

Impact of Phenol-Enriched Virgin Olive Oils on the Postprandial Levels of Circulating microRNAs Related to Cardiovascular Disease

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Scope: We investigate the postprandial modulation of cardiovascular-related microRNAs elicited by extra virgin olive oil (EVOOs) containing different levels of their own polyphenols.

Methods and results: It is randomized, postprandial, parallel, double-blind study. Twelve healthy participants consumed 30 mL of EVOO containing low (L-EVOO; 250 mg total phenols kg⁻¹ of oil), medium (M-EVOO; 500 mg total phenols kg⁻¹ of oil), and high (H-EVOO; 750 mg total phenols kg⁻¹ of oil) enriched EVOOs. Postprandial plasma microRNAs levels are analyzed by real-time quantitative PCR. The results show that L-EVOO intake is associated with decreased let-7e-5p and miR-328a-3p levels and increased miR-17-5p and miR-20a-5p, concentrations. M-EVOO decreases plasma let-7e-5p and increases miR-17-5p, miR-20a-5p, and miR-192-5p levels. Finally, H-EVOO decreases let-7e-5p, miR-10a-5p, miR-21-5p, and miR-26b-5p levels.

Conclusion: During the postprandial state, the levels of let-7e-5p decrease with EVOO regardless of polyphenol content suggesting a general response to the fatty acid composition of EVOO or/and the presence of at least 250 mg polyphenol kg⁻¹ olive oil. Moreover, the miR-17-92 cluster increases by low and medium polyphenol content suggesting a role in fatty acid metabolism and nutrient sensing. Thus, postprandial modulation of circulating microRNAs levels could be a potential mechanism for the cardiovascular benefits associated with EVOO intake.

1. Introduction

Extra virgin olive oil (EVOO) is a cornerstone of the Mediterranean diet (MD), and its health properties were initially attributed to its fatty acid composition and later to its unique mix of minor components (i.e., polyphenols).^[1,2] Observational studies, such as The Three-City Study, found an association between olive oil consumption and a lower incidence of stroke in older subjects.^[3] This was also the outcome of the randomized controlled trial Prevención con Dieta Mediterránea (PREDIMED) Study that showed a lower incidence of cardiovascular disease (CVD) in the group following the traditional MD supplemented with EVOO in comparison with the low-fat diet control group.^[4]


Oleic acid has been associated with an increase in HDL-cholesterol levels and a decrease in LDL oxidation.^[5,6] Additionally, some EVOO polyphenols have been suggested to function as chemoprotective agents.^[7] Hydroxytyrosol, the most

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representative polyphenol of EVOO, is a phenolic alcohol that functions as a scavenger of free reactive oxygen species and has antioxidant properties.^[8] Moreover, EVOO also has anti-inflammatory, antiplatelet, anti-atherogenic,^[9] anti-tumor,^[10] and neuroprotective properties.^[11]

Despite numerous reports describing the beneficial effect of EVOO consumption, knowledge about the molecular mechanisms explaining such properties is still sparse. Most researchers have focused on how EVOO modulates gene expression in cell lines and murine models^[12] with less information being available in humans. One of such studies is the Virgin Olive Oil and HDL Functionality (VOHF) Study.^[13] This is a randomized, double-blind, crossover, controlled trial that compared the effect of three different levels of endogenous polyphenols using the same EVOO on HDL-cholesterol changes in hypercholesterolemic patients.^[6] Following a nutrigenomics approach, other interventional studies with olive oil have reported changes in the expression of genes related to inflammation, oxidative stress, lipid metabolism, cellular apoptosis, and DNA damage.^[14,15] However, the influence of three different olive oil endogenous polyphenol concentrations on microRNAs is unknown. MicroRNAs are non-coding RNA sequences of 22 to 24 nucleotides whose primary function is to “fine-tune” gene expression and are critical regulators of many cellular processes. MicroRNAs have been associated with many diseases such as cancer, CVD, diabetes, and neurological disorders.^[16,17] Interestingly, microRNAs can be found in circulation,^[18] and they could serve as disease or nutritional status biomarkers.^[19] Several studies in cell and animal models have shown that dietary compounds can modulate the levels of microRNAs.^[20,21] Specifically in a murine model, it was found that enriched polyphenol EVOO intake modified the expression of brain microRNAs related to the age-related decline of motor coordination and contextual memory.^[22] However, although a few studies have investigated the modulation of microRNAs by dietary factors in humans,^[23,24] no studies have reported on the postprandial modulation of circulating microRNAs by EVOO intake.

The objective of this research was to examine postprandial changes in circulating microRNAs levels after acute intake of three phenol enriched EVOOs containing different concentrations of polyphenols from olive oil in healthy volunteers and to identify metabolic pathways targeted by modified microRNAs.

2. Results

2.1. Postprandial Modification of Plasma microRNA Levels with Functional Olive Oils

Four plasma microRNAs were significantly modulated by L-EVOO (Figure 1A). Let-7e-5p showed a bimodal distribution with two peaks of repression at 1 Fold change abbreviation to be added (FC = -2.147 ± 0.473 , $p = 0.007$) and 4 (FC = -1.879 ± 0.637 , $p =$ non significant) h. miR-17-5p and miR-20a-5p were upregulated after consumption of L-EVOO along the whole postprandial curve with a maximum at 4 (FC = 0.724 ± 0.247 , $p = 0.031$) h and 1 (FC = 0.615 ± 0.162 , $p = 0.006$) and 2 (FC = 0.724 ± 0.205 , $p = 0.007$) h, respectively. On the other hand, miR-328a-3p was downregulated after L-EVOO intake with a minimum of expression at 1h (FC = -1.179 ± 0.172 , $p = 0.006$).

Three microRNAs were modulated by M-EVOO (Figure 2A). Let-7e-5p showed a similar pattern than with L-EVOO ($p = 0.025$), although these results were not significant after Bonferroni correction in intra-subject comparisons. The miR-17-5p linear trend was not significantly modified, although we observed a significant overexpression at 6 h when comparing with 2 h (FC = -0.2 , $p = 0.05$). Finally, miR-192-5p was significantly upregulated by the M-EVOO. miR-328a-3p and miR-20a-5p showed the same trend observed with L-EVOO, without reaching statistical significance (Figure S1, Supporting Information).

Four microRNAs were significantly modified by H-EVOO (Figure 3); all of them were downregulated after 2 h of the H-EVOO ingestion. We also observed a bimodal distribution of postprandial circulating let-7e-5p levels with a minimum at 2 h (FC = -1.181 , $p = 0.02$).

2.2. In Silico Functional Analyses

We selected the genes that are predicted and validated targets of at least 2/4 microRNAs in L-EVOO, 2/3 mRNAs in M-EVOO, and 2/4 mRNAs in H-EVOO.

Functional analyses showed that 1361 genes were targets of microRNAs modulated by L-EVOO, 1076 of them were targets of miR-17-5p and miR-20a-5p, which belong to the same cluster (Figure 1B). Targeted genes were mainly linked to different types of cancer and related processes (Table S3, Supporting Information). Among the enriched pathways, we also observed mTOR, AMPK, FOXO, and insulin signaling pathways as significantly modulated by miR-17-5p and miR-20a-5p and fatty acid biosynthesis as significantly modulated by let-7e-5p (Figure 1C). Fourteen genes were predicted or validated targets of all those miRNAs (Figure 1B and Table S4, Supporting Information). Interestingly, 30 genes were predicted or validated targets of let-7e-5p and miR-328a-3p, both downregulated at 1 h (Figure 1B and Table S4, Supporting Information). Three hundred eighty-one genes were validated or predicted targets of microRNAs modulated by M-EVOO (Figure 2B). These microRNAs were involved in different types of cancer and related processes (Figure 2C and Table S3, Supporting Information). Interestingly, they were also involved in fatty acid biosynthesis (let-7e-5p), FOXO and PI3K/AKT signaling (let-7e-5p and miR-17-5p), AMPK and mTOR signaling (miR-17-5p), and steroid and folate biosynthesis (miR-192-5p) (Figure 2C). Twenty-five of these

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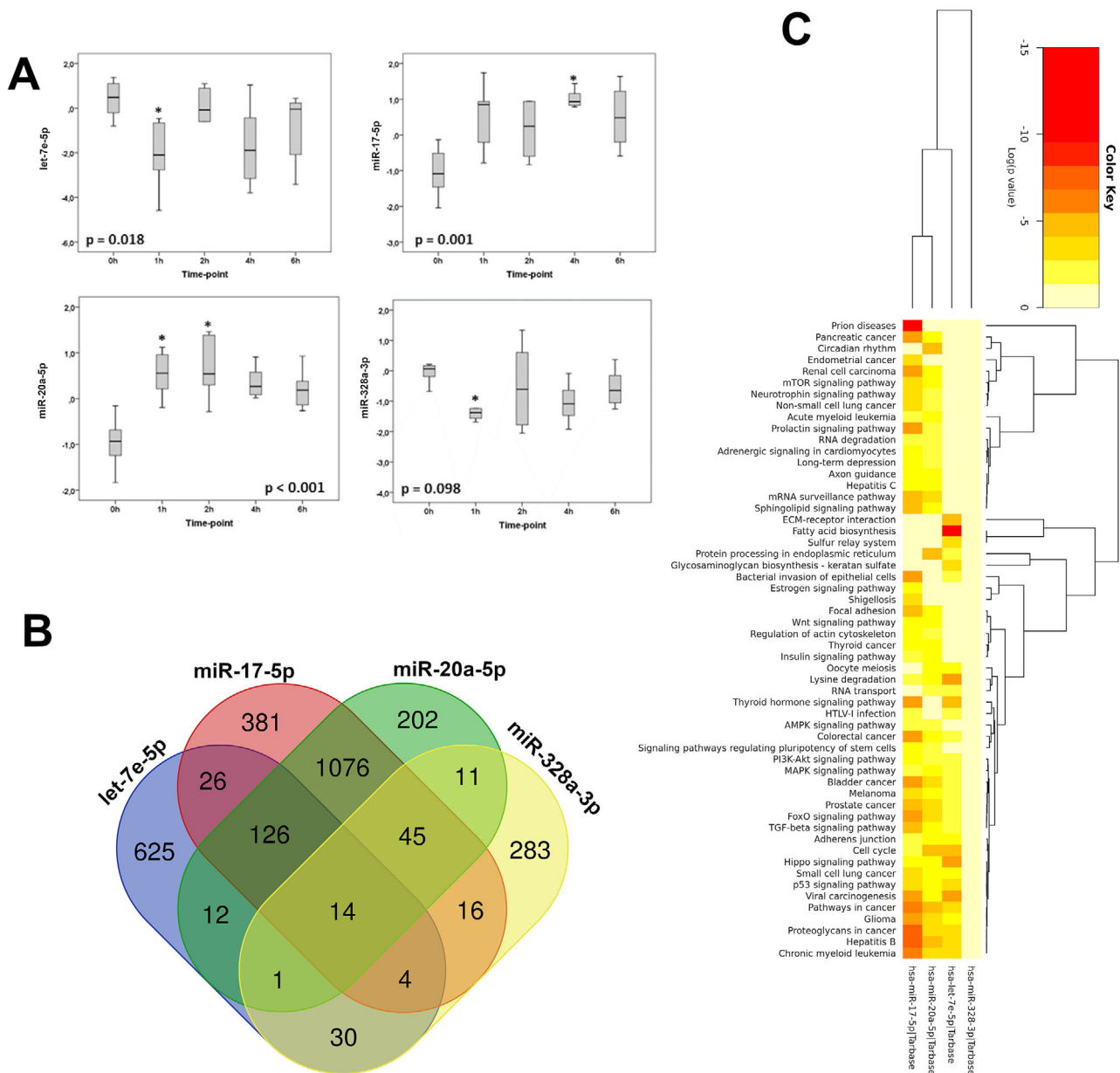


Figure 1. microRNAs modulated by L-EVOO. A) Box plots showing Log₂ transformed relative quantification levels of indicated microRNAs along the postprandial phase ($n = 8$). p -value refers to the intra-subject's comparison of the paired-repeated measures ANOVA with Bonferroni correction. * $p < 0.05$. B) Venn diagram showing common targets of the modulated microRNAs. C) Heatmap plot of significant KEGG pathways (Log₁₀).

genes were predicted targets for all three microRNAs (Figure 2B and Table S5, Supporting Information).

Four hundred fifty-four genes were validated or predicted targets of microRNAs modulated by H-EVOO (Figure 3B). These microRNAs were involved in different types of cancer and related pathways. However, they were also involved in fatty acid metabolism: fatty acid biosynthesis (let-7e-5p and miR-10a-5p), synthesis of unsaturated fatty acids (miR-21-5p and miR-26b-5p), and fatty acid elongation (miR-21-5p) (Figure 3C and Table S3, Supporting Information). YOD1 gene was the only predicted

target of all four microRNAs. YOD1 is a protein deubiquitinase involved in many cellular processes, including cell cycle, signal transduction, inflammation, and synthesis of unsaturated fatty acids.^[25]

2.3. microRNA Levels According to the Type of Functional Oil

Next, we performed interaction analyses to compare microRNA levels across the functional olive oils under investigation

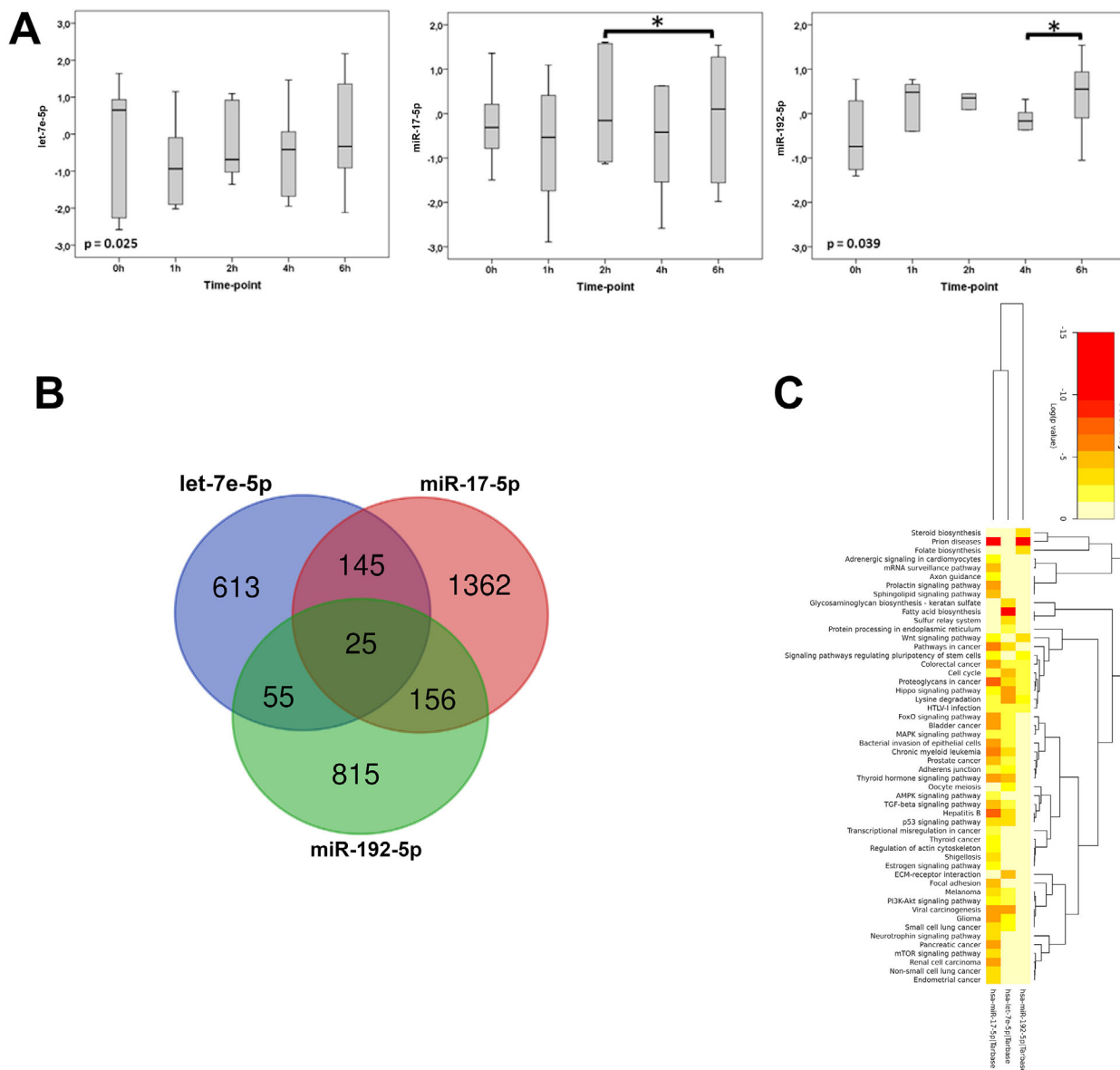


Figure 2. microRNAs modulated by M-EVOO. A) Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the postprandial phase ($n = 11$). p -value refers to the intra-subject's comparison of the paired-repeated measures ANOVA with Bonferroni correction. * $p < 0.05$. B) Venn diagram showing common targets of the modulated microRNAs. C) Heatmap plot of significant KEGG pathways (Log_{10}).

(Figure 4A). Interestingly, let-7e-5p showed the same bimodal response to all functional oils, although the trend was delayed for 1 h with the H-EVOO. Interaction analyses for miR-126-3p, miR-24-3p, and miR-328a-3p levels were not significant. However, it is worth mentioning that circulating levels of these microRNAs with L-EVOO and M-EVOO showed a more similar trend between them than with H-EVOO. The interaction between circulating miR-26b-5p levels and the type of functional oil was significant ($p = 0.043$), with a similar pattern found with low and medium EVOO than with H-EVOO. More specifically, miR-26b-5p circulating levels barely changed along the postprandial phase with L-EVOO and M-EVOO, while they were significantly downregulated after 2 h of the ingestion of H-EVOO. H-EVOO and M-EVOO modified circulating levels of miR-192-

5p oppositely. While M-EVOO leads to a postprandial increase, H-EVOO resulted in a postprandial decrease of circulating miR-192-5p (FC (2 h) ≈ 2.0 ; $p = 0.041$). Finally, miR-20a-5p plasma levels were similarly modified by L-EVOO and M-EVOO. However, the early increase observed was more pronounced with L-EVOO, while the late decrease was more pronounced with M-EVOO. After 6 h, levels had come back to basal levels in the two cases.

We then performed an in silico analysis to define which genes are frequent targets of microRNAs modulated by the three functional oils (Figure 4B). Forty-four genes were predicted and validated targets of microRNAs modified by all three oils. Those genes were involved in different types of cancers, as well as in AMPK, MAPK, and FOXO signaling and the circadian system (Figure S2 and Table S6, Supporting Information).

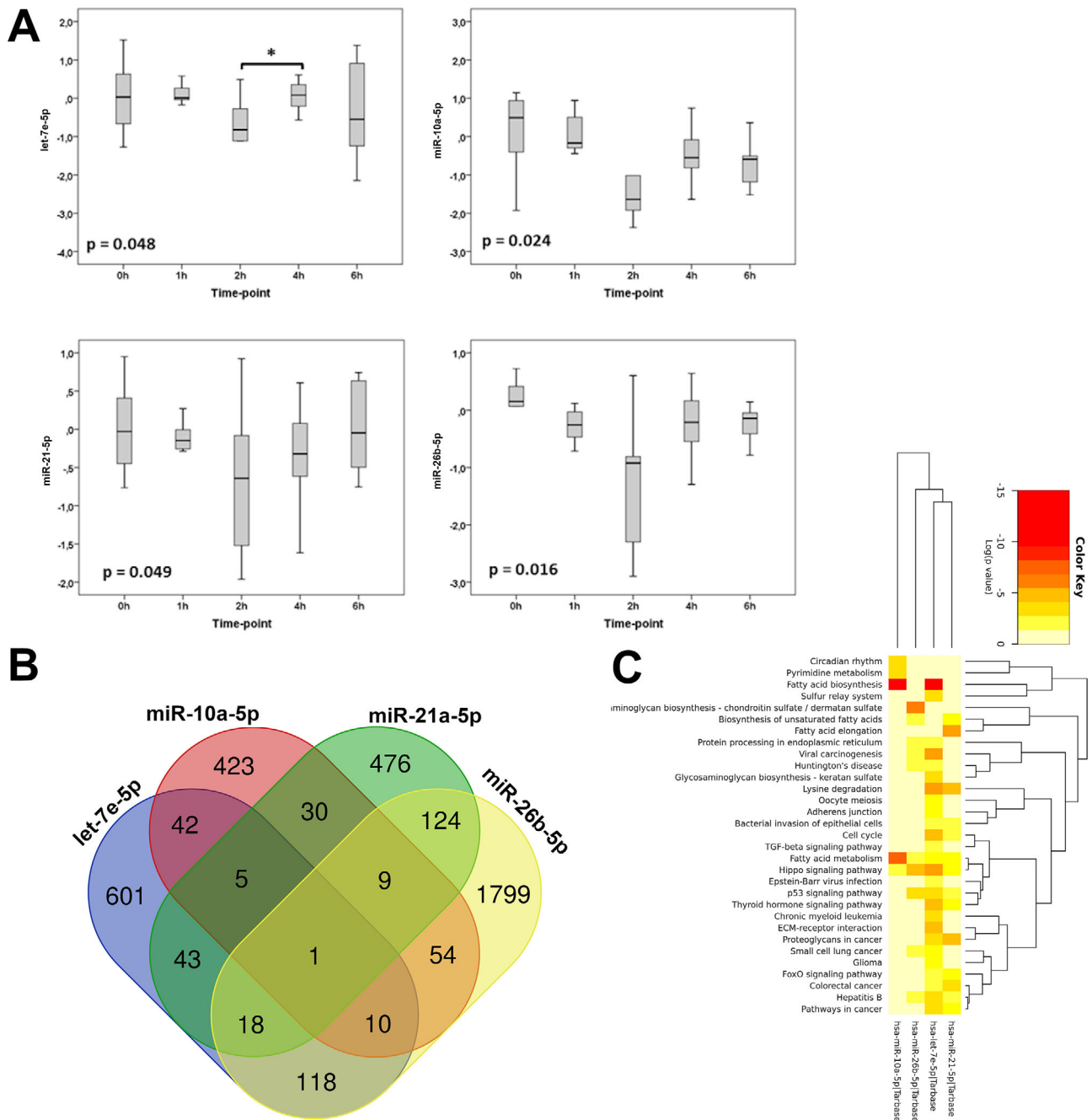


Figure 3. microRNAs modulated by H-EVOO. A) Box plots showing Log₂ transformed relative quantification levels of indicated microRNAs along the postprandial phase ($n = 10$). p -value refers to the intra-subject's comparison of the paired-repeated measures ANOVA with Bonferroni correction. $^*p < 0.05$. B) Venn diagram showing common targets of the modulated microRNAs. C) Heatmap plot of significant KEGG pathways (Log₁₀).

2.4. Correlation between Circulating Levels of Modified microRNAs and Plasma Lipid and Glucose Levels

We aimed to define if changes in circulating levels of the differentially modulated microRNAs were correlated with changes in circulating levels of biochemistry parameters used as biomarkers of metabolic disorders like lipid and glucose levels. We found that the decrease in miR-328a-3p after 4 h of L-EVOO ingestion

correlated with a decrease in HDL plasma levels at the same time-point ($R = 0.68$, $p = 0.033$) (Figure S3C, Supporting Information and Figure 5) and the increase in miR-20a-5p after 6 h of L-EVOO ingestion correlated with a decrease in total cholesterol ($R = -0.68$, $p = 0.033$) (Figure S3D, Supporting Information and Figure 5).

The increase in miR-17-5p levels correlated with a decrease in total cholesterol 2 h after the ingestion of M-EVOO ($R = -0.5$,

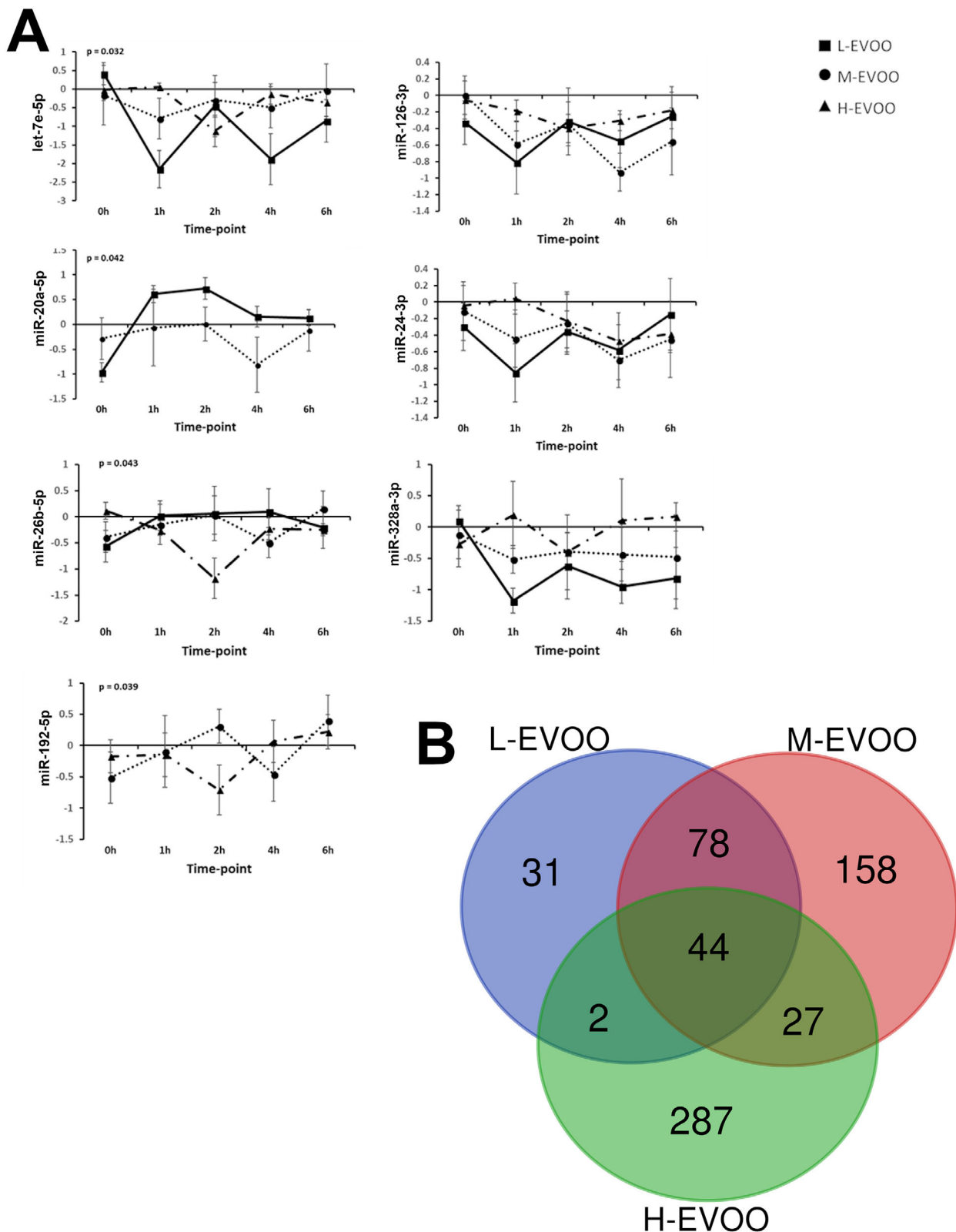


Figure 4. Differentially modulated microRNAs according to the type of functional oil. A) Interaction plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the postprandial phase in the three functional olive oils. p -value refers to analyses of the paired-repeated measures ANOVA with oil type as interaction parameter and Bonferroni correction. * $p < 0.05$, ** $p < 0.001$. B) Venn diagram showing common targets of the modulated microRNAs in the three functional oils.

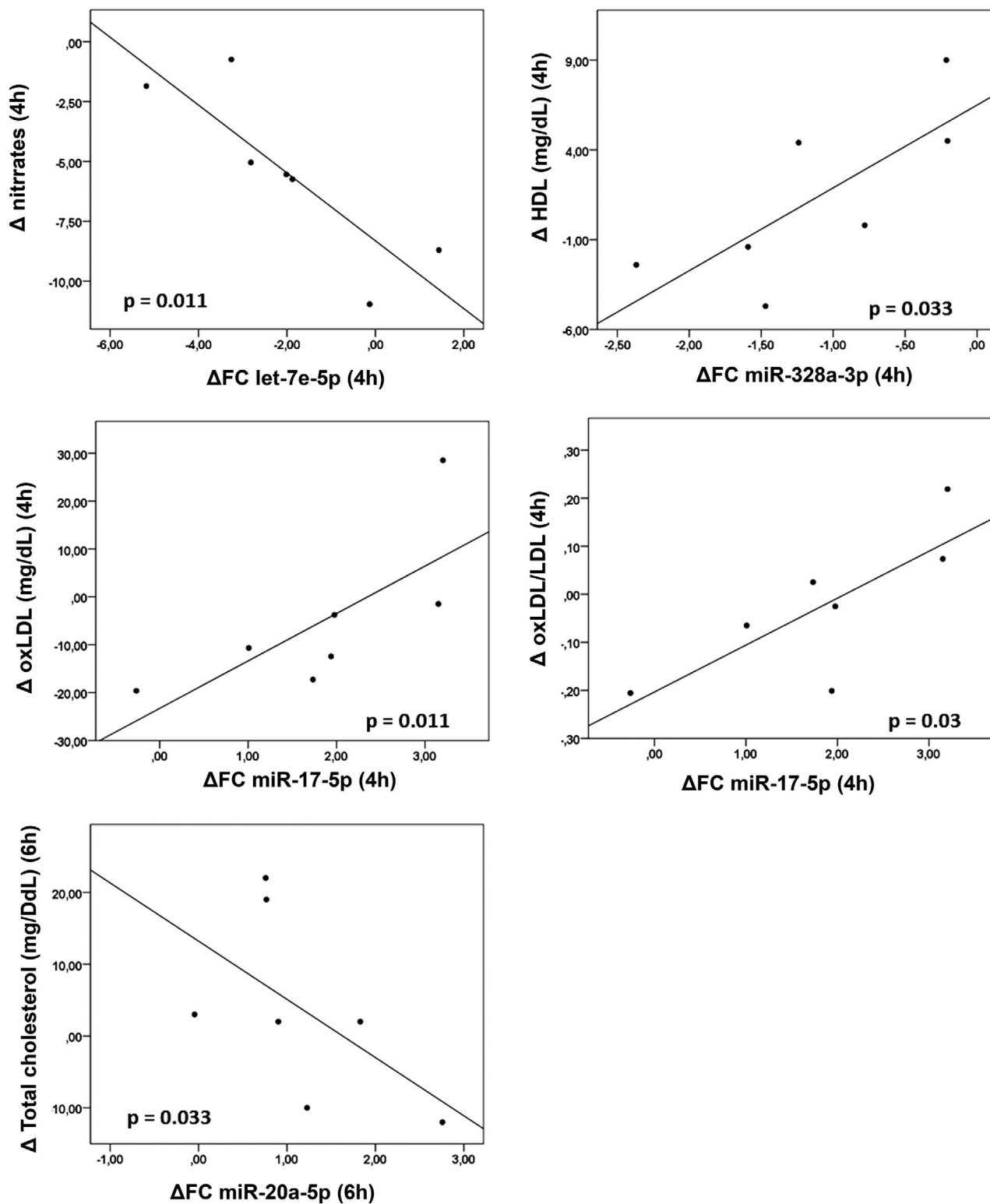


Figure 5. Correlation plots of microRNAs modulated by L-EVOO. Correlation with lipid and glucose metabolic markers and endothelial function markers was calculated by Tau Kendall test.

$p = 0.034$) (Figure S4B, Supporting Information and Figure 6A) and the increase in miR-192-5p levels correlated with increased levels of triglycerides (TG) after 6 h of M-EVOO ingestion ($R = 0.59$, $p = 0.01$) (Figure S4D, Supporting Information and Figure 6C). Finally, the decrease in miR-26b-5p levels correlated with a decrease in glucose level after 2 h of H-EVOO ingestion ($R = 0.6$, $p = 0.016$) (Figure S5B, Supporting Information and Figure 7A).

2.5. Correlation between Circulating Levels of Modified microRNAs and Markers of Endothelial Function

Finally, we investigated if changes in microRNA levels correlated with changes in circulating levels of two markers of endothelial function and atherosclerosis: endothelin and oxidized-LDL. We found interesting correlations between changes in microRNA levels after 4 h of L-EVOO ingestion and changes in total oxLDL and nitrites levels (Figure S3C, Supporting Information). We found that a higher decrease of let-7e-5p levels correlated with a lower decrease in nitrates levels at 4 h ($R = -0.81$, $p = 0.011$) (Figure S3C, Supporting Information and Figure 5). We observed that the increase in circulating levels of miR-17-5p mediated by L-EVOO correlated with an increase in oxLDL ($R = 0.81$, $p = 0.011$) and in oxLDL/LDL ratio ($R = 0.71$, $p = 0.03$) after 4 h (Figure S3C, Supporting Information and Figure 5). The increase in miR-192-5p levels correlated with an increase in endothelin levels at 4 h of ingestion of M-EVOO ($R = 0.6$, $p = 0.01$) (Figure S4C, Supporting Information and Figure 6B). Finally, the decreased in miR-21-5p levels observed 2 h after the ingestion of H-EVOO correlated with increase in both, LDLox ($R = -0.56$, $p = 0.029$) and the ratio LDLox/LDL ($R = -0.51$, $p = 0.047$) at the same time-point (Figure S5B, Supporting Information and Figure 7A). On the other hand, the decrease in miR-10a-5p levels after 4 h of H-EVOO ingestion correlated with higher endothelin levels at the same time-point ($R = -0.6$, $p = 0.017$) (Figure S5C, Supporting Information and Figure 7B).

3. Discussion

Our study shows the postprandial modification of circulating microRNAs after intake of three different functional olive oils enriched with their own polyphenols. Some recent reports have shown the change of postprandial levels of circulating microRNAs in response to fat challenges or mixed meal challenges in humans.^[26,27] Although these reports only compare basal fasting levels to one postprandial timepoint. The study reported by Quintanilha et al. analyzed the effect of a high-fat breakfast on circulating levels of microRNAs after 1, 3, and 5 h (4 timepoints, up to 5 h post-ingestion), although they did not conduct time-dependent analyses.^[28] We studied circulating microRNAs levels at basal time and after 1, 2, 4, and 6 h (5 timepoints, up to 6 h post-ingestion) of ingestion of 30 mL of three functional extra virgin olive oils with different polyphenolic content. Thus, to the best of our knowledge, this is the first study analyzing the postprandial behavior of human plasma microRNAs with this level of detail.

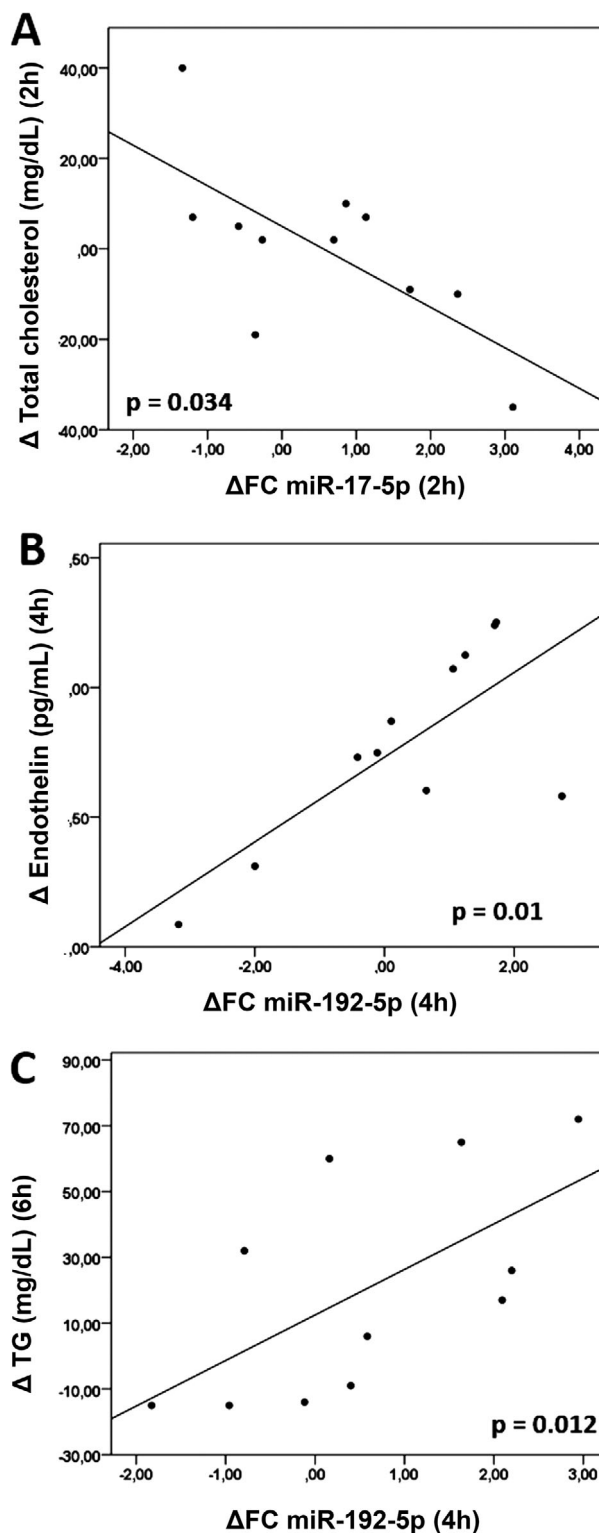


Figure 6. Correlation plots of microRNAs modulated by M-EVOO. Correlation with lipid and glucose metabolic markers and endothelial function markers was calculated by Tau Kendall test. A) Correlation between FC in miR-17-5p and in total cholesterol after 2 h. B) Correlation between FC in miR-192-5p and endothelin after 4 h. C) Correlation between miR-192-5p and TG after 4 h.

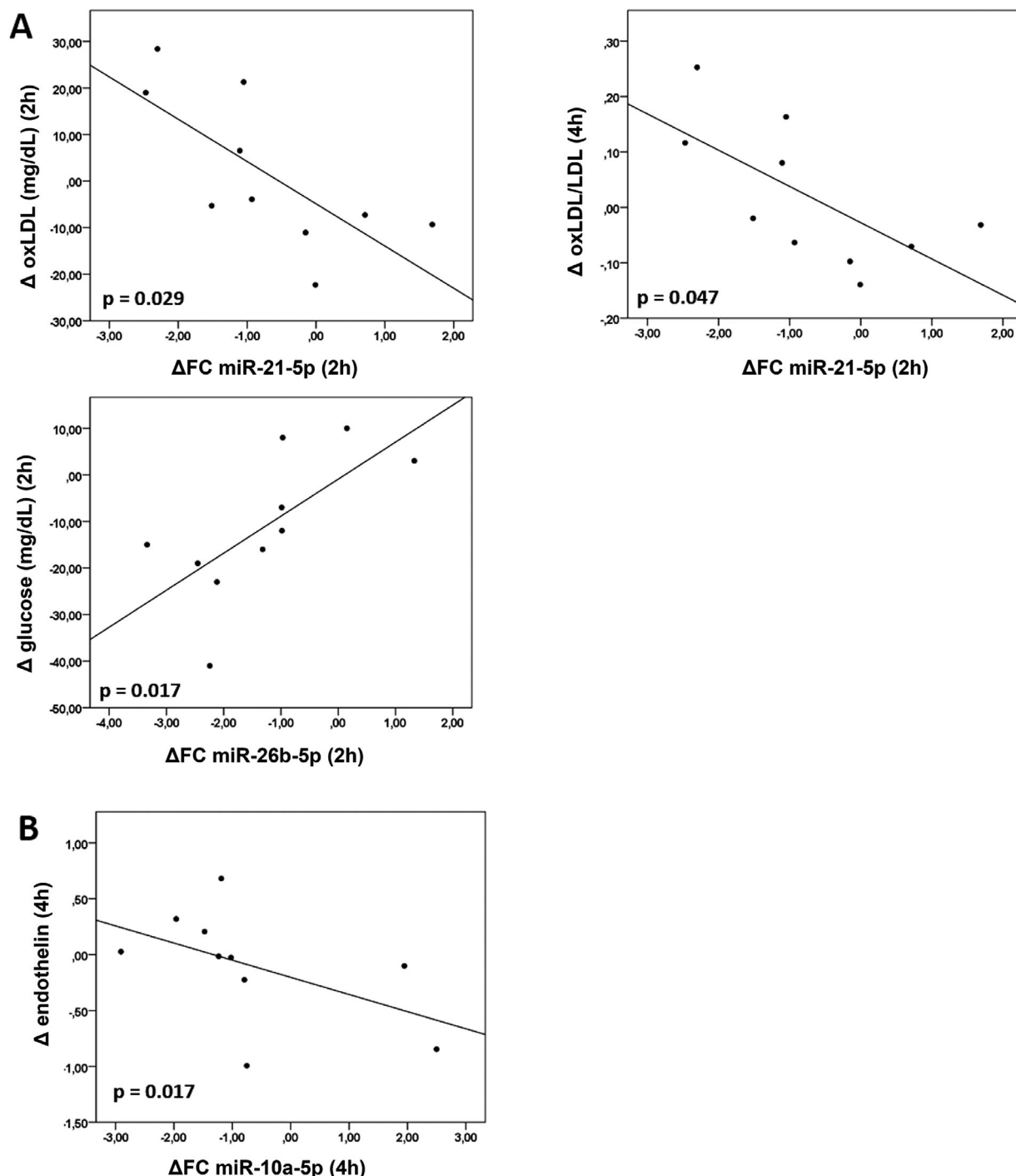


Figure 7. Correlation plots of microRNAs modulated by H-EVOO. Correlation with lipid and glucose metabolic markers and endothelial function markers was calculated by Tau Kendall test. A) Correlation between FC in miR-21-5p and in oxLDL and oxLDL/LDL ration and between FC in miR-26b-5p and glucose after 2 h. B) Correlation between FC in miR-10a-5p and endothelin after 4 h.

Some previous studies have reported postprandial changes in microRNA levels in fish tissues (liver and muscle), suggesting that early changes in microRNA levels as a response to diet are biologically plausible.^[29,30] López et al. studied the postprandial response of microRNAs to a high-saturated fat meal in peripheral blood mononuclear cells of nine healthy young men^[31]

and showed nine regulated microRNAs after 2 h of the fat load. Rodríguez-Ayala et al. found that the circulating levels of some microRNAs related to adipose tissue function were changed after 3 h of a balanced mixed meal.^[27] Mantilla-Escalante et al. studied in detail the postprandial response of circulating microRNAs to a high-fat diet consisted of the administration (oral gavage) of

250 μ L olive oil enriched with 40 mg of cholesterol to wild-type and DICER knock out mice.^[26] None of their significantly modulated microRNAs were in our panel. Although we cannot rule out the fact that these microRNAs could also be modulated in our volunteers, we must bear in mind that postprandial microRNAs modulated in mice can significantly differ from those modulated in humans. Besides, the fat challenge was based on olive oil (authors do not specify the type of olive oil) and cholesterol. Finally, this report measures three of the modified microRNAs in ten healthy subjects at baseline and after 3 h of a fat load not containing olive oil.^[26] Differences in the experimental design and the microRNAs analyzed between this report and ours make difficult to comparatively discuss the results. Quintanilha et al. also analyzed circulating plasma microRNA levels along 3 h after the ingestion of a high-saturated fat breakfast.^[28] They found up to 33 differently expressed microRNAs, comparing each time-point to basal level. Among them, miR-145-5p and miR-200 were modulated at all timepoints. They also found an increase in miR-143-3p, miR-375-3p, miR-33b-5p, and a decrease in miR-92b-3p. We did not find changes in these microRNAs in our postprandial challenge. The discordant results could be due to the difference between Quintanilha et al. challenge, rich in saturated fats and ours, based on extra-extra virgin olive oil enriched in polyphenols. This suggests that circulating levels of microRNAs we identified are modulated specifically by unsaturated fats and polyphenols.

Our design and analysis method allow us to define more precise postprandial kinetics of microRNAs. In this regard, different behaviors have been found with our different functional oils, such as bimodal (let-7e-5p), increased (miR-17-5p, miR-20a-5p, and miR-192-5p), or decreased (miR-328a-3p, miR-10a-5p, miR-21-5p, and miR-26b-5p) responses.

One of our most striking findings is the consistent increase of plasma miR-17-5p and miR-20a-5p, two members of the miR-17-92 cluster (also known as oncomiR-1), by L-EVOO and M-EVOO. Specifically, both microRNAs presented an increasing postprandial curve with peaks at 2 or 4 h. These results point toward a key role of this cluster in the postprandial response to these functional olive oils. These results are in agreement with those from Lopez et al.^[31] who found that miR-18a-3p and miR-19b-1-5p (two other members of the miR17-92 cluster) were upregulated in peripheral mononuclear cells 2 h after the fat load, and with those reported by Ortega et al.,^[23] who found miR-18a and miR-19, other members of the cluster, upregulated in obese individuals after 8 weeks of an isocaloric diet enriched in ω -3 and ω -6 polyunsaturated fatty acids. The increased expression of the miR-17-92 cluster has been associated with different types of cancer, where it plays a key role in the metabolic reprogramming of tumour cells.^[32] The overexpression of the miR-17-92 cluster has been found to enhance glycolysis and oxidative phosphorylation in tumor cells, with different members of the cluster having different effects.^[32] In this regard, the lack of miR-92a has been found to increase glycolytic and oxidative metabolism, whereas the lack of miR-17 and miR-20a is most effective in reducing glycolytic and oxidative metabolism.^[32] Lack of miR-17 and miR-20a has also been found to increase AMPK and decrease mTOR signaling pathways.^[32] Our *in silico* analysis showed that these nutrient-sensing pathways were significantly enriched among the processes regulated by miR-17 and miR-20a. Apart from its role in cancer, the cluster miR-17-92

also plays important roles in lipid and glucose metabolism,^[33] endothelial cell function,^[34] and macrophage differentiation^[35] all of them related to CVD. Interestingly, circulating levels of miR-17 and miR-20a have been found upregulated. In contrast, circulating levels of the other members of the miR-17-92 cluster have been found downregulated in patients with coronary artery disease and miR-17 levels have been positively associated with hyperlipidaemia and total cholesterol levels.^[36] We found interesting correlations between changes in circulating miR-17-5p levels and changes in CVD biomarkers such as oxLDL and total cholesterol after the ingestion of L-EVOO and M-EVOO, supporting a role of this cluster in CVD, and suggesting that the impact of our enriched EVOO on CVD biomarkers could be mediated by this microRNA. Plasma let-7e-5p was reduced by all three functional oils, suggesting that it is a primary target of dietary modification either by monounsaturated fatty acids or oil polyphenols. Let-7e is downregulated after intensive exercise^[37] and upregulated in coronary artery disease patients.^[36] Plasma let-7e has been found to increase in children with metabolic syndrome and correlated with waist circumference, insulin resistance, and with the accumulation of metabolic syndrome traits.^[38] Moreover, in children with metabolic syndrome, a negative association of plasma let-7e and HDL was found.^[38] We also found a significant negative correlation between let-7e with total cholesterol, LDL-c, and HDL-c at 1 h after the ingestion of L-EVOO. Interestingly, serum let-7e levels are also increased in ischemic stroke patients^[39,40] and varied in different stages, being higher in the acute phase and lower in the recovery phase.^[39] As Let-7e is a key regulator of endothelial function and immune activity and promotes inflammation,^[41] low levels of plasma let-7e can indicate a good health condition produced by EVOO, by olive oil or/and at least a 250 mg polyphenol kg^{-1} olive oil.

Another interesting finding refers to the different postprandial microRNA profile associated with H-EVOO ingestion in comparison with L-EVOO (250 mg polyphenol kg^{-1} EVOO) and M-EVOO (500 mg polyphenol kg^{-1} EVOO) that showed a more similar pattern. It is worth mentioning that the three functional oils share the same matrix and the only difference among them is their polyphenolic content. It is possible that a higher polyphenol enrichment has different effects on molecular pathways and a different biological impact. In this regard, it has been shown that an excess of polyphenols could produce pro-inflammatory effects.^[42] Moreover, preliminary studies to select the dosage that provided the highest beneficial effects on cardiovascular and endothelial parameters showed that EVOO enriched with 500 mg kg^{-1} provided additional benefits in comparison with those enriched with 250 and 750 mg kg^{-1} .^[43] This study also showed that plasma phenolic concentration was not linearly correlated with dosage after 500 mg kg^{-1} .^[43] Phenolic concentration in virgin olive oil is very variable (0.02 to 600 mg kg^{-1}) and it depends on different factors like cultivation area, irrigation techniques, the age of the olive tree, maturation of the olive fruit at harvest, the oil extraction process, storage conditions, and cooking techniques.^[44] According to the Commission Regulation (EU) 432/2012, the health claim regarding the protection of lipid from oxidative stress attributed to olive oil “*may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil.*” Thus, 250 mg of polyphenol kg^{-1} is considered healthy extra virgin oil.

Our results suggest that an enrichment with up to 500 mg kg⁻¹ of polyphenols may improve the benefits associated with olive oil consumption. Our results with H-EVOO suggest a microRNA profile more associated with hypertension. These results suggest that the different effects of H-EVOO on microRNA levels could be, in part, due to a detrimental effect of an excess of polyphenols and thus, high concentration of polyphenols should be carefully considered.

This study has some limitations. First, the small sample size calls for validation in a larger clinical trial. Second, although circulating plasma microRNAs have a high potential as biomarkers, we cannot assert the impact of changes in circulating microRNA levels on target cells. We cannot discern the tissue of origin nor the target tissue of the microRNAs differentially modulated. In this regard, further studies in animal models where the postprandial expression of these microRNAs in different tissues were assessed are needed. We should also bear in mind that this is an acute postprandial study and some changes produced by EVOO intake could need a more extended exposure. Moreover, participants drank 30 mL of EVOO that is a dose which is not usually consumed in only one ingestion. It would be necessary to carry out another intervention study with doses of EVOO ingested regularly. Furthermore, further studies specifically designed to analyze the effect of each specific polyphenol in every concentration on the expression of the modulated microRNA are needed to discern which component of our functional EVOOs is affecting each microRNA concentration and regulation. Also, we analyzed a selected panel of microRNAs. Therefore, we cannot rule out the possibility that these functional oils modulate circulating levels of other microRNAs associated with other conditions. Finally, some technical limitations linked to the detection and normalization of microRNAs in plasma could interfere with the results by reducing sensitivity.

However, the microRNA results offer promising evidence that paves the way for future studies addressing the biological impact of microRNAs modification by olive oil and polyphenols in humans. Notably, this study points to the decrease let-7e-5p with low, medium, and high olive oil polyphenol content consumption and the decrease miR-17-92 cluster after low and medium polyphenol content as a potential mechanism of action and biomarkers of the effect polyphenol content of extra virgin olive oil postprandial intake.

4. Experimental Section

Study Design: The VOHF study was carried out in the Hospital Universitari Sant Joan de Reus (Spain). The study design and characteristics of the volunteers have been described in detail elsewhere.^[43] The study was a postprandial, randomized, controlled, crossover trial with twelve healthy volunteers (50% women, 22–60 years) consisting of the intake of 30 mL of the functional EVOOs after 12 h of fasting. Three polyphenols-enriched EVOOs differing in phenolic content were assessed: Low-EVOO (L-EVOO, 250 mg total phenols kg⁻¹ of oil), Medium-EVOO (M-EVOO, 500 mg total phenols kg⁻¹ of oil), and High-EVOO (H-EVOO, 750 mg total phenols kg⁻¹ of oil). Composition of the three functional oils is detailed in Table S1, Supporting Information. Blood samples were collected at 1, 2, 4, and 6 h after the ingestion. The intervention was carried out according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local IRB (Clinical Research Ethical Committee,

University Hospital Sant Joan, Reus, Spain (Ref 09-02-26/2proj2) and was registered at ClinicalTrials.gov (Identifier: NCT01347515). All participants provided informed written consent.

Dosage Information: The volunteers ingested 30 mL of the functional EVOOs after 12 h of fasting. The three polyphenols-enriched EVOOs differed in phenolic content: L-EVOO (250 mg total phenols kg⁻¹ of oil), M-EVOO (500 mg total phenols kg⁻¹ of oil), and H-EVOO (750 mg total phenols kg⁻¹ of oil). The oil matrix of the three oils was the same. The preparation of the functional oils has been previously detailed.^[43] They were prepared by adding an extract obtained from freeze-dried olive cake rich in the main olive oil phenolic compounds to an extra virgin olive oil matrix with low phenolic content (80 mg total phenols kg⁻¹ of oil).^[43] This dose of these functional oils ingested in a single dose after fasting did not result in adverse events and previous results suggest that the M-EVOO could provide additional cardiovascular benefits.^[43] In the Mediterranean population, the mean intake of EVOO is 40 g per day (≈3 tablespoons per day).^[4] This study reduced the intake to 30 mL because this dose was ingested in a single intake after 12 h fasting.

Analyses of microRNAs Circulating Levels: Plasma was obtained from fresh blood by centrifugation. MicroRNA-enriched total RNA from 200 μL of plasma was isolated with miRCURY RNA Isolation Kit – Biofluids (Exiqon, A/S, Vedbaek, Denmark) using manufacturer's instructions and were diluted in 50 μL of RNase-free water.

A panel of 53 microRNAs related to CVD was selected by using prediction algorithms such as miRWalk^[45] and miRbase^[46] and a literature search (Table S2, Supporting Information).

MicroRNA levels were analysed by Real-Time quantitative PCR (RT-qPCR). Isolated microRNAs were retro transcribed with the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Before retro transcription, 5 fmol of single-strand cel-miR-54 was added to control for retro transcription variability. 5 μL of RNA were used as a template. Resulting cDNA was pre-amplified with the TaqMan PreAmp Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and amplified by RT-qPCR with TaqMan OpenArray PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a TaqMan custom OpenArray loaded using an OpenArray AccuFill System (Thermo Fisher Scientific, Waltham, MA, USA). The RT-qPCR was run in a QuantStudio 12K Flex Real-Time PCR System with OpenArray Block (Thermo Fisher Scientific, Waltham, MA, USA). The fold-change (FC) in circulating microRNAs levels was measured as relative quantification using the 2^{-ΔΔCt} method comparing each time-point to the basal point and was Log₂ transformed. The best two endogenous reference microRNAs were identified with the NormFinder algorithm.^[47] The quadratic average of the two endogenous controls plus the exogenous cel-miR-54 control was used for normalization.

In Silico Functional Analyses: MicroRNAs significantly modified in, at least, one time-point in each treatment were selected for further functional analyses. Enrichment analysis was carried out with DIANA miRPath v0.3^[48] and targets of these microRNAs were identified using the miRWalk tool^[45] and analyzed for functionality with Babelomics 5 and Software String.^[49,50]

Statistical Analyses: Changes in circulating microRNAs levels along the postprandial curve were analyzed by repeated-measurements ANOVA with Bonferroni correction. Missing values were imputed using a multivariate linear regression model. Imputed values accounted for less than 10% of all values. The interaction with the type of oil was included in the analysis. Data are shown as fold change (FC) ± standard error of the mean. A two-tailed *p*-value of 0.05 was considered significant. Statistical analyses were made with SPSS v24.

Statistical significance for enriched GO biological processes and KEGG pathways were analyzed with Babelomics 5, Diana miRPath v0.3, and Software String algorithms applying a False Discovery Rate adjustment. Overrepresented KEGG pathways are shown in heat maps with the color representing the log₁₀ of the *p*-value.

Venn diagrams were built with the Bioinformatics and Evolutionary Genomics application using default settings (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Changes in concentration of microRNAs with respect to basal time were correlated with changes in biochemical parameters with Tau Kendall

test and heatmap correlation plots were created using Corplot package in R i386 3.5.2 using RStudio.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.D., V.M., and R.M.V. contributed equally to this work. L.D. and J.M.O. conceived the study idea and designed the research. M.J.M., L.R., M.F., M.-I.C., R.M.V., A.P., and R.S. carried out the postprandial VOHF study and provided the plasma samples and data. L.D. and V.M. carried out the experimental procedures and analyzed microRNA and bioinformatics data. L.D. and V.M. wrote the manuscript, and all authors assisted in manuscript revision and approved it.

Keywords

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