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Modulation of miR-29a and miR-29b Expression by an Oral Nutritional Supplement Similar to the Mediterranean Diet Pattern with Probiotics in Malnourished Hemodialysis Patients

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Abstract: Malnutrition is prevalent in patients with chronic kidney disease (CKD), especially those on hemodialysis. Recently, our group described that a new oral nutritional supplement (ONS) for malnourished (or at-risk) hemodialysis patients with a "similar to the Mediterranean diet" model, improved caloric and protein intake, nutritional status and inflammation and oxidation biomarkers. Our aim in this study was to evaluate whether the new ONS, including probiotics, may produce changes in miRNAs expression and its target genes in malnourished hemodialysis patients, compared to individualized diet recommendations. We conducted a randomized, multicenter, parallel-group study in malnourished hemodialysis patients divided in 3 groups (1: Control -C- individualized diet 2: Oral nutritional supplement -ONS- + placebo -ONS-PL- and 3: ONS + probiotics -ONS-PR). Study registration: NCT03924089**.** The trial was open to the ONS diet or individual dietary advice, but double-blind for the intake of probiotics supplements. MiRNAs and gene expression levels were analyzed by RT-qPCR at baseline and after 3 and 6 months. We observed that the expression of miR-29a and miR-29b increased significantly in patients with ONS-PR at 3 months in comparison with baseline, stabilizing at the sixth month. Moreover, we observed differences between studied groups, where miR-29b expression levels were elevated in patients receiving ONS-PR compared to the control group in the third month. Regarding the gene expression levels, we observed a decrease in the ONS-PR group compared to the control group in the third month for *RUNX2* and *TNFα*. *TGFB1* expression was decreased in the ONS-PR group compared to baseline in the third month. *PTEN* gene expression was significantly elevated in the ONS-PR group at 3-months in comparison with baseline. *LEPTIN* expression was significantly increased in ONS-PL group at 3-month intervention compared to baseline. The new Oral Nutritional Supplement associated with probiotics; increases the expression levels of miR-29a and miR-29b after 3 months of intervention, modifying the expression of target genes with anti-inflammatory and anti-fibrotic actions. This study highlights the potential benefit of this Oral Nutritional Supplement, especially associated with probiotics, in malnourished patients with chronic renal disease on hemodialysis.

Keywords: chronic kidney disease; oral nutritional supplement; malnutrition; hemodialysis; probiotics; circulating miRNAs; gene expression; inflammation; renal fibrosis

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1. Introduction

Malnutrition is highly prevalent in patients with chronic kidney disease (CKD), particularly those on hemodialysis and is associated with increased mortality rates, decreased physical function and poorer outcomes. The etiology of malnutrition is multifactorial and includes decreased protein-calorie intake, inflammation, hypercatabolism, physical inactivity, nutrient loss into the dialysate, metabolic acidosis, uremic toxicity, nutrient malabsorption, among others [1]. Recently, our group described that a novel oral nutritional supplement (ONS), designed for malnourished (or at-risk) hemodialysis patients with a "similar to the Mediterranean diet" model (containing functional nutrients such as extra virgin olive oil, n-3 fatty acids, whey protein, fiber and antioxidants), improved caloric and protein intake, nutritional status (especially fat-free mass), and inflammation and oxidation biomarkers. Furthermore, the addition of probiotics may act synergistically with the ONS components to improve these biomarkers [2].

MicroRNAs (miRNAs) are a class of small, single-stranded, non-coding RNA molecules that play an important role in the regulation of gene expression. Most miRNAs are transcribed from DNA sequences as primary miRNAs and processed into precursor miRNAs and finally mature miRNAs [3]. In general, miRNAs interact with the 3' untranslated region (3' UTR) of target mRNAs, causing mRNA degradation and translational repression, but under certain conditions miRNAs can activate translation or regulate transcription [4]. miRNAs are very stable in human biological fluids, including blood and serum, as they are packaged in the blood vessel membrane (such as exosomes, microparticles, and apoptotic bodies) and associated with RNA-binding proteins. Many studies highlight the role of circulating microRNAs as biomarkers for several diseases [5]. miRNAs are involved in the normal kidney function and development. They are also implicated in several renal diseases, including CKD, where patients show specific circulating miRNA expression profiles associated with the regulation of metabolic, muscle and inflammation function and specific miRNAs have been identified as key players in the fibrosis process [6,7]. The expression of miRNAs is potentially altered by external factors such as diet [8,9] or the microbiota [10]. These miRNA profiles do not seem to vary before or after dialysis since most of them are not removed by hemodialysis membranes [11,12]. This makes miRNAs potential biomarker candidates even in this stage of kidney disease. Therefore, it would be interesting to evaluate the effect of a nutritional intervention on protein energy malnutrition associated with CKD. For example, miR-21 is overexpressed in CKD in animal models and humans. It also regulates metabolic pathways involved in fibrogenesis, inflammation, oxidation-reduction activity and cell proliferation [13,14]. Recent studies have described the role of miR-29 family as a strong regulator in several diseases such as obesity and diabetes [15] and a regulator of lipids metabolism during renal fibrosis [12]. Specifically, miR-29a and miR-29b have been associated with various protective effects in renal disease, particularly in the context of fibrosis, inflammation, apoptosis and kidney injury [16,17]. Furthermore, circulating levels of mir-155 (related to inflammatory pathways) and miR-126 (involved in vascular homeostasis) are reduced in patients with hemodialysis [12,13,18]. Other miRNAs have been related with CKD, such as miR-128a intervenes in mechanisms of muscle atrophy [19], meanwhile miR-223 intervenes in inflammation processes and is associated with vascular complications [18]. Finally, miR-378 is involved in angiogenic processes and adipogenesis, and can be modulated by inflammatory cytokines such as TNFalpha, IL6 and leptin [20].

MiRNAs have been suggested as potential diagnostic and therapeutic tools for CKD, however further research is needed to fully understand the role of miRNAs in CKD and its potential as a biomarker or therapeutic target [21,22]. Furthermore, it is known that miRNA levels change in response to different dietary interventions [23]: miRNAs levels can be regulated by carbohydrates, protein, fat, vitamins, minerals, dietary fiber or even isolated nutrients or bioactive compounds [9,24]. Further studies are needed to fully understand the mechanisms underlying these interactions and to develop effective strategies for using oral nutrition supplements to modulate miRNA expression. Our aim in this study was to evaluate whether the new ONS, with the action of probiotics, may produce changes in miR-21, miR-29a, miR-29b, miR-126, miR-128, miR-155, miR-223 and miR-378 expression

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and its target genes in malnourished hemodialysis patients, in comparison to individualized diet recommendations.

2. Results

A total of 31 patients (11 corresponding to group C, 10 to ONS-PL, and 10 to ONS-PR) completed the 6-months trial (Figure 1) [2].

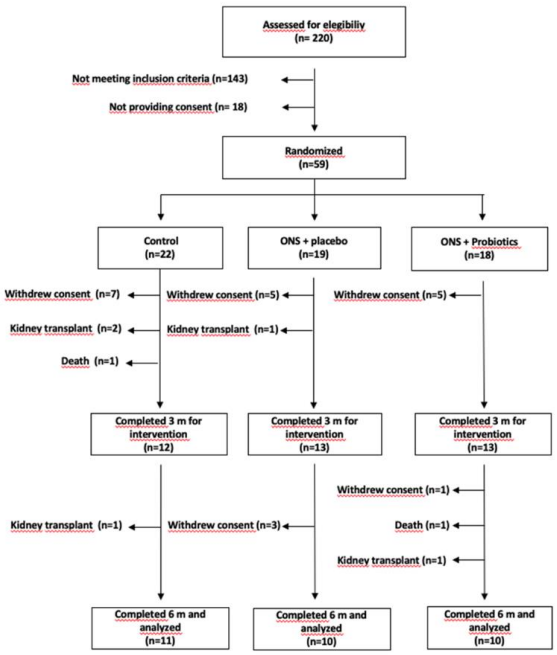

Figure 1. *Study flowchart.* ONS: Oral nutritional supplement.

At the baseline visit there were no basal significant differences between groups regarding age, sex, diabetes, Charlson comorbidity index, or intake of fermented milk or antibiotics during the month prior to inclusion. Furthermore, there were no baseline differences in any of the parameters for the nutritional assessment, dietary intake or biochemical data [2].

2.1. Circulating miRNAs expression levels before and after intervention

The changes in miRNAs expression levels are represented in Figure 2.

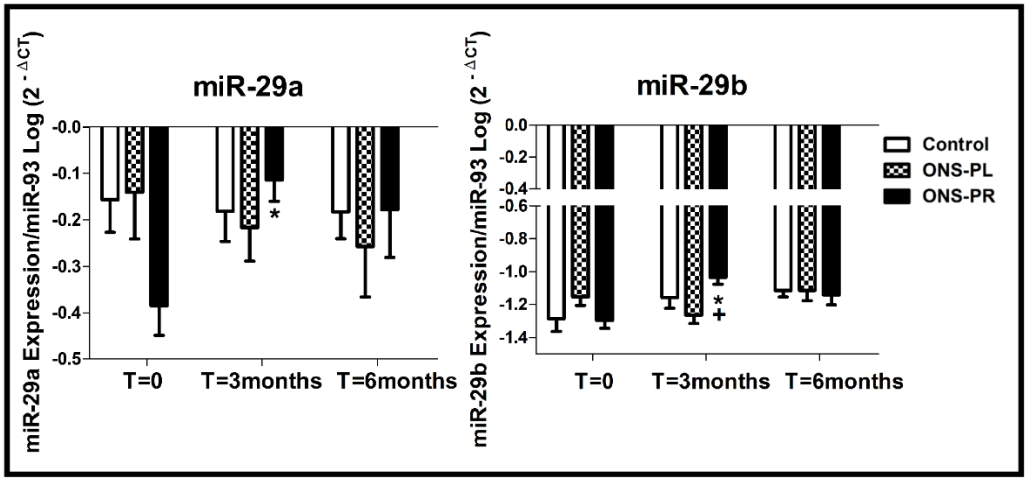

Figure 2. *MiR-29a and miR-29b expression levels.* Examinations were carried out at baseline and after 3 and 6 months. All the values come from the logarithm of the correspondent parameter. Data are

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presented as mean ± standard deviation. *p<0,05 versus baseline. +p<0,05 versus control. ONS-PL= oral nutritional supplement with placebo; ONS-PR= oral nutritional supplement with probiotics. Note: the negative values in miRNAs expression result from the fact that the $2^{-\Delta Ct}$ value is lower than 1.

We analyzed the miR29-a expression levels between the groups and we found a significant increase in the ONS-PR group in the 3-month intervention compared to baseline (p=0.03).

Regarding miR29-b expression levels, we found an increase in the ONS-PR group at 3-month intervention compared to baseline (p=0.023) and compared to the control group (p=0.027).

We did not find significant differences between the groups and between times of intervention for miR-21, miR-126, miR-128, miR-155, miR-233 and miR-378 expression levels.

2.2. In silico identification of predicted and validated miR-29a and miR-29b target genes

The bioinformatic analysis has revealed 2900 potential TGs for miR-29a and 2600 miR-29b. Moreover, Panther and DAVID database have shown the implication of the examined miRNAs in various biological processes. We accorded priority to TGs and biological processes linked with our ONS and renal function including inflammation, lipid metabolism and bone formation (Table 3, Figure 3). Then, we chose 9 important TGs that regulate 2 or more of selected processes.

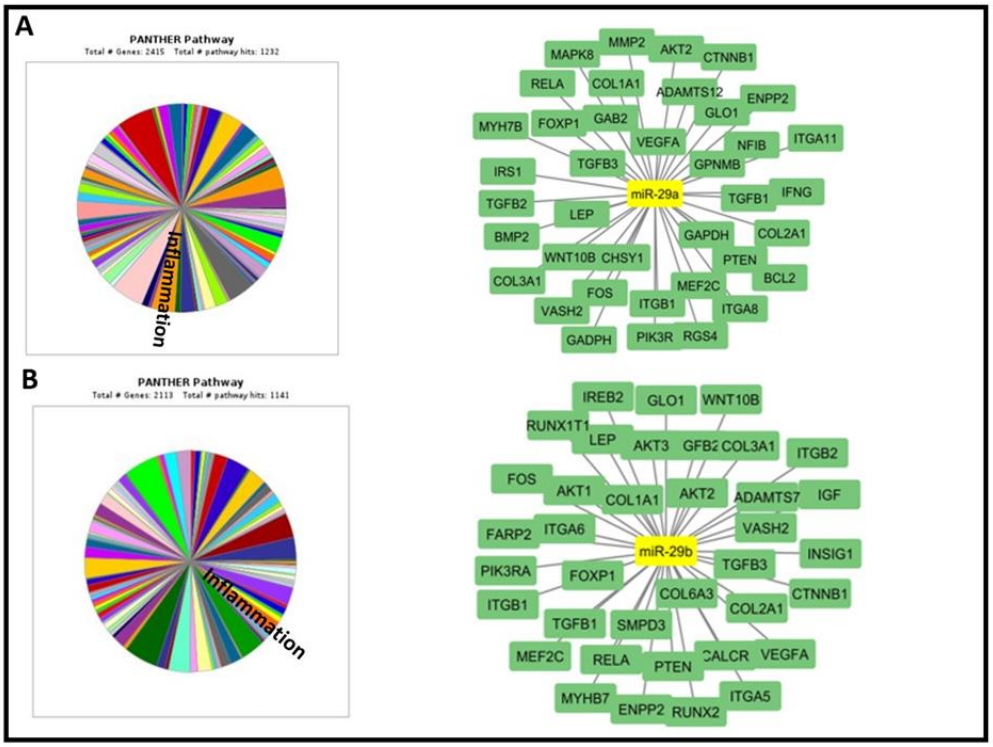

Figure 3. In silico analysis and schema recapitulating the interactions between miR-29a, miR-29b and TGs involved in inflammation, lipid metabolism and bone formation. (A-B) miRTarbase 9.0, Tarbase v.8 and targetScan 8.0 were used to identify possible TGs of miR-29a and miR-29b (yellow nodes). Panther and DAVID database were carried out to identify TGs (green nodes) implicated in inflammation, lipid metabolism and bone formation. The interactions among miRNAs and TGs were visualized using Cytoscape v.3.10.1.

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Table 3. Panther and DAVID bioinformatic analysis. Biological processes associated with the TGs of the miR-29a and miR-29b.

MiRNA	Database	Biological Process	Genes
MiR-29a	Panther	Inflammation	IFNG, ITGB1, **RELA**, RGS4
	DAVID	Osteoclast differentiation	**TGFB1**, GLO1, PIK3R1, FOS, GAB2, FOXP1, BMP2, CALCR, **CTNNB1**
		Chondrocyte differentiation	WNT10B, **MEF2C, TGFB1**, COL3A1, BMP2, COL2A1, NFIB, **CTNNB1**, ADAMTS12
		Ossification	**MEF2C**, SFRP1, **TGFB1**, CALCR, CHSY1, BCL2
		Cardiomyopathy	IRS1, **PTEN, RELA**, MAPK8, AKT2, TGFB2, TGFB1, TGFB3, MMP2, **MYH7B**, COL1A1, COL3A1, GAPDH
		Regulation of angiogenesis	GPNMB, **LEP**, ENPP2, VASH2, **CTNNB1**, VEGFA
		Cell-matrix adhesion	ADAMTS12, COL3A1, COL2A1, ITGA11, ITGA8, ADAM9, **CTNNB1**
MiR-29b	Panther	Inflammation	ITGB1, **RELA, AKT1**, AKT2, AKT3, COL6A3
	DAVID	Osteoclast differentiation	FARP2, **TGFB1**, CALCR, GLO1, IREB2, **CTNNB1**, FOS, PIK3R1, FOXP1
		Chondrocyte differentiation	WNT10B, **MEF2C,** COL3A1, COL2A1, **TGFB1**, **RUNX2**, ADAMTS7
		Ossification	SMPD3, COL1A1, **MEF2C**, COL2A1, **RUNX2**
		Cardiomyopathy	ITGB1, TGFB2, **TGFB1**, **MYH7B**, TGFB3, IGF, **RELA**, AKT2, AKT3, ITGA6, ITGA5, **AKT1**
		Regulation of angiogenesis	**LEP**, ENPP2, VASH2, **CTNNB1, PTEN**, VEGFA

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		Cell-matrix adhesion	FGB, ITGB1, COL3A1, COL5A3, ITGA11, **CTNNB1**, ITGA6, ITGA5
		Regulation of fat cell differentiation	WNT10B, **TGFB1**, INSIG1, RUNX1T1

2.3. Gene expression levels from human blood before and after intervention

The changes in miR-29a and miR-29b target genes expression are represented in Figure 4.

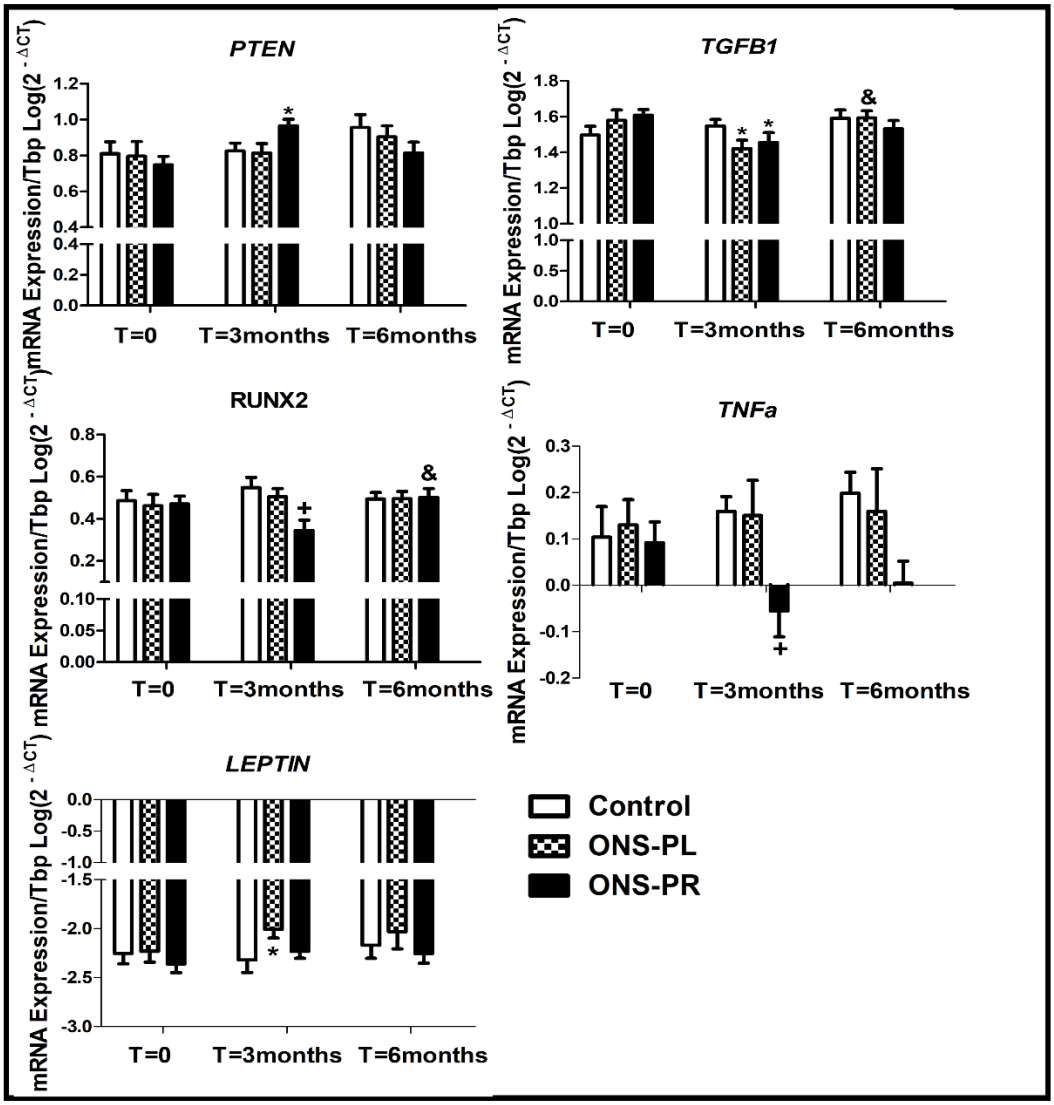

Figure 4. *mRNA expression levels from human blood*. Examinations were carried out at baseline and after 3 and 6 months. All the values come from the logarithm of the correspondent parameter. *p<0,05 versus baseline. +p<0,05 versus control. ONS-PL= oral nutritional supplement with placebo; ONS-PR= oral nutritional supplement with probiotics. *PTEN*: Phosphatase and tensin homolog, *TGFB1:* Transforming growth factor beta 1, *RUNX2*: Runt-related transcription factor 2, *LEPTIN*, *TNFa*: Tumor necrosis factor a.

PTEN expression was increased at 3-month intervention compared to baseline in the ONS-PR group (p=0.023) and a slight but not significant increased expression compared to the control group (p=0.080). We found no differences between *PTEN* expression in 6-month intervention and baseline.

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The expression of *TGFB1* was decreased at 3-month intervention compared to baseline in the ONS-PR (p=0.019) and ONS-PL group (p=0.027). We observed an increase in *TGFB1* expression at 6-month intervention in the ONS-PL group, when compared to 3-month intervention (p=0.016).

Regarding RUNX2 expression, we observed a decrease in the ONS-PR group compared to the control group at 3-month intervention (p=0.012). In the ONS-PR group, we observed a slight decrease in *RUNX2* expression at 3-month intervention compared to baseline (p=0.079) and a later increase in 6-month intervention (p=0.023).

The *LEPTIN* expression was increased at 3-month intervention compared to baseline in the ONS-PL group (p=0.04). We found no significant differences between 6-month intervention and baseline.

Regarding *TNFa* expression, we observed a decrease in ONS-PR group at 3- month intervention compared to the control group (p=0.041). Furthermore, we observed a slight but not significant decrease in the ONS-PR group at 3-month intervention compared to baseline (p=0.098).

We also measured *AKT1*, *RELA*, *IL1B*, *IL6*, *MYH7B*, *HEF2C*, *CTNNB1*, *GHRL*, *RASA1* and *PPARG* expression gene levels, for being target genes of the studied miRNAs, but we found no significant differences between the groups and the times of intervention.

2.4. Correlations between miRNAs levels with serum biomarkers and expression levels of target genes.

We have previously described significant changes in inflammation biomarkers in the groups receiving supplements along the intervention [2]. Now we analyzed the correlations between the percentage change between these variables and miRNAs levels, represented in Figure 5.

When comparing baseline and 3-month intervention, we found a significant negative correlation between the expression of miR-29b with TNFα (p=0.017) in the ONS-PL group. In the ONS-PR group, we found a significant positive correlation between miR-29a and IL-10 (p=0.025) and a significant negative correlation between miR-29b with IL-8 (p=0.020).

Regarding the correlations between miRNAs levels and expression levels of target genes we did not find significant results.

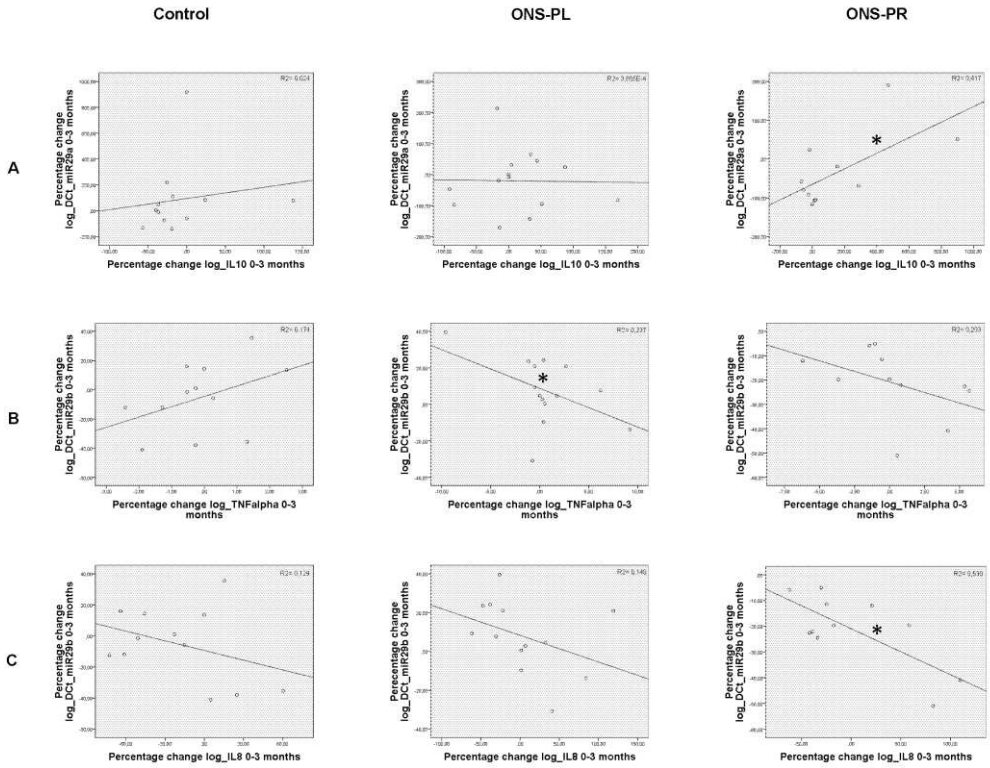

Figure 5. *Correlations between miR-29a and miR-29b expression levels and serum biomarkers*. Examinations were carried out at baseline and after 3 months. Correlations were analyzed using Pearson's

 doi:10.20944/preprints202312.1566.v1

correlation coefficient using the percentage change of the logarithm of the variables between 3 months and baseline in each group. A) Correlation between miR-29a and IL-10. B) Correlation between miR-29b and TNFα. C) Correlation between miR-29b and IL-8. ONS-PL= oral nutritional supplement with placebo; ONS-PR= oral nutritional supplement with probiotics. *p<0,05.

3. Discussion

In this study we analyzed for the first time the effects of a new oral supplement combined or not with probiotics on miRNA expression and its target genes in malnourished hemodialysis patients. The results provide valuable insights into the intricate relationship between nutritional interventions, miRNA regulation, and potential therapeutic outcomes. Notably, we observed that the expression of miR-29a and b increased significantly in patients with ONS-PR at 3 months in comparison with baseline. Moreover, miR-29b expression levels were elevated in patients receiving ONS-PR compared to control at month 3. Our previous study has shown that ONS improved body composition by inducing anti-inflammatory and antioxidant processes [2]. It is well known that miR-29a and miR-29b are implicated in the pathophysiology of various diseases, including obesity, diabetes, cardiovascular diseases and cancers [15,25–27]. Also, recent studies highlighted the protective effects of miR-29a and miR-29b against chronic kidney disease (CKD) and renal fibrosis, which are characterized by excessive accumulation of collagen and fibronectin in extracellular matrix (ECM), inflammation and oxidative stress [28–31]. Moreover, in the present study, we revealed changes in *TNFα* expression and a positive correlation between miR-29a and miR-29b with anti-inflammatory markers such as IL-10, and a negative correlation between miR-29b with pro-inflammatory markers TNFα and IL-8. All these findings may underscore the potential anti-inflammatory effects of the oral supplement and probiotics within miR-29a and miR-29b regulation. The decrease in *TNFα* expression aligns with the notion that these supplements might contribute to decreasing the chronic inflammatory state associated with malnutrition and renal dysfunction. Specially, the decreased *TNFα* expression observed in the ONS-PR group could be indicating a possible synergistic nutrition-probiotic effect. We observed that the expression of miR-29a and miR-29b increased significantly in patients with ONS-PR at 3 months in comparison with baseline, stabilizing at the sixth month.

Regarding the expression levels of *TGFB1*, we observed a decrease of this transcription factor in both ONS groups at month 3 with respect to control. Consistent with these results, several studies, have emphasized the role of *TGFB1* in the induction of renal fibrosis and the progression of CKD by promoting inflammation and ECM modulation [32,33]. Moreover, *RUNX2*, well-known for its crucial role in osteoblast differentiation and bone formation, has also been shown to be involved in fibrosis induction in various organs [34,35]. Furthermore, some studies have reported a tight correlation between *RUNX* family and *TGFB1* with fibrosis and CKD development [36–39]. In our study, we observed a decrease in *RUNX2* expression in the ONS-PR group compared to the control group at 3-month, followed by a slight decrease at 3-month compared to baseline, and a subsequent increase at 6-month intervention. In addition, Wang and collaborators have revealed that miR-29a attenuates kidney fibrosis in Unilateral Ureteral Obstruction (UUO) mice by inhibiting fibrotic proteins and *TGFB1* [17]. Moreover, *in vitro* upregulation of miR-29b inhibited the expression of *TGFB1* [30]. These observations are in accordance with our results, suggesting that these microRNAs could exert protective effects in the context of CKD by inhibiting a pro-fibrotic state mediated by *TGFB1* and *RUNX2.*

Concerning *PTEN* expression, we found that it was significantly elevated in the ONS-PR group at 3-months in comparison with the control group. Higgins and collaborators have demonstrated that *PTEN* prevents kidney fibrosis and renal injury by inhibiting PI3K/Akt and by counteracting the fibrotic effect of *TGFB1* in cultured renal cells and UUO model [40]. In addition, another study has revealed that increased *TGFB* expression during acute kidney injury (AKI) was associated with the loss of *PTEN* expression [41]. Also, the recovery of *PTEN* was accompanied with normal tubule repair and less fibrosis. Furthermore, inhibition of *PTEN* expression increased renal fibrosis in AKI mice model [36]. Additionally, other researchers have found that *PTEN* improves cellular fibrotic changes and renal fibrosis via inhibiting FAK/AKT signaling pathway [42]. In line with these results, one

 doi:10.20944/preprints202312.1566.v1

study has shown that Homeobox transcript antisense RNA (HOTAIR) downregulates *PTEN* via miR-29b inhibition during liver fibrosis [43]. Moreover, other study has revealed that miR-29b contributed to the regulation of renal interstitial fibrosis via targeting PI3K/Akt signaling pathway and *PTEN* expression [44]. These findings suggest the role that could play miR-29a and b and its target genes in kidney fibrosis and renal injury within CKD.

In relation to *LEPTIN* levels, the increase of its expression in the ONS-PL group raises interesting questions about the possible link between the oral supplement and appetite regulation in malnourished patients. Leptin is a peptide hormone primarily synthesized in adipose tissue and plays an important role in regulating appetite and energy balance [45]. Moreover, leptin exerts a pivotal role in inducing angiogenesis and adipogenesis in adipose tissue [46]. In addition, it has been described that low levels of leptin are associated with protein energy wasting in people with CKD and hemodialysis and with a worse prognosis [47–49], even though a correlation with the amount of fat mass is maintained [50]. In our previous study [2] both groups that received the ONS increased weight at the end of the intervention, improving the nutritional status at the expense of, specially fat-free mass, but also with an increase in fat mass (around 1kg). Furthermore, dietary total fat increased significantly especially in the ONS-PL group [2]. In this context, the significant increase in fat intake could have stimulated the *LEPTIN* expression as an appetite-regulating response also associated with the increase in body fat mass. Furthermore, the probiotics in the ONS-PR group may have influenced the gut microbiota composition, mitigating a significant leptin response through various mechanisms [51].

A major limitation of this study was the relatively high dropout rate, which was primarily due to the challenges posed by the SARS-CoV-2 pandemic and the difficulty of maintaining ongoing patient follow-up [2], which has contributed to not finding significant changes in other circulating miRNAs. We highlight the study's strengths as being a randomized clinical trial (double-blind in the case of probiotics), long-term (6 months) follow-up, multicenter characteristics, and multiple parameter measurements, the measurement of multiple parameters (including biomarkers, miRNAs and its target genes), and the comparison to a control group following a personalized diet prescribed by certified dietitians.

4. Materials and Methods

4.1. Design

The trial design is set out in the previous publication [2]. Briefly: it is a randomized, multicenter, parallel-group study divided in 3 groups, open for the intake of ONS or individualized dietary advice, but double-blind for probiotics consumption. Patients were assigned to one of the following three groups (using a computer-generated random number table):

1: Control (C): received individualized dietary recommendations (IDR).
2: ONS + placebo (ONS-PL): received ONS + IDR.
3: ONS + probiotics (ONS-PR): received ONS + probiotics + IDR.

The Renacare® ONS was designed for malnourished hemodialysis patients. It is a high-calorie (2 kcal/mL) and high-protein supplement containing several functional nutrients (extra virgin olive oil, omega 3 fatty acids, whey protein, antioxidants, low-glycemic index carbohydrates, fiber and carnitine). The composition is shown on the website: https://adventiapharma.com/nutricion-clinica/productos/enteral-oral/bi1-renacare-dial

Inclusion and exclusion criteria are detailed in our previous publication [2]. Comprised adult subjects (>18 years) undergoing hemodialysis (standard therapy or on-line hemodiafiltration with high-reinfusion rate therapy not being modified within 3 months before inclusion for more than 6 months before inclusion) and who were malnourished. Written informed consent was obtained from all patients.

Nutritional requirements were assessed according to the International Society of Renal Nutrition and Metabolism guidelines. The target protein intake was at least 1.2 g/kg/day [52]. All participants completed a personal interview with a nutritionist at baseline and 3 and 6 months after. Patients

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randomly assigned to the ONS group were advised to consume two bricks of 400 ml per day (minimum 1 day - 200 ml). Patients' daily ONS intakes were recorded on a data sheet. Probiotics and placebo were delivered in visually indistinguishable capsules (one 380g tablet). Each probiotic capsule contains the following live bacteria: *Bifidobacterium breve* CNCM I-4035 (1.00E+09 Colony Forming Units -CFU-), *Bifidobacterium animalis lactis* BPL1 CECT 8145 (3.50E+09 CFU), and *Lactobacillus paracasei* CNCM I-4034 (5.00E+08 CFU).

The Research Ethics Committee of the Province of Malaga approved this study and the procedure follows the ethical standards of the Declaration of Helsinki. Study registration: NCT03924089.

https://clinicaltrials.gov/study/NCT03924089?term=OLVEIRA%20G&rank=2

4.2. Outcomes

Examinations were performed at baseline and after 3 and 6 months. Blood samples were taken before starting dialysis; plasma and serum were aliquoted and stored at −80 °C in the Hospital-IBIMA biobank, Andalusian Public Health System Biobank, which belongs to the National Biobank Platform (exp. PT20/00101).

4.3. miRNA and RNA extraction

miRNAs were extracted from plasma samples by means of automated method with Maxwell® 16 miRNA Tissue Kit (Promega Biotech Ibérica S.L., Madrid, Spain) according to the manufacturer's protocol. For each sample, 300 µL of plasma were consequently mixed with the following reagents: 200 µL of homogenization solution, 200 µL of lysis buffer and 15 µL of Proteinase K. Samples were incubated for 20 min on a heat block at 37 °C, 300 rpm. After the incubation, samples were transferred to RSC cartridges followed by automated RNA extraction with the Maxwell instrument. Finally, total RNA was eluted in 30 µL nuclease-free water and stored at −80 °C until use.

Total RNA was extracted from PAXgene Blood RNA Tubes (Qiagen, USA). Briefly, the the content of the thawed tube was transferred to a 50 ml conical tube with 3 ml 1X phosphate buffered serum (PBS), then vortexed for 30 seconds, and later centrifuged at 4000xg in 4 °C for 20 min. The RNA pellet was further lysed with QIAzol Lysis reagent (Qiagen, USA), followed by incubation with 200 µL chloroform. After the phase separation, the upper aqueous phase was mixed with isopropanol and incubated for 10 minutes on ice. After centrifugation, the RNA pellet was washed with 75% ethanol. After centrifugation, the RNA pellet was air-dried at room temperature for 10 minutes. Total RNA was resuspended in 25 µL of nuclease-free water and stored at −80 °C until use.

The purity of miRNA and total RNA was determined by the absorbance 260/280 ratio on a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

4.4. RT-qPCR

cDNA was synthesized from miRNAs and mRNAs by reverse transcription with the Universal cDNA Synthesis Kit (Exiqon, Denmark) following the manufacturer's recommendations for each kit.

Measurements of miRNA expression levels (miR-21-5p, miR-29a-3p, miR-29b-3p, miR-126-3p, miR-128-3p, miR-155-5p, miR-233-3p and miR-378-3p) were performed in duplicated by qPCR real time in 384 plates in a LightCycler 480 (Roche Diagnostics, S.L, Barcelona, Spain) at the Genomics platform of IBIMA-Plataforma Bionand. The master mix was prepared following the guidelines of Exiqon with GoTaq® qPCR Master Mix (Promega Biotech Ibérica S.L., Madrid, Spain) and the specific LNA™ miRNA PCR primer Assy (Exiqon A/S Vedbaek, Denmark) (Table 1).

Gene expression levels (TBP, *TGFB, RUNX2, PTEN, AKT1, RELA, TNF, IL1B, IL6, MYH7B, HEF2C, CTNNB1, LEP, GHRL, RASA1* and *PPARG*) were analyzed in duplicate by qPCR using QuantStudio 12K Flex Real Time PCR 384 system (Thermo Fisher Scientific Inc., USA) with TaqMan technology. Predesigned Thermo Fisher primers were used to detect the canonical isoform of the gene of interest in humans, built based on the GRCh38 reference genome (Table 2). These primers have been validated in silico and in vitro by Thermo Fisher

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(https://www.thermofisher.com/taqman/gene-expression/assay). To carry out the qPCR assays, the recommendations established by the manufacturer were followed, using TaqMan® Fast Advanced Master Mix.

Quantitative measures were obtained using the $2^{-\Delta Ct}$ method and expression results were given as the expression ratio relative to miR-93 for miRNAs after an analysis with RefFinder (https://blooge.cn/RefFinder/), found it to be the most stable miRNA. TBP was used as the normalizing gene for gene expression, selected after performing a screening (TaqMan™ Array Human Endogenous Controls Plate, Fast 96-well), with 32 possible constituent genes in triplicate, in a study subpopulation made up of 3 subjects from each of the 3 groups, a total of 9 subjects. Also, in order to detect the most stable gene, the RefFinder program was used.

For data normalization, negative controls and a specific calibrator in all plates for interplate normalization were included. Those determinations with Ct >38 were considered under the limit of detection and were taken out of the analysis.

Table 1. Primers used for amplification miRNAs: miRNA quantification using LNA-optimized, SYBR® Green-based miRNA PCR.

miRNA Assay ID	miRCURY LNA miRNA PCR Assays	Mature miRNA sequence	miRBase accession
hsa-miR-21-5p	YP00204230	5'UAGCUUAUCAGACUGAUGUUGA	MIMAT0000076
hsa-miR-29a-3p	YP00204698	5'UAGCACCAUCUGAAAUCGGUUA	MIMAT0000086
hsa-miR-29b-3p	YP00204679	5'UAGCACCAUUUGAAAUCAGUGUU	MIMAT0000100
hsa-miR-93-5p	YP00204715	5'CAAAGUGCUGUUCGUGCAGGUAG	MIMAT0000093
hsa-miR-126-3p	YP00204227	5'UCGUACCGUGAGUAAUAAUGCG	MIMAT0000445
hsa-miR-128-3p	YP00205995	5'UCACAGUGAACCGGUCUCUUU	MIMAT0000424
hsa-miR-155-5p	YP02119311	5'UUAAUGCUAAUCGUGAUAGGGGUU	MIMAT0000646
hsa-miR-223-3p	YP00205986	5'UGUCAGUUUGUCAAAUACCCCA	MIMAT0000280
hsa-miR-378a-3p	YP00205946	5'ACUGGACUUGGAGUCAGAAGGC	MIMAT0000732

Table 2. Primers used for amplification: genes.

Gene	Ref_Seq	Assay_ID	Dye Label	Chromosome Location
TBP	NM_001172085.1	Hs00427620_m1	FAM-MGB	Chr.6 170554333 – 170572870
TGFB1	NM_000660.5	Hs00998133_m1	FAM-MGB	Chr.19: 41330531 - 41353933
RUNX2	NM_001015051.3	Hs01047973_m1	FAM-MGB	Chr.6: 45328142 - 45664032
PTEN	NM_000314.6	Hs02621230_s1	FAM-MGB	Chr.10: 87863438 - 87971930
AKT1	NM_001014431.1	Hs00178289_m1	FAM-MGB	Chr.14: 104769349 - 104795743
RELA	NM_001145138.1	Hs01042014_m1	FAM-MGB	Chr.11: 65653596 - 65662972

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TNF	NM_000594.3	Hs00174128_m1	FAM-MGB	Chr.6: 31575567 - 31578336
IL1 B	NM_000576.2	Hs01555410_m1	FAM-MGB	Chr.2: 112829758 - 112836842
IL6	NM_000600.4	Hs00174131_m1	FAM-MGB	Chr.7: 22725889 - 22732002
MYH7B	NM_020884.4	Hs00293096_m1	FAM-MGB	Chr.20: 34955835 - 35002437
MEF2C	NM_001131005.2	Hs00231149_m1	FAM-MGB	Chr.5: 88718241 - 88904105
CTNNB1	NM_001098209.1	Hs00355045_m1	FAM-MGB	Chr.3: 41199451 - 41240448
LEP	NM_000230.2	Hs00174877_m1	FAM-MGB	Chr.7: 128241201 - 128257629
GHRL	NM_001134941.2	Hs01074053_m1	FAM-MGB	Chr.3: 10285750 - 10292947
RASA1	NM_002890.2	Hs00243115_m1	FAM-MGB	Chr.5: 87267801 - 87391926
PPARG	NM_005037.5	Hs01115513_m1	FAM-MGB	Chr.3: 12287850 - 12471054

4.5. In silico identification of predicted and validated miRNA target genes

The miRTarBase 9.0 (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php), TarBase v.8 (https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=tarbasev8) and TargetScan 8.0 (https://www.targetscan.org/vert_80/) databases were used to identify the validated and predicted target genes (TGs) of miR-29a and miR-29b. The Protein Analysis Through Evolutionary Relationships (PANTHER) Classification System (http://www.pantherdb.org/) and the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) were carried out to annotate the biological processes and pathways of the predicted TGs. Then, we focused our study on TGs involved in inflammation, lipid metabolism and bone formation, as these are associated with our ONS and renal function. The interactions between miRNAs and the TGs were visualized with Cytoscape software v.3.10.1 (https://cytoscape.org/).

4.6. Biomarkers

Blood samples were taken before starting dialysis; plasma and serum were aliquoted and stored at −80°C in the Hospital-IBIMA Biobank until analysis. IL-10, IL-8 and TNFα were measured in 25 ul of serum using the ProcartaPlex multiplex Immunoassay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions [2]. All measurements were performed in duplicate and a standard curve was used to obtain serum concentrations.

4.7. Statistical Analysis

Data analysis was performed with the IBM SPSS Statistics software version 26 (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). Data are expressed as the mean ± SEM. Comparisons between multiple groups were performed using one-

way ANOVA, and for comparisons between two groups Student's t test was used. To compare variables by the different groups and the changes over time (baseline, 3, and 6 months), ANOVA for repeated variables was used. The level of significance considered was 5%. For multiple comparisons (post-hoc) Bonferroni's correction was used. The percentage of increased or decreased expression of miRNAs or serum parameters with respect to the basal levels were calculated as follows:

Percentage increase/decrease = ((log parameter at 3 months - log parameter at basal time) / log parameter at basal time) X 100

Correlations were analyzed using Pearson's correlation coefficient using the percentage change between times of intervention between the groups. Normality was assessed by Shapiro–Wilk test and all the variables that did not presented normal distribution ($p > 0.05$) were log transformed in order to normalize; thus, we performed parametric tests for our analysis. GraphPad Prism version 9.0.0 for Windows, GraphPad Software (Boston, Massachusetts USA, www.graphpad.com) was used for miRNAs expression graphics.

5. Conclusions

The new Oral Nutritional Supplement specifically designed for malnourished (or at risk) hemodialysis patients with a "similar to the Mediterranean diet" pattern, associated with probiotics; increases the expression levels of miR-29a and miR-29b after 3 months of intervention, modifying the expression of target genes with anti-inflammatory and anti-fibrotic actions.

This study highlights the potential benefit of this Oral Nutritional Supplement, especially associated with probiotics, in malnourished patients with chronic renal disease on hemodialysis.

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Institutional Review Board Statement: This study was approved by the Provincial Research Ethics Committee of Málaga. The ethical principles stated in the latest revision of the Declaration of Helsinki and good clinical practice standards were applied. The study was registered with the following code: NCT03924089.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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