00034), p53 pathway (P04398), apoptosis signaling (P00006), inflammation (P00031), hypoxia response (P00030), angiogenesis (P00005), and Alzheimer disease–presenilin pathway (P00004), among others (Fig. 3b and Online Resource Table S3). To determine the possible origin or contribution of tissue miRNAs to exosome circulating levels, we analyzed their expression pattern using the TissueAtlas database [40] (Fig. 4S-Online Resource). We found that miR-32-5p is enriched in the thyroid, muscle and epididymis, while miR-29b-3p is enriched in the thyroid, muscle and brain.

Walnut supplementation does not change the lipid profile of exosomes

No previous studies of lipidomics of circulating exosomes after long-term dietary supplementation have been conducted to date. To address this issue, 126 lipid species were analyzed (Fig. 5S-Online Resource) in pooled samples ($n = 10$ pools) of participants of both intervention groups.

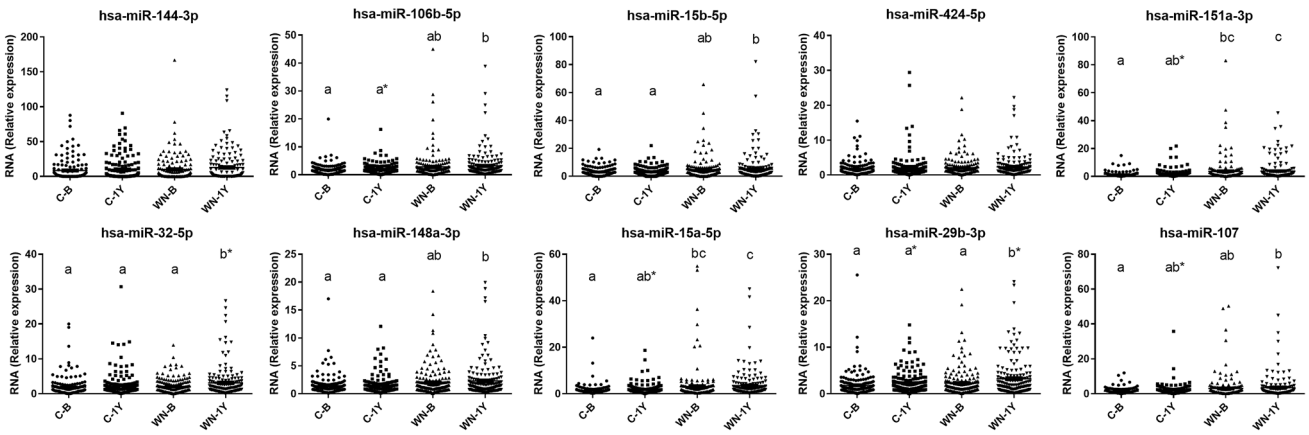

Fig. 2 Variation of c-miRNA expression of miRNAs candidates in each individual ($n = 333$) selected from screening. a–d indicate the levels that contain a significant difference at the 95.0% confidence level compared with others in the same row. In addition, *indicates statistically significant differences in the same intervention group (control or walnuts) between basal and after 1-year of intervention ($p < 0.05$) by paired *t*-test. *C-B* control individuals at baseline, *C-1Y* control individuals after 1 year of intervention. *WN B* Walnut group individuals at baseline, *WN 1Y* Walnut group individuals after 1 year of intervention

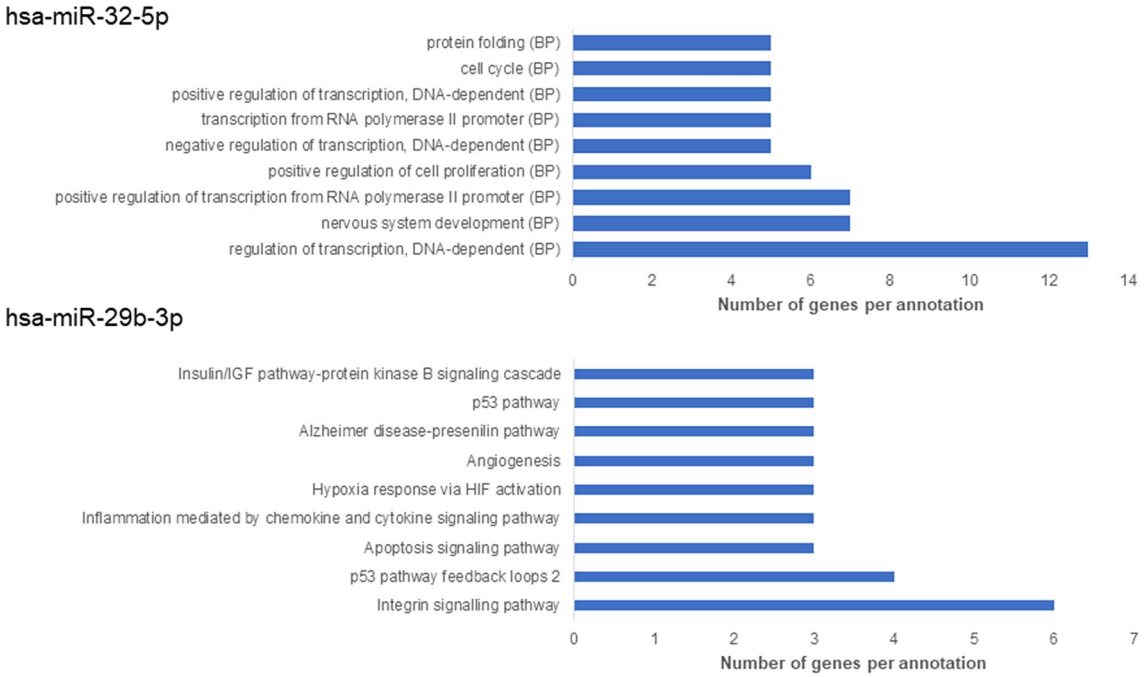

Fig. 3 Gene Ontology (GO) analysis of over-represented pathways for **a** hsa-miR-32-5p and **b** hsa-miR-29b-3p

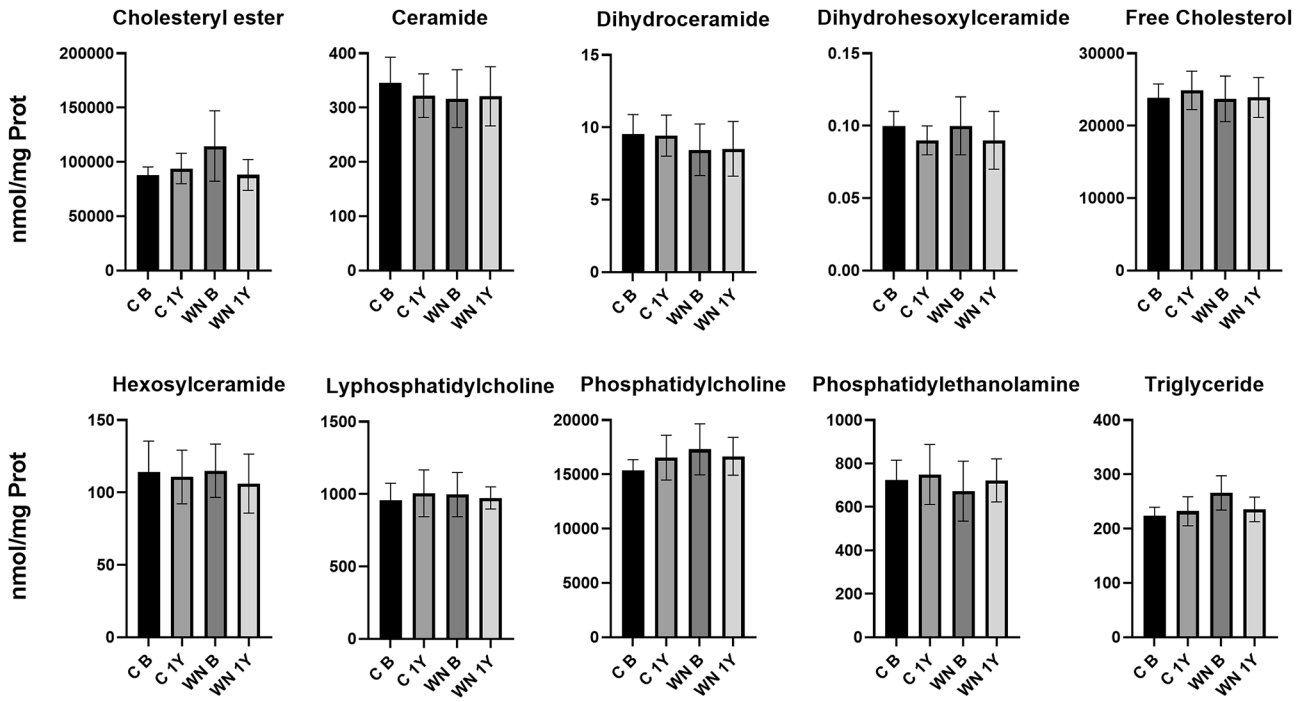

Fig. 4 Lipidomic composition of different families in exosomes from participants in the walnut group or control group. Exosomes were isolated from pooled samples ($n \geq 6$ subjects per pool). Levels of lipids classes containing different lipids as lateral chains. *C-B* control individuals at baseline, *C-1Y* control individuals after 1 year of intervention. *WN B* Walnut group individuals at baseline, *WN 1Y* Walnut group individuals after 1 year of intervention

No statistically significant differences were found for the major lipid families analyzed (Fig. 4), so we were not able to validate in the whole cohort.

Discussion

Previous experimental studies suggest that certain dietary polyphenols (i.e., curcumin) could promote exosome secretion in vitro [21] acting on signaling pathways that interfere with the biogenesis of EVs [22]. Whether this phenomenon can be observed upon long-term consumption of a certain food is unknown. We first evaluated whether consumption of walnuts, a food naturally rich in polyphenolic compounds, could influence the size and concentration of exosomes. Unfortunately, the supplementation level of nearly 15% of energy, walnuts or their polyphenols do not influence exosome characteristics (Fig. 2S-Online Resource and Table 3). Maybe, the lack of differences could be attributed to the use of pooled plasma samples, if very small changes are produced by interindividual differences they cannot be identified. In addition, we did not find differences in particle quantification between treatments (Fig. 1S). A recent comparison between NanoSight NS300 and ZetaView suggest that NTA devices differ strongly in their hardware and software, which influences measurements [44].

miRNAs are found in almost all biological fluids [15] and are mainly transported in EVs [45], lipoproteins [46] or associated to Ago2 proteins [47]. Exosomes as transporter of circulating miRNAs and their ability to cross different biological barriers, including the blood–brain barrier have special relevance [48]. Interestingly, no previous studies have evaluated the influence of walnut supplementation on c-miRNAs transported in exosomes. However, Ortega and colleagues [20] evaluated the profile of 192 common miRNAs in response to a 8-week trial with a normocaloric diet enriched in polyunsaturated fatty acids (PUFAs) (30 g/day of almonds and walnuts) in a smaller population sample ($n = 10$ in the screening phase and $n = 30$ in the full cohort). Interestingly, two miRNAs (hsa-miR-106 and hsa-miR-130) were commonly modulated (upregulated) in both studies; however, only miR-32-5p and miR-29b-3p were validated in the whole cohort ($n = 210$). The small number of validated miRNAs in our study might be influenced by the limited variety of miRNAs content in exosomes (compared to that of whole plasma), larger number of subjects analyzed, and its common in free-living population studies researching on biomarkers.

PUFAs have been also reported to modulate the expression of certain tissues miRNAs, both in vitro [49] and in vivo [50]. Walnuts are not only rich in PUFAs, but are also a source of the dietary polyphenols ellagitannins [23]. Increasing evidence suggest that polyphenols can modulate the expression of miRNAs [18]. Indeed, in vivo studies wherein ellagitannins or food containing ellagitannins were administered showed modulation of certain tissue miRNAs, both in animal models [51, 52] and humans [51]. Whether the changes observed are due to walnut PUFAs or polyphenols or due to bioactive synergy cannot be ascertained from our results and deserves further investigation.

Recently, the number of studies reporting the possibility that miRNAs may be transfer horizontally across species and kingdoms and induce gene silencing in trans, because they are deeply conserved over long evolutionary distances, has increased [53, 54]. However, to regulate the host gene expression dietary miRNAs (xenomiRs) should resist the gastrointestinal digestion process, cross the intestinal barrier, arrive at the appropriate target cell, and in the enough copy numbers to surpass the threshold required for a biological effect [55]. These difficulties support the hypothesis that this transference does not exist [56, 57]. By contrast, recent evidences suggest that exosomes from edible plants may protect their miRNA content during gastrointestinal digestion [58]. In addition, plant miRNAs are methylated on the 3′-nucleotide ribose which confer protection against hard conditions like cooking and gastrointestinal digestion [55]. Although in the present paper we did not evaluate the possible passage of walnuts miRNAs to the human circulation, we cannot discard this possibility and it deserves further investigation.

Regarding the target genes modulated by exosomal c-miRNAs affected by the dietary intervention, miR-32-5p targets are involved in the regulation of apoptosis [59], and their overexpression is associated with reduced apoptosis [60]. Nuts contain a plethora of components with recognized bioactivity and some of them (phenolic compounds, vitamin E, choline and arginine) are associated with increased neurogenesis [61]. miR-29b contributes to osteoblast differentiation [62] via regulating IGF-1 secretion and is responsive for the mechanical tensile strain [63]. Epidemiological studies suggest that the incidence of CVD and postmenopausal osteoporosis is low in the Mediterranean area [64]. As recently reviewed [65], dietary patterns based on fruit and vegetables, whole grains, poultry and fish, nuts and legumes, and low-fat dairy products are beneficial for bone health [65]. Thus, it is feasible that the consumption of nuts (i.e., walnuts) in the Mediterranean area may contribute to decrease osteoporosis and fracture risk through the upregulation of circulating miR-29b. Indeed, higher levels of miR-29b has been strongly associated to increased calcification [66] and osteogenic differentiation [67]. Whether these tissues directly contribute to the secretion of these miRNAs cannot be ascertained from our study and deserves further investigation. Indeed, walnut supplementation in animal models has been reported to exert protective effects on age-related neurodegenerative disorders via a reduction of oxidative stress [68, 69]. Other tissues might also contribute to the secretion of these miRNAs. For

example, walnut supplementation has been shown to induce the expression miR-29b in colorectal cancer model [70].

Exosomes and other EVs transport a myriad of molecules, including nucleic acids, proteins, lipids, and different metabolites [71], most of them can be employed as biomarkers of disease [72]. Regarding the exosomal lipidomic hallmark, after long-term dietary supplementation with walnuts none of the 126 species analyzed showed statistical difference between the 4 groups analyzed (Fig. 5S-Online Resource). Although a unique lipid signature has been found in EVs (i.e., exosomes) from different cell types [73] both in health and disease [74], highlighting their possible role as biomarkers of disease [72]. At this stage, we cannot ascertain whether this finding is either due to a real lack of effect of the tested walnut doses or to methodological issues, namely the limited amount of circulating exosomes compared to those derived from cell lines. Whether a higher dose of walnuts can modify the lipid composition of exosomes deserves further investigation.

Limitations and future perspectives

Although almost 180 highly expressed circulating miRNAs were analyzed during the screening phase, screening did not include other miRNAs reported to be transported in exosomes. The validation of c-miRNAs in the whole cohort was performed only for a reduced number of miRNA candidates ($n = 10$). The lipidomic and nanoparticle tracking analyses were performed in pooled samples, as discovery phase, which may mask a small effect on these parameters at an individual level. The reduced amount of plasma used for lipidomic analysis of exosomes is also a limiting factor for the individualized analysis. Despite these limitations, the effect of foods on the modulation of gene expression by epigenetic mechanisms is little known, thus our work contributes to this concept and opens up new perspectives for understanding the mechanisms of regulation. Further studies are needed, not only to validate more exosomal c-miRNAs, but also to better delineate their putative role in mediating the benefits ascribed to sustained walnut consumption.

Conclusions

In summary, we provide novel insights into the biology underlying the effect of walnut consumption on the concentration, size, miRNA, and lipidomic content of exosomes. Our results show that long-term (1 year) consumption of walnuts, a food rich in PUFA and polyphenols, affects the signature of miRNAs transported in circulating exosomes. Modulation of miRNAs by dietary factors provides adjuvant to the ongoing therapy against miRNA function. Moreover, the use of miRNAs in circulating exosomes provides potentially useful biomarkers to predict dietary effects in different tissues, including those protected by the blood–brain barrier. Future studies of the tissue of origin and fate of the secreted exosomes containing miRNAs and whether changes in miRNAs correspond to transcriptome changes of specific pathways connecting disease outcome are clearly needed. Our results provide additional evidence on the potential mechanism of the beneficial effects of walnut consumption.

Acknowledgements We thank the volunteers who participated in this study, the Hector Peinado Lab (Madrid) for the use of NanoSight equipment and the Solmeglas S. L. company (Madrid) for the use of ZetaView® equipment. CIBEROBN is an initiative of Instituto de Salud Carlos III, Spain. We thank the Quantification and Molecular Characterization Unit (IRYCIS) for their technical help.

Author contributions JS, SR, ER, AS-V and AD designed the study. MC, MS-M, IR, T-MF-S, MD, CV-P recruited and obtained the samples. MCLH, JG-Z, DME, LDP, AGR and AD, performed miRNA analysis. MCLH, CM, and MYM contributed to the NTA analysis, MCLH, JG-Z and DCME analyzed data. OP perform the lipidomic analysis. MCLH and DCME performed bioinformatic analysis. MCLH, ER, AS-V and AD wrote the manuscript. All authors reviewed and accepted the manuscript.

Funding This research was supported in part by a grant from the California Walnut Commission, Folsom, CA, USA. The funding agency had no involvement in any stage of the study design, research or writing of the manuscript. The work is also supported by Fundación Ramón Areces (CIVP18A3888) Madrid, Spain; the Instituto de Salud Carlos III-Fondo de Investigación Sanitaria–Fondo Europeo de Desarrollo Regional (grant PI15/01014 and PI18/01152), and the Spanish Agencia Estatal de Investigación and European Feder Funds (AGL2016-78922-R, PID2019-109369RB-I00, RTI2018-093873-A-I00 and BIO2017-86500-R). AS-V is recipient of the Instituto de Salud Carlos III Miguel Servet II fellowship (grant CP II 17/00029). MCLH and LdP were supported by a postdoctoral research contract funded by the community of Madrid and European Union (PEJD-2016/BIO-2781 and PEJD-2017-PRE/BIO-5100, respectively). D.C.M.-E. is a fellow of "Centro de Estudios Interdisciplinarios Básicos y Aplicados" (CEIBA), Colombia, through the program "Bolívar Gana con Ciencia". Also, A.G.-R. acknowledges the Marie Curie AMAROUT-II Europe Program (Grant Agreement No. 291803).

Compliance with ethical standards

Conflict of interest AS-V, SR, JS, and ER have received research funding through their institutions from the California Walnut Commission, Folsom, CA, USA. JS and ER were nonpaid members of California Walnut Commission Scientific Advisory Council. ER was a paid member of the California Walnut Commission Health Research Advisory Group. AS-V has received support from California Walnut Commission to attend professional meetings. All the other authors declare no competing financial interests.

References

1. Hayes D, Angove MJ, Tucci J, Dennis C (2016) Walnuts (*Juglans regia*) chemical composition and research in human

health. Crit Rev Food Sci Nutr 56:1231–1241. https://doi.org/10.1080/10408398.2012.760516
2. Li L, Tsao R, Yang R et al (2007) Fatty acid profiles, tocopherol contents, and antioxidant activities of heartnut (*Juglans ailanthifolia* Var. *cordiformis*) and Persian walnut (*Juglans regia* L.). J Agric Food Chem 55:1164–1169. https://doi.org/10.1021/jf062322d
3. Martínez ML, Mattea MA, Maestri DM (2006) Varietal and crop year effects on lipid composition of walnut (*Juglans regia*) genotypes. J Am Oil Chem Soc 83:791–796. https://doi.org/10.1007/s11746-006-5016-z
4. EFSA (2011) Scientific Opinion on the substantiation of health claims related to walnuts and maintenance of normal blood LDL-cholesterol concentrations (ID 1156, 1158) and improvement of endothelium-dependent vasodilation (ID 1155, 1157) pursuant to Article 13(1) of. EFSA J 9:2074. https://doi.org/10.2903/j.efsa.2011.2074
5. Nutrition C for FS and a constituent updates—FDA completes review of qualified health claim petition for oleic acid and the risk of coronary heart disease. https://www.fda.gov/food/cfsan-constituent-updates/fda-completes-review-qualified-health-claim-petition-oleic-acid-and-risk-coronary-heartdisease
6. Ros E (2015) Nuts and CVD. Br J Nutr 113:S111–S120. https://doi.org/10.1017/S0007114514003924
7. Zibaeenezhad MJ, Farhadi P, Attar A et al (2017) Effects of walnut oil on lipid profiles in hyperlipidemic type 2 diabetic patients: a randomized, double-blind, placebo-controlled trial. Nutr Diabetes 7:e259. https://doi.org/10.1038/nutd.2017.8
8. Arab L, Ang A (2015) A cross sectional study of the association between walnut consumption and cognitive function among adult us populations represented in NHANES. J Nutr Health Aging 19:284–290. https://doi.org/10.1007/s12603-014-0569-2
9. Toner CD (2014) Communicating clinical research to reduce cancer risk through diet: Walnuts as a case example. Nutr Res Pract 8:347–351. https://doi.org/10.4162/nrp.2014.8.4.347
10. Del Gobbo LC, Falk MC, Feldman R et al (2015) Effects of tree nuts on blood lipids, apolipoproteins, and blood pressure: systematic review, meta-analysis, and dose-response of 61 controlled intervention trials. Am J Clin Nutr 102:1347–1356. https://doi.org/10.3945/ajcn.115.110965
11. Barile L, Vassalli G (2017) Exosomes: therapy delivery tools and biomarkers of diseases. Pharmacol Ther 174:63–78. https://doi.org/10.1016/j.pharmthera.2017.02.020
12. Yáñez-Mó M, Siljander PR-M, Andreu Z et al (2015) Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 4:27066. https://doi.org/10.3402/jev.v4.27066
13. Skotland T, Hessvik NP, Sandvig K, Llorente A (2019) Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology. J Lipid Res 60:9–18. https://doi.org/10.1194/jlr.R084343
14. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
15. Weber JA, Baxter DH, Zhang S et al (2010) The MicroRNA spectrum in 12 body fluids. Clin Chem 56:1733–1741. https://doi.org/10.1373/clinchem.2010.147405
16. Gerhauser C (2018) Impact of dietary gut microbial metabolites on the epigenome. Philos Trans R Soc Lond B Biol Sci. https://doi.org/10.1098/rstb.2017.0359
17. Li R, Ibeagha-Awemu EM (2017) Altered gene expression of epigenetic modifying enzymes in response to dietary supplementation with linseed oil. J Dairy Res 84:119–123. https://doi.org/10.1017/S002202991700022X
18. Tomé-Carneiro J, Crespo MC, Iglesias-Gutierrez E et al (2016) Hydroxytyrosol supplementation modulates the expression of miRNAs in rodents and in humans. J Nutr Biochem 34:146–155. https://doi.org/10.1016/j.jnutbio.2016.05.009
19. Aganzo M, Montojo M-T, de Las L, Hazas M-C et al (2018) Customized dietary intervention avoids unintentional weight loss and modulates circulating miRNAs footprint in huntington's disease. Mol Nutr Food Res 62:1800619. https://doi.org/10.1002/mnfr.201800619
20. Ortega FJ, Cardona-Alvarado MI, Mercader JM et al (2015) Circulating profiling reveals the effect of a polyunsaturated fatty acid-enriched diet on common microRNAs. J Nutr Biochem 26:1095–1101. https://doi.org/10.1016/j.jnutbio.2015.05.001
21. Canfrán-Duque A, Pastor Ó, Quintana-Portillo R et al (2014) Curcumin promotes exosomes/microvesicles secretion that attenuates lysosomal cholesterol traffic impairment. Mol Nutr Food Res 58:687–697. https://doi.org/10.1002/mnfr.201300350
22. Soleti R, Andriantsitohaina R, Martinez MC (2018) Impact of polyphenols on extracellular vesicle levels and effects and their properties as tools for drug delivery for nutrition and health. Arch Biochem Biophys 644:57–63. https://doi.org/10.1016/j.abb.2018.03.004
23. González-Sarrías A, Giménez-Bastida JA, García-Conesa MT et al (2010) Occurrence of urolithins, gut microbiota ellagic acid metabolites and proliferation markers expression response in the human prostate gland upon consumption of walnuts and pomegranate juice. Mol Nutr Food Res 54:311–322. https://doi.org/10.1002/mnfr.200900152
24. Gurha P (2016) MicroRNAs in cardiovascular disease. Curr Opin Cardiol 31:249–254. https://doi.org/10.1097/HCO.0000000000000280
25. Rajaram S, Valls-Pedret C, Cofán M et al (2016) The walnuts and healthy aging study (WAHA): protocol for a nutritional intervention trial with walnuts on brain aging. Front Aging Neurosci 8:333. https://doi.org/10.3389/fnagi.2016.00333
26. Sala-Vila A, Valls-Pedret C, Rajaram S et al (2020) Effect of a 2-year diet intervention with walnuts on cognitive decline. The walnuts and healthy aging (WAHA) study: a randomized controlled trial. Am J Clin Nutr 111:590–600. https://doi.org/10.1093/ajcn/nqz328
27. Molina L, Sarmiento M, Peñafiel J et al (2017) Validation of the regicor short physical activity questionnaire for the adult population. PLoS ONE 12:e0168148. https://doi.org/10.1371/journal.pone.0168148
28. Joint FAO/WHO/UNU expert consultation on energy and protein requirements (1981 : Rome I (1985) energy and protein requirements : report of a joint FAO/WHO/UNU expert consultation. World Health Organization
29. Sodi R, Eastwood J, Caslake M et al (2017) Relationship between circulating microRNA-30c with total- and LDL-cholesterol, their circulatory transportation and effect of statins. Clin Chim Acta 466:13–19. https://doi.org/10.1016/j.cca.2016.12.031
30. Hoffmann J, Günther J, Stecher L et al (2019) Effects of a lifestyle intervention in routine care on short- and long-term maternal weight retention and breastfeeding behavior-12 months follow-up of the cluster-randomized gelis trial. J Clin Med 8:876. https://doi.org/10.3390/jcm8060876
31. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
32. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509
33. Pastor Ó, Guzmán-Lafuente P, Serna J et al (2019) A comprehensive evaluation of omega-3 fatty acid supplementation in cystic fibrosis patients using lipidomics. J Nutr Biochem 63:197–205. https://doi.org/10.1016/j.jnutbio.2018.09.026

34. Gardner MS, McWilliams LG, Jones JI et al (2017) Simultaneous quantification of free cholesterol, cholesteryl esters, and triglycerides without ester hydrolysis by UHPLC separation and in-source collision induced dissociation coupled MS/MS. J Am Soc Mass Spectrom 28:2319–2329. https://doi.org/10.1007/s13361-017-1756-2
35. Liebisch G, Vizcaíno JA, Köfeler H et al (2013) Shorthand notation for lipid structures derived from mass spectrometry. J Lipid Res 54:1523–1530. https://doi.org/10.1194/jlr.M033506
36. Sticht C, De La Torre C, Parveen A, Gretz N (2018) miRWalk: an online resource for prediction of microRNA binding sites. PLoS ONE 13:e0206239. https://doi.org/10.1371/journal.pone.0206239
37. Carmona-Saez P, Chagoyen M, Tirado F et al (2007) GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. Genome Biol 8:R3. https://doi.org/10.1186/gb-2007-8-1-r3
38. Nogales-Cadenas R, Carmona-Saez P, Vazquez M et al (2009) GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. Nucleic Acids Res 37:W317–W322. https://doi.org/10.1093/nar/gkp416
39. Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A (2012) GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics. Nucleic Acids Res 40:W478–W483. https://doi.org/10.1093/nar/gks402
40. Ludwig N, Leidinger P, Becker K et al (2016) Distribution of miRNA expression across human tissues. Nucleic Acids Res 44:3865–3877. https://doi.org/10.1093/nar/gkw116
41. Ioannidis JPA (2008) Why most discovered true associations are inflated. Epidemiology 19:640–648. https://doi.org/10.1097/EDE.0b013e31818131e7
42. Nair VS, Pritchard CC, Tewari M, Ioannidis JPA (2014) Design and analysis for studying microRNAs in human disease: a primer on-omic technologies. Am J Epidemiol 180:140–152
43. Kalra H, Simpson RJ, Ji H et al (2012) Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol 10:e1001450. https://doi.org/10.1371/journal.pbio.1001450
44. Bachurski D, Schuldner M, Nguyen P-H et al (2019) Extracellular vesicle measurements with nanoparticle tracking analysis—an accuracy and repeatability comparison between NanoSight NS300 and ZetaView. J Extracell Vesicles 8:1596016. https://doi.org/10.1080/20013078.2019.1596016
45. Valadi H, Ekström K, Bossios A et al (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654–659. https://doi.org/10.1038/ncb1596
46. Vickers KC, Palmisano BT, Shoucri BM et al (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 13:423–433. https://doi.org/10.1038/ncb2210
47. Arroyo JD, Chevillet JR, Kroh EM et al (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 108:5003–5008. https://doi.org/10.1073/pnas.1019055108
48. García-Romero N, Carrión-Navarro J, Esteban-Rubio S et al (2017) DNA sequences within glioma-derived extracellular vesicles can cross the intact blood-brain barrier and be detected in peripheral blood of patients. Oncotarget 8:1416–1428. https://doi.org/10.18632/oncotarget.13635
49. Gil-zamorano J, Martin R, Daimiel L et al (2014) Docosahexaenoic acid modulates the enterocyte caco-2 cell expression of MicroRNAs involved in lipid metabolism. J Nutr 1(3):575–585. https://doi.org/10.3945/jn.113.189050.575
50. Casas-Agustench P, Fernandes FS, Tavares do Carmo MG, et al (2015) Consumption of distinct dietary lipids during early pregnancy differentially modulates the expression of micrornas in mothers and offspring. PLoS ONE 10:e0117858. https://doi.org/10.1371/journal.pone.0117858
51. Nuñez-Sánchez MA, Dávalos A, González-Sarrías A et al (2015) MicroRNAs expression in normal and malignant colon tissues as biomarkers of colorectal cancer and in response to pomegranate extracts consumption: critical issues to discern between modulatory effects and potential artefacts. Mol Nutr Food Res 59:1973–1986. https://doi.org/10.1002/mnfr.201500357
52. Kim H, Banerjee N, Sirven MA et al (2017) Pomegranate polyphenolics reduce inflammation and ulceration in intestinal colitis—involvement of the miR-145/p70S6K1/HIF1α axis in vivo and in vitro. J Nutr Biochem 43:107–115. https://doi.org/10.1016/j.jnutbio.2017.02.005
53. Cai Q, He B, Weiberg A et al (2019) Small RNAs and extracellular vesicles: new mechanisms of cross-species communication and innovative tools for disease control. PLOS Pathog 15:e1008090. https://doi.org/10.1371/journal.ppat.1008090
54. Zhang L, Hou D, Chen X et al (2012) Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res 22:107–126. https://doi.org/10.1038/cr.2011.158
55. Dávalos A, Henriques R, Latasa MJ et al (2019) Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. EFSA Support Publ. https://doi.org/10.2903/sp.efsa.2019.en-1688
56. Liang H, Zhang S, Fu Z et al (2015) Effective detection and quantification of dietetically absorbed plant microRNAs in human plasma. J Nutr Biochem 26:505–512. https://doi.org/10.1016/J.JNUTBIO.2014.12.002
57. Dickinson B, Zhang Y, Petrick JS et al (2013) Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat Biotechnol 31:965–967
58. Xiao J, Feng S, Wang X et al (2018) Identification of exosome-like nanoparticle-derived microRNAs from 11 edible fruits and vegetables. PeerJ. https://doi.org/10.7717/peerj.5186
59. Köpke S, Buhrke T, Lampen A (2015) miRNA expression in human intestinal caco-2 cells is comparably regulated by *cis* - and *trans* -fatty acids. Lipids 50:227–239. https://doi.org/10.1007/s11745-015-3988-x
60. Wu W, Yang J, Feng X et al (2013) MicroRNA-32 (miR-32) regulates phosphatase and tensin homologue (PTEN) expression and promotes growth, migration, and invasion in colorectal carcinoma cells. Mol Cancer 12:30. https://doi.org/10.1186/1476-4598-12-30
61. Gorji N, Moeini R, Memariani Z (2018) Almond, hazelnut and walnut, three nuts for neuroprotection in Alzheimer's disease: a neuropharmacological review of their bioactive constituents. Pharmacol Res 129:115–127. https://doi.org/10.1016/j.phrs.2017.12.003
62. Li Z, Hassan MQ, Jafferji M et al (2009) Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem 284:15676–15684. https://doi.org/10.1074/jbc.M809787200
63. Zeng Q, Wang Y, Gao J et al (2019) miR-29b-3p regulated osteoblast differentiation via regulating IGF-1 secretion of mechanically stimulated osteocytes. Cell Mol Biol Lett 24:11. https://doi.org/10.1186/s11658-019-0136-2
64. Papoutsi Z, Kassi E, Chinou I et al (2008) Walnut extract (*Juglans regia* L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483. Br J Nutr 99:715–722. https://doi.org/10.1017/S0007114507837421
65. Movassagh EZ, Vatanparast H (2017) Current evidence on the association of dietary patterns and bone health: a scoping review. Adv Nutr An Int Rev J 8(1):2–16. https://doi.org/10.3945/an.116.013326

66. Jiang W, Zhang Z, Yang H et al (2017) The involvement of miR-29b-3p in arterial calcification by targeting matrix metalloproteinase-2. Biomed Res Int 2017:6713606. https://doi.org/10.1155/2017/6713606
67. Trompeter H-I, Dreesen J, Hermann E et al (2013) MicroRNAs miR-26a, miR-26b, and miR-29b accelerate osteogenic differentiation of unrestricted somatic stem cells from human cord blood. BMC Genomics 14:111. https://doi.org/10.1186/1471-2164-14-111
68. Haider S, Batool Z, Ahmad S et al (2018) Walnut supplementation reverses the scopolamine-induced memory impairment by restoration of cholinergic function via mitigating oxidative stress in rats: a potential therapeutic intervention for age related neurodegenerative disorders. Metab Brain Dis 33:39–51. https://doi.org/10.1007/s11011-017-0120-3
69. Hicyilmaz H, Vural H, Delibas N et al (2017) The effects of walnut supplementation on hippocampal NMDA receptor subunits NR2A and NR2B of rats. Nutr Neurosci 20:203–208. https://doi.org/10.1179/1476830514Y.0000000166
70. Tsoukas MA, Ko B-J, Witte TR et al (2015) Dietary walnut suppression of colorectal cancer in mice: mediation by miRNA patterns and fatty acid incorporation. J Nutr Biochem 26:776–783. https://doi.org/10.1016/j.jnutbio.2015.02.009
71. Pathan M, Fonseka P, Chitti SV et al (2019) Vesiclepedia 2019: a compendium of RNA, proteins, lipids and metabolites in extracellular vesicles. Nucleic Acids Res 47:D516–D519. https://doi.org/10.1093/nar/gky1029
72. Skotland T, Ekroos K, Kauhanen D et al (2017) Molecular lipid species in urinary exosomes as potential prostate cancer biomarkers. Eur J Cancer 70:122–132. https://doi.org/10.1016/j.ejca.2016.10.011
73. Haraszti RA, Didiot M-C, Sapp E et al (2016) High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. J Extracell Vesicles 5:32570. https://doi.org/10.3402/jev.v5.32570
74. Hough KP, Wilson LS, Trevor JL et al (2018) Unique lipid signatures of extracellular vesicles from the airways of asthmatics. Sci Rep 8:10340. https://doi.org/10.1038/s41598-018-28655-9

Affiliations

María-Carmen López de las Hazas[1] · Judit Gil-Zamorano[1] · Montserrat Cofán[2,3] · Diana C. Mantilla-Escalante[1] · Almudena Garcia-Ruiz[1] · Lorena del Pozo-Acebo[1] · Oscar Pastor[3,4] · María Yañez-Mo[5,6] · Carla Mazzeo[5,6] · Mercè Serra-Mir[2] · Monica Doménech[2] · Cinta Valls-Pedret[2] · Sujatha Rajaram[7] · Joan Sabaté[7] · Emilio Ros[2,3] · Aleix Sala-Vila[8,9] · Alberto Dávalos[1]

1 Laboratory of Epigenetics of Lipid Metabolism, Instituto Madrileño de Estudios Avanzados (IMDEA)-Alimentación, IMDEA Food Institute, CEI UAM+CSIC, Ctra. De Cantoblanco 8, 28049 Madrid, Spain

2 Lipid Clinic, Endocrinology and Nutrition Service, Institut d´Investigacions Biomèdiques August Pi i Sunyer, 08036 Barcelona, Spain

3 CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), 28029 Madrid, Spain

4 Servicio de Bioquímica Clínica (UCA-CCM), Hospital Ramón y Cajal-IRYCIS, 28034 Madrid, Spain

5 Department of Molecular Biology, UAM, 28049 Madrid, Spain

6 Centro de Biología Molecular Severo Ochoa (CBM-SO), Instituto de Investigación Sanitaria Princesa (IIS-IP), 28049 Madrid, Spain

7 Center for Nutrition, Healthy Lifestyle and Disease Prevention, School of Public Health, Loma Linda University, Loma Linda, CA 92350, USA

8 Barcelonaβeta Brain Research Center (BBRC), Pasqual Maragall Foundation, Barcelona 08003, Spain

9 Hospital del Mar Medical Research Institute, IMIM, Dr. Aiguader 88, 08003 Barcelona, Spain