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Next-generation sequencing reveals that miR-16-5p, miR-19a-3p, miR-451a, and miR-25-3p cargo in plasma extracellular vesicles differentiates sedentary young males from athletes

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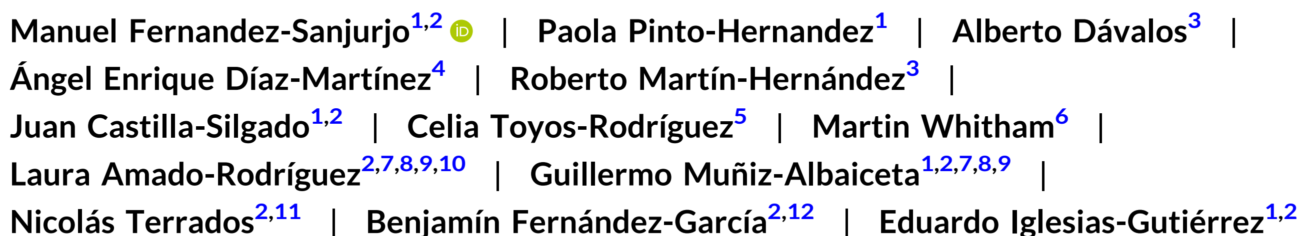

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Abstract

A sedentary lifestyle and Olympic participation are contrary risk factors for global mortality and incidence of cancer and cardiovascular disease. Extracellular vesicle miRNAs have been described to respond to exercise. No molecular characterization of young male sedentary people versus athletes is available; so, our aim was to identify the extracellular vesicle miRNA profile of chronically trained young endurance and resistance male athletes compared to their sedentary counterparts.

Benjamín Fernández-García and Eduardo Iglesias-Gutiérrez contributed equally as last authors.

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A descriptive case–control design was used with 16 sedentary young men, 16 Olympic male endurance athletes, and 16 Olympic male resistance athletes. Next-generation sequencing and RT-qPCR and external and internal validation were performed in order to analyze extracellular vesicle miRNA profiles. Endurance and resistance athletes had significant lower levels of miR-16-5p, miR-19a-3p, and miR-451a compared to sedentary people. Taking all together, exercise-trained miRNA profile in extracellular vesicles provides a differential signature of athletes irrespective of the type of exercise compared to sedentary people. Besides, miR-25-3p levels were specifically lower in endurance athletes which defines its role as a specific responder in this type of athletes. In silico analysis of this profile suggests a role in adaptive energy metabolism in this context that needs to be experimentally validated. Therefore, this study provides for the first time basal levels of circulating miRNA in extracellular vesicles emerge as relevant players in intertissue communication in response to chronic exercise exposure in young elite male athletes.

KEYWORDS
athletes, epigenetic modulation, exercise response, microRNA profile, sedentarism

Highlights

- An extracellular vesicle miRNA profile defined by next-generation sequencing differentiates athletes compared to sedentary young males.
- Extracellular vesicle miR-16-5p, miR-19a-3p, and miR-451a were lower in athletes irrespective of their sport disciplines compared to sedentary young males.
- Extracellular vesicle miR-25-3p was specifically lower in endurance athletes compared to sedentary young males.
- Energy metabolism and structural pathways are the main regulated ones based on extracellular vesicle miRNA profile.

1 | INTRODUCTION

Sedentarism is recognized as the fourth leading risk factor for global mortality by the World Health Organization (WHO, 2009). On the contrary, the effect of regular exercise on the prevention of a number of different pathologies has been clearly established (Pedersen et al., 2015). Furthermore, current studies have found an increase in longevity in Olympic athletes compared to the general population as well as a lower incidence of some prevalent conditions, such as cancer and cardiovascular disease (Antero et al., 2021; Takeuchi et al., 2019). Despite this wide-ranging preventive effect of regular training, there are different molecular gaps to be elucidated (Neufer et al., 2015). Historically, the main focus of exercise research was on the skeletal muscle as the primary effector system (Egan et al., 2013). However, intense interorgan crosstalk in response to exercise has been described, leading to coordinated systemic adaptations (Lavin et al., 2022; Verboven et al., 2023), in which extracellular vesicles play a key role (Whitham et al., 2018). The extracellular vesicles cargo comprises a variety of macromolecules, including microRNAs (miRNAs). miRNAs are small noncoding RNA molecules (≈22 nucleotides) with a post-transcriptional gene expression regulatory function by promoting mRNA degradation or by repressing protein translation (Ebert et al., 2012). They have a high level of interaction with coding gene sequences and their action is ubiquitous (Friedman et al., 2009). Although their activity was firstly defined as intracellular, they have been stably detected in different biological fluids, constituting the so-called circulating miRNAs (c-miRNAs) (Gupta et al., 2010). Studies published so far agree that both acute exercise and training modify c-miRNA profiles in healthy and diseased individuals (Fernández-Sanjurjo et al., 2018). This response has been found to be dependent on training status and exercise modality, intensity, and dose (de et al., 2018; Fernández-Sanjurjo et al., 2020; Fernández-Sanjurjo et al., 2020; Ramos et al., 2018; de et al., 2015). Most of these studies have analyzed total plasma c-miRNAs (Fernández-Sanjurjo et al., 2018), while less information was available on extracellular vesicle miRNAs (D'Souza et al., 2018; Just et al., 2020; Lai et al., 2023; Nair et al., 2020). Furthermore, the effect of exercise on basal miRNA expression in extracellular vesicles have been mainly studied in animal models (Nederveen et al., 2020). However, recent human studies have analyzed the whole basal miRNA profile in extracellular vesicles in older people depending on their physical activity (Nair et al., 2020) and in previously sedentary young people

in response to a concurrent training intervention (combining endurance and resistance sessions) (Garai et al., 2021). Garai et al., 2021) described that the modified miRNAs profile contained in plasma exosomes in young individuals who performed concurrent training for 6 months was very similar to the one observed in older men who had done regular exercise for 25 years.

Seeing that so far the analysis has been carried out in sedentary young people or in older people in relation to exercise, the aim of this study was first to analyze the basal miRNA profile of plasma extracellular vesicles in young male elite athletes from disciplines with a high endurance or a high resistance component in comparison with their sedentary counterparts. Then, to perform an in silico-based functional analysis of the extracellular vesicle miRNA signature identified.

This approach could provide insight into the role of extracellular vesicle miRNAs in the biological functions that differentiate these populations and thus provide a better understanding of the intertisular dialogue in the adaptation to training.

2 | MATERIALS AND METHODS

2.1 | Study design

A descriptive case–control design was used to analyze the extracellular vesicle miRNA profile in endurance and resistance male elite athletes compared to age-matched sedentary controls.

2.2 | Subjects

From Spanish Olympic national teams, 16 male athletes specialized in disciplines with a high endurance component and in this case long-distance runners and triathletes (END, 25.1 $\pm$ 7.0 years, weight: 64.2 $\pm$ 4.7 kg, height: 1.78 $\pm$ 0.05 m; and BMI: 20.1 $\pm$ 1.0), and 16 male athletes specialized in disciplines with a high resistance component, and in this case, weightlifters, throwing athletes, and gymnasts (RES, 22.1 $\pm$ 5.2 years, weight: 70.4 $\pm$ 10.3 kg, height: 1.67 $\pm$ 0.04 m; and BMI: 23.9 $\pm$ 2.0) volunteered to participate. Then, a total of 16 male sedentary University students (SED, 23.2 $\pm$ 2.9 years; weight: 76.3 $\pm$ 14.3 kg, height: 1.76 $\pm$ 0.06 m; and BMI: 24.5 $\pm$ 4.4), who performed less than 1 h per month of exercise for at least the last year, were recruited using flyers posted on campus and emails and agreed to participate in the study.

All experimental procedures were approved by the appropriate Research Ethics Committee of the Principality of Asturias, Spain (reference: 124/17) in accordance with the Declaration of Helsinki. All participants provided written informed consent.

2.3 | Blood sample collection

All blood samples were collected in fasted conditions, early in the morning (8––9 a.m.), at least 12 h after the last training session where applicable. On the same date, samples were obtained from the SED group. All samples were collected in EDTA-treated vacutainers (BD, USA). After gentle mixing, EDTA-treated tubes were centrifuged at 1800 $\times$ g for 15 min at 4°C to separate plasma. Plasma samples were aliquoted and immediately stored at −80°C for later analysis.

2.4 | Next-generation sequencing of miRNA cargo contained in extracellular vesicles

Of the 16 plasma samples from each group (SED, END, and RES), 8 were randomly selected and pooled resulting in three pooled samples for next-generation sequencing (NGS).

Starting with 500 µL of plasma from each of the three pooled samples, extracellular vesicle precipitation and subsequent RNA isolation were conducted using ExoRNeasy (Exiqon Services) according to the manufacturer's instructions. Total RNA was eluted in ultralow volume. The library preparation was done using the QIAseq miRNA Library Kit (QIAGEN). A total of 5 L total RNA was converted into microRNA NGS libraries. Adapters containing unique molecular identifiers were ligated to the RNA. Then, RNA was converted to cDNA using the miRCURY LNA RT kit (Qiagen). The cDNA was amplified using PCR (22 cycles), and during the PCR, indices were added. After PCR, the samples were purified. Library preparation QC was performed using either Bioanalyzer 2100 (Agilent) or TapeStation 4200 (Agilent). Based on quality of the inserts and the concentration measurements, the libraries were pooled in equimolar ratios. The library pool(s) were quantified using the qPCR ExiSEQ LNA™ Quant kit (Exiqon). The library pools were then sequenced on a NextSeq500 sequencing instrument according to the manufacturer instructions. Raw data were demultiplexed, and FASTQ files for each sample were generated using the bcl2fastq software (Illumina inc.). FASTQ data were checked using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bowtie2 was used for sequence alignment against reference miRNA sequences from humans. Feature counting was carried out using HTSeq-count. Before differential expression analysis, the obtained count matrix was normalized using the Trimed Mean of M-values method available within the edgeR library from the R coding environment. Raw data are available on ZENODO (https://doi.org/10.5281/zenodo.6531474).

For subsequent validation, a selection of miRNAs was carried out from NGS data. Two different criteria were applied: (1) miRNAs with a low expression but a high relative difference between, at least, two of the groups, that is, more than 30 and less than 1000 reads per million (RPM) and a fold change (FC) higher than 3 (overexpression) or lower than 0.3 (repression) and (2) highly expressed miRNAs with a moderate to high difference in expression, that is, at least 1000 RPM and a FC higher than 1.5 (overexpression) or lower than 0.6 (repression). This miRNA selection was composed of 50 members obtained from the sequencing data, miRNA identification, and LNA primer reference, and sequence can be found in Table S2 (https://doi.org/10.5281/zenodo.8143325).

2.5 | Internal and external validation of extracellular vesicles miRNA content by RT-qPCR

An internal validation using RT-qPCR was carried out for extracellular vesicle miRNAs differentially expressed between groups (SED, END, and RES) according to the criteria applied in the NGS analysis. This internal validation was performed individually on the same eight samples per group used for the NGS analysis. The remaining eight samples per group were used for an analogous external validation.

2.5.1 | Extracellular vesicles isolation and detection

200 µL of plasma from individual samples were pretreated with thrombin to convert fibrinogen to fibrin and clear fibrin, cells, and cell debris with the posterior centrifugation. Exoquick (System BioScience) product was used to extract extracellular vesicles following the manufacturer's instructions (Tang et al., 2017). Three aliquots were obtained and used for posterior size vesicle distribution analysis, microscopy, and miRNA isolation and analysis, respectively.

Size vesicles distribution was obtained through dynamic light scattering (DLS) from one of the aliquots using a Zetasizer Nano SZ system (Malvern Instruments) with an interval of measurement between 0.3 nm and 10 µm.

To validate these data, isolated extracellular vesicles were adsorbed on electron microscopy formvar/carbon-coated grids, negatively stained with 2% uranile acetate, washed with distilled water, and left air-dried overnight. Grids were visualized in a transmission electron microscope (TEM) JEOL 1011 operated at 110Kv.

2.5.2 | miRNA isolation from extracellular vesicles, reverse transcription, and amplification

After extracellular vesicle isolation, SeraMir (System BioScience) was used to extract miRNAs from extracellular vesicles obtained from 200 µL of plasma following the manufacturer's instructions (Tang et al., 2017). Synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) was added to the sample before isolation in order to be eventually used as a normalizer and an external reference. To monitor RNA extraction efficiency, RNA Spike-in kit containing UniSp2, UniSp4, and UniSp5 (Qiagen) was added. Extracellular vesicle RNA was eluted in 30 µL of RNase-free water. NanoDrop (ThermoFisher) was also used to confirm the presence of isolated RNA. For later analysis, RNA samples were immediately stored at −80°C.

miRCURY LNA RT kit (Qiagen) was used to perform reverse transcription (RT) from RNA to cDNA. LNA technology allowed a higher sensitivity, specificity, and reproducibility of the method. To check RT efficiency, an additional spike-in, UniSp6 (Qiagen), was added to each RNA sample before cDNA synthesis. The conditions of RT were as follows: incubation for 60 min at 42°C, heat-inactivation for 5 min at 95°C, and cooling immediately to 4°C.

For qPCR, miRCURY LNA miRNA Custom PCR Panels (Qiagen) were used, including those miRNAs selected from NGS. cDNA was diluted 80x and 4 L used in 10 µL qPCR reactions with miRCURY LNA SYBR Green PCR Kit master mix (Qiagen). PCR was performed on a 7900HT fast Real-Time PCR System (Applied Biosystems) following the manufacturer's conditions: 10 min at 95°C, 40 cycles of 10 s at 95°C, and 1 min at 60°C followed by a melting curve analysis. For both the analysis of the quantification cycle (Cq) and the melting curve, SDS v2.3 software was used. Cq was defined as the fractional cycle number at which fluorescence exceeded a given threshold. Melting curve analysis was performed to corroborate the specificity of the amplification. The spike-in qPCR curves were checked confirming optimal efficiency of the different steps of the protocol. miRNAs were considered to be expressed when their Cq values were higher than 38 according to the manufacturer's recommendations. For normalization purposes, the 2^(−ddCq) method proposed by Livak et al. (2001) was used, where dCq = Cq[miRNA] − Cq[cel-miR-39-3p] was the normalization of miRNA expression and ddCq = dCq [miRNA] − dCq[mean miRNA SED] was the relative expression (Livak et al., 2001). In this case, cel-miR-39-3p expression levels were used for normalization, and miRNA expression was calculated relative to the SED group.

Raw data and the summary of RNAseq detected miRNAs and their validation data are available on ZENODO (https://doi.org/10.5281/zenodo.8143325).

2.6 | In silico target gene prediction and pathway analysis of validated extracellular vesicle miRNAs

Pathway analysis of the genes targeted by the extracellular vesicle miRNAs identified was performed to gain insight into their potential functional implication in the biological response to exercise. For each miRNA, experimentally validated targets were retrieved from miRTarBase and miRWalk databases. mirTarBase validated gene targets were used on DIANA TOOLS miRpath v.3 using KEGG pathways analysis (Vlachos, Paraskevopoulou et al., 2015; Vlachos, Zagganas et al., 2015). We used target mining analysis by miRWalk for gene ontology and gene interactions. The data downloaded from miRWalk were utilized on Pantherdb 17.0 tool to obtain the analysis of protein classes and molecular function. All data on the figures of in silico analysis were represented by a number of targeted genes.

2.7 | Statistical analysis

Normality of variables was tested using Shapiro–Wilk's test. Descriptive values were presented as boxplots. A one-way ANOVA was used to assess differences in extracellular vesicle miRNA

expression between groups (SED, END, and RES). Differences between samples were considered relevant if it satisfied the following criteria: (a) *p*-value below (0.05) and (b) difference between means larger than 1.5. Spearman's correlation coefficient was calculated to explore associations between variables.

The statistical significances ($p < 0.05$) were indicated in the text and in the figures. Graph Pad Prism 8 (Graph Pad Software) and a customized R (www.r-project.org) function were used for all processes.

3 | RESULTS

3.1 | Isolation of extracellular vesicles was verified by DLS and microscopy

To morphologically characterize the isolated EVs, DLS was performed. EVs obtained from SED, END, and RES samples showed a homogeneous pattern with a size distribution between 22 and 230 nm (Figure 1A). This range correlates with the expected size for EVs (Lyu et al., 2021) pointing out the isolation of these vesicles. Additionally, TEM was used for further characterizations. Micrographs obtained for all the samples showed the presence of vesicles with a cup-shaped morphology surrounded by a bilayer as previously described (Vestad et al., 2021). A diameter size of 112 ± 23 nm (Figure 1B) was obtained for the measured EVs which is in accordance with the size distribution obtained through DLS. All these data validated the presence of extracellular vesicles in our samples.

3.2 | miR-16-5p, miR-451a, and miR-19a-3p showed a lower abundance in plasma extracellular vesicles in athletes compared to sedentary people, irrespective of exercise modality, while miR-25-3p was specifically repressed in endurance athletes

After NGS, we detected 513, 293, and 332 miRNAs in SED, END, and RES, respectively. When applying the first selection criterion, only miR-19a-3p was confirmed as differentially expressed between SED versus END and RES ($p = 0.0034$ and $p = 0.0284$, respectively) both after internal and external validation (Figure 2A,B). Regarding the second criterion, 25 miRNAs met it and all of them were detected after internal validation (Figure 2A), although the differences between groups were only confirmed for miR-16-5p, miR-451a, and miR-25-3p after external validation. Compared to SED, a significantly lower expression of miR-16-5p and miR-451a was observed in END ($p = 0.0469$ and $p = 0.0002$, respectively) and RES ($p = 0.0027$ and $p = 0.0004$, respectively) (Figure 2C–D). Furthermore, these differences were even larger considering END and RES together (*p*-value < 0.0001). Interestingly, miR-25-3p was significantly downregulated in END compared to SED ($p = 0.0229$) but not in RES ($p = 0.1127$) (Figure 2E) after both internal and external validation.

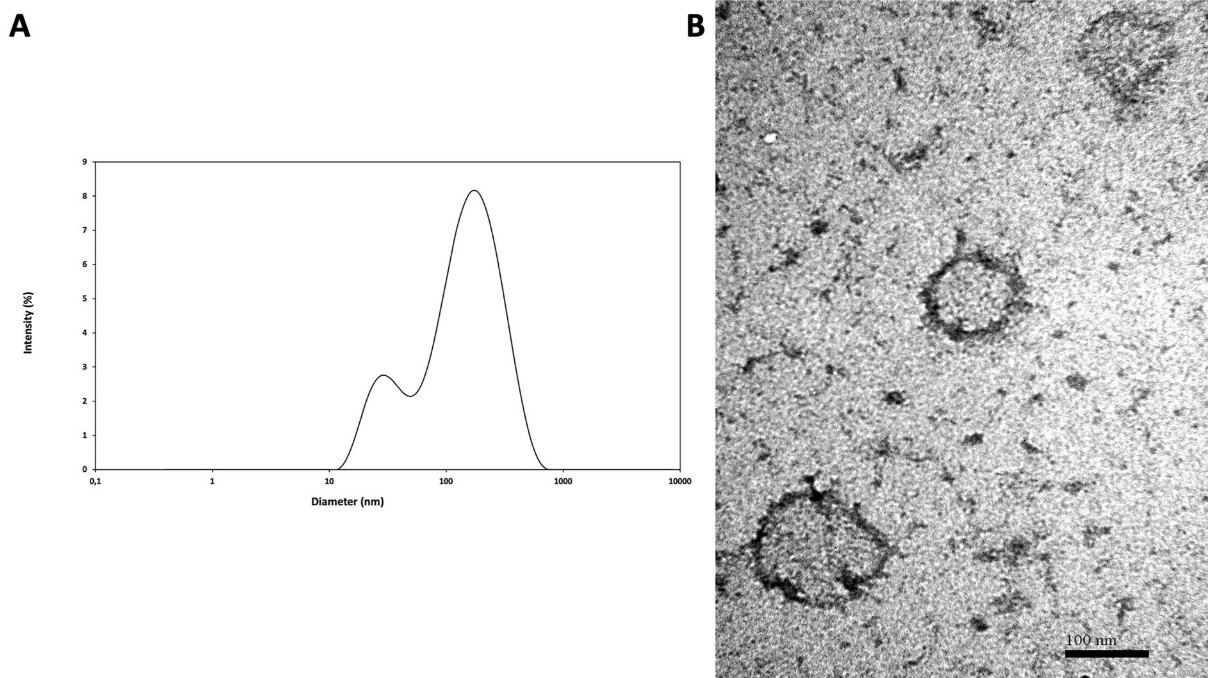

FIGURE 1 Extracellular vesicles isolation. (A) Histogram of hydrodynamic diameter of one extracellular vesicle aliquot observed through DLS obtaining a distribution in two peaks of 30 and 200 nm, respectively. (B) Image from transmission electron microscope JEOL 1011 of one extracellular aliquot defining a mean diameter of 112 nm. DLS, dynamic light scattering.

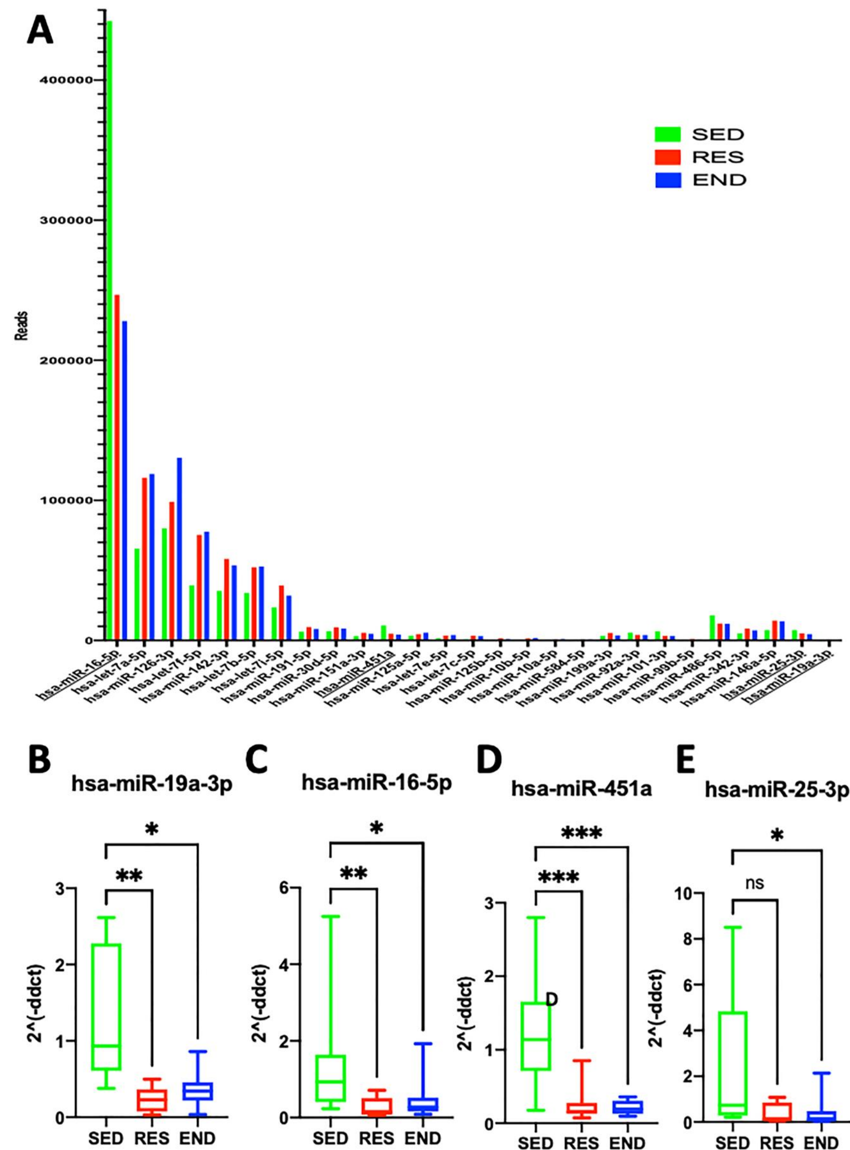

FIGURE 2 NGS screening and RT-qPCR internal and external validation of plasma extracellular vesicle miRNA signature in male athletes compared to sedentary counterparts. (A) NGS RPM of miRNAs selected for subsequent validation in extracellular vesicles. Underlined miRNAs are the ones that were finally validated after RT-qPCR. (B) Internal and external validation of extracellular vesicle hsa-miR-19a-3p content by RT-qPCR. (C) Internal and external validation of extracellular vesicle hsa-miR-16-5p content by RT-qPCR. (D) Internal and external validation of extracellular vesicle hsa-miR-451a content by RT-qPCR. (E) Internal and external validation of extracellular vesicle hsa-miR-25-3 content *p* by RT-qPCR. Ns non-significant * *p*-value <0.05, ** *p*-value <0.01. NGS, next-generation sequencing.

3.3 | Extracellular vesicle miRNA signature is related to key pathways in the adaptative response to exercise

On one hand, validated target analysis with Tarbase.v7 showed a strong relationship of miR-19a-3p, miR-16-5p, and miR-451a with several pathways related to the exercise response (Egan et al., 2013), particularly FoxO signaling pathway, protein processing, fatty acid biosynthesis, Hippo signaling pathway, mTOR signaling pathway, and AMPK signaling pathway (Figure 3A). On the other hand, the main validated targets of miR-25-3p were related to structural and anatomical pathways, such us Hippo signaling pathway and regulation of actin cytoskeleton (Figure 3D). Moreover, by gene ontology, the four miRNAs are related with binding and catalytic activities and implicated on molecular function of metabolite interconversion enzymes. Specifically, catalytic activity related with molecular function of enzymes emerged as the main regulations of these four miRNAs (Figure 3B,C,E,F). This highlights their relationship with energy metabolism and active metabolic processes.

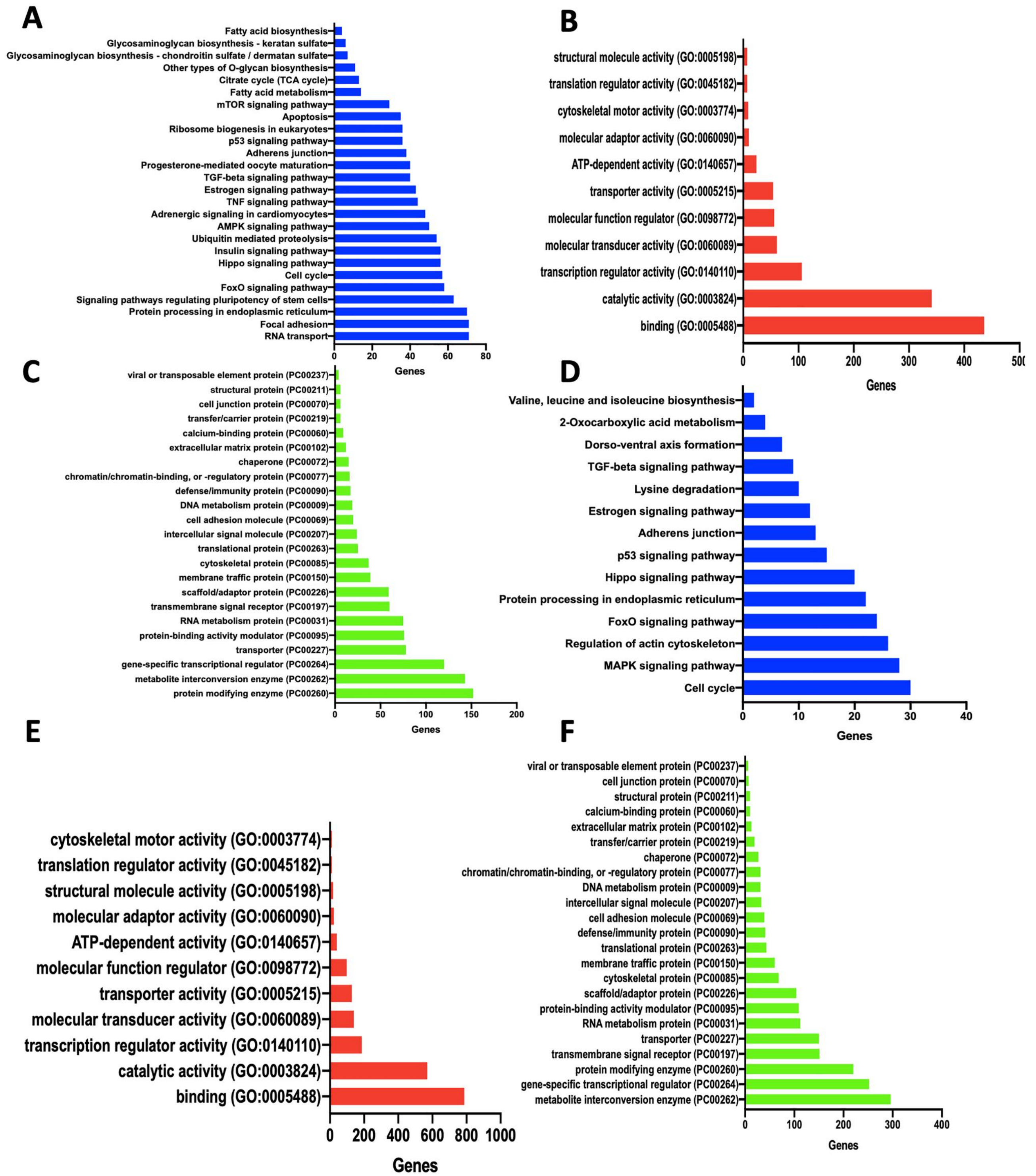

FIGURE 3 Target analysis of extracellular vesicle miRNA signature of male athletes compared to sedentary counterparts. (A) KEEG analysis of extracellular vesicle miRNAs with the same response in endurance and resistance training (miR-16-5p, miR-19a-3p, and miR-451a). (B) miR-19a-3p, miR-16-5p, and miR-451a target mining analysis for molecular function by Pantherdb 17. (C) miR-19a-3p, miR-16-5p, and miR-451a target mining analysis for protein class by Pantherdb 17. (D) KEEG analysis of specific endurance training extracellular vesicle microRNA (miR-25-3p). (E) miR-25-3p target mining analysis for molecular function by Pantherdb 17. (F) miR-25-3p target mining analysis for protein class by Pantherdb 17.

4 | DISCUSSION

For the first time, this study provides a comprehensive analysis of the basal extracellular vesicle miRNA profile of elite endurance and resistance male athletes compared to sedentary young males, describing a specific epigenetic signature, functionally related to the metabolic response to exercise. Although resistance and endurance exercise responses and adaptations involve different physiological and metabolic processes and pathways (Bangsbo, 2015; Hawley et al., 2014), it has also been described that the use of strength training is effective in improving endurance performance (Vorup et al., 2016). In this sense, our results highlight a new perspective of an epigenetic modulation mediated by miRNAs mostly common among different exercise models.

We have previously described miR-19a-3p, miR-16-5p, and miR-451a as responders to training when analyzed as total plasma content (Fernández-Sanjurjo et al., 2020). Thus, amateur runners showed a reduction in these miRNAs between their lowest and their highest performance peak during a season (Fernández-Sanjurjo et al., 2020). As far as we know, the extracellular vesicle expression of miR-16-5p and miR-451a has barely been analyzed in humans, mainly after an acute bout of exercise and with heterogeneous responses (D'Souza et al., 2018; Just et al., 2020; Lai et al., 2023; Nair et al., 2020), while no information was available for miR-19a-3p.

In a recent study, Garai et al. (2021) observed a decrease in extracellular vesicle miR-451a content after 6 months of regular moderate-intensity training, including resistance and aerobic activities, in healthy, young, and previously sedentary males with no changes in miR-16-5p. The authors observed the same trend in older and chronically endurance-trained males compared to sedentary young individuals. Surprisingly, a group of sedentary senior males was not included. This study suffers from other methodological limitations, such as the absence of internal and/or external validation after NGS analysis from pooled samples. Nair et al., 2020, using NGS analysis of individual samples, compared the basal profile of miRNA cargo in extracellular vesicles of active versus sedentary older individuals categorized according to their current exercise volume. However, no differences between groups were observed for miR-451a or miR-16-5p.

Regarding miR-25-3p content, our results show that this miRNA is specifically lower in endurance trained individuals compared to their sedentary counterparts but not in resistance trained athletes. This response was first described by Nielsen et al. (2014) in young males after 12 weeks of cycling training although in total plasma abundance. Moreover, Barber et al. described, also in total plasma levels, a decrease of miR-25-3p in response to 20-weeks of endurance training (Barber et al., 2019). Regarding its content in extracellular versicles, Garai et al. (2021) observed again a decrease in this miRNA in young males after 6 months of regular moderate-intensity concurrent training.

Surprisingly, only Barber et al. (2019) included women in their study, although they were analyzed jointly with men. Lamon et al. have shown that the profile of c-miRNAs varies throughout the menstrual cycle in healthy nonathletic women (Lamon et al., 2023). Interestingly, these authors described that total plasma levels of miR-19a-3p, miR-16-5p, miR-451a, and miR-25-3p were associated with ovarian cycle hormones. Besides, a previous study from our research group has also shown how progesterone levels modify the predictive role for performance of another c-miRNA, miR-106b-5p, in elite female athletes (Torres-Aguilera et al., 2023). Therefore, it is essential to extend this analysis to women taking into account the relevance of ovarian cycle hormones on the levels of c-miRNAs and that alterations in the menstrual cycle are not infrequent in female athletes.

Taking all together, extracellular vesicles derived from resting blood samples of trained humans appear to exert coordinated bioactive, and potentially beneficial, effects in several tissues compared to sedentary counterparts as it was previously described in rodents (Darragh et al., 2021). Moreover, exercise-induced miRNA cargo in extracellular vesicles may offer therapeutic potential in specific cases or may play a role in exercise adaptation and/or homeostatic maintenance (Darragh et al., 2021; Estébanez et al., 2021; Murphy et al., 2020).

Under this perspective, there is an opportunity to deepen the analysis and find a link between changes in plasma extracellular vesicle miRNAs and their possible role in gene targeting and subsequent bioactivity in response to training. Interestingly, miR-19a-3p, miR-16-5p, and miR-451a have been previously described as regulators of muscle function and metabolism (Csibi et al., 2012; Gnimassou et al., 2017). Thus, miR-451a has a key role in mTOR signaling and erythropoiesis (Minna et al., 2016; Rasmussen et al., 2010), and both miR-16-5p and miR-19a-3p target pathways are related to the cell cycle (Munson et al., 2019; Zhang et al., 2019) as well as Hippo signaling pathway. Interestingly, these miRNAs are highly expressed in the skeletal muscle (Kavakiotis et al., 2022). D'Souza et al. (D'Souza et al., 2019) have described an inverse relationship between muscle mass and miR-451a plasma levels. As for miR-19a-3p, Pinto et al. (Pinto et al., 2017) observed a repression in the skeletal muscle of animals with higher running capacity due to its target in citrate synthase. Finally, Silver et al. (Silver et al., 2020) analyzed extracellular vesicles and skeletal muscle miR-16-5p levels but failed to observe any relationship between them. According to miTED tool (Kavakiotis et al., 2022), miR-16-5p has its highest expression level in adipose tissue, which is in line with our in silico analysis, showing a relationship with fatty acid metabolism. Despite its relationship with structural and anatomical pathways, the expression of miR-25-3p in skeletal and cardiac muscle is low according to miTED tool (Kavakiotis et al., 2022). In contrast, very high levels of this miRNA have been described in serum and plasma (Kavakiotis et al., 2022) suggesting an important role in intertisular communication, although the specific functional relationship between the repression of miR-25-3p plasma levels and the aerobic response and adaptation to endurance exercise deserves further study. Taking into account the regulations described, the miRNAs identified are related to the greater aerobic, muscular, and metabolic capacity that

differentiates Olympic athletes from sedentary people. Therefore, they would constitute one more of the multiple modulable factors that condition these characteristics and that can be related to short- and long-term health status.

In conclusion, basal levels of c-miRNAs in extracellular vesicles emerge as relevant players in intertissue communication in response to chronic exercise exposure in young elite male athletes. In silico analysis suggests a role in adaptive energy metabolism in this context that needs to be experimentally validated.

5 | LIMITATIONS

This study suffers from some limitations, mainly the absence of women, which does not allow the results to be generalized. Furthermore, the data come from a young population in which the incidence rate of pathologies associated with lifestyle was lower than in other age groups, although the incidence of obesity is increasing. At a methodological level, the vesicle size analysis performed confirmed that miRNAs were isolated from extracellular vesicles, but the absence of a protein characterization does not allow for the identification of the specific family of vesicles. Additionally, blood samples were collected 12 h after the last training session. This determines a limitation as it might interfere with some blood parameters. However, it has been reported in humans that 4 h after acute exercise, the number of vesicles returns to basal levels after a significant increase is observed immediately post-exercise (Whitham et al., 2018). Therefore, it is an open question whether microRNA levels in extracellular vesicles may be affected especially when we have observed a decrease in levels compared to sedentary individuals. Finally, as our candidate miRNAs were highly expressed in a variety of cell types, their real source/s and target/s were not known, and it is out of the scope of this study to go deeper than an in silico analysis. Mechanistic *in vitro* and *in vivo* studies were necessary to experimentally validate these findings.

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CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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