Universidad de Oviedo
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Tesis

Caracterización y validación de microRNA circulantes como biomarcadores de la respuesta al ejercicio.

Characterization and validation of circulating microRNAs as biomarkers of exercise response.

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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RESUMEN (en español)

El estudio de la respuesta molecular al ejercicio quedó circunscrito históricamente al tejido muscular. Sin embargo, su efecto sistémico no está restringido a los tejidos implicados en la generación del movimiento, por lo que la respuesta al ejercicio implica una importante comunicación intertisular. Al margen de los mecanismos clásicos, ampliamente estudiados, mediados por hormonas o citocinas, en los últimos años ha destacado en este ámbito el papel de los exosomas y, encapsulados en ellos, distintas macromoléculas, incluidos los microRNA (miRNA), pequeñas moléculas de RNA no codificante con función reguladora post-trancripcional. Aunque su actividad es intracelular, se han detectado de forma estable en diferentes líquidos biológicos, constituyendo los denominados miRNA circulantes (c-miRNA). La presencia de c-miRNA en los exosomas plasmáticos sugiere que serían secretados de forma regulada en respuesta a una situación de estrés, actuando como un auténtico sistema de comunicación intercelular y regulando la expresión génica y el fenotipo de las células receptoras distantes.

Los estudios disponibles hasta la fecha concuerdan en que tanto el ejercicio agudo como el entrenamiento modifican los perfiles de c-miRNA.

Por ello, el objetivo principal de esta Tesis, en formato de compendio de publicaciones, es la caracterización y validación de los c-miRNA como biomarcadores de ejercicio y como reguladores de la respuesta y adaptación molecular al ejercicio.

En el primer artículo se lleva a cabo una revisión en la que se concluye que, en base al conocimiento disponible, el valor de los c-miRNA como biomarcadores en el ámbito del ejercicio no es sólido, por la falta de coherencia entre estudios en los resultados obtenidos y por las diferencias metodológicas, cuya influencia se analiza en profundidad.

En el segundo se analiza la respuesta a distintas dosis de ejercicio aeróbico de un panel amplio de c-miRNA, observando diferentes perfiles en función de la dosis de ejercicio, así como una progresión a lo largo de una temporada y el estado de entrenamiento.

La tercera publicación describe, por primera vez, subperfiles funcionales de c-miRNA por su correlación con marcadores fisiológicos y bioquímicos de respuesta a una prueba de esfuerzo máxima en laboratorio.

En la cuarta publicación se estudian, en paralelo, la respuesta a varias dosis de ejercicio aeróbico agudo de un panel restringido de c-miRNA descritos previamente en patología cardiaca, junto a otros marcadores clásicos. Nuestros resultados resaltan la relevancia de los c-miRNA como biomarcadores cardíacos emergentes, aun en ausencia de necrosis, sobrecarga o disfunción cardiaca, y su papel potencial en la respuesta cardíaca al ejercicio.

En el quinto estudio se plantea la necesidad de recurrir a modelos animales de ejercicio para el estudio de las potenciales fuentes y dianas tisulares de los c-mRNA detectados en respuesta al ejercicio, así como la posibilidad de estudiar modelos genéticamente modificados. Para ello se analizó el perfil de c-miRNA en personas y en animales de experimentación (ratones) crónicamente entrenados en fuerza y en resistencia, observándose perfiles específicos distintos, pero con un alto porcentaje de coincidencia

en dianas génicas y rutas metabólicas. A nivel de tejido se obtuvieron correlaciones entre los niveles circulantes de ciertos miRNA y su presencia en músculo e hígado. El perfil de miRNA en ambos tejidos se relacionó de manera significativa con la capacidad de adaptación al entrenamiento. Por último, en vista del aumento del miR-29a en circulación y su relación con las adaptaciones a nivel muscular, se analizó el rendimiento en ratones *KO* para este miRNA, que mostraron un claro deterioro tanto en fuerza como en resistencia.
En conclusión, los c-miRNA tienen un claro papel en la respuesta molecular y en la adaptación al ejercicio, emergiendo como potenciales biomarcadores en este contexto.

RESUMEN (en Inglés)

The study of the molecular response to exercise was historically limited to muscle tissue. However, its systemic effect is not restricted to the tissues involved in generating movement, so exercise response involves important intertisular communication. Apart from the classical mechanisms, widely studied, mediated by hormones or cytokines, in recent years the role of exosomes has been highlighted in this field. Encapsulated in these exosomes are different macromolecules, including microRNAs (miRNAs), small non-coding RNA molecules with a post-transcriptional regulatory function. Although their activity is intracellular, they have been stably detected in different biological fluids, constituting the so-called circulating miRNA (c-miRNA). The presence of c-miRNA in plasma exosomes suggests that they would be secreted in a regulated manner in response to a stress situation, acting as a true intercellular communication system and regulating gene expression and phenotype of distant receptor cells.
The studies available to date agree that both acute exercise and training modify c-miRNA profiles.
Therefore, the main objective of this Thesis, in the form of a compendium of publications, is the characterization and validation of c-miRNA as exercise biomarkers and as regulators of the response and molecular adaptation to exercise.
In the first paper, a review is carried out in which it was concluded that, based on the available knowledge, the value of c-miRNA as biomarkers in the field of exercise is not solid, due to the lack of coherence between studies in the results obtained and due to the methodological differences, whose influence is analysed in depth.
In the second study, the response to different doses of aerobic exercise from a broad panel of c-miRNA is analyzed, observing different profiles depending on the exercise dose, as well as a progression over a season and the state of training.
The third publication describes, for the first time, functional sub-profiles of c-miRNA by their correlation with physiological and biochemical markers of response to a maximal incremental exercise test.
In the forth publication, the response to various doses of acute aerobic exercise from a restricted panel of c-miRNA previously described in cardiac pathology is studied in parallel, together with other classical markers. Our results highlight the relevance of c-miRNA as emerging cardiac biomarkers, even in the absence of necrosis, overload or cardiac dysfunction, and their potential role in the cardiac response to exercise.
In the fifth study, the need to use animal exercise models for the study of potential sources and tissue targets of c-miRNA detected in response to exercise is raised, as well as the possibility of studying genetically modified models. To this end, c-miRNA profile was analyzed in humans and in experimental animals (mice) chronically trained in resistance and endurance, observing different specific profiles, but with a high percentage of coincidence in gene targets and metabolic pathways. At the tissue level, correlations were obtained between the circulating levels of certain miRNA and their presence in muscle and liver. The miRNA profile in both tissues was significantly related to the ability to adapt to training. Finally, in view of the increase in circulating miR-29a and its relationship with muscle-level adaptations, performance in KO mice was analyzed for this miRNA, which showed a clear deterioration in both resistance and endurance.
In conclusion, c-miRNAs have a clear role in molecular response and exercise adaptation, emerging as potential biomarkers in this field.

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SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y CELULAR

A los incondicionales,
Aguantar, luchar y crecer juntos

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Abreviaturas

RNA: acido ribonucleico

miRNA: microRNA, microARN

c-miRNA: microRNA circulante

CK: creatina quinasa

CKMB: creatina quinasa isoforma cardiaca

CRP: proteína C reactiva

myomiR: microRNA muscular

AGO: proteína argonauta

10K: 10 km

MM: media maratón

M: maratón

Índice

Reflexión inicial

Estamos en una sociedad donde nuestros pensamientos se reciclan al momento, se cambian en función de la influencia de un mundo que nos es constantemente atractivo y cambiante al mismo tiempo. Las modas se pasan con una velocidad inverosímil desde hace unos años. Sin embargo, nos olvidamos de lo que somos, animales. Animales regidos por ciclos, por adaptaciones fisiológicas, respuestas hormonales y expresiones génicas como todos nuestros acompañantes del reino animal. No dejamos de ser células, pero nos olvidamos. Nos olvidamos de que nuestra esencia es la actividad física, esa actividad que nos permite desplazarnos y avanzar. Todo lo que tenemos hoy en día parte de la capacidad de movimiento y la bipedestación del ser humano, pero nos olvidamos. La capacidad integradora del ejercicio físico no es suficiente para que todos tomemos conciencia de su importancia. El sedentarismo llena nuestras casas, pero nuestro organismo responde pidiendo actividad en respuesta al confinamiento. Hay luz al final del túnel.

Introducción

La actividad física ha sido postulada como "*the real polypill*"(1), su capacidad de prevenir patologías es inigualable dado que va desde síndromes metabólicos como la obesidad o la diabetes(1), hasta patologías netamente genéticas como el Huntington(2) o incluso en enfermedades con factores intrínsecos y extrínsecos como el cáncer(3). Además de esto, el ejercicio físico de alto rendimiento se correlaciona con una mayor longevidad(4, 5). Por lo que me gustaría dejar aquí una pregunta, ¿A qué esperamos los seres humanos para no perder los orígenes?

Por otra parte, el numero de participantes en eventos deportivos sube cada año produciéndose una polarización en la sociedad entre personas superactivas y sedentarias. Cuando el fin a alcanzar es un mayor estado de salud aparece la dualidad sistémica del ejercicio, en la que cohabitan el gasto energético y el fortalecimiento de aquellos que lo

practican. De este modo y ante esta disyuntiva, se abren los siguientes debates, ¿cuánto es suficiente? Y ¿cuál es el límite? Dos preguntas que hasta ahora no tienen respuesta concreta y que sirven de base hipotética (6).

El órgano efector del ejercicio físico es el músculo, con lo que la respuesta simple a ambas preguntas sería aquel ejercicio que generase una demanda muscular, pero sin llegar a producir un daño tan elevado que no permitiese el correcto funcionamiento del organismo. Asociado a este primer punto surgen los primeros análisis clásicos asociados al ejercicio. Ya en 1907 Fletcher determina como las concentraciones de lactato en el musculo son diferentes en condiciones anaeróbicas o en presencia de oxigeno (7). Además, el hecho se hace aún mas importante cuando se observa que a nivel sanguíneo estos niveles también se modifican con el ejercicio (8). Por otra parte, pero de manera análoga, se descubre la participación de los fosfatos en el metabolismo energético del músculo(9) y posteriormente, la presencia de la principal enzima de este proceso, la creatina kinasa(CK), en el flujo sanguíneo (10). Ambos parámetros, lactato y CK, representan en parte el tipo de ejercicio realizado metabólicamente hablando y la carga de dicho ejercicio a nivel muscular. Sin embargo, su secreción es diametralmente opuesta, en el caso del lactato es activa y regulada y en el caso de la CK es pasiva y mediada por la permeabilidad de la membrana. Por tanto, los procesos asociados al musculo esquelético comienzan a hacerse complejos y son valorados por Goldstein en 1961 en la regulación humoral del musculo en la respuesta a la glucemia (11). Por otro lado, el efecto de la permeabilidad de la membrana no se circunscribe al musculo esquelético sino que también se detecta en el musculo cardiaco con la presencia circulante de la isoforma cardiaca de la creatina kinasa (CKMB)(12). Este daño muscular observado, lleva consigo la inflamación que emerge como un proceso íntimamente relacionado con las adaptaciones al ejercicio. La medición de parámetros inflamatorios como la proteína C

reactiva (CRP) o la haptoglobina y su modificación en respuesta al ejercicio caracterizan también esta intrínseca unión entre ejercicio e inflamación(13). Esta relación no se queda en una relación acción-reacción, sino que es mucho más que eso. En 1998 se describe por primera vez por Ostrowski y cols. la secreción por parte del músculo esquelético de la citokina IL-6 (14). Con este hecho el músculo deja de ser solo un efector y pasa a ser un regulador más en la función endocrina de nuestro organismo. Por tanto, el análisis circulante de los marcadores inflamatorios pasa a ser un estudio del dialogo intertisular establecido(15). Ahondando en esta idea, se comienza la medición de biomarcadores específicos de ejercicio. Hasta ese momento los parámetros medidos eran consecuencias del ejercicio realizado, pero a partir de este punto el ejercicio pasa a ser un regulador de la respuesta sistémica y no un mero ejecutor con consecuencias.

Este dialogo intertisular del ejercicio, nos abre otra pregunta retornando a origen, ¿es el lactato el primer metabolito regulador intertisular descrito en el ejercicio?(16) La historia tiene muchos puntos de vista, si analizamos el lactato vemos un punto de partida, el musculo, y una diana, el hígado, o varias, intestino y músculos adyacentes; donde se resintetiza a glucosa para volver a ejercer su función energética(17-19). Dejando que la historia avanzase y con ello aumentara el conocimiento molecular del ejercicio, la intercomunicación emerge con Whitham y col. en 2018 describiendo como los exosomas actúan de motor de acción de esta regulación, no solo post ejercicio sino también durante(20). Su acción nos devuelve al mismo tejido que el lactato, el hígado, donde principalmente van a ser metabolizados dichos exosomas y microvesículas. Por otro lado, los exosomas tienen una mayor complejidad que el lactato, en ellos son transportados lípidos, proteínas, DNA y RNA (21, 22).

La utilización de un tejido como la sangre, por parte de nuestro organismo, para su intercomunicación en el ejercicio hace que emerja la opción de analizar dichas moléculas

como biomarcadores de ejercicio. Dentro de las moléculas presentes en circulación libres, en exosomas y microvesiculas se presentan los microRNAs(miRNA) como posibles biomarcadores de patologías(23) y en el ámbito del ejercicio como ya propuso Baggish y cols. en 2011(24)y analizó Fernández-Sanjurjo y cols. en 2018(25).

Los miRNA son pequeñas moléculas de RNA entre 19-25 nucleótidos no codificantes que participan en la regulación negativa a nivel post-transcripcional de la expresión génica (26). La importancia de su papel regulador de la expresión génica queda de manifiesto por el hecho de que el conjunto de los miRNA expresados en humanos, más de 2600 según miRBase, la principal base de datos de miRNA(27), tiene diana en aproximadamente el 60 % de las secuencias codificantes del genoma(28), jugando un papel fundamental en el desarrollo, el mantenimiento de la homeostasis y la respuesta al estrés fisiológico y fisiopatológico (23) incluido el ejercicio.

Los genes que contienen los miRNAs pueden estar localizados en zonas intergénicas, donde la regulación de su expresión es producida por sus propios elementos, o bien en regiones intrónicas o exónicas, donde la expresión del miRNA está íntimamente relacionada con la expresión del propio gen en cuestión(29).

La biogénesis de los miRNA comienza en el núcleo celular y finaliza en el citoplasma. En el núcleo, la transcripción de los miRNA se produce mediante la RNA polimerasa II, produciéndose un transcrito primario de cientos de nucleótidos denominado pri-miRNA. Los pri-miRNAs tiene una estructura secundaria de horquilla que tiene en el extremo 3’ una cadena de poliA y en el extremo 5’ una caperuza de 7-metil-guanosina(29). El pri-miRNA es reconocido por un complejo formado por la ribonucleasa tipo III (Drosha) y su proteína de ligación DGCR8 (Región Crítica 8 del síndrome de DiGeorge) o Pasha. La ribonucleasa Drosha tiene una doble función, la de estabilización de la estructura en horquilla y la de realizar el corte de los extremos 3’ y 5’

del pri-miRNA. De este modo, queda solo una secuencia de unos 70 nucleótidos, de estructura en horquilla que pasa a denominarse pre-miRNA. Una vez llegado este punto, el pre-miRNA es exportado al citoplasma mediante la exportina 5 (EXP5) que crea un complejo con la proteína RAN-GTP(30). Este complejo utiliza el consumo de uno de los fosfatos pasando de GTP a GDP+P y permitiendo la exportación del pre-miRNA al citoplasma celular.

En el citoplasma celular, se produce el siguiente paso de la biosíntesis del miRNA. Al pre-miRNA se une la proteína DICER, una RNA endonucleasa III que producirá un procesamiento posterior. En la selección de los transcritos participa la proteína TRBP la cual a través de sus tres dominios realiza una labor de selección, desechando aquellos pre-miRNA con uniones no canónicas y seleccionando para su procesamiento aquellos con una estructura secundaria correcta. A estas dos proteínas se asocia la proteína Argonauta, que participará en la función reguladora posterior de los miRNAs(31). Una vez procesado el pre-miRNA se obtiene el miRNA de doble cadena o dúplex. Dicho miRNA de doble cadena queda, por criterio termodinámico, reducido a una sola cadena siendo aquel que tiene una unión más inestable en el extremo 5' el que queda como miRNA maduro. En algunos casos como ocurre en la Drosophila melanogaster, la selección es realizada por una proteína denominada R2D2(30).

Con todos estos pasos, se obtiene el miRNA maduro unido a un complejo denominado miRISC formado en mayor parte por las proteínas Argonautas. En el caso de los mamíferos tiene especial importancia la proteína Argonauta 2(AGO2) con su ayuda en la función reguladora del miRNA sobre el RNA mensajero (mRNA)(32).

La función ejecutiva de los miRNAs no es otra que la represión de la traducción de los mRNAs bien por degradación de los mismo, si la complementariedad es total, o bien por inhibición de la traducción, si la complementariedad no es total. La degradación es

llevada a cabo por el dominio de AGO2 con función endonucleasa(32). Cuando la complementariedad no es total la degradación del mRNA se produce por la deadenilización de la cola de poli-A mediante la interacción del complejo miRISC con la proteína de unión al poli(A) (PABP)(33) y el reclutamiento de las proteínas deadenilasas CAF1 y CCR4(34). Dado que la mayoría de los sitios diana en el mRNA sólo tienen complementariedad de bases parcial con cada miRNA, un mismo miRNA puede interaccionar con más de 100 mRNAs diferentes. Además, cada mRNA puede contener múltiples sitios de unión para diferentes miRNAs, dando lugar a una compleja red de regulación de la expresión génica (35, 36).

Aunque los miRNAs son reguladores intracelulares de la expresión génica, también se han detectado en forma estable en diferentes fluidos corporales. En 1999 Kopreski y cols. (37) describen por primera vez la presencia en suero de moléculas de RNA que tienen la capacidad de no ser degradados por las RNAsas presentes en el flujo sanguíneo. Los miRNAs circulantes (c-miRNAs), como tales, fueron descritos por primera vez en 2008 por Mitchell y cols. (38) también en sangre, si bien posteriormente han sido descritos en los demás fluidos corporales (39). Los c-miRNAs están presentes en los fluidos corporales bien transportados como ya se ha introducido anteriormente en exosomas o microvesículas(22, 40), bien asociados a proteínas necesarias en su procesamiento (como Ago2 o Npm1)(41, 42), a lipoproteínas (43) o bien incluso a cuerpos apóptóticos (35). Todo ello sugiere que los c-miRNAs son secretados de forma regulada en respuesta a una situación de estrés, actuando como un auténtico sistema de comunicación intercelular, autocrina, paracrina y endocrina, regulando la expresión

génica y el fenotipo de las células receptoras (23, 44). Además de poder ser liberados de forma pasiva por células dañadas o necróticas (45).

De forma análoga a sus formas intracelulares, los c-miRNAs participan tanto en respuestas fisiológicas y adaptativas, como en el inicio y en el desarrollo de estados patológicos(46).

En respuesta al ejercicio son descritos por primera vez por Baggish y cols. en 2011(24) en entrenamiento y ejercicio agudo. Seguidamente se realizó la aproximación histórica al ejercicio centrada en el músculo, que ha sido presentada con anterioridad, donde se realizaron búsquedas concretas en circulación de aquellos miRNA más expresados en el músculo esquelético (*myomiRs*)(47-52), llegando a ser propuestos como biomarcadores de daño muscular al igual que la CK(53). Sin embargo, son ya los propios autores de este artículo Siracusa y cols. los que proponen que no son los solo los *myomiRs* los que han de ser estudiados en respuesta al daño muscular(54). En este sentido, Sawada y cols. y Nielsen y cols. son los primeros en estudiar una aproximación global de c-miRNAs en respuesta a ejercicio de fuerza y ejercicio agudo y entrenamiento de resistencia, respectivamente (55, 56). De este modo, se remarca la labor con anterioridad mencionada del ejercicio como regulador y no como mero efector, dado que se definen perfiles específicos en función del ejercicio realizado. La presencia de c-miRNAs en respuesta al ejercicio detalla el hecho de una funcionalidad en una localización distante del tejido secretor(57).

La propuesta de los c-miRNAs como biomarcadores de ejercicio es algo ya estudiado hasta el momento en diferentes revisiones(58, 59). El Grupo de Trabajo Internacional de Definición de Biomarcadores define biomarcador como un marcador biológico que pueda ser objetivamente medido y evaluado como un indicador de normalidad de los procesos biológicos, de procesos patológicos o de respuesta

farmacológica a una intervención terapéutica(60). Además, desde un punto de vista clínico, es aceptado que los biomarcadores tengan un papel biológico en la patología estudiada, pero también podrían ser aceptados aquellos en los que no existe una relación de causa-efecto estudiada. La correlación de los biomarcadores clásicos de ejercicio con los c-miRNAs fue definida por primera vez por Bye y cols. observando un perfil específico de miRNA asociado al consumo máximo de oxígeno (VO_2max). Mayores niveles de miR-21, miR-210 y miR-222 fueron observados en aquellos sujetos con menor capacidad aeróbica (61). En el mismo sentido, Mooren y cols. obtuvieron una relación positiva en respuesta a una maratón entre la expresión de miR-1, miR-133a y miR-206, el VO_2max y la velocidad de carrera(48). Por otra parte, realizando comparativas entre poblaciones, Clauss y cols obtuvieron perfiles específicos en respuesta a una maratón en función de que los atletas fueran amateur o elite(62) y Wardle y cols. definieron perfiles basales específicos en poblaciones altamente entrenadas en resistencia y en fuerza(63).

Sin embargo, como se ha analizado en la revisión de Fernández-Sanjurjo y cols. la heterogeneidad de los resultados hasta el momento no permite concretar si realmente los c-miRNAs son buenos biomarcadores de ejercicio. La falta de control de los factores externos que influyen sobre el perfil de c-miRNA como la dieta y la obtención de muestras, la heterogeneidad de los sujetos analizados, así como la metodología dificultan la extracción de conclusiones al respecto. Además, la falta de datos sobre la funcionalidad o no de dichos c-miRNA en los tejidos diana o en sus dianas génicas relacionadas con el ejercicio nos hace perder de vista la definición de un buen biomarcador. Con esta base teórica, nuestra hipótesis es que los miRNA modulan las diferentes respuestas agudas y adaptativas al ejercicio, pudiendo tener un valor añadido como biomarcadores de esta respuesta.

Los objetivos para la determinación de dicha hipótesis son:

1. Definición teórica del potencial de los miRNA circulantes como biomarcadores de ejercicio en sanos y enfermos.
2. Analizar la respuesta aguda de c-miRNA a diferentes volúmenes e intensidades de ejercicio de resistencia, establecimiento de dosis de ejercicio.
3. Caracterizar la respuesta aguda de los c-miRNA en esfuerzo aeróbico máximo y la generación de subperfiles asociados a parámetros fisiológicos.
4. Determinar si la carga cardiaca en distintas dosis de ejercicio se caracteriza mejor mediante los microRNA circulantes o mediante biomarcadores proteicos clásicos.
5. Analizar el perfil basal de miRNA circulantes en personas entrenadas y sedentarias.
6. Comparar el perfil basal descrito de c-miRNA en humanos con el perfil de ratones entrenados y sedentarios.
7. Analizar los posibles tejidos diana y secretores de los miRNA presentes en exosomas en respuesta al entrenamiento.
8. Determinar el papel regulador de los miRNA sobre el rendimiento físico

ARTICLE

Circulating microRNA as Emerging Biomarkers of Exercise

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FERNÁNDEZ-SANJURJO, M., D. DE GONZALO-CALVO, B. FERNÁNDEZ-GARCÍA, S. DÍEZ-ROBLES, Á. MARTÍNEZ-CANAL, H. OLMEDILLAS, A. DÁVALOS, and E. IGLESIAS-GUTIÉRREZ. Circulating microRNA as emerging biomarkers of exercise. *Exerc. Sport Sci. Rev.*, Vol. 46, No. 3, pp. 160–171, 2018. *An interest has recently emerged in the role of circulating microRNAs (c-miRNAs) as posttranscriptional regulators, intercellular communicators and, especially, as potential biomarkers of the systemic response to acute exercise and training. We propose that, with the limited, heterogeneous, and mainly descriptive information currently available, c-miRNAs do not provide a reliable biomarker of exercise in healthy or diseased individuals.* **Key Words:** circulating microRNAs, biomarkers, gene expression regulators, acute exercise, training

Key Points

- microRNAs regulate gene expression; some of them can be found in plasma.
- Plasma levels of circulating microRNAs (c-miRNAs) change in response to acute exercise and training in healthy and diseased people, and so c-miRNAs have been proposed as biomarkers of exercise response.
- However, their value as exercise biomarkers is not solid and still controversial, mainly because the number of articles published to date on this topic show inconsistent results and use a limited number of subjects, preventing a validation step or a generalization to a larger population.
- Clarifying important unanswered questions could shed some light on their value as biomarkers of exercise response in health and disease: what are their secretory tissues, how it is the way they are transported, are they released in a meaningful amount to exert a significant and essential biological effect, and which are their target tissues and genes.

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INTRODUCTION

Regular exercise is one of the main determinants of health because it is strongly associated with a lower risk of mortality and lower incidences of the most prevalent chronic pathologies in developed countries, such as cardiovascular disease; stroke; metabolic syndrome; type 2 diabetes; some types of cancer: prostate, colon, or breast cancer; and depression (1).

The beneficial effect of exercise on organic health is systemic and is not restricted to those tissues that are most actively involved in generating movement (2). Countless investigations have demonstrated that the response to acute exercise and training involves a complex cross-communication between tissues and has profound effects on gene expression. The study of the molecular response to exercise is, therefore, an essential tool for understanding how this systemic response is integrated and how it relates to health status (3). This understanding will allow for the discovery of new potential mechanisms involved in prevention of pathophysiological alterations associated to disease processes as well as new therapeutic targets (1). From a practical perspective, understanding the insights of the molecular response to exercise is essential to optimize performance and exercise recommendations and to explore the limits of healthy exercise.

Molecular adaptive responses to exercise are largely determined by altered gene expression and subsequent protein synthesis, although the exact mechanisms that orchestrate this systemic regulation remain mostly unknown (2). In recent years, an increasing interest has emerged in analyzing the influence of exercise on the profile of circulating microRNAs

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(c-miRNAs), which are intercellular communicators with a posttranscriptional regulatory role (4).

MicroRNAs (miRNAs) are small noncoding RNAs that regulate more than 60% of human protein-coding genes (5) and play a critical role in the regulation of homeostasis and the stress response (4).

In addition to the intracellular locations in which miRNA biogenesis occurs, miRNAs have been detected in different body fluids, including blood. c-miRNAs can be actively or passively released from tissues and regulate the gene expression of distant cells, acting as a true intercellular communication mechanism (4). miRNAs also have been described as powerful emerging and minimally invasive biomarkers in a number of physiological and pathological conditions and define disease-specific profiles (6).

In recent years, an increasing number of methodically diverse studies have analyzed the effects of acute exercise and training on the c-miRNA profile of active healthy people (7–22). In addition, a limited number of studies have been published about the effects of acute exercise and training programs on the altered c-miRNA profiles described in several pathological situations for which exercise is used as an adjunctive therapy, such as diabetes and obesity (23–28). Most of these studies demonstrate that acute exercise and training elicit changes in the expression, type, and circulation appearance-clearance kinetics of certain c-miRNAs in both health and disease. This profile has been suggested to depend on the mode, intensity, and dose of exercise (29), and c-miRNAs have been repeatedly proposed as promising biomarkers in this context (30,31). However, the results obtained are heterogeneous, most likely due to huge differences in the methodological and experimental approaches. Furthermore, validation studies are virtually nonexistent and the information available is mainly observational. Finally, the concept of an exercise biomarker is vague, and the actual value of c-miRNAs in this context is still controversial.

All this makes it difficult to obtain general conclusions that allow for a clearer view of the role of these regulators of gene expression in the molecular response to exercise, its possible practical implications for health and performance, and their value as biomarkers in this context.

Our hypothesis is that, considering the limited, heterogeneous, and mainly descriptive information currently available, c-miRNAs do not provide a reliable biomarker of exercise response in healthy or diseased individuals.

For these reasons, the aims of this review are the following: 1) to synthesize the information that is available about the effects of acute exercise and training protocols on the c-miRNA profiles of healthy and diseased people, 2) to identify the determinants of the heterogeneity of the results obtained and analyze their influences on this profile, and 3) to clarify the value of c-miRNAs as biomarkers of exercise response.

CIRCULATING MIRNAS IN RESPONSE TO EXERCISE IN HEALTH AND DISEASE

The effect of acute exercise on the plasma profile of c-miRNAs was first described in 2011 by Baggish *et al.* (8). Since then, an increasing number of studies have been published about the c-miRNA response to acute exercise and training in healthy and diseased individuals. A number of reviews have successively compiled the updated information available, although none of them has considered the inclusion of studies about patients.

Tables 1 and 2 summarize the main characteristics of the studies that have analyzed the effects of acute exercise and training programs on the c-miRNA profiles of healthy volunteers. Studies with apparently similar experimental designs have failed to find the same behaviors of specific miRNAs. Thus, whereas Mooren *et al.* (18), Baggish *et al.* (8) and Clauss *et al.* (11) observed increases in miR-1 and miR133a immediately after a marathon, de Gonzalo-Calvo *et al.* (15) describe no changes in these miRNAs, and only Mooren *et al.* (18) observed increases in miR-206 and miR-208b (Table 1). Similarly, after a period of training with a cycle ergometer, no common changes in the miRNA profile were detected by Nielsen *et al.* (19) and Aoi *et al.* (7) (Table 2). In contrast, similar changes for the same miRNAs have been observed in response to exercises of very different natures. For example, increases in miR-133a and miR-133b were observed 2 and 6 h posteccentric downhill running by Banzet *et al.* (10), immediately after a sprint interval cycling test by Cui *et al.* (13), or immediately after a marathon and after acute resistance exercises (lateral pulldown, leg press, and butterfly) by Uhlemann *et al.* (21), although this latter author used the imprecise nomenclature miR-133. However, other authors failed to find changes in these miRNAs after acute bouts of endurance (7,8,15,19,21), resistance (17,20), and vigorous-intensity continuous exercise (14). Some authors describe opposite results, such as Cui *et al.* (12), who found a significant decrease in miR-133a immediately after muscular hypertrophy and maximum strength resistance exercises (Table 1). Similarly, Baggish *et al.* (8) and Nielsen *et al.* (19) observed increases in miR-21 both after 90 d of rowing training and after 12 wk of cycling training, respectively, but no other common changes were observed, despite the fact that Nielsen *et al.* (19) analyzed 160 different microRNAs.

The scenario is even more complex and heterogeneous for studies that have analyzed the effect of acute exercise and training programs on the c-miRNA profiles of diseased volunteers for whom exercise is recommended as a positive adjunctive therapy (Tables 3, 4). Each study included in this review focused on a different pathological situation, and not a single coincident change in any miRNA was described despite the considerable number of common miRNAs that were analyzed.

An additional confounding factor is the controversy in miRNA nomenclature among studies. This situation is evident, for example, in light of the study of Uhlemann *et al.* (21), who used the nomenclature miR-133, without specifying whether they refer to miR-133a or miR-133b and with their respective -3p and -5p chains. The same situation occurs for cel-miR-39, which is commonly used for normalization purposes and for which no information about the -3p or -5p chains often is provided by most authors (Table 5).

Summarizing, together with the diverse approaches used by the different authors, it also is evident the heterogeneity of the results obtained. This situation raises doubts not only about the lack of coincidence in the response of certain miRNAs but also about the coincidences themselves. Therefore, the notion that the diverse methodological approaches are not responsible for the heterogeneous observed results cannot be excluded.

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TABLE 1. Studies on the profile of circulating microRNAs in response to acute exercise in healthy people.

Type of Exercise		Subjects	miRNAs Analyzed	Sampling Points	Results		Reference
					Increase	*Decrease*	
Endurance aerobic exercise	Exercise in cycle ergometer. Incremental test (25 W·min^{-1}) until extenuation, before and after a 90-d rowing training period.	10 male university rowers (19.1 ± 0.6 yr).	miR-20a, miR-21, miR-133a, miR-146a, miR-210, miR-221, miR-222, and miR-328.	Before exercise, immediately after (within 1 min of completion), and after 1 h of rest postexercise.	Pretraining period, immediately postacute exercise: miR-21, miR-146a, miR-221, and miR-222. Pretraining period, 1 h postexercise: miR-221. Posttraining period, immediately postacute exercise: miR-146a and miR-222.		Baggish *et al.* (8)
	Exercise in cycle ergometer. 60 min at 70% of $\dot{V}O_{2max}$.	11 males without training background (21.5 ± 4.5 yr).	miR-1, miR-133a, miR-133b, miR-206, miR-208b, miR-486, and miR-499.	Before and immediately after, 3 h, and 24 h postexercise.		Postexercise: miR-486.	Aoi *et al.* (7)
	Exercise in cycle ergometer. 60 min at 65% of peak power output.	13 trained males (28 ± 8 yr).	752 miRNAs in a commercial miRNA PCR panel.	Immediately before, immediately after, 1 h, and 3 h postexercise.	1 h postexercise: miR-139-5p, miR-143, miR-223, miR-330-3p, and miR-338-3p. 3 h postexercise: miR-1.	Immediately postexercise: miR-30b, miR-106a, miR-146, miR-151-3p, miR-151-5p, miR-221, miR-652, and let-7i.	Nielsen *et al.* (19)
	Exercise in cycle ergometer. 4 h at 70% of individual anaerobic threshold.	12 trained males (32.4 ± 2.3 yr).	miR-126 and miR-133.	Before, during the race (5, 10, 15, 30, 60, 120, 180, 240 min), 1 h, and 24 h postexercise.	Increased 30 min after the start and remained elevated until the end of the test: miR-126.		Uhlemann *et al.* (21)
	Marathon	14 trained male endurance runners (42.8 ± 6.0 yr).	miR-1, miR-21, miR-133a, miR-155, miR-206, miR-208b, and miR-499.	2 d before, immediately after, and 24 h postexercise.	Immediately postexercise: miR-1, miR-133a, miR-206, miR-208b, and miR-499. 24 h postexercise: miR-208b and miR-499.		Mooren *et al.* (18)
	Marathon	22 male marathon runners (56.8 ± 5.2 yr).	miR-126 and miR-133.	Immediately before and after finishing.	Immediately postexercise: miR-126 and miR-133.		Uhlemann *et al.* (21)
	Marathon	21 male marathon runners (51.8 ± 1.4 yr).	miR-1, miR-126, miR-133a, miR-134, miR-146a, miR-208a, miR-422b, and miR-499-5p.	The day before, immediately before, and the following day.	Immediately postexercise: miR-1, miR-126, miR-133a, miR-134, miR-146a, miR-208a, and miR-499-5p.		Baggish *et al.* (9)
	Marathon and 10-k running.	9 male amateur runners (39.1 ± 2.2 yr).	106 inflammation-related miRNAs and 4 myomiRs (miR-1, miR-133a, miR-133b, and miR-206).	Before, immediately after, and 24 h postexercise.	Immediately after marathon: miR-29a-3p, miR-34a-5p, miR125b-5p, miR-132-3p, miR-143-3p, mirR-148a-3p, miR-223-3p, miR-223-5p, miR-424-3p, miR-424-5p, let-7d-3p, and let-7f-2-3p. Immediately after 10 k: miR-150.		de Gonzalo-Calvo *et al.* (15)

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	Marathon	30 male marathon runners: 15 amateur (40.1 ± 1.4 yr) and 15 elite (40.0 ± 1.7 yr).	miR-1, miR-26a, miR-29b, miR-30a, and miR-133a.	2–5 d before and immediately postexercise.	Immediately postexercise: miR-1, miR-30a, and miR-133a in elite runners and miR-30a in amateur runners.	24 h postexercise: miR-26a in elite runners.	Clauss *et al.* (11)
	Half marathon	5 male amateur runners (31.6 ± 4.4 yr).	miR-1, miR-133a, miR-206.	Before warming and up to 10 min postexercise.	Immediately postexercise: miR-1, miR-133a, and miR-206.		Gomes *et al.* (16)
	Uphill and downhill walking on treadmill 30 min at at a constant velocity of 1 $m \cdot s^{-1}$, with a grade of 25%, and an additional load carriage of 12% of body weight (loaded backpack).	9 active males (27–36 yr).	miR-1, miR-133a, miR-133b, miR-208a, miR-208b, and miR-499.	Before, immediately after, and 2, 6, 24, 48, and 72 h postexercise.	2 and 6 h postdownhill exercise: miR-1, miR-133a, miR-133b, and miR-208b. Immediately postuphill exercise: miR-181b and miR-214.		Banzet *et al.* (10)
	Vigorous-intensity continuous exercise, running.	26 active males (20.38 ± 0.12 yr).	TaqMan Low-Density Array of 754 miRNAs with subsequent validation by qPCR.	Before and immediately postexercise.	Immediately postexercise: miR-1, miR-133a, miR-133b, miR-206, miR-485-5p, miR-509-5p, miR-517a, miR-518f-3p, miR-520f, miR-522, miR-553, and miR-888.		Cui *et al.* (14)
	High-volume training on cycle ergometer. 2 h at 55% of peak power output, with 10 min of warm-up at 50% of peak power output.	12 trained males (24.7 ± 3.4 yr).	miR-16, miR-21, and miR-126.	Before and 0, 30, 60, and 180 min postexercise.	Immediately postexercise: miR-21 and miR-126.		Wahl *et al.* (22)
Resistance exercise	Bench press and leg press. 5 sets of 10 repetitions at 70% of 1-RM.	12 active males (29.9 ± 1.2 yr).	More than 3000 miRNA probes in a microarray and miR-20a, miR-21, miR-133a, miR-146a, miR-149[a], miR-1908, miR-210, miR-221, miR-222, and miR-328.	Before, immediately after, and 60 min, 1, and 3 d after exercise.	3 d postexercise: miR-149a.	3 d postexercise: miR-146a and miR-221.	Sawada *et al.* (20)
	Lateral pulldown, leg press, and butterfly. 3 sets of 15 repetitions, with a 1-min pause between sets, and an increased eccentric load of 25% of the concentric load, determined 1 wk before.	11 trained volunteers (37 ± 2 yr): 4 males and 7 females.	miR-126 and miR-133.	Before (undetermined, on the same day), immediately after, and 1 h after exercise.	Immediately postexercise: miR-133.		Uhlemann *et al.* (21)
	Sprint interval cycling modeled on the Wingate Anaerobic test. Two 30-s all-out sprints against a force load of approximately 7.5% of subject's body weight, with 4 min of active recovery between.	18 active males (20.23 ± 0.97 yr).	miR-1, miR-16, miR-122, miR-133a, miR-133b, miR-206, and miR-499.	Before and immediately after (within 1 min of completion).		Immediately postexercise: miR-1, miR-16, miR-122, miR-133a, and miR-133b.	Cui *et al.* (14)

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TABLE 1. *(Continued)*

Type of Exercise		Subjects	miRNAs Analyzed	Sampling Points	Results		Reference
					Increase	*Decrease*	
	Bilateral knee extension exercise and bilateral leg press exercise. 3 sets of 10 repetitions at 80% of 1-RM.	18 sedentary male volunteers: 9 young (22 ± 1 yr) and 9 old (74 ± 2 yr).	372 miRNAs in a commercial miRNA PCR array and miR-34a, miR-181, miR-206, miR-208b, miR-324, and miR-486.	24 h before, immediately after, and at 6 h postexercise.	6 h postexercise, younger participants: miR-18a-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, miR-195-5p, miR-206, miR-221-3p, and miR-486.		Margolis *et al.* (17)
	Strength endurance. 3 sets of 16–20 repetitions at 40% of 1-RM, with a 1-min rest interval.	15 active males (19.36 ± 0.14 yr).	TaqMan Low-Density Array of 754 miRNAs with subsequent validation by qPCR.	Before, immediately after, and 1 and 24 h postexercise.	1 and 24 h postexercise: miR-532.	Immediately postexercise and 1 h postexercise: miR-208b.	Cui *et al.* (12)
	Muscular hypertrophy. 3 sets of 12 repetitions at 70% of 1-RM, with a 2-min rest Interval.	15 active males (19.72 ± 0.20 yr).	TaqMan Low-Density Array of 754 miRNAs with subsequent validation by qPCR.	Before, immediately after, and 1 and 24 h postexercise.	1 h postexercise: miR-21, miR-181a, and miR-206. 24 h postexercise: miR-133b.	Immediately postexercise: miR-21 and miR-133a.	Cui *et al.* (12)
	Maximum strength. 4 sets of 6 repetitions at 90% of 1-RM, with a 3-min rest interval.	15 active males (18.87 ± 0.12 yr).	TaqMan Low-Density Array of 754 miRNAs with subsequent validation by qPCR.	Before, immediately after, and 1 and 24 h postexercise.		Immediately postexercise: miR-133a.	Cui *et al.* (12)
Intermittent high-intensity exercise	High-intensity interval running exercise. 7 bouts of 4 min of high-intensity running (~85%–95% of HRmax), interspersed with 2 min of active recovery in addition to 10 min of warm-up and 5 min of cool down, for an average running time close to 1 h.	26 active males (20.38 ± 0.12 yr).	TaqMan Low-Density Array with subsequent validation of 754 miRNAs by qPCR.	Before and immediately postexercise.	Immediately postexercise: miR-1, miR-133a, miR-133b, miR-206, miR-485-5p, miR-509-5p, miR-517a, miR-518f, miR-520f, miR-522, miR-553, and miR-888.		Cui *et al.* (14)
	High-intensity training in cycle ergometer. 4 × 4 min at 90%–95% of peak power output, separated by 3 min of active recovery at 45% of peak power output each, with 10 min of warm-up at 50% of peak power output.	12 trained males (24.7 ± 3.4 yr).	miR-16, miR-21, and miR-126.	Before and 0, 30, 60, and 180 min postexercise.			Wahl *et al.* (22)
	Sprint-interval training in cycle ergometer. 4 × 30 s of maximal effort separated by 7:30 min of active recovery at 45% of peak power output each, with 10 min of warm-up at 50% of peak power output.	12 trained males (24.7 ± 3.4 yr).	miR-16, miR-21, and miR-126.	Before and 0, 30, 60, and 180 min postexercise.	Immediately postexercise: miR-21 and miR-126.		Wahl *et al.* (22)

1-RM indicates one-repetition maximum; HRmax, maximum heart rate; and $\dot{V}O_{2max}$, maximal oxygen consumption.

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TABLE 2. Studies on the profile of circulating microRNAs in response to training in healthy people.

Type of Exercise	Training Protocol	Subjects	miRNAs Analyzed	Results		Reference
				Increase	*Decrease*	
Rowing	90 d of team-based training in open water and indoor ergometer, 1–3 $h \cdot d^{-1}$, 5 $k \cdot d^{-1}$ at 20–24 strokes/min.	10 male university rowers (19.1 ± 0.6 yr).	miR-20a, miR-21, miR-133a, miR-146a, miR-210, miR-221, miR-222, and miR-328.	miR-21, miR-146a, miR-221, miR-222.		Baggish *et al.* (8)
Cycling	12 wk of training in cycle ergometer, 5 times/wk: Monday, maximal power output test (Pmax); Tuesday, intervals at 85%–91% Pmax for 70–80 min, Wednesday, continuous exercise at 60%–66% for 60–80 min; Thursday, intervals at 75%–81% for 75–81 min; Friday, continuous exercise at 55%–61% for 120–150 min.	7 trained males (28 ± 5 yr).	752 miRNAs in a commercial miRNA PCR panel.	miR-21, miR-25, miR-148a, miR-185, miR-342-3p, miR-766, let-7d.	miR-103, miR-107.	Nielsen *et al.* (19)
Cycling	4 wk of training in cycle ergometer, 3 times/wk, 30 $min \cdot d^{-1}$ at 70% $\dot{V}O_{2max}$.	11 males without training background (21.5 ± 4.5 yr)	miR-1, miR-133a, miR-133b, miR-206, miR-208b, miR-486, and miR-499.		miR-486.	Aoi *et al.* (7)

DIFFERENT METHODOLOGICAL APPROACHES AND EXPERIMENTAL DESIGNS: BEHIND THE SCENES

The results obtained in the different studies are hardly complementary or comparable, mainly due to huge differences in the methodologies used (detection technique, normalization strategy, and treatment of missing values), in the experimental design (timing of sampling and type and number of c-miRNAs analyzed), and in the characteristics of the participants (age, exercise background, dietary intake, and pathologic condition). Next, we analyze in detail potential confounding influences of these factors.

Methods of c-miRNA Analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) has been the technique of choice by most authors who have analyzed c-miRNAs in response to exercise. However, this does not rule the technique out as a potential source of confusion between studies mainly because of the lack of information on the different approaches used for qPCR raw data processing, particularly cycle threshold, handling of missing data, and normalization strategy. Information on the first two is practically nonexistent in the literature as is the influence of dissociation and melting curve analysis on the final inclusion of amplified miRNAs (12–14,17,19,24,25). As expected, a variety of normalization strategies has been used by different authors, as summarized in Table 5, because no stable constitutive or exogenous miRNA or group of miRNAs have been established or validated for the normalization of miRNA expression in this context (11). Most authors have used cel-miR-39 for normalization; cel-miR-39 is a *Caenorhabditis elegans* miRNA that is added in equal quantities to all samples (11,15,17,18,20–22,24,26,27). Other authors have used specific software tools to detect which gene or group of genes are expressed more stably in their specific samples and use these genes for normalization (10,19). The amount of RNA extracted from serum or plasma samples is small and difficult to accurately quantify. Therefore, the amount of RNA that is initially added for miRNA detection will hardly be the same for all samples. Consequently, normalizing by an exogenous miRNA that is added in equal amounts in all samples or by an endogenous miRNA for which the raw expression is stable seem to be questionable options. A solid and common standardization strategy would be desirable, as proposed by Lee *et al.* (32). However, considering the limited and heterogeneous information about c-miRNAs in the context of exercise, this is not realistic nowadays. Carrying out a pilot study using different methods for data normalization, including synthetic miRNAs, endogenous miRNAs, and the mean expression of all analyzed miRNAs to identify the combination of normalizers that best suit the specific characteristics of each study would be desirable (33), and seems to be the best option at present. Unfortunately, this is not easy to achieve, and the confounding influence of data processing would persist.

Another important point is how miRNA *levels* are estimated from qPCR formulas. Although several models can be used, the literature describes two that are widely applied: the efficiency calibrated model (34) and the $\Delta\Delta$CT model (35). How miRNA levels are analyzed using these formulas also might be relevant. Whereas absolute quantification uses an internal or external calibration curve to derive the input template copy number, relative quantification relies on the comparisons between expressions of target genes versus a reference gene (ΔCT) (36). Most studies of circulating miRNAs in response to exercise use relative quantification. However, the use of miRNAs as biomarkers will probably require the use of a quantitative value (*i.e.*, copy number) to be used routinely in the clinical practice. Regarding the data quality control, most studies of miRNA expression in the field do not report the estimation of the amplification efficiency. Thus, the lack of proper quality control also could have a significant impact on the final data analysis (36).

miRNAs Analyzed

Most authors have analyzed the circulating levels of a selection of one or a few miRNAs (typically between three and eight; Tables 1–4). In most cases, the selected miRNAs are among those that are enriched in skeletal muscle, *i.e.*, the so-called myomiRs: miR-1, miR-133a, miR-133b, miR-206a, miR-208b, miR-486, and miR-499. Notably, very few authors have analyzed all myomiRs (7,12,14,19), and the selections considered in the remaining studies are not always the same. In addition, several

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TABLE 3. Studies on the profile of circulating microRNAs in response to acute exercise in patients.

Condition	Type of Exercise	Subjects	miRNAs Analyzed	Sampling Points	Results		Reference
					Increased	*Decreased*	
Heart failure	Endurance aerobic exercise	Maximal symptom-limited incremental cardiopulmonary exercise test in cycle ergometer: 3 min walking on the treadmill, 3 min cycling at 60 rpm without any resistance, 2 min at 20 $J \cdot s^{-1}$ increasing by 5 $J \cdot s^{-1}$ every 30 s.	28 male patients (59.07 ± 1.79 yr).	miR-1, miR-21, miR-126, miR-133a, miR-133b, miR-146a, miR-155, miR-208a, miR-208b, miR-210, miR-221, miR-328, miR-378, miR-486, miR-499, and miR-940.	Before and immediately postexercise.	Immediately postexercise miR-21, miR-378, and miR-940.		Xu *et al.* (27)
Hypercholesterolemia	Endurance aerobic exercise	Marathon	56 male and female patients using statins (56.6 ± 8.2 yr) and not taking statins (53.0 ± 6.5 yr).	miR-1, miR-133a, miR-134, miR-206, and miR-499-5p.	The day before, immediately after, and approximately 24 h postexercise.	Immediately postexercise: miR-1, miR-133a, and miR-206 in both groups. 24 h postexercise: miR-499 in the runners using statins.		Min *et al.* (23)
Chronic kidney disease	Endurance aerobic exercise	Maximal symptom-limited cardiopulmonary exercise test in a cycle ergometer: starting with either 20 or 40 W and an incremental load of 10 or 20 $W \cdot min^{-1}$, to ensure 8–10 min of exercise.	32 male and female patients (49.6 ± 15.3 yr).	miR-17, miR-21, miR-24, miR-92a, miR-125b, miR-126, miR-130a, miR-145, miR-146a, miR-150, miR-155, and miR-210.	Immediately before and 10 min postexercise.	10 min postexercise: miR-150.	10 min postexercise: miR-146a.	Van Craenenbroeck *et al.* (26)

$J \cdot s^{-1}$ indicates Joules per second; RPM, revolutions per minute; and W, Watt.

authors have accompanied the analysis of myomiRs with a few other miRNAs that have previously been described as circulating markers of processes that are directly related to the response to exercise, such as inflammation or angiogenesis (8,18) (see Table, Supplemental Digital Content 1, http://links.lww.com/ESSR/A47). Furthermore, in studies with diseased people, miRNAs that are altered in a particular pathological situation or are related to metabolic pathways that are relevant to that condition also are frequently included (24,26). Limiting the analysis to a selection of miRNAs might provide an incomplete perspective of their holistic regulatory role, *i.e.*, the systemic nature of the response to acute exercise and training. Surprisingly, few authors have addressed wider screenings of more than 100 miRNAs in this situation (12,14,15,17,19,20,28), and again, they have done so with a variety of approaches (Tables 1–4): whereas some authors have used microarray or commercial qPCR panels, others have opted for customized panels of a group of miRNAs that are related to some biological processes, such as inflammation or metabolism. Apart from the complex handling of data, one limitation of this type of approach is that all variables are considered equally (un)related to each other when performing the statistical analysis, although they are not really unrelated. In fact, most human miRNAs are located in clusters, so those miRNAs in the same cluster are coordinately regulated and expressed (37). Still, this constitutes an interesting approach because, as proposed by Nielsen *et al.* (19), the origin and fate of circulating miRNAs seem to be diverse and not restricted to skeletal or cardiac muscle.

In this sense, the tissue or cell type from which c-miRNAs originate and whether they are actively or passively released into the circulation have barely been studied. Most authors agree that plasma myomiR appearance in response to exercise is not a consequence of passive leakage from damaged skeletal or cardiac muscle because neither their plasma levels nor their kinetics correlate with those of classic markers of muscle damage, such as plasma creatine kinase concentration (7,9,16,18,21). However, it is unknown how miRNAs are secreted in response to exercise or if they can be incorporated into some tissues. The only study to date that has attempted to elucidate this issue in the context of exercise was published by Wahl *et al.* in 2016 (22) and focused on vascular miRNAs, such as miR-16, miR-21, and miR-126, not on myomiRs. Although the authors do not provide *in vivo* evidence of endothelial damage after exercise, they suggest in light of their *in vitro* results that these miRNAs can be passively released into circulation packed in microparticles because of the exercise-induced apoptosis of endothelial cells and that they act as intercellular communicators.

The active secretion of exosomes and other extracellular vesicles, such as microparticles and apoptotic bodies, has been recognized because of their possible role in intercellular communication (38). Exosomes are nanovesicles originated by inward budding inside an intracellular endosome, leading to the formation of a multivesicular body, which could then fuse with the plasma membrane releasing the internal vesicles (39). The presence of miRNAs in the exosomes exported by cells was first described in 2007 (40). Since then, very few studies have focused on the influence of exercise on the secretion of miRNAs transported by exosomes or other extracellular vesicles (41). Most studies have analyzed total plasma miRNA levels. Whether exosomal miRNAs contribute to the biological effects of exercise

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TABLE 4. Studies on the profile of circulating microRNAs in response to training in patients.

Condition	Type of Exercise	Training Protocol	Subjects	miRNAs Analyzed	Results		Reference
					Increase	Decrease	
Obesity	Walking	5 mo of supervised treadmill walking, 4 $d \cdot wk^{-1}$, progressing from 15–20 min at 50% heart rate reserve (1st wk), to 30 min at 65%–70% (by the end of the 6th wk).	33 male and female patients (69.3 ± 3.6 yr).	800 human miRNAs using nCounter technology.	miR-376a-5p.	miR-16-5p, miR-27a-3p and miR-28-3p.	Zhang *et al.* (28)
Obesity (1–3 mo after bariatric surgery)	Cycling or walking	6 mo of semisupervised exercise in cycle ergometer, treadmill walking or cycling or walking outdoors, 3–5 sessions/wk, progressing from ~10–15 min/session at 60%–70% of their maximal heart rate (first 4 wk), to 120 $min \cdot wk^{-1}$ over 3 mo and maintained for the final 3 mo.	11 male and female patients (43.0, 36.7–49.3 yr).	96-feature TaqMan microRNA panel.	miR-15a and miR-149.	miR-34a, miR-122, miR-135b, miR-144, and miR-206.	Nunez Lopez *et al.* (24)
Chronic kidney disease	Cycling	12 wk of home-based exercise in cycle ergometer, 4 sessions/wk, 10 min/session at 90% of the heart rate at anaerobic threshold.	19 male and female patients (51.5 ± 14.1 yr).	miR-17, miR-21, miR-24, miR-92a, miR-125b, miR-126, miR-130a, miR-145, miR-146a, miR-150, miR-155, and miR-210.			Van Craenenbroeck *et al.* (26)
Prediabetes	Cycling and resistance exercise	16 wk, 2 sessions/wk: 1) exercise in cycle ergometer at 60%–85% of $\dot{V}O_{2max}$. 2) resistance exercise, 3–5 series of 8–15 repetitions at 50%–70% of 1-RM (first 8 wk), progressing to 4–5 series of 4–12 repetitions at 70%–80% of 1-RM (last 8 wk).	6 male and female patients (53.83–3.44 yr).	miR-150, miR-192, and miR-193b.		miR-192 and miR-193b.	Párrizas *et al.* (25).

One-repetition maximum, 1-RM; microRNAs, miRNAs; Maximal oxygen consumption, $\dot{V}O_{2max}$.

is completely unknown. It has been proposed that plasma miRNA and plasma-derived exosomal miRNA levels may not differ when evaluating healthy people (42). However, some studies suggest that they can be differentially regulated in disease conditions (43). Whether it also is the case for exercise activity — that is to say whether exercise condition regulates the exosomal miRNA profile — is poorly characterized. In this context, initial evidence suggests that acute aerobic exercise could influence the level of certain miRNAs in extracellular vesicles (41). Moreover, miRNAs encapsulated in extracellular vesicles seem to be more protected from degradation than those not encapsulated (44). Furthermore, whether the abundance of the miRNAs in body fluids reflects their abundance in cells or tissues is matter of debate. There are a number of publications that have suggested the existence of a selection mechanism for miRNA release and propose that the extracellular and cellular miRNA signatures differ (45). Indeed, the incorporation of miRNAs into exosomes is regulated by the presence of specific sequence motif overrepresented in miRNAs (46). Because the exposition to physiological and pathological stress may alter the miRNA content of the secreted vesicles (45), it seems that extracellular miRNAs could be biomarkers of the exercise response of the different tissues, more than surrogate biomarkers of miRNA tissue content.

Considering all of the aforementioned, the lack of coincidence in the results between studies could be partially due to the differences in the selected miRNAs, in the criteria used for this selection, and in the technical approaches used. Furthermore, deepening the study of the relation between circulating miRNA abundance and tissue content, as well as whether exercise-induced circulating miRNAs are encapsulated or not in extracellular vesicles, also could help in determining the search and use of circulating miRNAs as biomarkers or mediators of the systemic adaptations to exercise.

Exercise Models and Dietary Control

Another element of methodological divergence is the model of exercise performed by the volunteers. Most studies about acute exercise and training in health and disease consist of endurance aerobic exercise interventions (Tables 1–4), although the type, duration, and intensity of exercise varied between studies. Even in those studies in which the exercise model was the same, such as in those that have analyzed the acute response to a marathon, both the characteristics of the subjects (which we will analyze later) and the sampling points or dietary control differed between studies, which could influence the observed responses. Thus, in some cases, the baseline sample was drawn just before the start of the marathon (15,21), whereas in others,

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TABLE 5. Different strategies to normalize microRNA data in response to acute exercise and training in health and disease.

Normalizer	Reference
cel-miR-39	Clauss *et al.* (11)
	de Gonzalo-Calvo *et al.* (15)
	Mooren *et al.* (18)
	Sawada *et al.* (20)
	Uhleman *et al.* (21)
	Wahl *et al.* (22)
	Margolis *et al.* (17)
	Xu *et al.* (27)
	Van Craenebroeck *et al.* (26)
	Nunez Lopez *et al.* (24)
miR-422b	Baggish *et al.* (8)
	Baggish *et al.* (9)
	Min *et al.* (23)
miRNA U6	Cui *et al.* (13)
cel-miR-39 and miR-16	Aoi *et al.* (7)
cel-miR-54 and cel-miR-238	Gomes *et al.* (16)
Genorm: miR-20a, miR-103, miR-21, miR-192, and miR-185	Banzet *et al.* (10)
An endogenous c-miRNA selected using the Genorm and normfinder algorithms	Nielsen *et al.* (19)
let-7b, let-7 g, and let-7i	Párrizas *et al.* (25)
let-7d, let-7i, and let-7 g	Cui *et al.* (14)
let-7d, let-7i, and let-7 g	Cui *et al.* (12)
Mean abundance of the top 100 expressed plasma miRNAs	Zhang *et al.* (28)

it was taken one (9), two (18), or even between 2 and 5 d before (11). In these cases, the observed differences in the expression of c-miRNAs between the baseline and the postexercise levels did not allow for the isolation of the effect of exercise because of many uncontrolled factors, particularly food and nutrient intake.

There is increasing evidence about the influence of dietary components in the expression of miRNAs and in the levels of c-miRNAs (47) as well as a new, intriguing, and controversial relation between the ingestion of miRNAs from food sources and their absorption, appearance in biological fluids, and intracellular regulatory role (48). Despite this, very few authors have considered monitoring food or nutrient intake (7,15,17,19,22), and only de Gonzalo-Calvo *et al.* (15) and Wahl *et al.* (22) have used strict control and recording of food intake before, during, and after exercise. Surprisingly, no study of diseased people has provided this information despite the fact that studies of metabolic disorders have been performed.

Characteristics of the Subjects

As previously mentioned, huge differences in the characteristics of the subjects included in the different studies are evident, particularly in relation to age and exercise background (Tables 1–4). For example, whereas in the study by Baggish *et al.* (8), the participants were young male university rowers aged 19.1 yr on average, Uhlemann *et al.* (21) recruited adult male runners aged 56.8 yr, and Gomes *et al.* (16) analyzed the acute c-miRNA response to a half marathon in obese and overweight amateur runners, some of whom had only 6 mo of regular exercise background. Thus, both factors could introduce one more element of variability that explains the heterogeneity in the results.

Almost all of the studies have been performed with men or with men and women considered together. Information about the specific response of c-miRNAs to exercise in women is lacking, and studies would be desired because sex-related differences in the circulating levels of certain miRNAs, such as miR-125a or miR-34a, have been described (49). Therefore, it is unclear if sex influences c-miRNA response to exercise.

There is not much information about the effect of age on the c-miRNA profiles of humans. Noren Hooten *et al.* (50) observed that the expressions of miR-151a-5p, miR-181a-5p, and miR-1248 are significantly suppressed in older (64 yr old) versus younger (30 yr old) men and women. Furthermore, Zhang *et al.* (51) suggested that the circulating profiles of miR-29b and miR-92a should gradually change with the aging process based on observations of the differences between subjects aged 22, 40, 59, and 70 yr. Regarding the response to exercise, in a pioneering study, Margolis *et al.* (17) observed that the acute response of c-miRNAs to resistance exercise differs between young (22 ± 1 yr) and old (74 ± 2 yr) male volunteers (Table 1). Therefore, differences in the ages of subjects could determine differences not only in the response to exercise but also in the baseline levels of some c-miRNAs, which introduces another confounding factor.

For their part, Baggish *et al.* (9) suggested that systematic training may be associated with elevated basal levels of c-miRNAs *per se*, particularly some myomiRs, as observed by Nielsen *et al.* (52) in skeletal muscle cells. This elevation could mask the effect of acute exercise on these c-miRNAs and explain why, in some studies with trained individuals, no changes in the circulating levels of myomiRs are reported.

CIRCULATING MIRNAS AS BIOMARKERS OF EXERCISE RESPONSE?

Numerous studies have proposed the use of c-miRNAs as diagnostic, prognostic, and therapeutic biomarkers of numerous and diverse pathological processes (6). However, c-miRNAs also seem to be able to regulate various developmental and physiological processes (6). The potential of c-miRNAs as biomarkers lies in the fact that, on the one hand, they can be released into extracellular media, including blood, in response to cellular stress and damage, which would define specific profiles, and, on the other hand, they exhibit optimal biochemical and physiological properties to constitute excellent biomarkers (39).

Therefore, it is not surprising that, considering the aforementioned and the fact that c-miRNAs respond to exercise, they also have been proposed as biomarkers in the context of exercise (30,31). However, several questions should be addressed in this regard.

First, the commonly used concept of c-microRNAs as *exercise biomarkers* seems unclear. Alternative and more accurate expressions have been proposed by several authors, such as "biomarkers for exercise-related biological responses" (29) or simply "biomarkers of exercise response" (30), when referring to c-miRNAs as biomarkers of physical performance, physical fitness/capacity, training load, or exercise/training adaptations, response, and injury (29–31).

Furthermore, the National Institutes of Health working group (53) defined a biomarker as a biological marker that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to therapeutic interventions. From a clinical point of view, it is widely accepted that a biomarker should be implicated in the pathophysiology of a given disease. This situation is recommended; nonetheless, biomarkers do not necessary need to be mediators in the causal pathway of the outcome of interest to be useful. Biomarkers could merely be bystanders passively

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associated with the outcome, although they should strongly correlate with the endpoints of interest and be accurately and reproducibly measured (54).

In this sense, some articles have demonstrated that both the baseline c-miRNA profile and the response of c-miRNAs to acute endurance exercise are related to aerobic fitness in healthy but not in diseased volunteers, which suggests a specific value as a biomarker in this context. Bye *et al.* (55) observed a differentiated baseline c-miRNA profile in middle-aged (40–45 yr) healthy male and female volunteers depending on their $\dot{V}O_{2max}$ and demonstrated higher levels of miR-21 (only in men), miR-210, and miR-222 in those with low aerobic fitness. Similarly, Mooren *et al.* (18) found that the changes in the plasma concentrations of miR-1, miR-133a, and miR-206 in response to acute aerobic exercise (marathon) exhibited a strong correlation with classical aerobic performance parameters, such as $\dot{V}O_{2max}$ and running speed at the individual lactate threshold. In addition, Clauss *et al.* (11) reported that the responses to acute exercise (marathon) of plasma miR-1, miR-26a, miR-29b, miR-30, and miR-133a are different in elite compared with amateur runners. In contrast, regarding patients of chronic obstructive pulmonary disease (COPD), heart failure, and chronic kidney disease (CKD), although differentiated basal miRNA profiles compared with healthy controls were observed, no correlation was found in performance in a 6-min walk distance (56), parameters of cardiopulmonary exercise testing (57), or peak oxygen consumption ($\dot{V}O_{2peak}$) (58). Finally, Wardle *et al.* (59) demonstrated that the type of exercise background modified the baseline levels of certain c-miRNAs because miR-21, miR-146a, miR-221, and miR-222 expression differed between strength and endurance athletes.

Other authors have described c-miRNAs as promising biomarkers of acute exercise load as considered in terms of type, dose, and intensity of exercise. Thus, using a randomized crossover design, Banzet *et al.* (10) described different profiles of several c-miRNAs in response to concentric versus eccentric exercise. Using a repeated-measures design, de Gonzalo-Calvo *et al.* (15) and Wahl *et al.* (22) observed that the number, type, and kinetics of the c-miRNAs analyzed significantly differed according to the dose (10-km running vs marathon) and the intensity (high-volume, high-intensity, and sprint-interval exercise protocols) of exercise, respectively. In contrast, Cui *et al.* (14) compared two classical and opposite protocols for improving endurance capacity, *i.e.*, interval high-intensity and

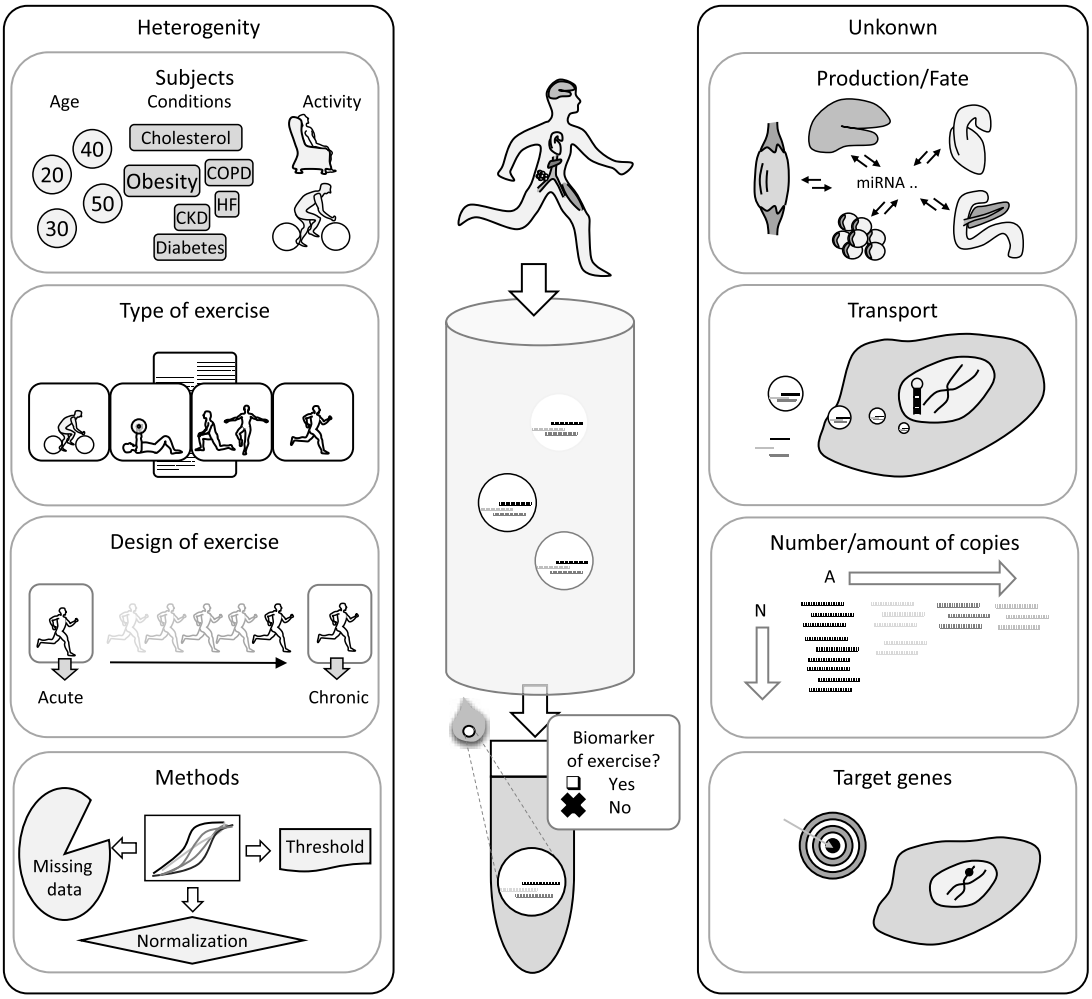

Figure. Acute exercise and training modify the circulating microRNA (c-miRNA) profile. However, huge differences in the methodologies used (detection technique, normalization strategy, and treatment of missing values), in the experimental design (timing of sampling and type and number of c-miRNAs analyzed), and in the characteristics of the participants (age, exercise background, dietary intake, and pathologic condition: COPD, Heart Failure (HF), CKD), together with a lack of information about the origin, the form of transport, the quantitative amount released, and the tissue and gene targets, do not currently support the idea of c-miRNAs as biomarkers of exercise response.

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moderate continuous exercise, and these produced similar changes in the c-miRNA profile. Several other authors have separately found exercise load-related c-miRNA profiles in response to acute exercise in healthy volunteers (Table 1), also suggesting an effect of exercise load. However, we have only highlighted these articles because of their solid experimental designs: crossover or repeated measures. Establishing such comparisons between separate studies is problematic because of the previously mentioned huge differences in the experimental designs, the methodological approaches, and the subjects' characteristics between studies or even between groups in the same study (21). A more complete overview of the methodology used and a stricter control of confusing factors may help comparisons between future studies (32).

Thus currently, the measurement of microRNAs is efficient, but it has not been demonstrated to be reproducible because of the plethora of uncontrolled and confusing elements, which weakens their current potential as biomarkers in the field of exercise. Therefore, observations of changes in the expression of c-miRNAs in response to exercise are not sufficient to define them as biomarkers.

An alternative that has barely been explored might be to analyze the response to exercise of extensive panels of miRNAs that have previously been described or validated as circulating biomarkers of physiological (coagulation, neurogenesis, inflammation, angiogenesis) or pathological (cardiac damage, endothelial dysfunction) processes related to exercise in crossover or repeated-measures designs.

Related and more important than their potential utility as biomarkers is the role of miRNAs as regulators of the molecular response to exercise (60). Most studies included in this review have a clear descriptive and associative character. Therefore, the origin, the form of transport, the quantitative amount released, and the tissue and gene targets of the c-miRNAs that respond to exercise remain to be known and validated. Additional studies using exercise animal models and *in vitro* approaches will provide insights to understand the extent and importance of their functional role in the molecular response to exercise and to determine their potential value as biomarkers in this context in health and performance applications, which for today is still unclear (Figure).

CONCLUSIONS

The studies available to date agree that both acute exercise and training modify the c-miRNA profiles of healthy and diseased volunteers. However, the low reproducibility of the results powerfully limited their usefulness as biomarkers in this context. The huge differences in methodology, in the experimental design, and in the characteristics of the participants, have strongly influenced the results obtained.

Instead of deepening the study of microRNAs as biomarkers, it seems to be a priority to study their regulatory role in the molecular response to acute exercise, as well as in recovery and adaptation in detail; such study will help to validate the use of c-miRNAs as biomarkers of exercise response.

The eventual validation of c-miRNAs as biomarkers in health and disease may allow for the development of more specific recommendations for the use of training as a therapeutic and preventive tool and for exploring the maximal limits of safe and healthy exercise. Understanding the role of exercise as a c-miRNA profile modulator also could set exercise as a valuable alternative or adjuvant to upcoming pharmacological and nutritional interventions based on miRNAs. However, we are still *miles away* from c-miRNAs being considered as validated biomarkers of exercise response in health and disease.

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ORIGINAL ARTICLE

WILEY

Exercise dose affects the circulating microRNA profile in response to acute endurance exercise in male amateur runners

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The systemic response to exercise is dose-dependent and involves a complex gene expression regulation and cross-talk between tissues. This context ARISES the need for analyzing the influence of exercise dose on the profile of circulating microRNAs (c-miRNAs), as emerging posttranscriptional regulators and intercellular communicators. Thus, we hypothesized that different exercise doses will determine specific c-miRNA signatures that will highlight its potential as exercise dose biomarker. Nine active middle-aged males completed a 10-km race (10K), a half-marathon (HM), and a marathon (M). Blood samples were collected immediately before and after races. Plasma RNA was extracted, and a global screening of 752 microRNAs was analyzed using RT-qPCR. Three different c-miRNA profiles were defined according to the three doses. In 10K, 14 c-miRNAs were found to be differentially expressed between pre- and post-exercise, 13 upregulated and 1 downregulated. Regarding

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HM, 13 c-miRNAs were found to be differentially modulated, in all the cases up-regulated. A total of 28 c-miRNAs were found to be differentially expressed in M, 21 overexpressed and 7 repressed after this race. We had also found 3 common c-miRNAs between 10K and M and 2 common c-miRNAs between 10K and HM. In silico analysis supported a close association between exercise dose c-miRNA profiles and cellular pathways linked to energy metabolism and cell cycle. In conclusion, we have observed that different exercise doses induced specific c-miRNA profiles. So, our results point to c-miRNAs as emerging exercise dose biomarkers and as one of regulatory mechanisms modulating the response to endurance exercise.

KEYWORDS

circulating microRNAs, endurance exercise, exercise biomarkers, exercise dose

1 | INTRODUCTION

The systemic response to acute exercise and training has profound effects on gene expression and involves a complex cross-talk between tissues.[1,2] Although these adaptations are dose-dependent and exert systemic beneficial effects for health,[3] adverse consequences of acute prolonged exercise and chronic excessive endurance exercise have also been reported, suggesting that a safe upper-dose limit potentially exists.[4] However, the exact mechanisms by which gene expression is regulated to orchestrate this response remain partially unknown.[5]

During the last decade, several authors have suggested a prominent role of the non-coding transcriptome in the regulation of the physiological responses to exercise.[6] In this context, there emerges the need to assess the role of circulating microRNAs (c-miRNAs), as new players for intercellular communication and gene expression regulation,[7] as well as potential biomarkers of exercise dose.[8]

MicroRNAs (miRNAs) are short (≈22 nucleotides) non-coding RNA molecules that regulate posttranscriptional gene expression by promoting mRNA degradation or by repressing protein translation,[9] and they are predicted to target >60% of protein-coding genes.[10] Although miRNAs are intracellular regulators of gene expression, they have been detected in different body fluids in a stable form, including bloodstream,[11] mainly transported in extracellular vesicles,[12] associated to proteins[13] or lipoproteins.[14] It has been proposed that c-miRNAs can be secreted in a regulated manner as a response to stress, constituting a true intercellular communication system, or be passively released from injured, necrotic, or apoptotic cells.[15]

To date, an increasing number of studies have been published about the effect of acute exercise on the c-miRNA profile (reviewed in[8]). However, the response to different doses of acute exercise has been barely explored.[16-18] Surprisingly, despite the systemic nature of the response to exercise, none of them have addressed a global c-miRNA screening in this situation. The analysis of limited miRNA panels provides an incomplete perspective of their holistic regulatory role.[8] Therefore, analyzing the influence of exercise dose on the global response of c-miRNAs will provide a better understanding of their potential regulatory role on the systemic effect of acute exercise, as well as analyzing their value as biomarkers of exercise dose, which may also help in exploring the maximal limit for a safe and healthy exercise.

Thus, we hypothesized that different exercise doses will determine specific c-miRNA signatures that will highlight its potential as exercise dose biomarker.

To test this hypothesis, we performed a global screening of plasma c-miRNAs in response to different doses of acute endurance exercise in a group of active middle-aged males.

2 | SUBJECTS AND METHODS

2.1 | Ethics statement

All experimental procedures were approved by the Research Ethics Committee of the Principality of Asturias, Spain (reference 124/17). All participants gave written informed consent.

2.2 | Experimental design

In an observational repeated measures design, all subjects completed a 10 km race (10K), a half-marathon (HM), and a marathon (M), separated by 1 month.

Although all races involved the same type of exercise (endurance running), they differed in terms of duration, intensity, and energy demands, and thus represent distinct exercise doses.[18-20]

2.3 | Subjects

Volunteers were recruited among the members of MAPOMA Sports Association, which includes professional

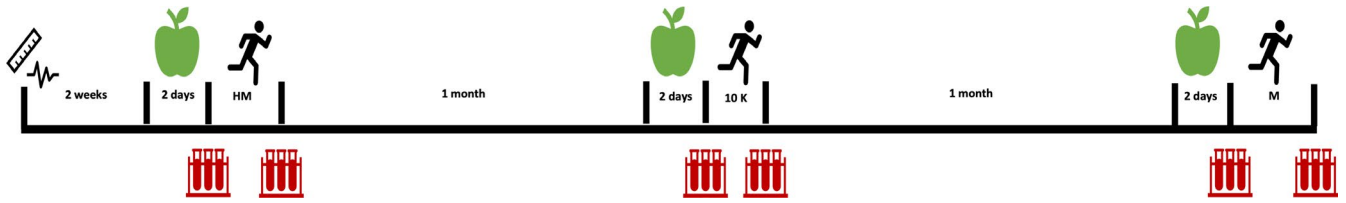

FIGURE 1 Study timeline

and amateur runners. A team of sports and health professionals design the training plans, promoting proper care and preparation.

All runners at the amateur training group (n = 35) were invited to an informative briefing. A member of the research team presented the aims and methodology of the study, and answered the questions of the potential participants. Finally, 18 (51%) agreed to participate. The rest showed interest in the study, but various reasons (family and professional commitments, travel, and injuries) did not allow them to participate. Prior to participation, each volunteer underwent a thorough medical screening to determine eligibility. A number of inclusion and exclusion criteria were also established. Inclusion criteria were as follows: (a) men over 18 years of age; only men were selected as subjects due to the stability of their hormonal status, (b) regular trained, at least 50 km/wk, (c) have previously participated in at least two marathons, (d) be officially registered for the Madrid Marathon, (e) signing written informed consent. Exclusion criteria were as follows: (a) smokers and frequent passive smokers, (b) suffering from any chronic disease, (c) body mass index (BMI) over 30 kg/m^2, (d) be under dietary or pharmacological treatment during the time of the study. Although 14 volunteers met these criteria and participated, finally 9 subjects completed the whole study. The remaining 5 subjects were unable to finish at least one of the races, and their samples were excluded from the final analyses.

2.4 | Aerobic capacity and body composition assessment

VO_2max was determined by indirect calorimetry (Oxycon Pro, Jaeger) using an incremental protocol till exhaustion on a treadmill (LE- 600 C, Jaeger -HP Cosmos). Maximal heart rate (HRmax) and Maximal Aerobic Speed (MAS) were also recorded.

Before the test, the participants had their body composition assessed. The same, ISAK Level III certified anthropometrist measured height and body mass using a combined medical scale (model 778, Seca Ltd; precision 0.1 cm for height and 0.1 kg for weight). Body mass index (BMI) was then calculated from these measurements. The equation of Kyle et al[21] was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance system device (Total Body Scan, Bio-Logic®). This equation was considered the most appropriate according to the position stand of the Spanish Group of Kinanthropometry.[22]

2.5 | Training schedule, athletic background, and in-race measurements

Volunteers were interviewed in order to determine their training history (years of training, number of 10K, HM and M races previously finished, and personal bests) and the volume of training in, at least, the last 3 months (days per week, hours per day, and km per week). Subjects were asked not to alter their usual training schedule, and all of them performed low intensity training the day before each race.

Mean speed and mean heart rate during each race were measured. From these data, the ratios Mean HR/HR max and Mean Speed/MAS were calculated.

2.6 | Dietary control

Participants were asked to keep a food diary 2 days before the first exercise bout and also on the test day (Figure 1). All foods and beverages were recorded using standard culinary measures[23]; for information about packed-foods and snacks, food labels were collected. Volunteers received specific oral guidelines and detailed written instructions about how to put this method into practice. A telephone number was available for them to answer any queries about the recording of their diets. In order to minimize the impact of food intake on the results, volunteers were asked to repeat the same intake pattern on the following races. They were also asked to keep a food diary, which were compared in terms of energy and macronutrients in order to certify that the same food and nutrient intake pattern was followed. Subjects were clearly asked not to alter their usual dietary pattern during the recording periods. No limitations for the type or the amount of food or beverages consumed were established at any time during the food-recording period. None of the volunteers reported the use of nutritional supplements. Food records were carefully reviewed immediately after completion, and subjects were contacted to clarify ambiguous information. Dietary records were analyzed using a software program for nutrient intake analysis (DIAL®, Alce Ingeniería).

2.7 | Blood sampling

Two blood samples were drawn, before and after the end of each exercise bout (Figure 1). In all cases, experienced technical staff, using standardized techniques and materials, obtained the samples. Subjects had their first blood sample taken about 1 hour and a half before the race, in fasting state, and before starting warming-up (Pre). Subjects then consumed their breakfast and performed warm-up exercises, after which point the race started. Another blood sample was drawn within 15 minutes after the cessation of exercise (Post). Blood draws and the immediate processing of the samples were carried out in a field laboratory installed near the starting and finishing lines, with the permission and cooperation of the organization staff.

The total volume of blood taken per exercise bout was <20 mL per individual. Blood samples were collected in vacutainers (No Additive (Z), Becton Dickinson), stored at room temperature, at least 15 minutes to allow clot formation, and immediately centrifuged at 1600 *g* for 15 minutes at 10°C. Serum samples were then aliquoted, immediately preserved in dry ice, and finally stored at −80°C for later analysis.

2.8 | Estimations of exercise intensity

All participants used heart rate (HR) monitors during the races. The ratio (%) between the mean HR during each race and the maximal HR (HRmax) during the incremental protocol on a treadmill was used to calculate the percent of HRmax (%HRmax), in order to determine the individual exercise intensity during all exercise bouts.[24]

Besides taking into account the VO_2max test and the mean speed of each race, the exercise intensity was also estimated as the percentage of the Maximal Aerobic Speed (MAS) for each race.

Time spent in completing each race was also recorded and compared with their personal best race time to calculate the percentage of personal best (%PB) as follows: race time 100/personal best.

2.9 | RNA isolation and qRT-PCR

Total circulating RNA from 200 μL of serum was isolated using the miRCURY RNA isolation kit (Exiqon) following the manufacturer's instructions. For ulterior normalization, synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p), lacking sequence homology to human miRNAs, was added as an external reference. The mixture was supplemented with 1 μg of MS2 carrier RNA (Roche) to improve extracellular miRNA yield. The RNA Spike-in kit with synthetic RNA spike-in templates (UniSp2, UniSp4, UniSp5) (Exiqon) was also used in all extractions to monitor RNA isolation efficiency. RNA was eluted in 30 μL RNase-free H_2O and stored in a −80°C freezer.

For miRNA quantification, cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon). The miRCURY LNA Universal RT microRNA PCR System offers a high sensitivity, specificity, and reproducibility.[25] Briefly, 10 μL RNA samples were reverse transcribed in 50 μL reactions. Additional spike-in (UniSp6) (Exiqon) was added to the cDNA synthesis reaction to check for RT and PCR inhibitors. RT reaction was performed with the following conditions: incubation for 60 minutes at 42°C, heat-inactivation for 5 minutes at 95°C, immediately cool to 4°C. Then, cDNA was stored at −80°C. For qPCR, cDNA was diluted 80x and 4 μL used in 10 μL qPCR reactions with ExiLENT SYBR Green master mix (Exiqon) on a 7900HT fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 10 minutes at 95°C, 40 cycles of 10 seconds at 95°C and 1 minutes at 60°C, followed by a melting curve analysis. To discard the presence of nucleases, inhibitors, or hemolysis, the miRCURY miRNA Quality Control PCR Panel (Exiqon) was used before miRNA analysis. For whole genome screening, miRNAs were quantified using the human miRNome panels (752 human mature miRNAs) Version 3 (Exiqon). miRNA relative expression analysis was performed using the GenEx software (MultiD Analyses AB).

To ensure good quality data, synthetic spike-in RNA templates were analyzed to monitor the uniformity of the RNA extraction procedure and efficiency of RT and PCR reactions. The synthetic spike-in UniSp3 was also analyzed as an interplate calibrator. The SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and for the melting curve analysis. The Cq was defined as the fractional cycle number at which the fluorescence exceeded a given threshold. The specificity of the PCR reaction was corroborated by melting curve analysis. The dCq (miR-23a-3p – miR-451a) method was used to confirm that none of the samples were affected by hemolysis (all samples had dCq value below 6). miRNAs were considered to be expressed when Cq values < 37 or were detected with at least 5 Cq below the negative control, as recommended by the manufacturer. For normalization purposes, the dCq method was used, where dCq = Cq[miRNA] − Cq[cel-miR-39]. Relative quantification to basal samples was performed using the 2^{-ddCq} method, where ddCq = dCq[miRNA] − dCq[mean miRNA prerace].[26]

2.10 | Pathway analysis and prediction

Pathway analysis of target genes of modulated c-miRNAs was performed to determine their possible implication in the biological response to exercise.

The R Bioconductor package mdgsa (version 1.8.0) was used for pathway analysis of differentially expressed miRNAs

in an integrative manner. This method allows for an accurate modeling of multiple miRNA's regulatory processes at a time, accounting for additive/cancelation effects of several miRNAs targeting the same gene based on the direction of their expression profile. For each miRNA, experimentally validated targets were retrieved from miRTarBase and miRWalk databases. Pathway annotations for each gene were retrieved from KEGG pathways. Thus, we obtained gene sets from both annotation databases, linked to the miRNAs targeting those genes. The results output a log odds ratio for each interrogated gene set, along with raw and false discovery adjusted *P*-values.

2.11 | Statistical analysis

Normality of variables was tested using Shapiro-Wilk's test. In light of the results obtained, descriptive values for anthropometric, dietetic, and performance variables are presented as means and standard deviations, and parametric methods were used for analytical statistics. General additive linear models, in which subject was considered as a random-effects covariate, were used for studying the effect of race length (10K, HM, M) on dietetic and performance variables. A multiple paired samples *t* test was performed and the *P*-values adjusted by the false discovery rate (FDR) criterion in order to compare pre- and post-exercise miRNA expression levels. Differences between pre- and post-samples were considered relevant if satisfied one of the following criteria: (a) *P*-value below (0.05) (computed by using the paired Student's *t*-test) and (b) difference between means larger than 1.5. A one-way repeated measures ANOVA was used to assess differences in c-miRNA expression between races. A customized R (www.r-project.org) function was used for all process.

3 | RESULTS

3.1 | Physical characteristics, training habits, and dietary control

The subject characteristics and training habits were previously described elsewhere.[16] No differences were observed in the daily energy and macronutrient intake of volunteers during five consecutive days before, during, and after the exercise bouts (Table 1).

3.2 | Performance parameters

As expected, performance parameters defined three doses of maximal endurance exercise determined by significant differences in relative intensity for the different volumes (Table 2).

TABLE 1 Nutritional intake of volunteers (n = 9) before and after the race days

	10K	Half-marathon	Marathon
Energy (kcal)	2533 ± 525	2620 ± 528	2581 ± 530
Carbohydrates			
g	321 ± 75	332 ± 74	335 ± 81
%energy	51 ± 4	51 ± 4	52 ± 5
Proteins			
g	90 ± 19	93 ± 21	90 ± 20
%energy	14 ± 1	14 ± 1	14 ± 1
Lipids			
g	98 ± 22	102 ± 23	98 ± 31
%energy	35 ± 4	35 ± 5	35 ± 5

Note: Data are presented as mean ± standard deviation of 3 d food records. %energy: Percentage of the total daily energy intake provided by the different macronutrients.

TABLE 2 Performance parameters of participants in a 10K race, a half-marathon and a marathon

	10 km	Half-marathon	Marathon
Race time (h:min)	0:42 ± 0:03	1:37 ± 0:10	3:54 ± 0:36
Personal best (h:min)	0:42 ± 0:04	1:35 ± 0:08	3:35 ± 0:32
Mean HR (bpm)	169 ± 3[a]	162 ± 4	157 ± 7[b]
Mean HR/HR max (%)	92.3 ± 2.1[a]	90.2 ± 0.3	86.7 ± 2.8
Mean speed (km/h)	14.3 ± 1.0[a]	13.1 ± 1.3	10.9 ± 1.6[b]
Mean speed/MAS (%)	83.9 ± 4.1[a]	76.1 ± 4.6	64.1 ± 5.9[b]

Note: Data are presented as mean ± standard deviation.

Abbreviations: bpm, beats per minute; HR max, Maximal Heart Rate; HR, Heart rate; MAS, Maximum Aerobic Speed.

[a]Significantly different ($P < 0.05$) from half-marathon and marathon races;

[b]Significantly different ($P < 0.05$) from 10-km and half-marathon.

Participants performed close to their personal best in 10K (%PB: 100.2 ± 2.5%) and HM (%PB: 102.7 ± 3.0%), while performed worse in M (%PB: 109.1 ± 8.8%), maybe due to adverse weather conditions, although the difference to their personal bests was not significant.

3.3 | Circulating miRNAs

To explore the effect of acute endurance exercise on the non-coding miRNA transcriptome, the expression levels of 752

human circulating miRNAs, under three races representing three differing doses of maximal endurance exercise, were screened by qRT-PCR (Table 3). A total of 378 individual miRNAs were detected in at least one of the races. If we differentiate by race, we find that 321 miRNAs were detected in 10K race samples (Table S1). From these, 14 c-miRNAs were found to be differentially expressed between pre- and post-exercise, 13 upregulated and 1 downregulated. Regarding HM, 266 c-miRNAs were detected (Table S1), from which 13 c-miRNAs were found to be differentially modulated, in all the cases upregulated. Finally, 339 c-miRNAs were detected in M (Table S1), from which a total of 28 c-miRNAs were found to be differentially expressed, 21 overexpressed and 7 repressed after race. A total of 239 microRNAs were detected in all the races (Table S1), which represents 74% of those detected in the basal samples of 10K, 90% in HM, and 70% in M.

The expression of 168 of the common baseline c-microRNAs detected was significantly different between races (Table S1).

We have used a Venn diagram to represent the profile of c-miRNAs differentially expressed between pre- and post-exercise samples in each race, as well as the overlapping between races (Figure 2). No common c-miRNAs were found for all races. However, 2 common miRNAs were observed between 10K and HM: miR-33a-5p and miR-505-3p, both upregulated. Comparing 10K and M races, 3 common miRNAs were observed: miR-1260a, miR-345-5p, and miR-424-3p, all of them were upregulated in both races. Figure 3 shows the acute response (pre-post)

TABLE 3 Significant circulating miRNAs after a 10 km race, a half-marathon, and a marathon

10K			HM			M		
c-miRNA	**FC**	***P***	**c-miRNA**	**FC**	***P***	**c-miRNA**	**FC**	***P***
miR-199b-5p	22.81	.02	miR-425-3p	22.08	.02	miR-1972	71.65	.03
miR-424-3p	9.74	.03	miR-33a-5p	16.52	.05	miR-940	13.72	.02
miR-33a-5p	8.26	.03	miR-338-3p	11.65	.05	miR-424-3p	12.75	.04
miR-551a	7.75	.04	miR-339-5p	11.10	.02	miR-130b-5p	11.06	.04
miR-1537	4.52	.02	miR-106b-3p	10.02	.00	miR-223-5p	8.67	.04
miR-223-5p	3.94	.02	miR-502-3p	8.57	.04	miR-145-3p	8.43	.02
miR-1260a	3.29	.04	miR-27a-3p	6.37	.05	miR-181c-3p	8.42	.05
let-7b-3p	3.04	.04	miR-660-5p	5.85	.05	miR-501-3p	7.40	.04
miR-150-5p	2.55	.04	miR-505-3p	5.60	.03	miR-1260a	7.40	.01
miR-423-5p	2.17	.04	miR-100-5p	5.25	.05	miR-675-3p	7.25	.04
miR-223-3p	2.13	.03	miR-22-3p	4.40	.05	miR-345-5p	5.96	.04
miR-345-5p	2.04	.02	miR-30e-5p	4.38	.05	miR-424-5p	5.38	.04
miR-505-3p	1.94	.04	miR-497-5p	2.76	.05	miR-1-3p	4.62	.03
miR-346	−4.69	.02				miR-34a-5p	3.74	.03
						miR-629-5p	3.17	.04
						miR-30a-5p	3.01	.02
						miR-148a-3p	2.68	.02
						miR-596	2.46	.00
						miR-10b-5p	2.37	.01
						miR-30d-5p	1.93	.05
						miR-320d	1.60	.04
						miR-192-5p	−1.52	.04
						miR-20b-5p	−1.72	.04
						miR-103a-3p	−1.74	.04
						miR-106b-5p	−1.89	.01
						miR-144-3p	−2.69	.01
						miR-665	−3.26	.04
						miR-486-3p	−3.85	.04

Abbreviations: 10K, 10 km race; FC, Fold change; HM, Half- marathon;M, Marathon; *P*-V, *P*-Value.

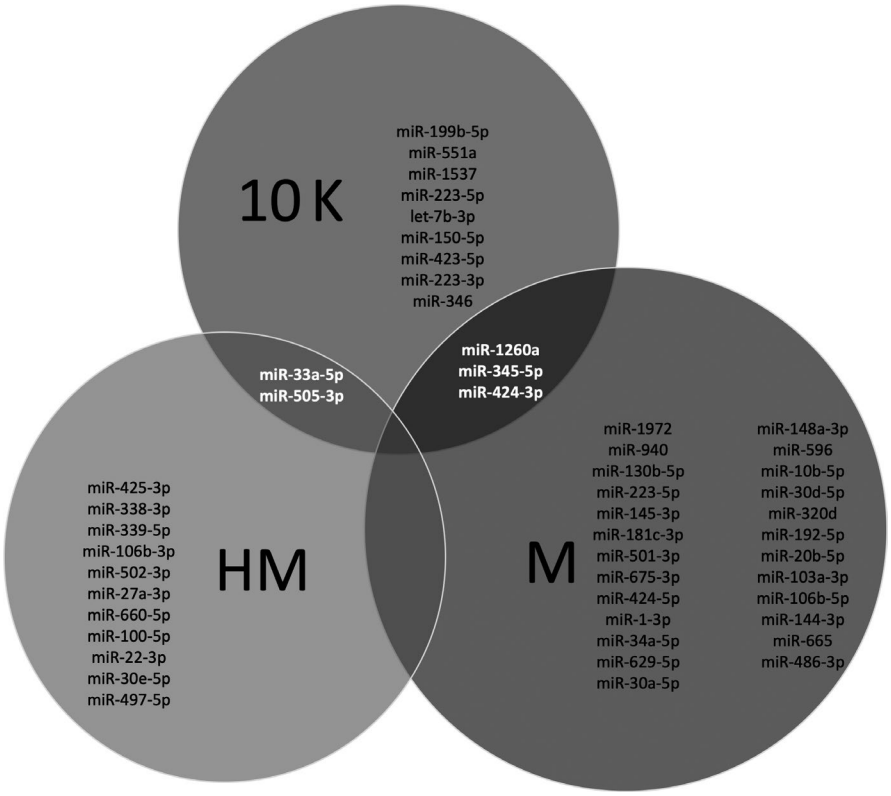

FIGURE 2 Venn diagram analysis of modulated (overexpressed and repressed) circulating miRNAs in response to different doses of acute exercise. In black bold font, repressed miRNAs. In white bold font, common miRNAs, all of them overexpressed

of significantly changed common circulating miRNAs, as well as the differences between exercise doses. No common c-miRNAs were found to change in response to HM and M. Furthermore, the magnitude of change for miR-33a-5p, miR-505-3p, miR-1260a, and miR-345-5p was significantly different between races, being highest for M in most cases, except for miR-33a-5p.

3.4 | Target pathways analysis

Pathway analyses of target genes of modulated c-miRNAs were performed to determine their possible implication in the biological response to exercise. Using experimentally validated miRNA-target interaction databases (TarBase v7),[27] we performed pathway analysis using KEGG database.

The specific miRNA profiles detected for each exercise dose have a specific validated target signature (Table S2). These profiles have 44, 65, and 57 significant validated targets for upregulated miRNAs and 3, 0, and 64 significant validated targets for downregulated miRNAs, considering 10K, HM, and M, respectively. The large number of validated targets supports the hypothesis that c-miRNA has potentially enormous influence on gene responses to exercise. Pathways related to energy metabolism and cell cycle control are the most represented. Interestingly, AMPK and mTOR pathways are targeted by most of the up and downregulated miRNAs in the acute response to HM and M.

Considering only the common miRNAs between races, which are shown in Table S3, all of them target lysine degradation pathway and cell cycle or p53. All these common miRNAs were upregulated.

4 | DISCUSSION

We have found that, according to the aim proposed, different exercise doses define specific c-miRNA profiles. Three doses of exercise in-field conditions were assayed in the same subjects. All the races were maximal endurance efforts at different volumes, with an inverse relationship with intensity. This approach, together with the global c-miRNA screening performed (>750 miRNAs analyzed), provided a new perspective on this field, enhancing the value of c-miRNAs as biomarkers of endurance exercise dose.

Other authors have previously analyzed the acute effect of different doses of endurance exercise on the c-miRNA profile. de Gonzalo-Calvo et al[17] described a relationship between two doses of endurance exercise (10 km and Marathon) and the c-miRNA response, although using a selection of 106 specific inflammatory miRNAs instead of a global screen. Furthermore, Ramos et al[18] defined, under laboratory conditions, the isolated effect of intensity or duration of treadmill running on the c-miRNA profile. A very restricted selection of 7 miRNAs was analyzed in two separate groups of volunteers: variable intensity and variable duration cohorts. However, most of the intensities tested in the variable

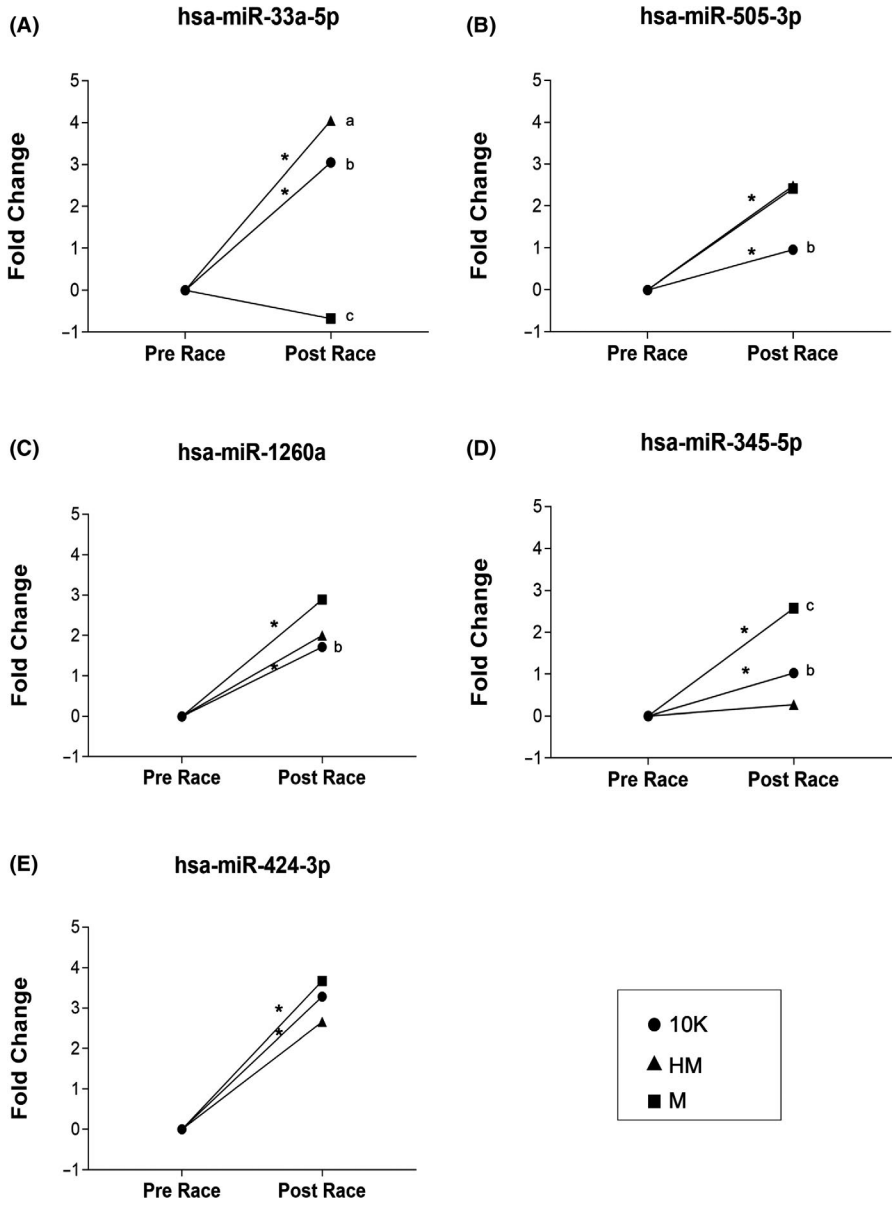

FIGURE 3 Acute response (pre-post) of significantly changed common circulating miRNAs (A-E) between exercise doses. Data are presented as means. **P*-values adjusted by the false discovery rate (FDR) <0.05 and pre-post fold change >1.5. [a]Significant differences (*P*-vale < 0.05) between 10 km and Half-Marathon. [b]Significant differences (*P*-vale < 0.05) between 10 km and Marathon. [c]Significant differences (*P*-vale < 0.05) between Half-Marathon and Marathon

intensity group were submaximal. Furthermore, in the variable duration cohort, a submaximal intensity was also fixed. Interestingly, Ramos et al[18] described that miR-21 and miR-210 did not respond to any of the intensities and durations tested, which is in accordance with our results. However, although they describe that miR-1, miR-24, miR-146a, miR-133a, and miR-222 increase at different volumes and/or intensities, we found no significant changes in these miRNAs, except for the overexpression of miR-1-3p in response to M. This result suggests that, under laboratory conditions, miR-1-3p is a good biomarker of both exercise volume and intensity, but in field situations, this miRNA only changes in high-volume conditions at maximal efforts. The upregulation of miR-1-3p in response to marathon had been previously described by Baggish et al,[28] Mooren et al,[29] and Clauss et al[30] although no other exercise doses were tested. Here, we provide data to confirm that miR-1-3p is a good biomarker of high-volume maximal endurance exercise, while in low-volume doses, there is an absence of response for this c-miRNA.

It was also clear that an adaptive response of c-microRNAs occurred during the season. The objective of the athletes who participated in this study was to run a marathon, for which they followed a structured training plan, complemented by participation in different races. In this case, the first race was HM, followed a month later by the 10K, which took place a month before M (Figure 1). The expression levels of 168 out of 239 c-miRNAs detected in the pre-exercise samples of all races were significantly different between races, which represents 70% of all the baseline c-miRNAs detected (Table S1), although more than 70% of the miRNAs detected were the same. Therefore, the baseline miRNA profiles were very similar, but not the expression levels of the miRNAs detected. These results suggest that the differences observed in the basal expression levels of certain c-miRNAs

in the different races may be related to an adaptive response to training. In this sense, it should be taken into account that the races were separated by 1 month. Furthermore, potential confounding factors, such as previous food intake or training, were strictly controlled. Very few authors have analyzed the response of c-miRNAs to a training intervention,[8] and heterogeneous results were observed. Thus, Aoi et al[31] described a decrease in miR-486-5p after 4 weeks of training in cycle ergometer, while Nielsen et al[32] observed no changes in this miRNA, and Baggish et al[33] did not analyze it. We have observed a significant decrease in the baseline levels of expression of this miRNA between HM and 10K, with no differences between HM and M (Table S1). On the other hand, Nielsen et al[32] described a decrease in miR-21 in response to 12 weeks of training in cycle ergometer, while Baggish et al[33] described an increase after 90 days of team-based rowing training in open water and indoor ergometer, and Aoi et al[31] did not analyze it. Our results show a significantly lower baseline level of expression of this miRNA in 10K and M compared to HM (Table S1). The heterogeneity in the catalogue of miRNAs analyzed by each author makes it difficult to establish further comparisons, but what seems clear is that the c-miRNA profile varies in response to training. In this sense, the changes that we have observed in the basal levels of expression of a significant number of c-miRNAs in the different races could also be indicating an adaptation to training. It should also be noted that, in order to compare pre-post changes in c-miRNA expression, we used the $2^{(-\Delta\Delta ct)}$ method defined by Livak and Schmittgen in 2001,[26] for which the relative expression of a gene of interest is calculated relative to some internal control gene and to a reference sample (in this case, basal sample). Therefore, the differences described in c-miRNA expression in basal samples from different races would be normalized by using this method, and therefore, comparisons between races can be carried out. Thus, we have observed that in response to M not only is higher the number of c-miRNAs that change significantly, but also the magnitude of that change (Figure 3), which could be indicating a greater regulatory role for c-miRNAs in response to this exercise dose.

Another interesting result of the present study was that, at the same exercise dose, up- and downregulated c-miRNAs were observed (Table 3). This response is evident at 10K (lowest volume, highest relative intensity) and at M (highest volume, lowest relative intensity). This suggests that there is an interaction between volume and intensity with the c-miRNA response and could be indicating an active secretion and absorption of c-miRNAs in the acute response to exercise. Since secretion and uptake of c-miRNAs are thought to be facilitated by extracellular vesicle (EV) carriers and EVs have been hypothesized to carry out inter-tissue cross-talk during exercise,[2] it is conceivable that assessments of c-miRNAs in serum reflect a snapshot of this complex process.

At this point, the following question arises: which tissues or cell types could have released the c-miRNAs detected? Although the cell origin of c-miRNAs in response to exercise is unknown, but potentially diverse,[32] most authors analyzing exercise-induced c-miRNAs in humans have focused on muscle-specific miRNAs, the so-called myomiRs: miR-1-3p, miR-1-5p, miR-133a-3p, miR-133a-5p, miR-133b, miR-206, miR-208a-3p, miR-208a-5p, miR-208b-3p, miR-208b-5p, miR-486-3p, miR-486-5p, miR-499a-3p, miR-499a-5p, and miR-499b-5p.[34] Although the results obtained for circulating myomiRs in humans in response to an acute bout of exercise are inconsistent or diverse in the different studies, mainly due to different methodological approaches and experimental designs,[8] there is unanimous agreement that their circulating levels are not influenced by passive release from damaged muscle tissue.[35] In the present study, all myomiRs were analyzed, except miR-208a and miR-499a, and detected at every sampling point. However, only miR-1-3p and miR-486-3p changed in the acute response to M. Interestingly, miR-1-3p was overexpressed, while miR-486-3p was repressed. It has been described that miR-1 is actively secreted to the bloodstream in EVs.[35] Furthermore, a large proportion of EVs secreted in response to exercise are taken by the liver.[2] Therefore, it is reasonable thinking that miR-1-3p is actively secreted by the skeletal muscle during exercise in a regulated manner, depending on the dose, with the liver being a potential target.

Regarding miR-486-3p, it has been barely analyzed in the different studies, but the acute repression observed is in accordance with what Aoi et al[31] described in response to a 1 hour of exercise on cycle ergometer at 70% VO_2max. This lower plasma level might be indicating a repressed expression or secretion of this specific miRNA in skeletal muscle, but also an intense uptake by some unknown tissue.

As mentioned before, little information is available about the target tissues of the circulating miRNAs that respond to exercise, which limits its understanding as inter-tissue communicators.[8] However, a pathway analysis of validated gene targets provides interesting information about their potential regulatory role (Tables S2 and S3). In this sense, the extensive number of validated targets on AMPK and mTOR, pathways, particularly of those miRNAs that respond to higher exercise doses, highlights the potential systemic regulatory action of these miRNAs on energy metabolism. AMPK and mTOR activities have been widely studied in the context of exercise metabolism.[1,36] Surprisingly, their acute response to marathon or half-marathon has not been explored in any tissue, although a significant response is plausible. Based on our data, in which both acutely overexpressed and suppressed circulating miRNAs target those pathways, an inter-tissue balanced regulation of energy metabolism mediated by these miRNAs could be proposed. This regulatory effect of circulating miRNAs may not be restricted to active muscle tissue. Previous studies on

obese mice have shown a regulatory role of certain miRNAs on hepatic energy metabolism both when associated with[37] and without EVs.[38] These findings, alongside data suggesting uptake of EVs to the liver with exercise,[2] may support a role of miRNAs on hepatic metabolism during exercise. Lysine degradation, a cross-cutting target of the miRNAs described, is a mainly liver process.[39] Lysine cannot be used in the muscle as a metabolic fuel, as happens with other amino acids, like branched-chain amino acids.[40] These facts contrast with a decrease of lysine plasma concentration in response to marathon[41] so our data suggested a cross-talk between tissues and regulatory role of miRNAs in response to exercise dose.

In summary, our results add novel evidence that acute endurance exercise induces specific c-miRNA profiles depending on exercise dose. Moreover, we provide evidence that certain c-miRNAs are overexpressed and repressed, which points out their possible balanced regulatory role during acute exercise and training adaptations.

5 | STRENGTHS AND LIMITATIONS

The strengths of our study are the strict control and characterization of the subjects, including dietary habits, the repeated measures experimental design, in which the same subjects performed the different exercise doses tested, and the global c-miRNA screening performed, which boosts the discovery component of this study. Considering the enormous heterogeneity that has been described in the c-miRNA response between different individuals, the repeated measures nature of this study helps in reducing the variability in the response and strengthening the ulterior statistical analysis. Furthermore, we recruited amateur athletes, who represent a great proportion of participants in endurance events and for which exploring the maximal limit of healthy exercise dose is relevant. Some limitations should also be noted. First, a larger number of volunteers would have been desirable, although, in the repeated measures design of the present study, the same 9 subjects participated in three different exercise trials and provided two samples, one before and one after each race, which led us to analyze more than 50 samples. Second, this study was performed in male subjects; whether sex difference may exert a different response is not known. Third, no samples were taken during exercise. We do not discard that exercise-induced changes in miRNA levels are not lineal, as it happens for other plasma biomarkers in response to exercise.[42] Finally, as our candidate c-miRNAs are highly expressed in a variety of cell types, their real source/s and target/s are 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 are necessary to experimentally validate these findings.

6 | PERSPECTIVE

There is indubitable evidence about the beneficial systemic effects of regular exercise to health throughout the lifespan. However, controversy persists on the effect of acute exercise, even for trained individuals. Thus, elucidating the molecular signaling pathways and effectors of acute exercise is important for the development of further healthy exercise recommendations that may include a maximal safe exercise dose.[43] In this sense, our data suggest that the modulation of certain c-miRNAs could be also achieved by physical exercise. Therefore, c-miRNAs emerge as exercise dose biomarkers.[8,16] Therapeutic modulation of miRNA function involves both, the inhibition or gain of function of a particular miRNA, and both features have been observed in the c-miRNA response to endurance exercise in active middle-aged individuals.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary Table S1. Baseline circulating microRNA analysis at dCt level. The whole microRNA profile analysed, the circulating microRNAs detected at baseline, as well as the mean dCt value are shown, together with the statistical analysis of the differences between races determined by one-way ANOVA paired test.

Whole microRNA profile	HM	dCt, HM	10K	dCt, 10K	M	dCt, M	HMvs10K	10KvsM	HMvsM
hsa-let-7a-2-3p									
hsa-let-7a-3p			hsa-let-7a-3p	17,01594815	hsa-let-7a-3p	16,64819636		ns	
hsa-let-7a-5p	hsa-let-7a-5p	6,3825495	hsa-let-7a-5p	7,41943475	hsa-let-7a-5p	6,7349195	*	ns	ns
hsa-let-7b-3p	hsa-let-7b-3p	11,03044926	hsa-let-7b-3p	13,85703458	hsa-let-7b-3p	13,9636245	ns	ns	ns
hsa-let-7b-5p	hsa-let-7b-5p	5,10035125	hsa-let-7b-5p	8,28316475	hsa-let-7b-5p	5,4686245	*	*	ns
hsa-let-7c-5p	hsa-let-7c-5p	10,09983375	hsa-let-7c-5p	11,30282275	hsa-let-7c-5p	10,198966	ns	ns	ns
hsa-let-7d-3p	hsa-let-7d-3p	6,54437875	hsa-let-7d-3p	8,86270725	hsa-let-7d-3p	8,26184325	*	ns	ns
hsa-let-7d-5p	hsa-let-7d-5p	9,3438205	hsa-let-7d-5p	10,14018075	hsa-let-7d-5p	8,82085375	ns	ns	ns
hsa-let-7e-3p									
hsa-let-7e-5p	hsa-let-7e-5p	12,22624239	hsa-let-7e-5p	12,2025515	hsa-let-7e-5p	11,73562925	ns	ns	ns
hsa-let-7f-1-3p			hsa-let-7f-1-3p	17,41353565	hsa-let-7f-1-3p	16,25652289		ns	
hsa-let-7f-2-3p			hsa-let-7f-2-3p	17,0552849	hsa-let-7f-2-3p	16,96023325		ns	
hsa-let-7f-5p	hsa-let-7f-5p	9,792604712	hsa-let-7f-5p	11,385365	hsa-let-7f-5p	10,0241995	ns	ns	ns
hsa-let-7g-3p			hsa-let-7g-3p	16,76001256	hsa-let-7g-3p	15,78165156		ns	
hsa-let-7g-5p	hsa-let-7g-5p	6,57592175	hsa-let-7g-5p	7,384584	hsa-let-7g-5p	5,60171925	ns	*	*
hsa-let-7i-3p			hsa-let-7i-3p	16,83772124	hsa-let-7i-3p	16,15337611		ns	
hsa-let-7i-5p	hsa-let-7i-5p	7,49607475	hsa-let-7i-5p	9,362731	hsa-let-7i-5p	7,15037875	ns	*	ns
hsa-miR-1	hsa-miR-1	12,25131393	hsa-miR-1	15,71974566	hsa-miR-1	14,76516094	*	ns	*
hsa-miR-100-3p	hsa-miR-100-5p	11,56017876	hsa-miR-100-5p	11,97939175	hsa-miR-100-5p	11,472086	ns	ns	ns

hsa-miR-100-5p								
hsa-miR-101-3p	hsa-miR-101-3p	5,99684275	hsa-miR-101-3p	9,7119075	hsa-miR-101-3p	7,65998	*	*	*
hsa-miR-101-5p			hsa-miR-101-5p	17,25499806	hsa-miR-101-5p	17,59251492		ns	
hsa-miR-103a-3p	hsa-miR-103a-3p	4,61915325	hsa-miR-103a-3p	6,45637225	hsa-miR-103a-3p	4,80717975	*	*	ns
hsa-miR-103b									
hsa-miR-105-3p									
hsa-miR-105-5p									
hsa-miR-106a-3p									
hsa-miR-106a-5p	hsa-miR-106a-5p	4,199966093	hsa-miR-106a-5p	5,94885425	hsa-miR-106a-5p	4,5392175	*	*	ns
hsa-miR-106b-3p	hsa-miR-106b-3p	12,08119214	hsa-miR-106b-3p	15,09533183	hsa-miR-106b-3p	15,05162211	*	ns	*
hsa-miR-106b-5p	hsa-miR-106b-5p	6,58469775	hsa-miR-106b-5p	8,22597025	hsa-miR-106b-5p	6,40652125	ns	*	ns
hsa-miR-107	hsa-miR-107	7,01862975	hsa-miR-107	8,44238725	hsa-miR-107	6,54428975	*	*	ns
hsa-miR-10a-3p									
hsa-miR-10a-5p			hsa-miR-10a-5p	16,79650257	hsa-miR-10a-5p	16,27686547		ns	
hsa-miR-10b-3p									

hsa-miR-10b-5p	hsa-miR-10b-5p	9,46351575	hsa-miR-10b-5p	11,6271075	hsa-miR-10b-5p	11,80864	*	ns	*
hsa-miR-1178-3p									
hsa-miR-1179									
hsa-miR-1181									
hsa-miR-1182									
hsa-miR-1183			hsa-miR-1183	17,73130122					
hsa-miR-1184									
hsa-miR-1185-5p									
hsa-miR-1200									
hsa-miR-1203									
hsa-miR-1204									
hsa-miR-1205									
hsa-miR-1206									
hsa-miR-1207-5p	hsa-miR-1207-5p	12,0875318	hsa-miR-1207-5p	18,23130122	hsa-miR-1207-5p	17,84079883	*	ns	*
hsa-miR-1208									
hsa-miR-122-3p			hsa-miR-122-3p	17,26837865					
hsa-miR-122-5p	hsa-miR-122-5p	5,076987	hsa-miR-122-5p	6,637715	hsa-miR-122-5p	6,984494	ns	ns	ns
hsa-miR-1224-3p					hsa-miR-1224-3p	18,13137794			
hsa-miR-1227-3p			hsa-miR-1227-3p	17,98130122					

hsa-miR-1237-3p									
hsa-miR-1238-3p									
hsa-miR-124-3p									
hsa-miR-124-5p									
hsa-miR-1243									
hsa-miR-1244									
hsa-miR-1245a			hsa-miR-1245a	17,07874306	hsa-miR-1245a	17,13137794		ns	
hsa-miR-1247-5p									
hsa-miR-1248									
hsa-miR-1249	hsa-miR-1249	12,48146089	hsa-miR-1249	16,01770516	hsa-miR-1249	16,42802464	*	ns	*
hsa-miR-1252-5p									
hsa-miR-1253									
hsa-miR-1254									
hsa-miR-1255b-5p									
hsa-miR-1256									
hsa-miR-1258									
hsa-miR-125a-3p									
hsa-miR-125a-5p	hsa-miR-125a-5p	9,15075725	hsa-miR-125a-5p	10,1998095	hsa-miR-125a-5p	9,9031065	ns	ns	ns

hsa-miR-125b-1-3p									
hsa-miR-125b-2-3p									
hsa-miR-125b-5p	hsa-miR-125b-5p	7,73044725	hsa-miR-125b-5p	9,458653	hsa-miR-125b-5p	9,44704475	ns	ns	*
hsa-miR-126-3p	hsa-miR-126-3p	3,4819585	hsa-miR-126-3p	5,54295825	hsa-miR-126-3p	5,44316675	*	ns	*
hsa-miR-126-5p	hsa-miR-126-5p	8,04667475	hsa-miR-126-5p	7,61015475	hsa-miR-126-5p	7,609249	ns	ns	ns
hsa-miR-1260a	hsa-miR-1260a	9,14085525	hsa-miR-1260a	12,07189558	hsa-miR-1260a	12,18980175	ns	ns	*
hsa-miR-1264									
hsa-miR-1265									
hsa-miR-1267									
hsa-miR-1269a									
hsa-miR-127-3p	hsa-miR-127-3p	11,28929542	hsa-miR-127-3p	14,29437066	hsa-miR-127-3p	13,57475725	ns	ns	*
hsa-miR-127-5p			hsa-miR-1271-5p	16,47929683	hsa-miR-1271-5p	17,43661008		ns	
hsa-miR-1270									
hsa-miR-1271-5p									
hsa-miR-1272									
hsa-miR-128-3p	hsa-miR-128-3p	11,05060209	hsa-miR-128-3p	11,56722025	hsa-miR-128-3p	9,91547625	ns	*	ns
hsa-miR-129-1-3p									

hsa-miR-129-2-3p									
hsa-miR-129-5p									
hsa-miR-1296-5p			hsa-miR-1296-5p	18,23130122					
hsa-miR-130a-3p	hsa-miR-130a-3p	9,671389	hsa-miR-130a-3p	10,14460925	hsa-miR-130a-3p	9,85461975	ns	ns	ns
hsa-miR-130a-5p									
hsa-miR-130b-3p	hsa-miR-130b-3p	11,83348926	hsa-miR-130b-3p	12,25406175	hsa-miR-130b-3p	12,36766225	ns	ns	ns
hsa-miR-130b-5p	hsa-miR-130b-5p	11,16665217	hsa-miR-130b-5p	16,71908624	hsa-miR-130b-5p	17,64785008	*	ns	*
hsa-miR-132-3p	hsa-miR-132-3p	9,626944	hsa-miR-132-3p	12,37146367	hsa-miR-132-3p	11,07815772	*	ns	*
hsa-miR-132-5p									
hsa-miR-133a-3p	hsa-miR-133a-3p	11,77829576	hsa-miR-133a-3p	14,76455824	hsa-miR-133a-3p	13,61708825	*	ns	ns
hsa-miR-133b	hsa-miR-133b	9,4747665	hsa-miR-133b	15,16519591	hsa-miR-133b	14,78445169	*	ns	*
hsa-miR-134-5p	hsa-miR-134-5p	11,99785439	hsa-miR-134-5p	12,08661825	hsa-miR-134-5p	13,98325075	ns	ns	ns
hsa-miR-135a-3p									
hsa-miR-135a-5p			hsa-miR-135a-5p	16,77298557					

hsa-miR-135b-3p								
hsa-miR-135b-5p								
hsa-miR-136-3p	hsa-miR-136-3p	12,75131393	hsa-miR-136-3p	13,90214908	hsa-miR-136-3p	15,50190236	ns	ns	ns
hsa-miR-136-5p	hsa-miR-136-5p	12,75131393	hsa-miR-136-5p	13,212418	hsa-miR-136-5p	14,87869869	ns	ns	ns
hsa-miR-137								
hsa-miR-138-1-3p								
hsa-miR-138-2-3p								
hsa-miR-138-5p								
hsa-miR-139-3p					hsa-miR-139-3p	18,13137794		
hsa-miR-139-5p	hsa-miR-139-5p	10,7534978	hsa-miR-139-5p	11,0680895	hsa-miR-139-5p	10,6595395	ns	ns	ns
hsa-miR-140-3p	hsa-miR-140-3p	5,878628	hsa-miR-140-3p	8,63859075	hsa-miR-140-3p	6,58309625	*	*	ns
hsa-miR-140-5p	hsa-miR-140-5p	9,72486975	hsa-miR-140-5p	11,60341625	hsa-miR-140-5p	11,999984	ns	ns	*
hsa-miR-141-3p	hsa-miR-141-3p	11,52622634	hsa-miR-141-3p	16,07075107	hsa-miR-141-3p	15,12320678	*	ns	ns
hsa-miR-141-5p								

hsa-miR-142-3p	hsa-miR-142-3p	6,09887375	hsa-miR-142-3p	10,373974	hsa-miR-142-3p	9,2370645	*	ns	*
hsa-miR-142-5p	hsa-miR-142-5p	9,1208815	hsa-miR-142-5p	8,51409675	hsa-miR-142-5p	8,00592	ns	ns	ns
hsa-miR-143-3p	hsa-miR-143-3p	8,032769	hsa-miR-143-3p	12,90333525	hsa-miR-143-3p	11,04910625	*	*	*
hsa-miR-143-5p									
hsa-miR-144-3p	hsa-miR-144-3p	6,329151	hsa-miR-144-3p	7,93638525	hsa-miR-144-3p	6,258045	*	ns	ns
hsa-miR-144-5p	hsa-miR-144-5p	8,997946	hsa-miR-144-5p	11,94600408	hsa-miR-144-5p	12,61419875	*	ns	*
hsa-miR-145-3p	hsa-miR-145-3p	12,75131393	hsa-miR-145-3p	17,08795431	hsa-miR-145-3p	17,63137794	*	ns	*
hsa-miR-145-5p	hsa-miR-145-5p	9,002677	hsa-miR-145-5p	10,15773125	hsa-miR-145-5p	9,70404875	ns	ns	ns
hsa-miR-1468-5p									
hsa-miR-146a-3p									
hsa-miR-146a-5p	hsa-miR-146a-5p	7,066933	hsa-miR-146a-5p	7,64684575	hsa-miR-146a-5p	7,6978005	ns	ns	ns
hsa-miR-146b-3p									
hsa-miR-146b-5p	hsa-miR-146b-5p	11,57117984	hsa-miR-146b-5p	11,75367375	hsa-miR-146b-5p	11,57048725	ns	ns	ns
hsa-miR-1471	hsa-miR-1471	11,72555805	hsa-miR-1471	16,80327206	hsa-miR-1471	17,16857108	*	ns	*
hsa-miR-147a									

hsa-miR-147b								
hsa-miR-148a-3p	hsa-miR-148a-3p	7,425091	hsa-miR-148a-3p	9,156563	hsa-miR-148a-3p	8,81887875	*	ns	ns
hsa-miR-148b-3p	hsa-miR-148b-3p	6,84620225	hsa-miR-148b-3p	8,9869945	hsa-miR-148b-3p	8,19567575	*	ns	ns
hsa-miR-148b-5p									
hsa-miR-149-3p	hsa-miR-149-3p	12,75131393							
hsa-miR-149-5p									
hsa-miR-150-5p	hsa-miR-150-5p	4,66055225	hsa-miR-150-5p	6,8350805	hsa-miR-150-5p	6,38944925	*	ns	ns
hsa-miR-151a-3p	hsa-miR-151a-3p	7,983318	hsa-miR-151a-3p	11,0886315	hsa-miR-151a-3p	9,79803425	*	ns	ns
hsa-miR-151a-5p	hsa-miR-151a-5p	7,47901725	hsa-miR-151a-5p	8,8062645	hsa-miR-151a-5p	8,13964075	ns	ns	ns
hsa-miR-152-3p	hsa-miR-152-3p	9,909696628	hsa-miR-152-3p	11,1372505	hsa-miR-152-3p	11,57216225	ns	ns	ns
hsa-miR-153-3p									
hsa-miR-1537-3p	hsa-miR-1537-3p	12,13070555	hsa-miR-1537-3p	17,98130122	hsa-miR-1537-3p	16,359911	*	*	*
hsa-miR-1538			hsa-miR-1538	17,98130122	hsa-miR-1538	17,63137794		ns	
hsa-miR-1539									
hsa-miR-154-3p									

hsa-miR-154-5p	hsa-miR-154-5p	10,47644055	hsa-miR-154-5p	14,02854616	hsa-miR-154-5p	12,65225	*	ns	ns
hsa-miR-155-3p									
hsa-miR-155-5p	hsa-miR-155-5p	11,7184258	hsa-miR-155-5p	14,35292825	hsa-miR-155-5p	14,58108972	ns	ns	ns
hsa-miR-15a-3p	hsa-miR-15a-3p	12,75131393							
hsa-miR-15a-5p	hsa-miR-15a-5p	3,8181955	hsa-miR-15a-5p	5,96974575	hsa-miR-15a-5p	3,733122	*	*	ns
hsa-miR-15b-3p	hsa-miR-15b-3p	8,31579375	hsa-miR-15b-3p	11,98936633	hsa-miR-15b-3p	10,599063	*	ns	*
hsa-miR-15b-5p	hsa-miR-15b-5p	7,981549	hsa-miR-15b-5p	8,689415	hsa-miR-15b-5p	7,631864	ns	ns	ns
hsa-miR-16-1-3p	hsa-miR-16-1-3p	11,53325639	hsa-miR-16-1-3p	12,1704075	hsa-miR-16-1-3p	11,9413245	ns	ns	ns
hsa-miR-16-2-3p	hsa-miR-16-2-3p	8,41726575	hsa-miR-16-2-3p	13,35709333	hsa-miR-16-2-3p	11,15281375	*	ns	*
hsa-miR-16-5p	hsa-miR-16-5p	-0,5364245	hsa-miR-16-5p	1,78535775	hsa-miR-16-5p	0,321314	*	*	ns
hsa-miR-17-3p	hsa-miR-17-3p	11,52155434	hsa-miR-17-3p	16,85687707	hsa-miR-17-3p	15,92491961	*	ns	*
hsa-miR-17-5p	hsa-miR-17-5p	11,24340576	hsa-miR-17-5p	13,3731365	hsa-miR-17-5p	10,78720725	ns	*	ns
hsa-miR-181a-2-3p			hsa-miR-181a-2-3p	16,39826715					
hsa-miR-181a-3p									
hsa-miR-181a-5p	hsa-miR-181a-5p	8,495398	hsa-miR-181a-5p	9,4302205	hsa-miR-181a-5p	8,98600925	ns	ns	ns

hsa-miR-181b-5p	hsa-miR-181b-5p	12,11355339	hsa-miR-181b-5p	15,32738975	hsa-miR-181b-5p	13,55620475	*	*	*
hsa-miR-181c-3p			hsa-miR-181c-3p	16,48184499	hsa-miR-181c-3p	18,13137794		ns	
hsa-miR-181c-5p			hsa-miR-181c-5p	16,3563144	hsa-miR-181c-5p	14,60228494		ns	
hsa-miR-181d-5p					hsa-miR-181d-5p	16,34899978			
hsa-miR-182-3p									
hsa-miR-182-5p			hsa-miR-182-5p	17,83591706	hsa-miR-182-5p	13,66597175		*	
hsa-miR-183-3p									
hsa-miR-183-5p			hsa-miR-183-5p	14,91893725	hsa-miR-183-5p	13,55600475		ns	
hsa-miR-184									
hsa-miR-185-3p									
hsa-miR-185-5p	hsa-miR-185-5p	3,29996875	hsa-miR-185-5p	9,3140005	hsa-miR-185-5p	6,095248	*	*	*
hsa-miR-186-5p	hsa-miR-186-5p	10,90981317	hsa-miR-186-5p	10,3536855	hsa-miR-186-5p	9,2524015	ns	ns	ns
hsa-miR-187-3p									
hsa-miR-187-5p									

hsa-miR-188-3p									
hsa-miR-188-5p	hsa-miR-188-5p	11,8719008	hsa-miR-188-5p	17,24344881			*		
hsa-miR-18a-3p	hsa-miR-18a-3p	11,90085867	hsa-miR-18a-3p	16,26996266	hsa-miR-18a-3p	12,78270825	*	*	ns
hsa-miR-18a-5p	hsa-miR-18a-5p	7,26576625	hsa-miR-18a-5p	9,84433975	hsa-miR-18a-5p	8,6330305	*	ns	ns
hsa-miR-18b-3p									
hsa-miR-18b-5p	hsa-miR-18b-5p	8,706353295	hsa-miR-18b-5p	9,73658775	hsa-miR-18b-5p	8,33107	ns	ns	ns
hsa-miR-1908-5p	hsa-miR-1908-5p	12,75131393	hsa-miR-1908-5p	16,30347483	hsa-miR-1908-5p	14,90395161	*	ns	ns
hsa-miR-1909-3p									
hsa-miR-190a-5p									
hsa-miR-190b									
hsa-miR-191-3p			hsa-miR-191-3p	17,19248199	hsa-miR-191-3p	18,13137794		ns	
hsa-miR-191-5p	hsa-miR-191-5p	6,287696	hsa-miR-191-5p	8,0718295	hsa-miR-191-5p	7,36755275	ns	ns	ns
hsa-miR-1911-3p									
hsa-miR-1911-5p									
hsa-miR-1912									

hsa-miR-1913	hsa-miR-1913	11,1824173	hsa-miR-1913	17,38262032	hsa-miR-1913	15,59199286	*	ns	*
hsa-miR-1914-3p									
hsa-miR-1914-5p					hsa-miR-1914-5p	17,71822383			
hsa-miR-192-3p					hsa-miR-192-3p	17,19713758			
hsa-miR-192-5p	hsa-miR-192-5p	6,9660715	hsa-miR-192-5p	8,48530625	hsa-miR-192-5p	8,0359455	ns	ns	ns
hsa-miR-193a-3p									
hsa-miR-193a-5p	hsa-miR-193a-5p	11,53499322	hsa-miR-193a-5p	14,89998566	hsa-miR-193a-5p	14,68978494	*	ns	ns
hsa-miR-193b-3p	hsa-miR-193b-3p	11,64576422	hsa-miR-193b-3p	14,12164291	hsa-miR-193b-3p	13,42870525	ns	ns	ns
hsa-miR-193b-5p			hsa-miR-193b-5p	16,20307324					
hsa-miR-194-3p	hsa-miR-194-3p	11,8294608							
hsa-miR-194-5p	hsa-miR-194-5p	9,26329075	hsa-miR-194-5p	11,39952725	hsa-miR-194-5p	10,378309	*	ns	ns
hsa-miR-195-3p									
hsa-miR-195-5p	hsa-miR-195-5p	12,24196639	hsa-miR-195-5p	13,19572275	hsa-miR-195-5p	13,05622775	ns	ns	ns
hsa-miR-196a-5p									

hsa-miR-196b-3p									
hsa-miR-196b-5p					hsa-miR-196b-5p	14,71350019			
hsa-miR-197-3p	hsa-miR-197-3p	8,260763	hsa-miR-197-3p	11,6349185	hsa-miR-197-3p	10,32836375	*	ns	*
hsa-miR-1972	hsa-miR-1972	9,88789625	hsa-miR-1972	15,30895099	hsa-miR-1972	15,79533536	*	ns	*
hsa-miR-198									
hsa-miR-199a-3p	hsa-miR-199a-3p	7,71183275	hsa-miR-199a-3p	8,594936	hsa-miR-199a-3p	8,21854975	ns	ns	ns
hsa-miR-199a-5p	hsa-miR-199a-5p	9,74873934	hsa-miR-199a-5p	10,4046775	hsa-miR-199a-5p	10,42646075	ns	ns	ns
hsa-miR-199b-5p	hsa-miR-199b-5p	11,05035317	hsa-miR-199b-5p	17,04704757	hsa-miR-199b-5p	13,75136175	*	*	*
hsa-miR-19a-3p	hsa-miR-19a-3p	4,334113	hsa-miR-19a-3p	5,81560125	hsa-miR-19a-3p	5,020445	*	ns	*
hsa-miR-19a-5p									
hsa-miR-19b-1-5p					hsa-miR-19b-1-5p	18,13137794			
hsa-miR-19b-2-5p									
hsa-miR-19b-3p	hsa-miR-19b-3p	3,368682	hsa-miR-19b-3p	4,946033	hsa-miR-19b-3p	3,5424595	*	*	ns
hsa-miR-200a-3p					hsa-miR-200a-3p	13,79551025			
hsa-miR-200a-5p									

hsa-miR-200b-3p			hsa-miR-200b-3p	16,78273307	hsa-miR-200b-3p	16,01840178	ns	
hsa-miR-200b-5p	hsa-miR-200b-5p	11,64940672			hsa-miR-200b-5p	17,49080433		*
hsa-miR-200c-3p	hsa-miR-200c-3p	11,85541701	hsa-miR-200c-3p	14,65952025	hsa-miR-200c-3p	13,965654	ns	ns	*
hsa-miR-200c-5p								
hsa-miR-202-3p								
hsa-miR-202-5p								
hsa-miR-203a	hsa-miR-203a	12,75131393						
hsa-miR-204-5p	hsa-miR-204-5p	11,97175539	hsa-miR-204-5p	15,47879432	hsa-miR-204-5p	15,84894672	ns	ns	ns
hsa-miR-205-3p	hsa-miR-205-5p	11,11511326	hsa-miR-205-5p	14,06560949	hsa-miR-205-5p	12,80251825	ns	ns	ns
hsa-miR-205-5p								
hsa-miR-2053								
hsa-miR-206	hsa-miR-206	11,8748103	hsa-miR-206	13,7461015	hsa-miR-206	15,4154865	ns	ns	ns
hsa-miR-208a-3p								
hsa-miR-208b-3p			hsa-miR-208b-3p	17,98130122	hsa-miR-208b-3p	16,30362736		*
hsa-miR-20a-3p			hsa-miR-20a-3p	17,11900082	hsa-miR-20a-3p	15,79055672		ns

hsa-miR-20a-5p	hsa-miR-20a-5p	3,86018375	hsa-miR-20a-5p	5,8597765	hsa-miR-20a-5p	4,39894425	*	*	ns
hsa-miR-20b-3p									
hsa-miR-20b-5p	hsa-miR-20b-5p	11,75152505	hsa-miR-20b-5p	16,077693	hsa-miR-20b-5p	13,81855675	*	*	ns
hsa-miR-21-3p			hsa-miR-21-3p	16,565036	hsa-miR-21-3p	15,58643319		ns	
hsa-miR-21-5p	hsa-miR-21-5p	2,99603	hsa-miR-21-5p	4,89567325	hsa-miR-21-5p	4,9769135	*	ns	*
hsa-miR-210-3p	hsa-miR-210-3p	10,96264609	hsa-miR-210-3p	14,41744691	hsa-miR-210-3p	10,71782875	*	ns	ns
hsa-miR-211-5p									
hsa-miR-2110	hsa-miR-2110	12,25131393	hsa-miR-2110	14,97728608	hsa-miR-2110	13,92431325	*	ns	*
hsa-miR-2113									
hsa-miR-212-3p	hsa-miR-212-3p	12,75131393			hsa-miR-212-3p	18,13137794			*
hsa-miR-212-5p									
hsa-miR-214-3p	hsa-miR-214-3p	11,95140489	hsa-miR-214-3p	15,31421966	hsa-miR-214-3p	15,54988419	ns	ns	*
hsa-miR-214-5p									
hsa-miR-215-5p	hsa-miR-215-5p	8,4431395	hsa-miR-215-5p	9,65821325	hsa-miR-215-5p	9,210854	ns	ns	*
hsa-miR-216a-5p									
hsa-miR-216b-5p									

hsa-miR-217									
hsa-miR-218-1-3p									
hsa-miR-218-2-3p									
hsa-miR-218-5p									
hsa-miR-219a-1-3p									
hsa-miR-219a-2-3p									
hsa-miR-219a-5p									
hsa-miR-22-3p	hsa-miR-22-3p	7,33123975	hsa-miR-22-3p	8,044287	hsa-miR-22-3p	7,1667395	ns	ns	ns
hsa-miR-22-5p	hsa-miR-22-5p	12,75131393	hsa-miR-22-5p	12,01332875	hsa-miR-22-5p	11,2453775	ns	ns	*
hsa-miR-221-3p	hsa-miR-221-3p	6,227121	hsa-miR-221-3p	6,7877925	hsa-miR-221-3p	7,14822125	ns	ns	ns
hsa-miR-221-5p			hsa-miR-221-5p	15,92636483	hsa-miR-221-5p	17,20811433		ns	
hsa-miR-222-3p	hsa-miR-222-3p	7,062510646	hsa-miR-222-3p	8,977329	hsa-miR-222-3p	8,241616	*	ns	*
hsa-miR-222-5p									
hsa-miR-223-3p	hsa-miR-223-3p	1,02679525	hsa-miR-223-3p	2,95966625	hsa-miR-223-3p	2,93942075	ns	ns	*
hsa-miR-223-5p	hsa-miR-223-5p	9,952321712	hsa-miR-223-5p	14,57423533	hsa-miR-223-5p	13,56554625	*	ns	*

hsa-miR-224-3p								
hsa-miR-224-5p			hsa-miR-224-5p	15,40867474	hsa-miR-224-5p	16,21308281	ns	
hsa-miR-23a-3p	hsa-miR-23a-3p	2,88248725	hsa-miR-23a-3p	4,54360575	hsa-miR-23a-3p	4,471084	*	ns	*
hsa-miR-23a-5p	hsa-miR-23a-5p	11,46460272	hsa-miR-23a-5p	16,16873216	hsa-miR-23a-5p	16,69301375	*	ns	*
hsa-miR-23b-3p	hsa-miR-23b-3p	5,035522	hsa-miR-23b-3p	7,40843175	hsa-miR-23b-3p	6,8292925	*	ns	*
hsa-miR-23b-5p								
hsa-miR-24-1-5p								
hsa-miR-24-2-5p			hsa-miR-24-2-5p	16,0925139	hsa-miR-24-2-5p	15,45358614		ns	
hsa-miR-24-3p	hsa-miR-24-3p	3,731313	hsa-miR-24-3p	6,01348225	hsa-miR-24-3p	5,73389025	*	ns	*
hsa-miR-25-3p	hsa-miR-25-3p	3,96759075	hsa-miR-25-3p	6,92476075	hsa-miR-25-3p	4,94572175	*	*	ns
hsa-miR-25-5p								
hsa-miR-26a-1-3p								
hsa-miR-26a-2-3p								
hsa-miR-26a-5p	hsa-miR-26a-5p	7,1875295	hsa-miR-26a-5p	6,59486125	hsa-miR-26a-5p	6,4578505	ns	ns	*
hsa-miR-26b-3p	hsa-miR-26b-3p	12,46282614	hsa-miR-26b-3p	16,41420908	hsa-miR-26b-3p	15,66130678	*	ns	*

hsa-miR-26b-5p	hsa-miR-26b-5p	8,961636	hsa-miR-26b-5p	10,3091775	hsa-miR-26b-5p	8,8590675	ns	ns	ns
hsa-miR-27a-3p	hsa-miR-27a-3p	7,074054	hsa-miR-27a-3p	8,6683495	hsa-miR-27a-3p	8,60344425	*	ns	ns
hsa-miR-27a-5p	hsa-miR-27a-5p	8,02538225	hsa-miR-27a-5p	12,66409108	hsa-miR-27a-5p	15,39097	ns	*	*
hsa-miR-27b-3p	hsa-miR-27b-3p	5,48705825	hsa-miR-27b-3p	7,2055805	hsa-miR-27b-3p	7,61067575	*	ns	ns
hsa-miR-27b-5p									
hsa-miR-28-3p	hsa-miR-28-3p	9,444585962	hsa-miR-28-3p	12,29580708	hsa-miR-28-3p	12,022989	ns	ns	ns
hsa-miR-28-5p	hsa-miR-28-5p	10,13230946	hsa-miR-28-5p	11,8274345	hsa-miR-28-5p	10,5247	ns	ns	ns
hsa-miR-296-3p									
hsa-miR-296-5p			hsa-miR-296-5p	15,22644865					
hsa-miR-298									
hsa-miR-299-3p									
hsa-miR-299-5p									
hsa-miR-29a-3p	hsa-miR-29a-3p	7,277133	hsa-miR-29a-3p	8,19054675	hsa-miR-29a-3p	8,59467425	*	ns	ns
hsa-miR-29a-5p					hsa-miR-29a-5p	16,81240158			
hsa-miR-29b-2-5p			hsa-miR-29b-2-5p	15,75613907	hsa-miR-29b-2-5p	14,13391078		ns	

hsa-miR-29b-3p	hsa-miR-29b-3p	8,8995655	hsa-miR-29b-3p	9,9293965	hsa-miR-29b-3p	9,248284	*	ns	ns
hsa-miR-29c-3p	hsa-miR-29c-3p	7,30065775	hsa-miR-29c-3p	7,87788425	hsa-miR-29c-3p	7,9156495	ns	ns	ns
hsa-miR-29c-5p	hsa-miR-29c-5p	11,57193209	hsa-miR-29c-5p	17,98130122	hsa-miR-29c-5p	15,79902486	*	*	*
hsa-miR-300	hsa-miR-300	12,50131393	hsa-miR-300	18,23130122	hsa-miR-300	17,4768445	*	ns	*
hsa-miR-301a-3p	hsa-miR-301a-3p	10,31216601	hsa-miR-301a-3p	10,865902	hsa-miR-301a-3p	9,637507	ns	ns	ns
hsa-miR-301b			hsa-miR-301b	16,24037557	hsa-miR-301b	15,16990194		ns	
hsa-miR-302a-3p									
hsa-miR-302b-3p									
hsa-miR-302b-5p									
hsa-miR-302c-3p									
hsa-miR-302c-5p									
hsa-miR-302d-3p									
hsa-miR-302d-5p									
hsa-miR-302e									
hsa-miR-302f									
hsa-miR-30a-3p			hsa-miR-30a-3p	15,24000383	hsa-miR-30a-3p	16,22399656		ns	

hsa-miR-30a-5p	hsa-miR-30a-5p	10,508753	hsa-miR-30a-5p	12,307111	hsa-miR-30a-5p	12,1632205	*	ns	*
hsa-miR-30b-3p									
hsa-miR-30b-5p	hsa-miR-30b-5p	6,862244	hsa-miR-30b-5p	7,8404105	hsa-miR-30b-5p	7,24454025	*	ns	ns
hsa-miR-30c-1-3p									
hsa-miR-30c-2-3p									
hsa-miR-30c-5p	hsa-miR-30c-5p	8,20007125	hsa-miR-30c-5p	9,03074325	hsa-miR-30c-5p	8,86921925	ns	ns	ns
hsa-miR-30d-3p	hsa-miR-30d-3p	12,3559653							
hsa-miR-30d-5p	hsa-miR-30d-5p	5,5377775	hsa-miR-30d-5p	13,386378	hsa-miR-30d-5p	11,6989695	*	ns	*
hsa-miR-30e-3p	hsa-miR-30e-3p	10,79928521	hsa-miR-30e-3p	13,344523	hsa-miR-30e-3p	12,7105685	ns	ns	*
hsa-miR-30e-5p	hsa-miR-30e-5p	5,53189225	hsa-miR-30e-5p	7,4616865	hsa-miR-30e-5p	6,52379125	ns	ns	ns
hsa-miR-31-3p					hsa-miR-31-3p	17,74712517			
hsa-miR-31-5p	hsa-miR-31-5p	11,55995938	hsa-miR-31-5p	15,7566754	hsa-miR-31-5p	14,80993619	ns	ns	ns
hsa-miR-32-3p									
hsa-miR-32-5p	hsa-miR-32-5p	9,402599	hsa-miR-32-5p	10,90077525	hsa-miR-32-5p	10,419454	ns	ns	ns
hsa-miR-320a	hsa-miR-320a	4,94452125	hsa-miR-320a	7,85990275	hsa-miR-320a	6,11453	*	*	ns
hsa-miR-320b	hsa-miR-320b	5,511994	hsa-miR-320b	10,76271983	hsa-miR-320b	9,47773225	*	ns	*
hsa-miR-320c	hsa-miR-320c	7,19454175	hsa-miR-320c	11,23226083	hsa-miR-320c	9,9623345	*	*	*
hsa-miR-320d	hsa-miR-320d	8,2740895	hsa-miR-320d	11,70805483	hsa-miR-320d	10,46728925	*	*	*

hsa-miR-323a-3p			hsa-miR-323a-3p	15,31162116	hsa-miR-323a-3p	15,42732597		ns	
hsa-miR-323a-5p									
hsa-miR-323b-5p									
hsa-miR-324-3p	hsa-miR-324-3p	8,3869855	hsa-miR-324-3p	11,0161655	hsa-miR-324-3p	8,9892825	*	*	ns
hsa-miR-324-5p	hsa-miR-324-5p	8,73364325	hsa-miR-324-5p	13,27333075	hsa-miR-324-5p	10,7155635	*	*	*
hsa-miR-325									
hsa-miR-326	hsa-miR-326	9,302215878	hsa-miR-326	14,48088999	hsa-miR-326	14,26908494	ns	ns	*
hsa-miR-328-3p	hsa-miR-328-3p	9,462477295	hsa-miR-328-3p	12,6994575	hsa-miR-328-3p	11,6371795	*	ns	ns
hsa-miR-329-3p	hsa-miR-329-3p	12,25131393	hsa-miR-329-3p	13,66802975	hsa-miR-329-3p	13,502412	ns	ns	ns
hsa-miR-330-3p			hsa-miR-330-3p	16,84675948	hsa-miR-330-3p	16,58579439		ns	
hsa-miR-330-5p									
hsa-miR-331-3p	hsa-miR-331-3p	8,5398345	hsa-miR-331-3p	14,17714225	hsa-miR-331-3p	11,78068125	*	ns	*
hsa-miR-331-5p									
hsa-miR-335-3p	hsa-miR-335-3p	12,75131393	hsa-miR-335-3p	16,86922324	hsa-miR-335-3p	15,86422706	*	ns	ns
hsa-miR-335-5p	hsa-miR-335-5p	11,75155755	hsa-miR-335-5p	13,4699315	hsa-miR-335-5p	11,2724795	ns	ns	ns

hsa-miR-337-3p	hsa-miR-337-3p	12,50131393	hsa-miR-337-3p	13,27992325	hsa-miR-337-3p	15,19244494	ns	ns	ns
hsa-miR-337-5p	hsa-miR-337-5p	11,90457764	hsa-miR-337-5p	15,30652174	hsa-miR-337-5p	15,42604425	*	ns	*
hsa-miR-338-3p	hsa-miR-338-3p	11,19442946	hsa-miR-338-3p	13,04641075	hsa-miR-338-3p	12,9384975	ns	ns	ns
hsa-miR-338-5p			hsa-miR-338-5p	17,73130122					
hsa-miR-339-3p	hsa-miR-339-3p	11,42803101	hsa-miR-339-3p	15,32105699	hsa-miR-339-3p	12,704696	*	ns	ns
hsa-miR-339-5p	hsa-miR-339-5p	11,63156089	hsa-miR-339-5p	13,58126525	hsa-miR-339-5p	13,53565925	ns	ns	ns
hsa-miR-33a-3p									
hsa-miR-33a-5p	hsa-miR-33a-5p	12,50131393	hsa-miR-33a-5p	16,81008531	hsa-miR-33a-5p	15,12193844	*	ns	ns
hsa-miR-33b-3p									
hsa-miR-33b-5p			hsa-miR-33b-5p	17,11980415	hsa-miR-33b-5p	17,41622367		ns	
hsa-miR-340-3p	hsa-miR-340-5p	11,24069101	hsa-miR-340-3p	17,00000449	hsa-miR-340-3p	17,18744417	*	ns	*
hsa-miR-340-5p					hsa-miR-340-5p	17,3395875			
hsa-miR-342-3p	hsa-miR-342-3p	6,61060075	hsa-miR-342-3p	8,38723025	hsa-miR-342-3p	7,728422	*	ns	ns
hsa-miR-342-5p	hsa-miR-342-5p	11,42051617	hsa-miR-342-5p	15,58818808	hsa-miR-342-5p	15,80030861	*	ns	*

hsa-miR-345-5p	hsa-miR-345-5p	7,48916575	hsa-miR-345-5p	17,98130122	hsa-miR-345-5p	16,22196428	*	*	*
hsa-miR-346			hsa-miR-346	14,33088125	hsa-miR-346	14,70512425		*	
hsa-miR-34a-3p	hsa-miR-34a-3p	8,21156325			hsa-miR-34a-3p	16,48802756	*	*	*
hsa-miR-34a-5p	hsa-miR-34a-5p	10,80379755	hsa-miR-34a-5p	14,51837425	hsa-miR-34a-5p	12,5358765	*	ns	ns
hsa-miR-34b-3p									
hsa-miR-34b-5p									
hsa-miR-34c-3p									
hsa-miR-34c-5p					hsa-miR-34c-5p	18,13137794			
hsa-miR-361-3p	hsa-miR-361-3p	11,38572109	hsa-miR-361-3p	14,744759	hsa-miR-361-3p	12,8578685	*	ns	ns
hsa-miR-361-5p	hsa-miR-361-5p	7,33341275	hsa-miR-361-5p	9,255522	hsa-miR-361-5p	9,57798925	*	ns	ns
hsa-miR-362-3p	hsa-miR-362-3p	10,05579063	hsa-miR-362-3p	12,30306683	hsa-miR-362-3p	11,50911025	*	*	ns
hsa-miR-362-5p			hsa-miR-362-5p	17,28330281	hsa-miR-362-5p	16,84039344		ns	
hsa-miR-363-3p	hsa-miR-363-3p	7,77370425	hsa-miR-363-3p	10,53009575	hsa-miR-363-3p	8,3078475	*	*	ns
hsa-miR-363-5p									
hsa-miR-365a-3p	hsa-miR-365a-3p	9,524459545	hsa-miR-365a-3p	13,2796105	hsa-miR-365a-3p	11,7576465	ns	ns	ns

hsa-miR-365b-5p									
hsa-miR-367-3p									
hsa-miR-367-5p									
hsa-miR-369-3p	hsa-miR-369-3p	12,50131393	hsa-miR-369-3p	16,83034174	hsa-miR-369-3p	16,22693736	*	ns	*
hsa-miR-369-5p					hsa-miR-369-5p	17,42223522			
hsa-miR-370-3p			hsa-miR-370	15,88568365	hsa-miR-370	16,27610072		ns	
hsa-miR-371a-3p									
hsa-miR-371a-5p	hsa-miR-371a-5p	12,75131393							
hsa-miR-372-3p									
hsa-miR-373-3p	hsa-miR-373-3p	11,34970076							
hsa-miR-373-5p									
hsa-miR-374a-5p	hsa-miR-374a-5p	10,68470071	hsa-miR-374a-5p	11,10116275	hsa-miR-374a-5p	10,6196615	ns	ns	ns
hsa-miR-374b-3p									
hsa-miR-374b-5p	hsa-miR-374b-5p	9,63681175	hsa-miR-374b-5p	10,59117875	hsa-miR-374b-5p	9,928304	ns	ns	ns

hsa-miR-375	hsa-miR-375	8,20378575	hsa-miR-375	11,94413025	hsa-miR-375	11,07950925	*	ns	*
hsa-miR-376a-3p	hsa-miR-376a-3p	10,99536805	hsa-miR-376a-3p	12,9632515	hsa-miR-376a-3p	12,046511	ns	ns	ns
hsa-miR-376a-5p									
hsa-miR-376b-3p	hsa-miR-376b-3p	12,24503497	hsa-miR-376b-3p	13,726563	hsa-miR-376b-3p	14,56120469	ns	ns	ns
hsa-miR-376c-3p	hsa-miR-376c-3p	9,0162065	hsa-miR-376c-3p	10,2961945	hsa-miR-376c-3p	10,42595175	*	ns	ns
hsa-miR-377-3p	hsa-miR-377-3p	11,81769217	hsa-miR-377-3p	14,6517885	hsa-miR-377-3p	16,11150347	*	ns	*
hsa-miR-377-5p					hsa-miR-377-5p	18,13137794			
hsa-miR-378a-3p	hsa-miR-378a-3p	7,93049125	hsa-miR-378a-3p	10,61006	hsa-miR-378a-3p	10,077065	*	ns	*
hsa-miR-378a-5p			hsa-miR-378a-5p	15,36348491	hsa-miR-378a-5p	15,48617175		ns	
hsa-miR-379-3p									
hsa-miR-379-5p			hsa-miR-379-5p	14,97956391	hsa-miR-379-5p	15,86151197		ns	
hsa-miR-380-3p									
hsa-miR-380-5p									
hsa-miR-381-3p	hsa-miR-381-3p	12,75131393	hsa-miR-381-3p	14,300606	hsa-miR-381-3p	16,97201575	*	ns	ns

hsa-miR-381-5p									
hsa-miR-382-3p			hsa-miR-382-3p	16,77045115	hsa-miR-382-3p	16,90569747		ns	
hsa-miR-382-5p	hsa-miR-382-5p	10,38484205	hsa-miR-382-5p	14,47010991	hsa-miR-382-5p	13,42418219	ns	ns	ns
hsa-miR-383-5p									
hsa-miR-384									
hsa-miR-409-3p	hsa-miR-409-3p	8,5184995	hsa-miR-409-3p	10,83386125	hsa-miR-409-3p	12,40228469	*	ns	ns
hsa-miR-409-5p									
hsa-miR-410-3p	hsa-miR-410-3p	11,95581817	hsa-miR-410-3p	13,2220405	hsa-miR-410-3p	12,9329255	*	ns	ns
hsa-miR-411-3p									
hsa-miR-411-5p			hsa-miR-411-5p	16,23914724	hsa-miR-411-5p	15,97876097		ns	
hsa-miR-412-3p									
hsa-miR-421	hsa-miR-421	10,61589613	hsa-miR-421	14,38608541	hsa-miR-421	12,59344625	*	ns	ns
hsa-miR-422a									
hsa-miR-423-3p	hsa-miR-423-3p	8,84425575	hsa-miR-423-3p	10,25677875	hsa-miR-423-3p	8,666757	*	ns	ns
hsa-miR-423-5p	hsa-miR-423-5p	8,039793	hsa-miR-423-5p	8,625193	hsa-miR-423-5p	6,875161	ns	*	*

hsa-miR-424-3p			hsa-miR-424-3p	17,98130122	hsa-miR-424-3p	17,30984183		ns	
hsa-miR-424-5p	hsa-miR-424-5p	8,25921425	hsa-miR-424-5p	10,972949	hsa-miR-424-5p	9,75133525	*	ns	*
hsa-miR-425-3p	hsa-miR-425-3p	12,75131393	hsa-miR-425-3p	12,15305525	hsa-miR-425-3p	10,332131	ns	ns	*
hsa-miR-425-5p	hsa-miR-425-5p	6,09037025	hsa-miR-425-5p	8,01600775	hsa-miR-425-5p	6,6002495	*	*	ns
hsa-miR-429									
hsa-miR-431-3p									
hsa-miR-431-5p	hsa-miR-431-5p	12,20310572	hsa-miR-431-5p	14,050064	hsa-miR-431-5p	13,72247525	*	ns	ns
hsa-miR-432-3p	hsa-miR-432-5p	11,77530567	hsa-miR-432-5p	14,42429716	hsa-miR-432-5p	13,0918775	ns	ns	ns
hsa-miR-432-5p									
hsa-miR-433-3p			hsa-miR-433-3p	16,08748991	hsa-miR-433-3p	14,96840744		ns	
hsa-miR-448									
hsa-miR-449a									
hsa-miR-449b-3p									
hsa-miR-449b-5p									
hsa-miR-450a-5p	hsa-miR-450a-5p	12,75131393							

hsa-miR-450b-3p									
hsa-miR-450b-5p									
hsa-miR-451a	hsa-miR-451a	-0,81817625	hsa-miR-451a	0,275336	hsa-miR-451a	-1,83916525	*	*	ns
hsa-miR-452-3p									
hsa-miR-452-5p									
hsa-miR-454-3p	hsa-miR-454-3p	11,17193051	hsa-miR-454-3p	11,821537	hsa-miR-454-3p	10,5878635	ns	ns	ns
hsa-miR-454-5p									
hsa-miR-455-3p			hsa-miR-455-3p	16,15755774	hsa-miR-455-3p	16,50667406		ns	
hsa-miR-455-5p									
hsa-miR-483-3p	hsa-miR-483-3p	12,24842014	hsa-miR-483-3p	14,4918545	hsa-miR-483-3p	13,94522225	ns	ns	ns
hsa-miR-483-5p	hsa-miR-483-5p	9,909238628	hsa-miR-483-5p	14,62974558	hsa-miR-483-5p	15,61003436	*	ns	*
hsa-miR-484	hsa-miR-484	8,692463795	hsa-miR-484	8,953336	hsa-miR-484	7,951321	ns	ns	ns
hsa-miR-485-3p	hsa-miR-485-3p	12,75131393							
hsa-miR-486-3p	hsa-miR-486-3p	12,00131393	hsa-miR-486-3p	17,21963848	hsa-miR-486-3p	14,10686175	*	*	*
hsa-miR-486-5p	hsa-miR-486-5p	4,18872625	hsa-miR-486-5p	7,096211	hsa-miR-486-5p	4,863781	*	*	ns

hsa-miR-487a-3p			hsa-miR-487a-3p	17,4305824	hsa-miR-487a-3p	16,841986		ns	
hsa-miR-487b-3p	hsa-miR-487b-3p	12,27681589	hsa-miR-487b-3p	12,61561125	hsa-miR-487b-3p	12,230135	ns	ns	ns
hsa-miR-488-3p									
hsa-miR-488-5p									
hsa-miR-489-3p									
hsa-miR-490-3p			hsa-miR-490-3p	10,31417925	hsa-miR-490-3p	10,80646625		ns	
hsa-miR-490-5p									
hsa-miR-491-3p									
hsa-miR-491-5p	hsa-miR-491-5p	11,42238889	hsa-miR-491-5p	17,68741481	hsa-miR-491-5p	13,63068625	*	*	*
hsa-miR-492									
hsa-miR-493-3p			hsa-miR-493-3p	16,24742557	hsa-miR-493-3p	15,07101528		ns	
hsa-miR-493-5p			hsa-miR-493-5p	16,86933065	hsa-miR-493-5p	16,61197281		ns	
hsa-miR-494-3p			hsa-miR-494-3p	17,34813632					
hsa-miR-495-3p	hsa-miR-495-3p	11,30088717	hsa-miR-495-3p	11,405483	hsa-miR-495-3p	10,500301	ns	ns	ns
hsa-miR-496			hsa-miR-496	16,08814899	hsa-miR-496	16,60280081		ns	

hsa-miR-497-5p	hsa-miR-497-5p	10,09696325	hsa-miR-497-5p	13,75907674	hsa-miR-497-5p	12,326489	ns	ns	*
hsa-miR-498									
hsa-miR-499a-3p									
hsa-miR-499a-5p			hsa-miR-499a-5p	17,32633356					
hsa-miR-500a-5p			hsa-miR-500a-5p	17,17196032	hsa-miR-500a-5p	14,160371		*	
hsa-miR-501-3p	hsa-miR-501-3p	12,75131393	hsa-miR-501-3p	16,41430674	hsa-miR-501-3p	15,44414411	*	ns	*
hsa-miR-501-5p			hsa-miR-501-5p	17,20260581	hsa-miR-501-5p	14,37968969		ns	
hsa-miR-502-3p	hsa-miR-502-3p	11,54730214	hsa-miR-502-3p	14,66110408	hsa-miR-502-3p	12,744931	*	*	ns
hsa-miR-502-5p	hsa-miR-502-5p	11,51377939	hsa-miR-502-5p	17,06660515			*		
hsa-miR-503-5p			hsa-miR-503-5p	15,8605869					
hsa-miR-504-5p									
hsa-miR-505-3p	hsa-miR-505-3p	10,62101971	hsa-miR-505-3p	12,756609	hsa-miR-505-3p	12,60675725	ns	ns	*
hsa-miR-505-5p			hsa-miR-505-5p	17,98130122	hsa-miR-505-5p	14,6662375		*	
hsa-miR-506-3p									
hsa-miR-507									

hsa-miR-508-3p					
hsa-miR-508-5p					
hsa-miR-509-3-5p					
hsa-miR-509-3p	hsa-miR-509-3p	10,62998617			
hsa-miR-510-5p					
hsa-miR-511-5p	hsa-miR-511-5p	12,02168055	hsa-miR-511-5p	16,58793889	*
hsa-miR-512-3p					
hsa-miR-512-5p					
hsa-miR-513a-3p					
hsa-miR-513a-5p					
hsa-miR-513b-5p					
hsa-miR-513c-5p					
hsa-miR-514a-3p					
hsa-miR-515-3p					

hsa-miR-515-5p
hsa-miR-516a-3p
hsa-miR-516a-5p
hsa-miR-516b-5p
hsa-miR-517-5p
hsa-miR-517a-3p
hsa-miR-517c-3p
hsa-miR-518a-3p
hsa-miR-518b
hsa-miR-518c-3p
hsa-miR-518c-5p
hsa-miR-518d-3p
hsa-miR-518d-5p
hsa-miR-518e-3p

hsa-miR-517a-3p 17,88137794

hsa-miR-518e-5p					
hsa-miR-518f-3p					
hsa-miR-518f-5p	hsa-miR-518f-5p	12,75131393	hsa-miR-518f-5p	16,641337	*
hsa-miR-519a-3p					
hsa-miR-519b-3p					
hsa-miR-519c-3p					
hsa-miR-519d-3p					
hsa-miR-519e-3p					
hsa-miR-519e-5p					
hsa-miR-520a-3p					
hsa-miR-520a-5p					
hsa-miR-520b					
hsa-miR-520c-3p					
hsa-miR-520d-3p					

hsa-miR-520d-5p									
hsa-miR-520e									
hsa-miR-520f-3p									
hsa-miR-520g-3p									
hsa-miR-520h			hsa-miR-520h	15,22277508	hsa-miR-520h	14,562719		ns	
hsa-miR-521									
hsa-miR-522-3p									
hsa-miR-523-3p									
hsa-miR-524-3p									
hsa-miR-524-5p	hsa-miR-524-5p	10,83893476							
hsa-miR-525-3p									
hsa-miR-525-5p									
hsa-miR-526b-3p									
hsa-miR-526b-5p	hsa-miR-526b-5p	12,75131393							
hsa-miR-532-3p	hsa-miR-532-3p	8,93778425	hsa-miR-532-3p	13,66099133	hsa-miR-532-3p	12,76975125	*	ns	*

hsa-miR-532-5p	hsa-miR-532-5p	9,82020375	hsa-miR-532-5p	12,5846145	hsa-miR-532-5p	10,91228975	*	*	ns
hsa-miR-539-5p									
hsa-miR-541-3p									
hsa-miR-541-5p									
hsa-miR-542-5p			hsa-miR-542-5p	17,20614581	hsa-miR-542-5p	15,59651097		ns	
hsa-miR-543	hsa-miR-543	12,50131393	hsa-miR-543	13,77466833	hsa-miR-543	14,02223638	ns	ns	ns
hsa-miR-544a									
hsa-miR-545-3p					hsa-miR-545-3p	17,56894722			
hsa-miR-548a-3p	hsa-miR-548a-3p	11,47065864	hsa-miR-548a-3p	14,51309383	hsa-miR-548a-3p	13,94555536	ns	ns	ns
hsa-miR-548a-5p									
hsa-miR-548b-3p			hsa-miR-548b-3p	18,23130122					
hsa-miR-548c-3p									
hsa-miR-548c-5p	hsa-miR-548c-5p	9,09715475	hsa-miR-548c-5p	15,08670358	hsa-miR-548c-5p	15,71931875	*	ns	*
hsa-miR-548d-3p									
hsa-miR-548d-5p									

hsa-miR-548e-3p			hsa-miR-548e-3p	17,48130122	hsa-miR-548e-3p	17,38137794		ns	
hsa-miR-548h-5p	hsa-miR-548h-5p	11,38145305	hsa-miR-548h-5p	17,37380074	hsa-miR-548h-5p	17,04838747	*	ns	*
hsa-miR-548i									
hsa-miR-548j-5p					hsa-miR-548j-5p	16,15559219			
hsa-miR-548k									
hsa-miR-548l									
hsa-miR-548m									
hsa-miR-548n									
hsa-miR-549a									
hsa-miR-550a-3p			hsa-miR-550a-3p	16,07234624	hsa-miR-550a-3p	14,23660325		ns	
hsa-miR-550a-5p									
hsa-miR-551a	hsa-miR-551a	11,22571884	hsa-miR-551a	17,69231881	hsa-miR-551a	15,82112861	*	ns	*
hsa-miR-551b-3p			hsa-miR-551b-3p	15,26787391	hsa-miR-551b-3p	14,37981244		ns	
hsa-miR-551b-5p									
hsa-miR-552-3p					hsa-miR-552-3p	17,63137794			
hsa-miR-553									
hsa-miR-554									
hsa-miR-555			hsa-miR-555	17,48130122					
hsa-miR-556-3p					hsa-miR-556-3p	18,13137794			

hsa-miR-556-5p									
hsa-miR-557									
hsa-miR-558									
hsa-miR-559									
hsa-miR-561-3p									
hsa-miR-562									
hsa-miR-563									
hsa-miR-564									
hsa-miR-566	hsa-miR-566	12,75131393							
hsa-miR-567									
hsa-miR-569									
hsa-miR-570-3p	hsa-miR-570-3p	12,75131393	hsa-miR-570-3p	16,22649965	hsa-miR-570-3p	15,75447872	ns	ns	ns
hsa-miR-571									
hsa-miR-572									
hsa-miR-573									
hsa-miR-574-3p	hsa-miR-574-3p	11,7107478	hsa-miR-574-3p	11,4187935	hsa-miR-574-3p	12,09122025	ns	ns	ns
hsa-miR-576-3p									
hsa-miR-576-5p	hsa-miR-576-5p	12,75131393	hsa-miR-576-5p	17,13966582	hsa-miR-576-5p	14,72241025	*	*	*
hsa-miR-577									
hsa-miR-578									

hsa-miR-579-3p									
hsa-miR-580-3p									
hsa-miR-581									
hsa-miR-582-3p	hsa-miR-582-5p	11,77878314	hsa-miR-582-3p	17,58233273	hsa-miR-582-3p	18,13137794	*	ns	*
hsa-miR-582-5p			hsa-miR-582-5p	14,15833125	hsa-miR-582-5p	14,63634978		ns	
hsa-miR-583									
hsa-miR-584-5p	hsa-miR-584-5p	12,0204383	hsa-miR-584-5p	13,7836145	hsa-miR-584-5p	12,51942199	ns	ns	ns
hsa-miR-585-3p									
hsa-miR-586									
hsa-miR-587									
hsa-miR-588	hsa-miR-588	12,50131393							
hsa-miR-589-3p	hsa-miR-589-3p	10,21737696							
hsa-miR-589-5p					hsa-miR-589-5p	15,59487847			
hsa-miR-590-3p	hsa-miR-590-3p	12,2229278	hsa-miR-590-3p	15,77934733	hsa-miR-590-3p	15,44666319	*	ns	ns
hsa-miR-590-5p	hsa-miR-590-5p	8,9936535	hsa-miR-590-5p	11,879107	hsa-miR-590-5p	11,6134005	*	ns	*
hsa-miR-591									
hsa-miR-592									

hsa-miR-593-3p									
hsa-miR-593-5p									
hsa-miR-595									
hsa-miR-596					hsa-miR-596	18,13137794			
hsa-miR-597-5p									
hsa-miR-598-3p	hsa-miR-598-3p	10,2491958	hsa-miR-598-3p	14,61718466	hsa-miR-598-3p	15,260232	*	ns	*
hsa-miR-599									
hsa-miR-600									
hsa-miR-601									
hsa-miR-602									
hsa-miR-603									
hsa-miR-604			hsa-miR-604	17,36546381	hsa-miR-604	16,83476581		ns	
hsa-miR-605-5p					hsa-miR-605-5p	16,73362492			
hsa-miR-606									
hsa-miR-607									
hsa-miR-608									
hsa-miR-609									
hsa-miR-610									
hsa-miR-611									
hsa-miR-612									
hsa-miR-614									

hsa-miR-615-3p									
hsa-miR-615-5p									
hsa-miR-616-3p									
hsa-miR-616-5p									
hsa-miR-617									
hsa-miR-618									
hsa-miR-619-3p									
hsa-miR-620									
hsa-miR-621									
hsa-miR-622									
hsa-miR-623									
hsa-miR-624-3p									
hsa-miR-624-5p	hsa-miR-624-5p	12,2044098	hsa-miR-624-5p	16,92278374	hsa-miR-624-5p	15,27570975	*	ns	*
hsa-miR-625-3p			hsa-miR-625-3p	14,74506675	hsa-miR-625-3p	14,06873553		ns	
hsa-miR-626									
hsa-miR-627-5p					hsa-miR-627-5p	17,88137794			
hsa-miR-628-3p	hsa-miR-628-3p	11,9556718	hsa-miR-628-3p	15,28512575	hsa-miR-628-3p	15,14306544	*	ns	ns

hsa-miR-628-5p					hsa-miR-628-5p	18,13137794			
hsa-miR-629-3p									
hsa-miR-629-5p	hsa-miR-629-5p	11,57948039	hsa-miR-629-5p	13,0349325	hsa-miR-629-5p	12,17558975	ns	ns	ns
hsa-miR-630									
hsa-miR-631									
hsa-miR-632									
hsa-miR-634									
hsa-miR-635									
hsa-miR-636					hsa-miR-636	15,61398914			
hsa-miR-637									
hsa-miR-638			hsa-miR-638	17,98130122	hsa-miR-638	17,08408339			
hsa-miR-639					hsa-miR-639	17,82381992			
hsa-miR-640									
hsa-miR-641									
hsa-miR-642a-5p									
hsa-miR-643									
hsa-miR-645									
hsa-miR-646									
hsa-miR-647									
hsa-miR-649									
hsa-miR-650									
hsa-miR-651-5p									

hsa-miR-652-3p	hsa-miR-652-3p	7,58753975	hsa-miR-652-3p	9,46456475	hsa-miR-652-3p	8,05517175	*	*	ns
hsa-miR-653-5p	hsa-miR-653-5p	12,50131393							
hsa-miR-654-3p	hsa-miR-654-3p	11,53228009	hsa-miR-654-3p	16,31133699	hsa-miR-654-3p	17,52910408	*	ns	*
hsa-miR-654-5p					hsa-miR-654-5p	15,38058594			
hsa-miR-655-3p			hsa-miR-655-3p	16,99570482	hsa-miR-655-3p	16,20232622		ns	
hsa-miR-658									
hsa-miR-659-3p									
hsa-miR-660-5p	hsa-miR-660-5p	8,40018725	hsa-miR-660-5p	10,9205655	hsa-miR-660-5p	9,7856225	*	ns	ns
hsa-miR-661									
hsa-miR-662			hsa-miR-662	17,98130122					
hsa-miR-663a	hsa-miR-663a	11,32732134	hsa-miR-663a	14,74919275	hsa-miR-663a	14,57536572	*	ns	*
hsa-miR-663b	hsa-miR-663b	10,4261969							
hsa-miR-664a-3p	hsa-miR-664a-3p	11,98748664	hsa-miR-664a-3p	14,53529408	hsa-miR-664a-3p	14,59960775	*	ns	ns
hsa-miR-665	hsa-miR-665	12,63006906	hsa-miR-665	16,55392516	hsa-miR-665	16,02887603	*	ns	*
hsa-miR-668-3p									
hsa-miR-671-3p	hsa-miR-671-3p	11,99117939							
hsa-miR-671-5p			hsa-miR-671-5p	15,85868016					

hsa-miR-675-3p					hsa-miR-675-3p	17,88137794			
hsa-miR-675-5p									
hsa-miR-7-1-3p	hsa-miR-7-1-3p	11,88296214	hsa-miR-7-1-3p	14,29697833	hsa-miR-7-1-3p	14,57759125	ns	ns	ns
hsa-miR-7-2-3p									
hsa-miR-7-5p	hsa-miR-7-5p	11,40987434	hsa-miR-7-5p	15,29875366	hsa-miR-7-5p	12,8279525	*	*	ns
hsa-miR-708-3p	hsa-miR-708-3p	12,75131393	hsa-miR-708-3p	16,45501308	hsa-miR-708-3p	16,33089844	*	ns	*
hsa-miR-708-5p									
hsa-miR-744-3p			hsa-miR-744-3p	15,03333774	hsa-miR-744-3p	16,52999536		ns	
hsa-miR-744-5p	hsa-miR-744-5p	10,56583455	hsa-miR-744-5p	13,62696975	hsa-miR-744-5p	12,4353345	*	ns	ns
hsa-miR-758-3p									
hsa-miR-759									
hsa-miR-760	hsa-miR-760	12,2342268	hsa-miR-760	17,13611956	hsa-miR-760	17,88137794	*	ns	*
hsa-miR-761	hsa-miR-761	12,75131393							
hsa-miR-765									
hsa-miR-766-3p	hsa-miR-766-3p	10,92525859	hsa-miR-766-3p	11,632187	hsa-miR-766-3p	11,66481275	ns	ns	ns
hsa-miR-767-3p									
hsa-miR-767-5p									

hsa-miR-769-3p									
hsa-miR-769-5p			hsa-miR-769-5p	18,23130122	hsa-miR-769-5p	16,01863431		ns	
hsa-miR-770-5p									
hsa-miR-802									
hsa-miR-873-5p									
hsa-miR-874-3p	hsa-miR-874-3p	12,75131393	hsa-miR-874-3p	14,09185924	hsa-miR-874-3p	12,7111645	ns	ns	ns
hsa-miR-875-3p									
hsa-miR-875-5p									
hsa-miR-876-3p	hsa-miR-876-3p	12,75131393							
hsa-miR-876-5p									
hsa-miR-877-3p									
hsa-miR-877-5p	hsa-miR-877-3p	11,44511226	hsa-miR-877-5p	13,406359	hsa-miR-877-5p	12,796235	ns	ns	ns
hsa-miR-885-3p	hsa-miR-885-5p	11,93643322	hsa-miR-885-5p	12,72248325	hsa-miR-885-5p	12,9962985	ns	ns	ns
hsa-miR-885-5p									

hsa-miR-887-3p								
hsa-miR-888-3p								
hsa-miR-888-5p								
hsa-miR-889-3p			hsa-miR-889-3p	16,94799907	hsa-miR-889-3p	16,40723456		ns
hsa-miR-890								
hsa-miR-891a-5p								
hsa-miR-891b								
hsa-miR-892a					hsa-miR-892a	18,13137794		
hsa-miR-9-3p								
hsa-miR-9-5p								
hsa-miR-920								
hsa-miR-921								
hsa-miR-922								
hsa-miR-924								
hsa-miR-92a-1-5p								
hsa-miR-92a-2-5p								
hsa-miR-92a-3p	hsa-miR-92a-3p	3,147971	hsa-miR-92a-3p	5,2850395	hsa-miR-92a-3p	3,56330075	*	* ns
hsa-miR-92b-3p			hsa-miR-92b-3p	16,16752441	hsa-miR-92b-3p	14,94079553		ns

hsa-miR-92b-5p									
hsa-miR-93-3p	hsa-miR-93-3p	10,78957001	hsa-miR-93-3p	14,24400033	hsa-miR-93-3p	12,22966425	*	*	ns
hsa-miR-93-5p	hsa-miR-93-5p	4,0164435	hsa-miR-93-5p	6,52064	hsa-miR-93-5p	4,38687775	*	*	ns
hsa-miR-933									
hsa-miR-934			hsa-miR-934	14,8884235	hsa-miR-934	15,84870675		ns	
hsa-miR-935	hsa-miR-935	12,75131393							
hsa-miR-936									
hsa-miR-937-3p									
hsa-miR-938									
hsa-miR-940			hsa-miR-940	16,61367849	hsa-miR-940	17,74059625		ns	
hsa-miR-941	hsa-miR-941	12,00508014	hsa-miR-941	15,40611358	hsa-miR-941	15,93817736	*	ns	ns
hsa-miR-942-5p					hsa-miR-942-5p	16,19486447			
hsa-miR-943									
hsa-miR-944									
hsa-miR-95-3p			hsa-miR-95-3p	16,1041039	hsa-miR-95-3p	17,07110617		ns	
hsa-miR-96-3p									
hsa-miR-96-5p			hsa-miR-96-5p	16,62241557	hsa-miR-96-5p	13,53725825		ns	
hsa-miR-98-5p	hsa-miR-98-5p	11,48615764	hsa-miR-98-5p	13,4602155	hsa-miR-98-5p	12,99160825	ns	ns	ns
hsa-miR-99a-3p					hsa-miR-99a-3p	18,13137794			
hsa-miR-99a-5p	hsa-miR-99a-5p	10,13219413	hsa-miR-99a-5p	12,955146	hsa-miR-99a-5p	11,7105265	*	ns	ns
hsa-miR-99b-3p			hsa-miR-99b-3p	17,38036765	hsa-miR-99b-3p	17,20358925		ns	

hsa-miR-99b-5p	hsa-miR-99b-5p	10,70327076	hsa-miR-99b-5p	12,3336415	hsa-miR-99b-5p	10,7808975	ns	*	ns

HM: Half-Marathon; 10K: 10 km; M: Marathon.

ns: Non significant; *P-value < 0.05.

Suplemmentary Table S2. KEEG target analysis (Tarbase) of significantly changed microRNA in the three different doses. Pathway targeted by the microRNA, p-value of the probability of interaction between microRNA and pathway, number of genes regulated and number of miRNAs which have targets in this pathway.

M up-regulated

KEGG pathway	p-value	genes	miRNAs
Proteoglycans in cancer	4.89e-12	131	18
Adherens junction	6.11e-10	57	17
Cell cycle	3.22e-08	89	17
Colorectal cancer	7.42e-08	51	17
Protein processing in endoplasmic reticulum	7.42e-08	115	18
Pancreatic cancer	7.82e-08	54	15
Prostate cancer	7.82e-08	69	16
Hippo signaling pathway	7.82e-08	95	18
Pathways in cancer	7.82e-08	241	19
Viral carcinogenesis	1.89e-07	130	17
Chronic myeloid leukemia	1.89e-07	57	18
Non-small cell lung cancer	6.13e-07	43	15
Prion diseases	2.04e-06	20	16
Endometrial cancer	2.04e-06	42	16
Glioma	3.46e-06	46	17
p53 signaling pathway	3.97e-06	54	17
Thyroid cancer	4.67e-06	25	15
Endocytosis	8.41e-06	131	18
Renal cell carcinoma	1.17e-05	50	16
Lysine degradation	1.17e-05	33	17
Transcriptional misregulation in cancer	8.57e-05	107	17
Neurotrophin signaling pathway	8.57e-05	81	18
Central carbon metabolism in cancer	0.0001	48	16
Insulin signaling pathway	0.0001	93	18
FoxO signaling pathway	0.0002	89	17
Ubiquitin mediated proteolysis	0.0003	91	18
Bladder cancer	0.0003	31	15
N-Glycan biosynthesis	0.0003	34	14
Acute myeloid leukemia	0.0004	42	13
Spliceosome	0.0005	81	17
mTOR signaling pathway	0.0005	43	17
Hepatitis B	0.0006	89	17
HTLV-I infection	0.0015	153	18
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.0020	39	15
Regulation of actin cytoskeleton	0.0036	121	18
MAPK signaling pathway	0.0038	145	18

Apoptosis	0.0052	55	16
Bacterial invasion of epithelial cells	0.0063	49	18
Fatty acid biosynthesis	0.0064	6	10
Oocyte meiosis	0.0066	68	16
Small cell lung cancer	0.0085	55	17
Melanoma	0.0112	44	17
ErbB signaling pathway	0.0136	54	18
TGF-beta signaling pathway	0.0141	46	16
RNA transport	0.0173	98	19
Shigellosis	0.0179	41	17
Alcoholism	0.0197	103	17
Progesterone-mediated oocyte maturation	0.0225	55	16
Choline metabolism in cancer	0.0270	61	16
Dorso-ventral axis formation	0.0271	20	14
AMPK signaling pathway	0.0273	74	17
Gap junction	0.0287	52	17
DNA replication	0.0367	22	12
Axon guidance	0.0373	73	17
VEGF signaling pathway	0.0389	39	14
Steroid biosynthesis	0.0392	11	12
Estrogen signaling pathway	0.0480	58	17
M down-regulated			
Proteoglycans in cancer	2.19e-14	92	7
Protein processing in endoplasmic reticulum	1.75e-09	86	7
Prion diseases	2.37e-09	11	6
Viral carcinogenesis	1.72e-08	84	7
Cell cycle	4.68e-08	65	7
Lysine degradation	1.89e-07	25	6
Fatty acid biosynthesis	3.63e-07	4	4
Colorectal cancer	3.63e-07	35	7
Hippo signaling pathway	3.56e-06	69	7
Pancreatic cancer	1.42e-05	35	6
Chronic myeloid leukemia	1.60e-05	38	6
N-Glycan biosynthesis	1.84e-05	24	5
Hepatitis B	1.84e-05	62	7
Other types of O-glycan biosynthesis	4.27e-05	13	5
Fatty acid metabolism	5.80e-05	18	5
Thyroid hormone signaling pathway	0.0001	50	7
Steroid biosynthesis	0.0001	11	4
Signaling pathways regulating pluripotency of stem cells	0.0003	56	6
Endocytosis	0.0003	81	7
Wnt signaling pathway	0.0003	57	7

Endometrial cancer	0.0003	28	7
Prostate cancer	0.0003	44	7
TGF-beta signaling pathway	0.0006	33	6
p53 signaling pathway	0.0006	34	7
mTOR signaling pathway	0.0009	31	6
Non-small cell lung cancer	0.0009	27	6
Glioma	0.0012	29	7
DNA replication	0.0012	20	6
Homologous recombination	0.0013	15	4
Oocyte meiosis	0.0013	46	6
FoxO signaling pathway	0.0013	60	7
Adherens junction	0.0013	34	7
Pathways in cancer	0.0016	139	7
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	0.0016	9	4
HTLV-I infection	0.0020	99	7
Bladder cáncer	0.0024	21	6
Estrogen signaling pathway	0.0030	40	6
Ubiquitin mediated proteolysis	0.0030	60	7
Glycosaminoglycan biosynthesis - keratan sulfate	0.0035	5	4
Legionellosis	0.0042	27	6
Renal cell carcinoma	0.0042	31	6
mRNA surveillance pathway	0.0042	41	7
Progesterone-mediated oocyte maturation	0.0044	39	6
Acute myeloid leukemia	0.0049	28	7
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	0.0054	8	3
TNF signaling pathway	0.0056	48	7
Sphingolipid signaling pathway	0.0067	46	5
Focal adhesión	0.0067	79	7
Bacterial invasion of epithelial cells	0.0067	34	7
Small cell lung cancer	0.0075	37	7
Fatty acid elongation	0.0079	7	3
Thyroid cancer	0.0098	15	7
Melanoma	0.0098	28	7
AMPK signaling pathway	0.0121	54	7
Prolactin signaling pathway	0.0125	29	7
HIF-1 signaling pathway	0.0160	45	7
Apoptosis	0.0222	36	5
ErbB signaling pathway	0.0222	37	7
Measles	0.0222	54	7
Neurotrophin signaling pathway	0.0225	47	7
Spliceosome	0.0251	48	7

Regulation of actin cytoskeleton	0.0263	73	7
MAPK signaling pathway	0.0350	88	7
Fc gamma R-mediated phagocytosis	0.0355	36	7
HM up-regulated			
Proteoglycans in cancer	9.81e-14	117	13
Viral carcinogenesis	1.58e-08	107	12
Adherens junction	2.85e-08	49	12
Protein processing in endoplasmic reticulum	3.41e-08	99	12
TGF-beta signaling pathway	3.41e-08	49	13
Hippo signaling pathway	4.08e-08	79	13
Prion diseases	1.96e-07	16	8
Hepatitis B	1.60e-06	80	12
Pathways in cancer	1.84e-06	202	13
Prostate cancer	2.50e-06	58	12
Bacterial invasion of epithelial cells	4.30e-06	48	10
mTOR signaling pathway	6.70e-06	42	10
Glycosaminoglycan biosynthesis - keratan sulfate	7.50e-06	10	5
Ubiquitin mediated proteolysis	1.02e-05	84	12
ECM-receptor interaction	1.53e-05	38	12
Signaling pathways regulating pluripotency of stem cells	2.69e-05	76	11
Transcriptional misregulation in cancer	3.36e-05	90	13
Glioma	3.36e-05	39	13
FoxO signaling pathway	3.69e-05	76	12
Shigellosis	3.88e-05	41	11
Cell cycle	3.88e-05	73	12
Chronic myeloid leukemia	4.29e-05	47	12
TNF signaling pathway	4.70e-05	64	12
Neurotrophin signaling pathway	5.71e-05	71	12
Renal cell carcinoma	0.00011	43	12
Fatty acid biosynthesis	0.00018	5	7
Lysine degradation	0.00022	26	10
Focal adhesion	0.00024	110	13
Insulin signaling pathway	0.00025	79	12
Endocytosis	0.00025	106	13
AMPK signaling pathway	0.00026	71	12
p53 signaling pathway	0.00036	43	13
Axon guidance	0.00052	66	12
Small cell lung cancer	0.00052	51	12
Colorectal cancer	0.00053	38	12
Estrogen signaling pathway	0.00054	54	13
Oocyte meiosis	0.00055	61	12

Epstein-Barr virus infection	0.00093	104	13
Sphingolipid signaling pathway	0.0013	61	13
Thyroid hormone signaling pathway	0.0014	65	13
Pancreatic cancer	0.0024	37	12
Thyroid cancer	0.0026	19	8
Acute myeloid leukemia	0.0029	35	11
RNA transport	0.0039	85	13
Other types of O-glycan biosynthesis	0.0043	16	8
Endometrial cancer	0.0045	31	11
Regulation of actin cytoskeleton	0.0048	101	12
HIF-1 signaling pathway	0.0048	58	13
Wnt signaling pathway	0.0058	70	12
Spliceosome	0.0062	71	13
Non-small cell lung cancer	0.0079	31	13
N-Glycan biosynthesis	0.0110	26	9
Pathogenic Escherichia coli infection	0.0148	32	11
Prolactin signaling pathway	0.0192	39	12
Central carbon metabolism in cancer	0.0220	35	11
Apoptosis	0.0279	44	11
Chagas disease (American trypanosomiasis)	0.0279	52	13
Phosphatidylinositol signaling system	0.0326	42	13
Inositol phosphate metabolism	0.0328	32	11
Adipocytokine signaling pathway	0.0328	36	11
Salmonella infection	0.0328	44	12
Circadian rhythm	0.0384	19	9
Pantothenate and CoA biosynthesis	0.0424	8	7
MAPK signaling pathway	0.0469	117	13
ErbB signaling pathway	0.0490	47	12
10Km up-regulated			
Proteoglycans in cancer	6.52e-13	61	11
Adherens junction	2.67e-11	37	8
Fatty acid biosynthesis	2.09e-10	3	2
ECM-receptor interaction	1.38e-07	22	7
Hippo signaling pathway	2.13e-06	50	10
Lysine degradation	5.19e-06	17	8
Pathways in cancer	1.60e-05	113	11
Bacterial invasion of epithelial cells	0.00016	28	11
Central carbon metabolism in cancer	0.00016	25	10
Colorectal cancer	0.00022	26	11
Glioma	0.00036	24	9
Shigellosis	0.00054	27	9
Endocytosis	0.00076	59	11
Thyroid cancer	0.00089	13	6

Focal adhesion	0.00089	65	11
Signaling pathways regulating pluripotency of stem cells	0.00099	44	11
Bladder cancer	0.00114	19	9
Cell cycle	0.00215	44	10
Renal cell carcinoma	0.00240	25	10
Endometrial cancer	0.00284	20	9
Prostate cancer	0.00286	32	10
Chronic myeloid leukemia	0.00286	27	11
Protein processing in endoplasmic reticulum	0.00313	50	11
TGF-beta signaling pathway	0.00558	22	10
Hepatitis B	0.00558	42	11
Acute myeloid leukemia	0.00609	20	7
Regulation of actin cytoskeleton	0.00923	58	11
Oocyte meiosis	0.01235	35	10
Fatty acid metabolism	0.01290	10	8
p53 signaling pathway	0.01307	24	10
ErbB signaling pathway	0.01332	28	11
FoxO signaling pathway	0.01336	43	11
Thyroid hormone signaling pathway	0.01972	38	10
Fc gamma R-mediated phagocytosis	0.02002	29	11
Insulin signaling pathway	0.02270	42	11
Dorso-ventral axis formation	0.0236	12	8
Glycosaminoglycan biosynthesis - keratan sulfate	0.0256	5	4
Small cell lung cancer	0.0301	27	9
Wnt signaling pathway	0.0309	38	10
Neurotrophin signaling pathway	0.0323	37	11
Salmonella infection	0.0329	26	9
PI3K-Akt signaling pathway	0.0329	86	11
Non-small cell lung cancer	0.0430	17	9
Vasopressin-regulated water reabsorption	0.0431	17	7
10Km down-regulated			
Viral carcinogénesis	0.0135	6	1
Bile secretion	0.0148	3	1
Sulfur relay system	0.0283	1	1

Suplemmentary Table S3. KEEG target analysis (Tarbase) of common significantly changed microRNAs between races. Pathway targeted by common microRNA, p-value of the probability of interaction between microRNA and pathway, number of genes regulated and number of miRNAs which have targets in this pathway.

Common miRNAs 10K and M

KEGG pathway	p-value	genes	miRNAs
Steroid biosynthesis	0.0002	3	3
Lysine degradation	0.0002	7	3
Proteoglycans in cancer	0.0025	16	3
Huntington's disease	0.0029	12	3
Prostate cancer	0.0029	15	3
Viral carcinogenesis	0.0046	18	3
Adherens junction	0.0071	11	2
Colorectal cancer	0.0079	9	3
p53 signaling pathway	0.0080	11	3
Cell cycle	0.0171	16	3
Central carbon metabolism in cancer	0.0187	8	3
Endometrial cancer	0.0355	7	3
Wnt signaling pathway	0.0405	13	3

Common miRNAs 10K and HM

KEGG pathway	p-value	genes	miRNAs
Lysine degradation	9.7097e-07	9	2
Axon guidance	0.0004	19	2
Hippo signaling pathway	0.0016	17	2
Pantothenate and CoA biosynthesis	0.0020	3	2
Protein processing in endoplasmic reticulum	0.0020	22	2
Fc gamma R-mediated phagocytosis	0.0093	13	2
Galactose metabolism	0.0156	1	1
FoxO signaling pathway	0.0176	18	2
Adherens junction	0.0176	10	2
Circadian rhythm	0.0176	7	2
Chronic myeloid leukemia	0.0176	10	2
Oocyte meiosis	0.0247	13	2
p53 signaling pathway	0.0247	10	2
NOD-like receptor signaling pathway	0.0272	6	2
Glioma	0.0366	8	2
Sphingolipid metabolism	0.0455	6	2
Bacterial invasion of epithelial cells	0.0455	7	2
Shigellosis	0.0481	9	2
Choline metabolism in cancer	0.0481	11	2

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Circulating microRNA profiling reveals specific subsignatures in response to a maximal incremental exercise test

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Abstract:	Circulating microRNAs (c-miRNAs) have been described as emergent regulators and biomarkers of exercise. The aim of this study was to analyse the c-miRNA response to a maximal incremental exercise test (MIET) and its relationship with markers of exercise response and adaptation. Two blood samples were collected from 9 male amateur runners (31-50 y), before (Pre) and after (Post) a MIET. Maximal oxygen uptake (VO2max), maximum heart rate (HRmax), and maximal aerobic speed (MAS) were recorded. Lactate and creatine kinase (CK) plasma concentrations were measured. A panel of 752 miRNAs was analysed using standardized protocols and relative quantification to Pre. A total of 13 miRNAs were found significantly up-regulated at Post. By focusing on the exercise markers that correlate with the expression of these miRNAs, they were clustered into different functional groups or subsignatures. Thus, miR-21-5p, miR-29b-3p, and miR-183-5p, and showed a strong correlation with HRmax and a validated target signature related to fatty acid metabolism. Furthermore, let-7c-5p, miR-340-5p, miR-425-3p, and miR-629-5p, were significantly correlated with CK and the most significantly enriched pathways for this subsignature were Hippo signalling pathway and signalling pathways regulating

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	pluripotency of stem cells. Finally, Pre miR-106b-5p expression showed an inverse association with MAS and Post lactate concentration, which highlights its relevance as biomarker of training status and its predictive value for performance. No significant correlations were observed with VO2max. Our results define for the first time specific functional c-miRNA subsignatures, adding novel evidence about their potential regulatory role in exercise response.
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TITLE

Circulating microRNA profiling reveals specific subsignatures in response to a maximal incremental exercise test

RUNNING HEAD

c-miRNA subsignatures and exercise test

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TITLE

Circulating microRNA profiling reveals specific subsignatures in response to a maximal incremental exercise test

ABSTRACT

Circulating microRNAs (c-miRNAs) have been described as emergent regulators and biomarkers of exercise. The aim of this study was to analyse the c-miRNA response to a maximal incremental exercise test (MIET) and its relationship with markers of exercise response and adaptation. Two blood samples were collected from 9 male amateur runners (31-50 y), before (Pre) and after (Post) a MIET. Maximal oxygen uptake ($\dot{V}O_2max$), maximum heart rate (HRmax), and maximal aerobic speed (MAS) were recorded. Lactate and creatine kinase (CK) plasma concentrations were measured. A panel of 752 miRNAs was analysed using standardized protocols and relative quantification to Pre. A total of 13 miRNAs were found significantly up-regulated at Post. By focusing on the exercise markers that correlate with the expression of these miRNAs, they were clustered into different functional groups or subsignatures. Thus, miR-21-5p, miR-29b-3p, and miR-183-5p, and showed a strong correlation with HRmax and a validated target signature related to fatty acid metabolism. Furthermore, let-7c-5p, miR-340-5p, miR-425-3p, and miR-629-5p, were significantly correlated with CK and the most significantly enriched pathways for this subsignature were Hippo signalling pathway and signalling pathways regulating pluripotency of stem cells. Finally, Pre miR-106b-5p expression showed an inverse association with MAS and Post lactate concentration, which highlights its relevance as biomarker of training status and its predictive value for performance. No significant correlations were observed with $\dot{V}O_2max$. Our results define for the first time specific functional c-miRNA subsignatures, adding novel evidence about their potential regulatory role in exercise response.

KEY WORDS: Circulating microRNAs, Maximal incremental exercise test, Exercise biomarkers; Molecular response to exercise.

INTRODUCTION

Exercise response involves a complex cross-talk between tissues, with profound effects on gene expression (9). Circulating microRNAs (c-miRNAs) are intercellular communicators with a posttranscriptional regulatory role that have emerged during the last decade as biomarkers and regulators of exercise response and adaption (4). Some authors have described a strong relationship between maximal oxygen uptake ($\dot{V}O_2max$) and both the baseline levels of certain c-miRNAs or their changes in response to acute aerobic exercise (2). However, the response of c-miRNAs to a maximal incremental exercise test (MIET) has not been explored. Standardized laboratory tests are used to evaluate physical capacity (distance covered; maximal power output; etc.) and the cardiovascular and metabolic adaptation to exercise ($\dot{V}O_2max$; maximum heart rate, HRmax; lactate thresholds; etc.) (1). The aim of this study was to analyse the acute response to a MIET of a comprehensive panel of c-miRNAs and its relationship with physiological and biochemical markers of exercise response and adaptation.

METHODS

Ethics statement

Experimental procedures were approved by the Research Ethics Committee of the Principality of Asturias, Spain (reference: 124/17). All participants gave written informed consent.

Subjects

Volunteers were recruited among the amateur runners training group (n=35) of MAPOMA Sports Association, Spain. Each volunteer underwent a thorough medical screening to determine eligibility. A number of inclusion and exclusion criteria were also considered. Inclusion criteria: 1) Men >18 years; only men were selected due to the stability of their

hormonal status, 2) Training routine of >50 km/week. Exclusion criteria: 1) Smokers and frequent passive smokers, 2) Be under dietary or pharmacological treatment during the time of the study. Finally, 9 subjects (31-50 y) took part in the study.

Procedures

Maximal incremental exercise test

The test consisted on a ramp protocol on a treadmill (LE-600 C, Jaeger-HP Cosmos, Germany). The participants warmed up for 2 min at 4 km/h and 1% grade. Then the test began at 6 km/h, increasing by 0.25 km/h every 15 s until volitional exhaustion. $\dot{V}O_2max$ was determined (Oxycon Pro, Jaeger, Germany). Continuous 12-lead electrocardiographic monitoring was carried out (General Electric Medical Systems, USA). HRmax and Maximal Aerobic Speed (MAS) were recorded.

Blood sampling

Subjects had a blood sample taken in fasting state, 1 h before the test (Pre). Then consumed a standardized breakfast and performed warm-up exercises, after which point the test started. Another blood sample was drawn within 5 min after the cessation of exercise (Post). Blood samples (<10 ml) were collected in vacutainers (No Additive (Z), Becton Dickinson, USA), stored at room temperature for at least 15 minutes to allow clot formation, and immediately centrifuged at 4000 rpm for 15 min at 10ºC. Serum samples were then aliquoted and stored at -80ºC for later analysis.

Biochemical parameters analysis

Lactate and creatine kinase (CK) plasma concentrations were measured using standardized protocols (AU400, Beckman Coulter, USA). Haemoglobin and Haematocrit data (ADVIA 120, Siemens Healthcare Diagnostics, Germany) were used to account for plasma volume change after exercise (3). Post lactate and CK concentrations were adjusted accordingly.

MiRNA profiling

Total RNA was isolated from 200 µL of serum using the miRCURY RNA isolation kit (Exiqon). For ulterior normalization, synthetic cel-miR-39-3p was added. The mixture was supplemented with 1 µg of MS2 carrier RNA (Roche) to improve extracellular miRNA yield. The RNA Spike-in kit with synthetic RNA spike-in templates (UniSp2, UniSp4, UniSp5) (Exiqon) was also used to monitor RNA isolation efficiency. For miRNA quantification, cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon). Additionally, UniSp6 (Exiqon), was added to check for RT efficiency. For qPCR, cDNA was diluted 80x and 4 µl used in 10 µl qPCR reactions with ExiLENT SYBR Green master mix (Exiqon) on a 7900HT fast Real-Time PCR System (Applied Biosystems). To discard the presence of nucleases, inhibitors or hemolysis, the miRCURY miRNA Quality Control PCR Panel (Exiqon) was used before miRNA analysis. For whole genome screening, 752 mature miRNAs were quantified using human miRNome panels v4 (Exiqon). The synthetic spike-in UniSp3 was analysed as an interplate calibrator. SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and for melting curve analysis. The dCq(miR-23a-3p – miR-451a) method was used to confirm that none of the samples were affected by hemolysis (all samples had dCq value below 6). miRNAs were considered to be expressed when Cq<37 or were detected with at least 5 Cq below the negative control. Normalization to cel-miR-39-3p and relative quantification to Pre samples were performed using the 2^{-ddCq} method (8). GenEx software (MultiD Analyses AB, Sweden) was used for data processing and miRNA relative expression analysis.

Pathway analysis

The R Bioconductor package mdgsa (version 1.8.0) and DIANA path software were used for pathway analysis of differentially expressed miRNAs in an integrative manner. For each miRNA, experimentally validated targets were retrieved from miRTarBase v7 database. Pathway annotations for each gene were retrieved from KEGG pathways. Thus, we obtained

gene sets and metabolic pathways linked to miRNAs targeting genes. The results output a log odds ratio for each interrogated gene set, along with raw and false discovery adjusted *p*-values.

Statistical analyses

Normality of variables was tested using Shapiro Wilk's test. Data are expressed as Mean±standard deviation. Associations between variables were analysed using Pearson's correlation analysis. A multiple paired samples T-test was performed to compare Pre vs Post samples. *p*-values <0.05 were considered significant. For the analysis of changes in c-miRNA expression, the additional criterion of Pre-Post differences larger than 1.0 was considered. A customized R (www.r-project.org) function was used for all process.

RESULTS

Physiological and biochemical parameters

The results obtained for $\dot{V}O_2$max, HRmax, and MAS were 59.9±6.4 ml/kg/min, 175.5±10.8 bpm, and 17.4±1.4 km/h, respectively.

A significant Pre-Post increase was observed for lactate (1.7±0.6 vs. 11.5±4.9 mmol/l; *p*=0.0003) and CK (186.0±153.6 vs. 207.9±169.4 U/l; *p*=0.007).

Circulating miRNAs

A total of 226 miRNAs were detected in Pre and/or Post samples. From these, 13 c-miRNAs were found differentially expressed between Pre and Post, all of them up-regulated (Figure 1).

Relationship between circulating miRNA levels and physiological and biochemical parameters: target pathway analysis

No significant correlations were observed between the c-miRNA profile detected and $\dot{V}O_2$max. On the contrary, HRmax showed significant positive correlations with miR-21-5p

(r=0.750; *p*=0.02), miR-29b-3p (r=0.695; *p*=0.038), and miR-183-5p (r=0.798; *p*=0.01). This group of miRNAs have a specific validated target signature highly related to fatty acid metabolism (Figure 2A).

Significant positive correlations with the percentage of variation of CK were observed for Post levels of let-7c-5p (r=-0.773; *p*=0.01), miR-340-5p (r=-0.742; *p*=0.02), miR-425-3p (r=-0.781; *p*=0.01), and miR-629-5p (r=-0.706; *p*=0.03). Among the most significantly enriched pathways were the Hippo signalling pathway and Signalling pathways regulating pluripotency of stem cells (Figure 2B).

Interestingly, Pre levels of miR-106b-5p were significantly negatively associated with Post lactate concentration (r=-0.694; *p*=0.038), with the percentage of variation of lactate levels (r=-0.885; *p*=0.002), and with MAS (r=-0.688; *p*=0.041).

DISCUSSION

Our results show a specific c-miRNA signature in response to a MIET, which has no association with $\dot{V}O_2max$. Interestingly, several c-miRNA subsignatures were identified, considering their specific strong correlation with other exercise parameters and markers, like HRmax, CK, lactate or MAS. This is the first study in which functional c-miRNA subsignatures are identified in response to exercise.

Only Zhou et al. (14) have previously described the c-miRNA response to a maximal cardiopulmonary exercise test. The authors analysed a very restricted selection of 17 miRNAs, detecting a significant increase in miR-20a, which was not correlated with any of the cardiopulmonary function parameters measured. Although we have not detected changes in this specific miRNA, we agree in the absence of correlation of responsive c-miRNAs with $\dot{V}O_2max$. $\dot{V}O_2max$ is a measure for cardiorespiratory fitness, not a marker of acute exercise response (12). Therefore, it seems more likely that those miRNAs that significantly change in

response to MIET are associated with other responsive parameters. Interestingly, we have described a subset of c-miRNAs that correlate with HRmax. These miRNAs have been described as biomarkers of atrial fibrillation, the most common form of arrhythmia (6, 15). Furthermore, a relationship between atrial fibrillation and alterations in cardiomyocyte fatty acid metabolism has been observed (11). This highlights the potential role of this group of miRNAs in the cardiac muscle contractile activity during MIET, through regulating inotropic activity and energy metabolism. In turn, the most significantly enriched pathways for the subset of c-miRNAs associated with CK are strongly related to muscle mass response after exercise (5, 10, 13). This point to these miRNAs as candidate regulators of muscle adaption in response to exercise and even of the magnitude of exercise-induced muscle damage. Finally, our results highlight the potential of miR-106b-5p as a biomarker of training status, as well as its predictive value in physical performance, considering that these parameters are tightly related to MAS and to plasma lactate concentration changes in response to acute exercise (7).

The response to a MIET involves an intricate network of physiological, metabolic, and molecular processes. Our results add novel evidence about the potential regulatory role of c-miRNAs in this context, defining for the first time specific functional c-miRNA subsignatures.

PRACTICAL APPLICATIONS

Our data highlight the value of c-miRNAs as biomarkers of specific exercise-related processes, which might be used as a measurement of the magnitude of exercise response and training status, as well as predictive tool for physical performance.

ACKNOWLEDGEMENTS

This work was supported in part by the Ministerio de Economía y Competitividad (DEP2012-39262 and DEP2015-69980-P) to EI-G and BF-G. Also supported in part by the Agencia Estatal de Investigación and European FEDER funds to AD (AGL2016-78922-R and PID2019-109369RB-I00) and to AD, BF-G, EI-G, and MF-S (AGL2017-90623-REDT). AD lab is funded by Fundación Ramón Areces (CIVP18A3888), Spain. Also supported by Carlos III Health Institute, CIBERES to DdG-C (CB07/06/2008).

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FIGURE LEGENDS

Figure 1. Acute response (pre-post) of significantly changed circulating miRNAs in a maximal aerobic capacity test.

Data are presented as means. * Significant Pre-Post differences: *p*-value < 0.05 and Pre-Post fold-change > 1.0.

Figure 2. KEEG analysis of validated targets (Tarbase v7). A, validated targets of miR-21-5p, miR-183-5p, and miR-29b-3p. B, validated targets of miR-425-3p, miR-629-5p, let-7c-5p, and miR-340-5p.

Figure 1

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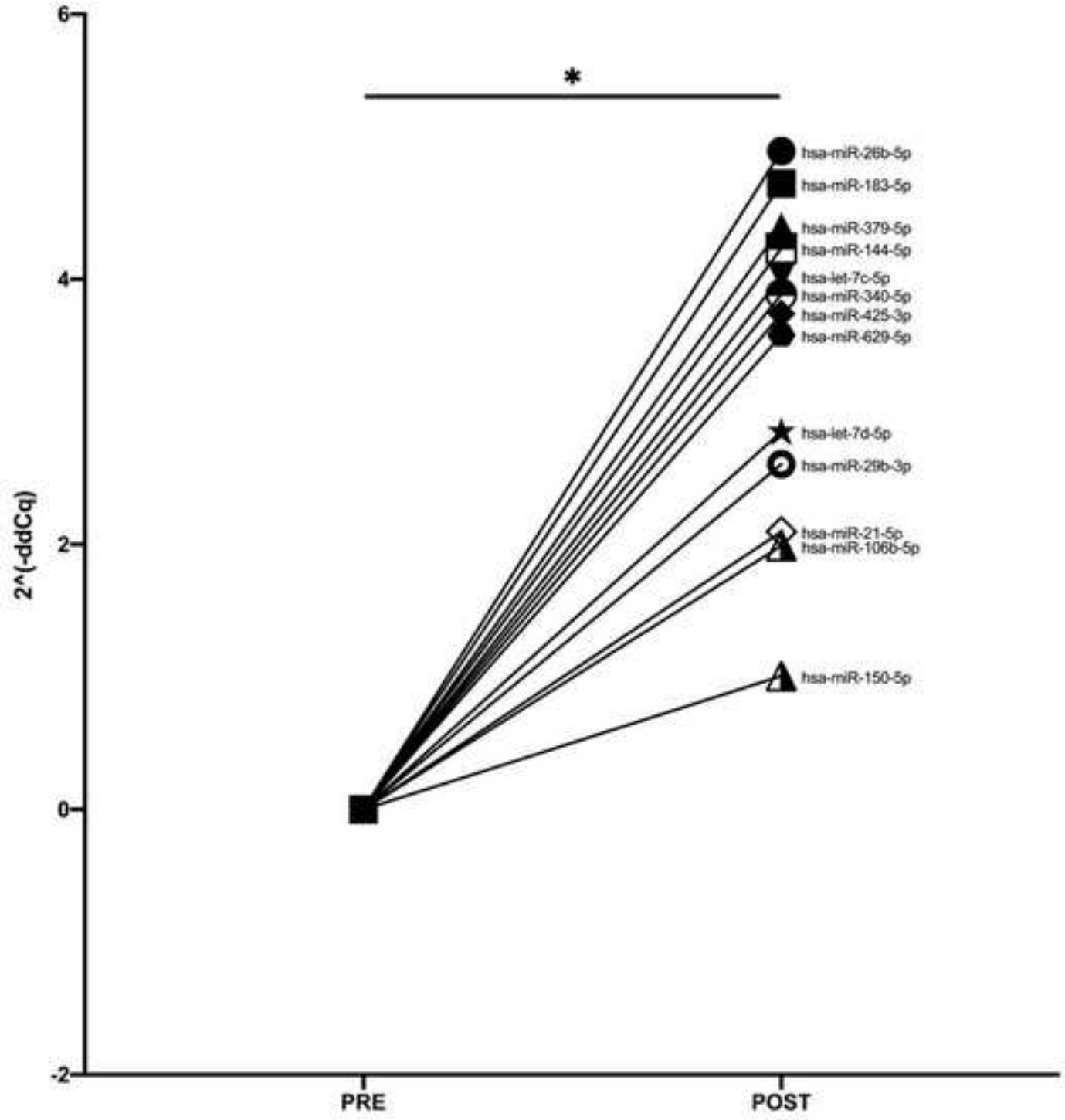

Figure 2

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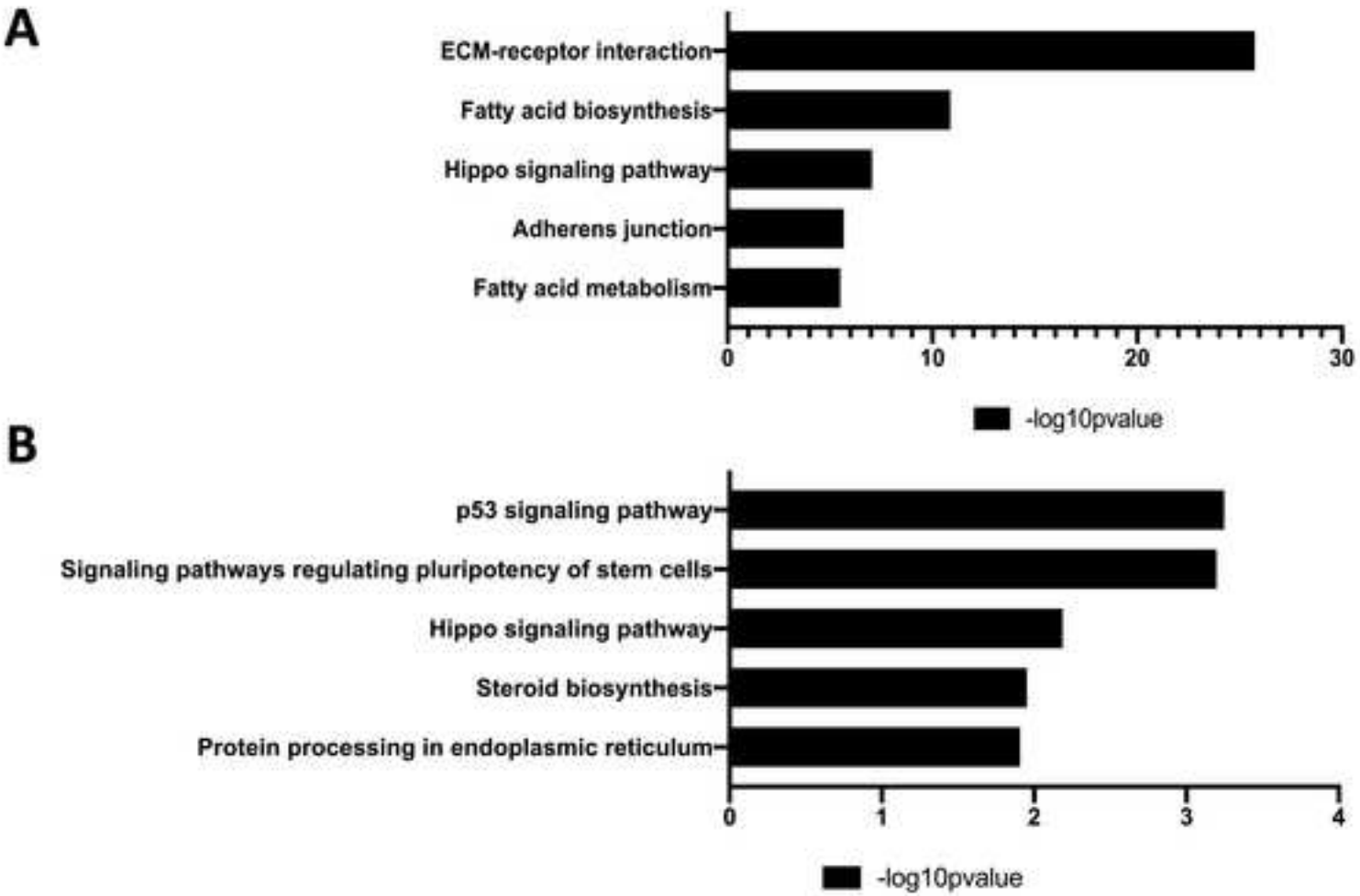

Nicholas A. Ratamess Jr., PhD, CSCS, FNSCA

Editor-in-Chief

Journal of Strength and Conditioning Research

Dear Prof. Ratamess:

Please find enclosed our manuscript entitled: **"Circulating microRNA profiling reveals specific subsignatures in response to a maximal incremental exercise test"** which we here submit as a brief report for consideration in the *Journal of Strength and Conditioning Research*.

This manuscript is original and not previously published in any form including on preprint servers, nor is it being considered elsewhere until a decision is made as to its acceptability by the JSCR Editorial Review Board

All previously published work by the authors of this manuscript and by other researchers has been fully cited in the text. All authors have contributed significantly to the research of the submitted manuscript and have read and approved it for submission. None of the authors have a conflict of interest.

This is the first study in which a comprehensive panel of >750 miRNAs is analysed in response to a maximal incremental exercise test. Our results show a specific c-miRNA signature in response to a MIET, which, surprisingly, has no association with $\dot{V}O_2max$. Interestingly, several c-miRNA subsignatures were identified, considering their specific strong correlation with other exercise parameters and markers, like maximum heart rate, maximal aerobic speed, and CK and lactate plasma concentrations. This is relevant considering that the response to exercise involves an intricate network of physiological, metabolic, and molecular processes. This is the first study in which functional c-miRNA subsignatures are identified in response to exercise. Our data

highlight the value of c-miRNAs as biomarkers of specific exercise-related processes, which might be used as a measurement of the magnitude of exercise response and training status, as well as predictive tool for physical performance.

We thank you for your time and effort.

Sincerely,

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Circulating microRNAs as emerging cardiac biomarkers responsive to acute exercise☆,☆☆

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ABSTRACT

Background: Circulating microRNAs (c-miRNAs) are mediators of intercellular communication with great potential as cardiac biomarkers. The analysis of c-miRNAs in response to physiological stress, such as exercise, would provide valuable information for clinical practice and a deeper understanding of the molecular response to physical activity. Here, we analysed for the first time the acute exercise response of c-miRNAs reported as biomarkers of cardiac disease in a well-characterized cohort of healthy active adults.

Methods: Blood samples were collected immediately before and after (0 h, 24 h, 72 h) a 10-km race, a half-marathon (HM) and a marathon (M). Serum RNA from 10-km and M samples was extracted and a panel of 74 miRNAs analysed using RT-qPCR. c-miRNA response was compared with a panel of nine cardiac biomarkers. Functional enrichment analysis was performed. Pre- and post-M echocardiographic analyses were carried out.

Results: Serum levels of all cardiac biomarkers were upregulated in a dose-dependent manner in response to exercise, even in the absence of symptoms or signs of cardiac injury. A deregulation in the profiles of 5 and 19 c-miRNAs was observed for 10-km and M, respectively. Each race induced a specific qualitative and quantitative alteration of c-miRNAs implicated in cardiac adaptions. Supporting their discriminative potential, a number of c-miRNAs previously associated with cardiac disease were undetectable or stable in response to exercise. Conversely, "pseudo-disease" signatures were also observed.

Conclusions: c-miRNAs may be useful for the management of cardiac conditions in the context of acute aerobic exercise.

Translational aspects of the work: Circulating microRNAs could offer incremental diagnostic value to established and emerging cardiac biomarkers, such as hs-cTnT or NT-proBNP, in those patients with cardiac

☆ These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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dysfunction symptoms after an acute bout of endurance exercise. Furthermore, circulating miRNAs could also show "pseudo-disease" signatures in response to acute exercise. Clinical practitioners should be aware of the impact caused by exercise in the interpretation of miRNA data.

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1. Introduction

How the type, duration and intensity of exercise affect different indicators of cardiac injury and overload is still a matter of debate. Strenuous exercise increases, even above the cardio-healthy reference limits, the concentrations of cardiac damage biomarkers widely used in clinical routine practice, such as high-sensitive cardiac troponin T (hs-cTnT) or N-terminal pro-brain natriuretic peptide (NT-proBNP) [1]. This situation not only raises doubts about the health benefits of intense exercise but also complicates the clinical management of patients after strenuous exercise due to the high risk of false positives.

microRNAs (miRNAs) are small non-coding RNAs involved in the regulation of gene expression at a post-transcriptional level. Genetic studies have demonstrated that miRNAs are essential for heart development and physiology and are differentially expressed in heart disease [2]. In addition to intracellular locations, miRNAs have been detected in different body fluids. Extracellular miRNAs participate in intercellular communication by regulating the gene expression of the recipient cells [3]. Similar to their intracellular forms, extracellular miRNAs are involved in the onset and development of cardiovascular disease [4]. Notably, circulating miRNAs (c-miRNAs) have been proposed as biomarkers of a wide array of cardiac conditions [5–7], in some cases with a higher diagnostic value than the established gold standard [8]. Indeed, Oerlemans et al. [9] showed that the combination of three miRNAs, miR-1, miR-21 and miR-499, has higher discriminative potential than hs-cTnT in patients with suspected acute coronary syndrome. Supporting these findings, Zeller et al. [10] demonstrated that the combination of miR-132, miR-150 and miR-186 has higher diagnostic accuracy for unstable angina than the combination of the clinical indicators hs-TnI, BNP, C-reactive protein and Cystatin C.

Identifying how acute exercise, including strenuous exercise, alters the c-miRNAs profile would provide valuable information on future clinical biomarkers. Additionally, this analysis will bring novel data about the molecular mechanisms involved in the cardiac response to exercise. Here, we analysed for the first time a panel of serum miRNAs proposed as biomarkers of heart disease in response to different doses of acute exercise in a strictly controlled and well-characterized cohort of healthy active adults.

2. Methods

2.1. Participants

Volunteers were recruited among the members of MAPOMA Sports Association. Patient recruitment and study procedures are described in the Supplemental Methods. Table 1 shows the characteristics of the study population: nine healthy, highly trained middle-aged amateur subjects. None of the participants had a medical history of cardiovascular disease. The participants completed three races: a 10-km race (10-km), a half-marathon (HM) and a marathon (M), each separated by one month. Although all races involved the same type of exercise (endurance running), they differed in terms of duration, intensity, and energy demands, and thus, represent distinct exercise doses [11,12]. Race times are shown in Supplemental Table 1. All experimental procedures were approved by the appropriate Research Ethics Committees of the CEU San Pablo University and Principado de Asturias, Spain, in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Table 1
Characteristics of study subjects.

Age (years)	39.1 ± 6.7
Body mass index (kg/m^2)	24.9 ± 2.5
VO_2max (ml·kg^{-1}·min^{-1})	59.9 ± 2.3
Training habits	
Training history (years)	6.6 ± 3.9
Training volume (days/week)	4.9 ± 0.5
Training volume (min/day)	88.3 ± 5.0

Data presented as mean ± SD.

2.2. Echocardiography

The echocardiographic evaluation was performed at two timepoints: a week before and immediately after M, the race with the greatest cardiac demand. Echocardiography was performed as described in Supplemental Methods.

2.3. Circulating cardiac biomarkers

A panel of biochemical indicators of cardiac necrosis, haemodynamic stress, ischemia and fibrosis: hs-cTnT, NT-proBNP creatine kinase (CK), cardiac MB isoform of creatine kinase (CK-MB), myoglobin (MGB), lactate dehydrogenase (LDH), heart-type fatty acid binding protein (h-FABP), galectin-3 and C-terminal pro arginine-vasopressin (CT-proAVP), was measured as described in Supplemental Methods.

2.4. Circulating microRNA expression

Quantitative miRNA analysis was restricted to a panel of 74 miRNAs proposed as circulating biomarkers of heart disease (Supplemental Table 2). Those miRNAs were selected by two independent and experienced researchers (DdG-C and EI-G) after an extensive review of the literature.

Total RNA was isolated from 200 μL of frozen serum samples using the miRCURY RNA isolation kit (Exiqon), according to the manufacturer's instructions. The RNA Spike-In Kit with synthetic RNA spike-in templates (UniSp2, UniSp4, UniSp5) (Exiqon) was used in all extractions to monitor RNA isolation efficiency. The mixture was also supplemented with 1 μg of MS2 carrier RNA (Roche) to improve extracellular miRNA yield. miRNA qPCR was performed according to the protocol for the miRCURY LNA Universal RT MicroRNA PCR System. The miRCURY LNA Universal RT microRNA PCR System offers high sensitivity, specificity and reproducibility [13]. RNA was reverse transcribed using the Universal cDNA Synthesis Kit II (Exiqon). Additional spike-in (UniSp6) (Exiqon) was added to the cDNA synthesis reaction to check for RT and PCR inhibitors. The RT reaction was performed under the following conditions: incubation for 60 min at 42 °C, heat inactivation for 5 min at 95 °C, and immediate cooling to 4 °C. miRNAs were quantified by quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the ExiLENT SYBR Green master mix (Exiqon) containing 1:50 diluted ROX (Invitrogen) in 384-well Pick-&-Mix microRNA PCR Plates (Exiqon). PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 10 min at 95 °C and 40 cycles of 10 s at 95 °C and 1 min at 60 °C, followed by a melting curve analysis. SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and melting curve analysis. The Cq was defined as the fractional cycle number at which the fluorescence exceeded a given threshold. The specificity of the PCR reaction was corroborated by melting curve analysis. To ensure the good quality of the data, we analysed spike-in RNA templates to monitor the uniformity of the RNA extraction procedure and the efficiency of the RT and PCR reactions. The ΔCq(miR-23a-3p – miR-451a) method was used to rule out the possibility of haemolysis contamination [14]. The miRNAs were considered to be expressed when Cq < 37 in 80% of samples. Relative quantification was performed using the 2^{-dCq} method. Fold change with respect to the Basal sample was determined to quantify the magnitude of c-miRNA response after each race (Post0 and Post24).

2.5. Functional enrichment analysis

The web-based computational tool DIANA-miRPath v3.0 was used for the identification of miRNA targets [15]. DIANA-miRPath v3.0 utilizes predicted miRNA targets from the DIANA-microT-CDS (v5.0) algorithm and combines the results with the pathway tool KEGG (Kyoto Encyclopaedia of Genes and Genomes) to identify possible molecular pathways. The level of significance was set at 0.050.

2.6. Statistical analysis

The statistical software R (www.r-project.org) was used for all statistical analyses. Descriptive statistics were used to characterize the study population. Data were presented as the mean ± SD. General additive linear models, in which subject was considered as a random-effects covariate, were used for studying the effect of race length (10-km, HM, M) and sampling timepoint (Basal, Post0, Post24 and Post72). Bonferroni correction was used to test for significant pairwise differences. For echocardiographic parameters, the comparison between variables was performed using the paired sample Student's *t*-test.

To estimate the effect of heart rate as a covariate for tricuspid and mitral E/A ratios, we used a generalized linear mixed model. Differences were considered statistically significant when $P < 0.050$.

3. Results

3.1. Echocardiographic findings

All subjects completed each race without symptoms of myocardial damage/dysfunction within 72 h after exercise. Relevant haemodynamic parameters and their behaviour in response to M are shown in Table 2. Echocardiographic measurements showed results within the limits of normality. All subjects showed a preserved ejection fraction with no alterations in global or segmental contractility after the M. LVEF was non-significantly augmented after exercise. However, there were significant increases in septal ($P < 0.0005$) and lateral ($P = 0.018$) mitral annulus s′ wave velocity. Regarding left ventricular diastolic function, a post-M decrease in E/A ratio ($P < 0.0005$) was observed due to a decreased E wave velocity ($P = 0.001$) compared with rest echocardiography. This finding persisted after these parameters were adjusted for heart rate ($P = 0.010$). Regarding right ventricular diastolic function, there was a significant increase in A wave velocity ($P = 0.049$), with a decrease in E/A ratio ($P = 0.044$) and an increase in tricuspid annulus a′ wave velocity ($P = 0.019$). However, after tricuspid E/A ratio was adjusted for heart rate, no significant differences were detected ($P = 0.297$).

3.2. Circulating cardiac biomarkers

All parameters showed significant increases immediately after all three races (Post0) (Fig. 1). The kinetics of the different biomarkers varied during the 72 h post-race, depending on the exercise dose. CK and CK-MB remained elevated 72 h after M and HM and 24 h after 10-km, recovering their baseline values within 72 h after the race. LDH remained elevated 72 h after M and 24 h after HM and 10-km, returning to baseline within 72 h after these races. CT-proAVP remained elevated 72 h after M and 24 h after HM, recovering its baseline value within 72 h. hs-cTnT and NT-proBNP remained elevated 24 h after M and HM, recovering basal values within 72 h after both races. Finally, galectin-3, MGB, and h-FABP remained increased 24 h after M, returning to baseline within 72 h. Notably, hs-cTnT levels exceeded the upper reference limit (99th percentile, 14 pg/mL) of our assay in one, five and eight participants immediately after the 10-km, MM and M races, respectively. As shown in Supplemental Table 3, the magnitude of the response observed for all biomarkers except CT-proAVP was higher after M than after 10-km or HM. We also observed a statistically significant increase in HM compared with 10-km for hs-cTnT, MGB, h-FABP and galectin-3.

Table 2
Hemodynamic and echocardiographic measurements.

	Basal	Post-marathon	*P*-value
SBP (mmHg)	125.6 ± 11.8	102.1 ± 9.3	<0.0005*
DBP (mmHg)	69.4 ± 10.1	68.3 ± 9.7	0.839
HR (bpm)	51.9 ± 3.9	103.4 ± 14.0	<0.0005*
RVDd (mm)	23.2 ± 3.3	24.8 ± 4.8	0.538
LVDd (mm)	52.7 ± 3.0	44.7 ± 7.3	0.010*
LVSd (mm)	33.0 ± 3.9	26.5 ± 5.4	0.019*
LVEDV (ml)	134.2 ± 17.4	94.0 ± 33.7	0.007*
LVESV (ml)	45.0 ± 12.6	27.5 ± 14.2	0.026*
FS (%)	0.4 ± 0.1	0.4 ± 0.1	0.406
CO (l/min)	4.7 ± 1.1	6.8 ± 2.6	0.057
LVEF (%)	66.4 ± 8.4	71.2 ± 8.0	0.327
Mitral E (cm/s)	74.5 ± 13.1	50.2 ± 9.1	0.001*
Mitral A (cm/s)	52.1 ± 11.4	55.7 ± 5.8	0.338
E/A Mitral	1.5 ± 0.2	0.9 ± 0.2	<0.0005*
Tricuspid E (cm/s)	49.2 ± 14.1	41.7 ± 6.5	0.201
Tricuspid A (cm/s)	28.7 ± 5.9	40.0 ± 13.3	0.049*
E/A Tricuspid	1.8 ± 0.6	1.2 ± 0.5	0.044*
Septal e´ (cm/s)	10.7 ± 2.1	9.6 ± 1.7	0.124
Septal a´ (cm/s)	8.1 ± 1.2	8.4 ± 1.9	0.535
Septal s´ (cm/s)	7.0 ± 0.6	10.1 ± 0.8	<0.0005*
Lateral e´ (cm/s)	11.8 ± 2.6	12.9 ± 3.8	0.312
Lateral a´ (cm/s)	7.3 ± 1.7	8.7 ± 2.4	0.056
Lateral s´ (cm/s)	8.8 ± 1.9	12.2 ± 3.0	0.018*
RV e´ (cm/s)	11.3 ± 3.5	10.3 ± 2.5	0.493
RV a´ (cm/s)	9.5 ± 2.4	14.8 ± 4.9	0.019*
RV s´ (cm/s)	12.2 ± 2.1	12.8 ± 3.3	0.682

* Statistically significant.

3.3. Circulating microRNAs associated with heart disease

The profiles of a panel of 74 c-miRNAs previously associated with different heart diseases were evaluated at Baseline, Post0 and Post24 at 10-km and M. These races constitute the highest and lowest doses of exercise considered in the current investigation. Due to the possible uncontrolled influence of lifestyle (particularly diet) and training or recovery strategies on the c-miRNA signature, the Post72 point was not analysed.

Seventeen miRNAs (22.9%) were below the limit of detection in both 10-km and M and were not considered for further analysis (Supplemental Table 4). Five (8.6%) and 19 (33.4%) c-miRNAs were up- or downregulated in response to the 10-km and M races, respectively (Fig. 2A). miR-103a-3p was the only miRNA that was regulated in both the 10-km and M races (Fig. 2A). Concerning 10-km, the serum levels of miR-132-3p and miR-150-5p showed a significant upregulation immediately after the race (2.92- and 4.38-fold change from baseline, respectively) (Fig. 2B). Conversely, the serum levels of miR-103a-3p and miR-139-5p were decreased immediately after the 10-km race (0.36- and 0.59-fold change from baseline, respectively) (Fig. 2B). The values of these miRNAs returned to baseline levels within 24 h after the race. Although no change was observed immediately after the race, a significant decrease in the circulating level of miR-590-5p 24 h after the 10-km race was also observed (0.45-fold change from baseline) (Fig. 2B). No differences were observed in the circulating levels of 52 miRNAs (Supplemental Table 4) after the 10-km race. Regarding M, miR-21-5p, miR-27a-3p, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-126-3p, miR-142-5p, miR-143-3p, miR-195-5p and miR-199a-3p peaked immediately after the race (Fig. 2C). These miRNAs returned to baseline levels by 24 h after the race. Again, independently of the circulating levels immediately after the M, we observed decreases in miR-25-3p, miR-29b-3p, miR-30b-5p miR-106b-5p, miR-107 and miR-497-5p (0.26- to 0.57-fold change from baseline) 24 h after the M race (Fig. 2C). Circulating levels of miR-103a-3p and miR-375-5p were downregulated immediately after the M race and remained decreased at the 24-hour sampling point (Fig. 2C). No differences with respect to corresponding baseline levels were observed in 38 miRNAs after the M race (Supplemental Table 4).

3.4. Functional enrichment analysis

The functional enrichment analysis identified 31 and 61 molecular pathways enriched with the targets of the miRNA profiles observed after the 10-km and M races, respectively (Supplemental Tables 5 and 6). These include molecular pathways closely linked to heart physiology and pathophysiology: hypertrophy, remodelling, function, fibrosis, metabolism, survival, proliferation, response to injury, pluripotency of stem cells and cell interactions.

4. Discussion

Here, we have analysed the circulating profile of a panel of miRNAs previously proposed as indicators of cardiac conditions in response to acute aerobic exercise, together with a panel of cardiac biomarkers and echocardiographic parameters, in healthy active subjects. Our

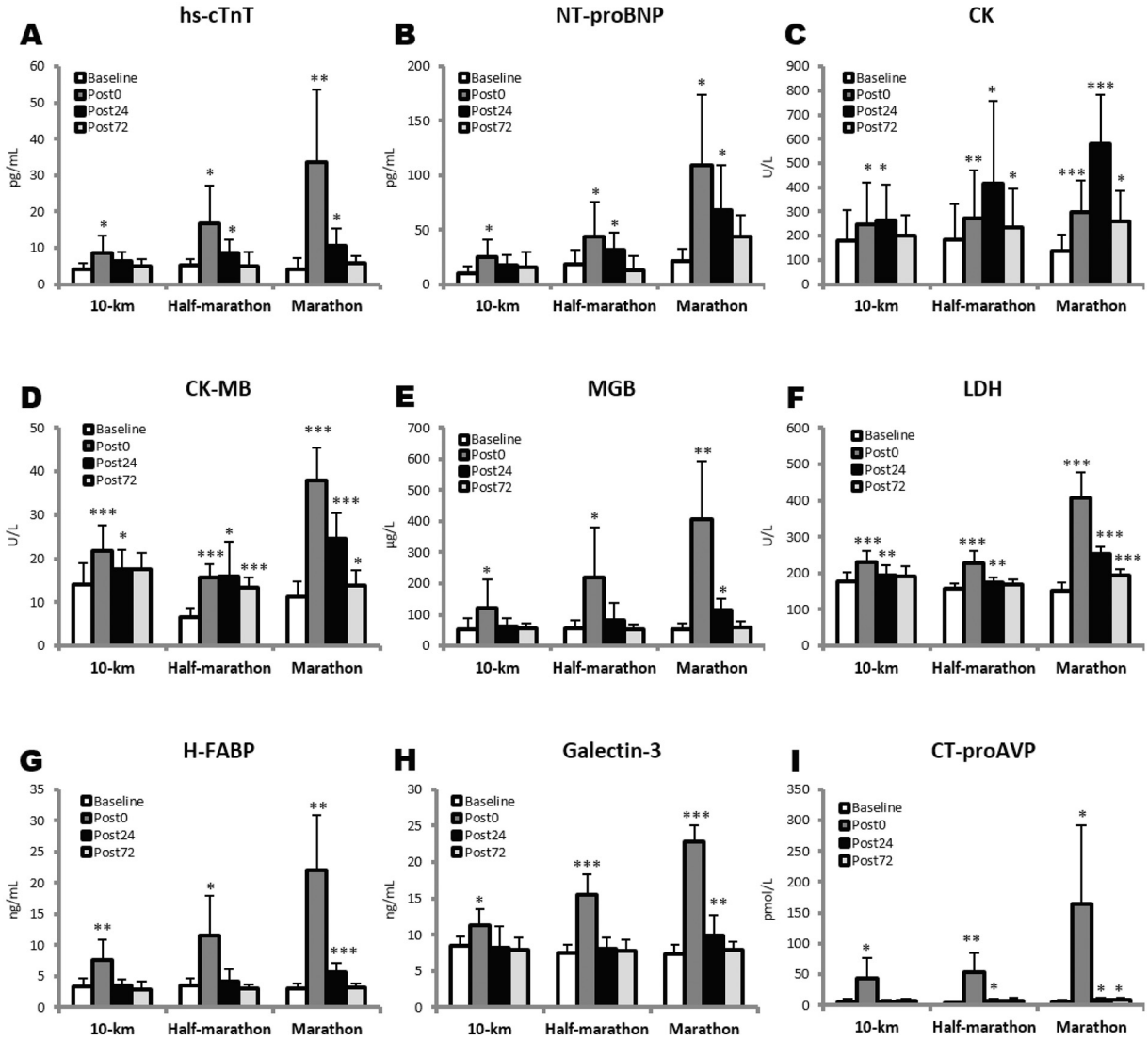

Fig. 1. Circulating profile of cardiac biomarkers. The concentrations of a panel of established and emerging biomarkers were evaluated before (Baseline), immediately after (Post0), and 24 (Post24) and 72 h after (Post72) each race. A) hs-cTnT: high-sensitive cardiac troponin T; B) NT-proBNP: N-terminal pro-brain natriuretic peptide; C) CK: creatine kinase; D) CK-MB: cardiac MB isoform of creatine kinase; E) MGB: myoglobin; F) LDH: lactate dehydrogenase; G) h-FABP: Heart-type fatty acid binding protein; H) Galectin-3; I) CT-proAVP: C-terminal pro arginine-vasopressin. Data were analysed using a one-way repeated-measures ANOVA with Bonferroni correction. The results are presented as the mean ± SD. $^{*}P < 0.050$, $^{**}P < 0.010$, $^{***}P < 0.001$ with respect to Basal samples.

results showed that different doses of acute endurance exercise induced specific signatures of these c-miRNAs providing valuable information on their role as cardiac biomarkers.

Acute exercise induced a dose-dependent increase in the serum concentrations of biomarkers of heart disease, followed by a recovery period during the next 24 and 72 h, in most cases. These results contribute to the controversy evidenced in the literature [16]. Strenuous endurance exercise was classically associated with myocardial damage compromising cardiac function and structure. Nevertheless, it has been proposed in recent years that the exercise-induced increase in biochemical indicators of cardiac injury has little or no clinical relevance [17]. The rapid normalization and the low levels, together with the echocardiographic findings and the absence of symptoms or signs of cardiac dysfunction, suggest the physiological and transitory nature of the increase observed. The changes observed in diastolic function were similar to those described in previous studies, which were shown to be transitory [18]. This hypothesis is supported by previous publications that demonstrated the absence of myocardial damage despite the increase in circulating cardiac biomarkers [19]. The magnitude and kinetics of hs-cTnT in response to acute exercise contrast with the kinetics described during irreversible myocardial necrosis (e.g., myocardial infarction), characterized by a prolonged elevation of hs-cTnT for four to ten days after the event [20]. The exercise-induced reversible elevation in parameters of myocardial damage and stress may be associated with physiological adaptations of the heart [21], similar to those that occur during training adaptation in skeletal muscle. Indeed, exercise-induced elevation in cTnT has been linked to reversible changes in cardiomyocyte structure in animal models [22].

Our data confirm and extend previous results that suggested the altered regulation of the c-miRNA signature in response to acute exercise [23]. As expected, the greatest disruption in the c-miRNA profile was observed after the race imposing the greatest cardiac stress, M. Whether these miRNA signatures are related to exercise-induced active (regulated secretion) or passive (cell necrosis) release of miRNAs is controversial [24]. Here, we evaluated in detail the circulating signature of myocardium-enriched miRNAs (i.e., miR-1, miR-133a-3p, miR-133b, miR-208a-3p, miR-208b-3p and miR-499a-5p) in response to acute exercise. The levels of these miRNAs, previously associated with

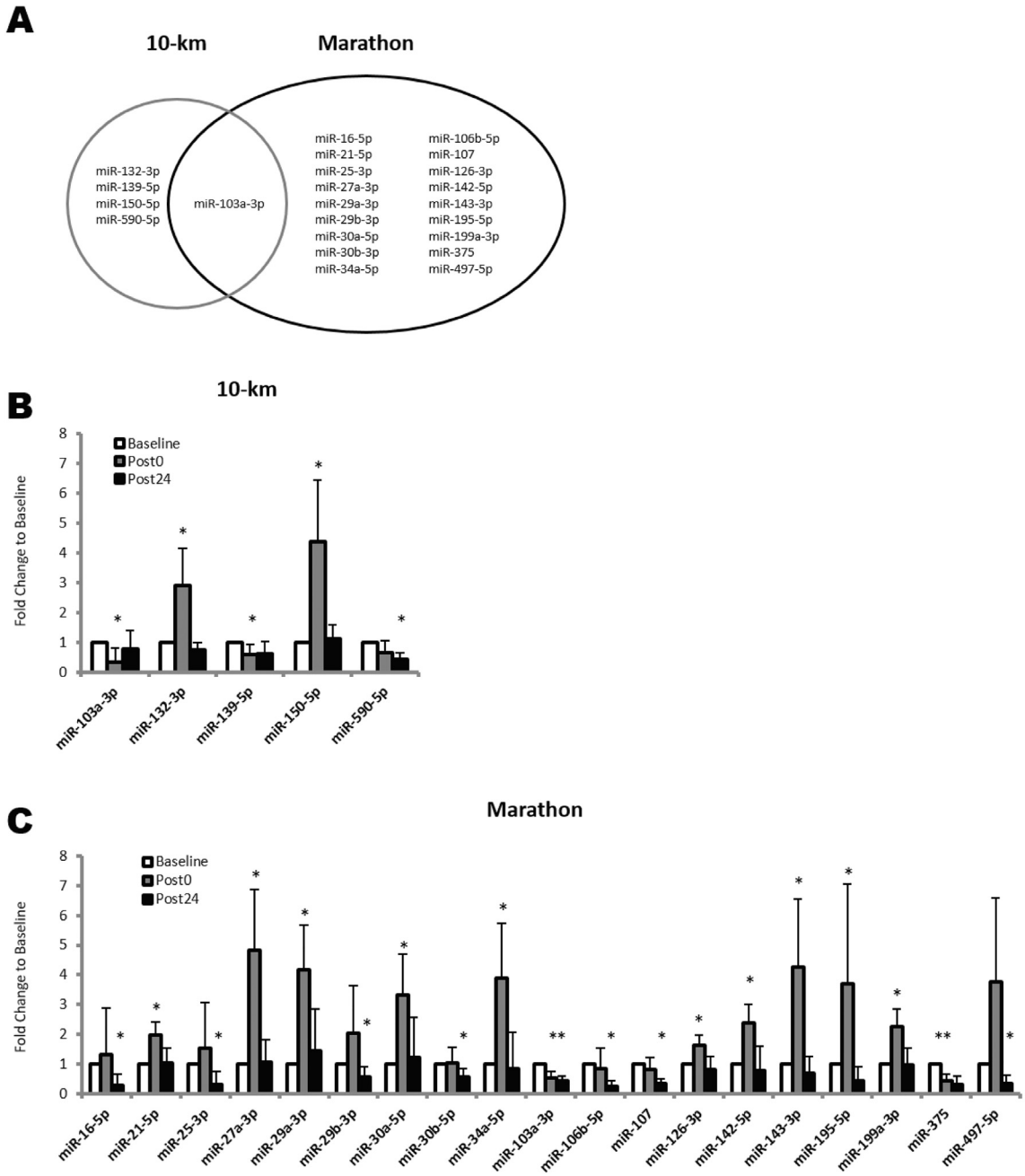

Fig. 2. Circulating microRNA profile in response to 10-km and marathon races. The expression levels were evaluated before (Baseline), immediately after (Post0) and 24 h after (Post24) each race. A) Circulating microRNAs with altered regulation in response to 10-km and M races; B) Circulating microRNA response to 10-km race; C) Circulating microRNA response to marathon race. Data were analysed using a one-way repeated measures ANOVA with Bonferroni correction. The results are presented as the mean ± SD with respect to Basal samples. $^{*}P < 0.050$.

myocardial necrosis in humans and animal models [25], failed to increase in serum or were below the detection limit of our high-sensitivity assay [13] in response to the races. These results rule out the non-specific release of miRNAs, at least from the myocardium. Additional data support this hypothesis. In general, c-miRNA kinetics were characterized by an increase immediately after the races followed by a rapid normalization within 24 h. Furthermore, we observed a specific and different c-miRNA profile for each race that is incompatible with passive release from the tissues. Rather than non-specific release linked to tissue damage, it seems that the miRNAs are selectively released into the circulation during and after the exercise. Overall, our data demonstrate that acute aerobic exercise induced a qualitative and quantitative alteration in the circulating profile of miRNAs previously associated with cardiac conditions.

4.1. Implications for the clinical application of circulating microRNAs as biomarkers

Beside detailed clinical assessment and electrocardiograms, cardiac biomarkers are key diagnostic tools for patients presenting with acute chest pain. The ideal biomarker should allow an accurate and immediate identification of acute coronary syndrome. Nevertheless, blood biomarkers of myocardial damage or dysfunction suffer from a lack of specificity since their elevation could be due to physiological causes. Elevations in cardiac biomarkers used in daily practice, even above the

clinical cut-off value, are common after acute endurance exercise in both professional and amateur athletes [26]. Furthermore, electrocardiographic abnormalities are typical in athletes and physically active subjects [27]. This situation complicates the management of those subjects with cardiac dysfunction symptoms after exercise since it could lead to overdiagnosis [28]. In the present study, all indicators of damage and dysfunction were significantly increased in response to the three races, even in absence of clinical evidence of cardiac pathology. However, as discussed above, the levels of the myocardium-enriched miRNAs, some of them recently proposed as suitable for use as diagnostic biomarkers (i.e., miR-133a and miR-499) [29], were undetected or stable after the 10-km and M races. Similar results were observed for circulating miR-423-5p, a biomarker of heart failure [30]. Exercise training blunts the miRNA response to an acute bout of exercise in both circulation and muscle [31,32]. Nonetheless, independently of the training status, our results suggest that the analysis of c-miRNAs may offer incremental diagnostic value to cardiac biomarkers currently used in clinical practice. A unique signature composed of c-miRNAs previously proposed as biomarkers of cardiac conditions that do not respond to exercise could discriminate between patients with cardiac conditions and healthy subjects after an acute bout of prolonged exercise. The addition of these c-miRNAs to the diagnostic algorithms could be considered as a way to improve the clinical management of those subjects for whom the diagnosis of myocardial injury is controversial.

In contrast, our results indicated that the applicability of certain c-miRNAs as biomarkers of heart disease should be taken with caution due to the risk of false positives. We have reported "pseudo-disease" miRNA signatures in response to acute exercise, including miRNAs associated with acute coronary syndrome, heart failure and different cardiomyopathies. These perturbations seem to be independent of the presence of cardiac necrosis or dysfunction. Thus, clinical practitioners should be aware of the impact caused by a recent bout of acute exercise in the interpretation of miRNA data.

4.2. Circulating miRNAs are potential mediators of the cardiac response to exercise

Since the mechanisms of exercise-modulated gene expression protect the heart against pathophysiological alterations, mimicking the response to exercise is an exciting approach for the development of cardioprotective strategies. However, the mechanisms that alter cardiac structure and function in response to an exercise bout are still unknown, especially when the evaluation of these adaptations with non-invasive imaging techniques does not provide information about the underlying molecular processes [33]. In addition, the alterations in current circulating biomarkers provide limited information about adaptive mechanisms related to physical activity. Although TnT and CK are key elements in contractile function and energy metabolism, respectively, they have limited functions as mediators of cellular responses [24]. By contrast, miRNAs are key elements of cellular homeostasis and regulate a number of processes that are essential for cardiac function [2].

Here, we could only speculate about the role of c-miRNAs as mediators of cardiac response to acute exercise. The cellular sources of c-miRNAs and the targeted tissues are unclear. In addition, we could not determine the extent to which the observed changes in the c-miRNA signature translate into the regulation of the recipient cell phenotype. It should be noted that the miRNAs evaluated are proposed as biomarkers of a wide array of cardiac conditions and they are not necessarily secreted by the heart. The influence of the c-miRNA profile on heart gene expression should be validated in future investigations. Nevertheless, results from the functional enrichment analysis suggest a potential role of miRNAs in the cardiac adaptions promoted by exercise. We have observed an association between the miRNAs that respond to the exercise bouts and molecular pathways related to cardiac hypertrophy, remodelling, function, fibrosis, metabolism, survival and response to injury. Different studies have experimentally validated our results. Aerobic swimming training induced physiological LV hypertrophy, at least in part, altering the cardiac expression of miR-21, miR-27a and miR-143 in *in vivo* models [34,35]. The same training program favoured cardiac angiogenesis through the regulation of miR-126 expression levels in heart muscle [36]. Importantly, these results support recent research linking miRNA expression induced by exercise to regenerative mechanisms in the heart [37]. Indeed, exogenous administration of miR-199a-3p promoted the cell cycle re-entry of adult cardiomyocytes in neonatal and adult mice [38]. Furthermore, molecular pathway analysis suggests an association between the c-miRNA profiles and pluripotency of stem cells. Since obtaining cardiomyocytes from stem cells is one of the most promising therapeutic strategies for patients suffering from heart disease [39], the analysis of the miRNA response to exercise emerges as an interesting source of information for this hot topic in cardiovascular research.

4.3. Strengths and Limitations

The strengths of our study are the strict control and characterization of the subjects, including dietary habits, and the experimental design. The repeated measures-nature of this study, barely used in the literature available, reduced the variability in the response to the different conditions tested (exercise doses), strengthening the statistical analysis. We recruited amateur athletes, who represent a great proportion of participants in endurance events. These amateur athletes are not usually under a strict medical control. Furthermore, participants did not alter their usual training schedule or food habits; therefore, the response to exercise was explored in "real-life" settings. Some limitations of the present study should be noted. First, the strict inclusion criteria and the invasive nature of the study, along with the inherent sampling techniques and logistical difficulties, limited the ability to analyse a larger number of subjects. These limitations are inherently linked to studies of this nature and cannot be attributed only to our study; similar sample sizes, typically 10–25, have been usually reported [31,40]. However, as mentioned before, in the repeated-measures design of the present study, the same 9 subjects participated in three different races and provided four samples before and after each race, which led us to analyse >100 samples. Second, generalization of results is limited by the characteristics of the study subjects: middle-aged experienced runners with moderately high workloads and no personal history of cardiovascular disease. Third, the presence of subclinical cardiac disease could not be ruled out. Forth, no samples were taken between Post0 and Post24 timepoints. Thus, we cannot rule out the possibility of exercise-induced changes in miRNA levels during this time window. Finally, we could only speculate about the potential role of c-miRNAs as mediators of the cardiac response to acute exercise. Mechanistic *in vitro* and *in vivo* studies are necessary to experimentally validate these findings.

5. Conclusions

Our results highlight the relevance of c-miRNAs as emerging cardiac biomarkers and their potential role in the cardiac response to exercise. The clinical evaluation of exercise-induced c-miRNAs related to cardiac pathology could contribute to improving the diagnosis and clinical management of heart disease. Furthermore, the identification of c-miRNA profiles induced by exercise allows the study of novel molecular mechanisms linked to the molecular response to exercise.

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Conflict of interest

Nothing to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2018.02.092.

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TITLE

Murine exercise models reflect the human circulating miRNA signature in response to training

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ABSTRACT

The study of the molecular response to exercise was historically limited to muscle tissue. However, its systemic effect is not restricted to the tissues involved in generating movement, so exercise response involves important intertisular communication. Apart from the classical mechanisms, widely studied, mediated by hormones or cytokines, in recent years the role of exosomes has been highlighted in this field. Encapsulated in these exosomes are different macromolecules, including microRNAs (miRNAs), small non-coding RNA molecules with a post-transcriptional regulatory function. Although their activity is intracellular, they have been stably detected in different biological fluids, constituting the so-called circulating miRNA (c-miRNA). The presence of c-miRNA in plasma exosomes suggests that they would be secreted in a regulated manner in response to a stress situation, acting as a true intercellular communication system and regulating gene expression and phenotype of distant receptor cells.The need to use animal exercise models for the study of potential sources and tissue targets of c-miRNA detected in response to exercise is raised, as well as the possibility of studying genetically modified models. The aim of this study was analyzed c-miRNA profile in humans and in experimental animals (mice) chronically trained in resistance and endurance, determining tissue expressions of these miRNAs and their role in exercise adaptations.

Plasma samples were collected in fasted conditions from 30 young males: 10 sedentary (HSED), and 20 elite athletes (10 weightlifters and 10 long-distance athletes). We had also taken basal plasma samples from 18 C57BL/6N mice, divided into three groups: sedentary, endurance training on a treadmill, and resistance training on a vertical ladder. The training design was 2 weeks of adaptation and 4 weeks of training, 5 days/week. Every session had fixed volumes, 1000m in endurance and 260mJ in resistance, but varying intensity between sesions. In order to analyse the role of miR-29 on performance,

8 C57BL6 knockout miR-29a/b1−/− mice (KO) and 8 wild type (WT) were used. Endurance and resistance maximal performance were tested by a treadmill ramp test protocol and a 10 repetition-maximum test, respectively.

Our results described that there were different specific profiles in human and mice, but with a high percentage of coincidence in gene targets and metabolic pathways between them based on miRWalk analysis. At the tissue level, correlations were obtained between the circulating levels of certain miRNA and their presence in muscle and liver. The miRNA profile in both tissues was significantly related to the ability to adapt to training. Finally, in view of the increase in circulating miR-29a and its relationship with muscle-level adaptations, performance in KO mice was analysed for this miRNA, which showed a clear deterioration in both resistance and endurance performance.

In conclusion, specific exosome miRNAs have a clear role in molecular response of exercise adaptation, emerging as potential intertissue and interspecies biomarkers in this field.

Keywords: circulating microRNA, biomarkers, training, interspecific differences

INTRODUCTION

In 2009, World health Organization recognized physical inactivity as the fourth leading risk factor in global mortality (WHO, 2009). The increasement in sedentary lifestyle is noticeable in the world, however, the number of papers which emphasized the role of high performance sport on longevity were also appearing (Sanchis-Gomar et al., 2011). All those factors contribute to a polarized society where the limit of exercise health benefits was not determined yet (Neufer et al., 2015). Physical activity is strongly correlated with our evolution as a specie. As the main activity of our lifestyle, if we changed in a completely sedentary way, consequences for human health would be terrible. The strong effect of physical exercise on pathologies is clearly established such as on diabetes, cancer, heart failure, … (Fiuza-Luces et al., 2013).

Despite the fact that exercise is a perfect prevention and treatment, physical exercise molecular determinants and pathway effects were not clearly explored (Neufer et al., 2015). Skeletal muscle as central tissue of physical activity was the main focus of sport science (Egan and Zierath, 2013). Researchers determined AMPK and mTOR as king of endurance and resistance exercise, respectively(Egan and Zierath, 2013). Nevertheless, exercise physiology not only regulates muscle metabolism, but also every single part of human metabolism. It is a brain health determinant(Mattson, 2012), a microbiota modifier (Scheiman et al., 2019), a circadian rhythm controlling method (Sato et al., 2019), an inflammatory response modulator(Pedersen et al., 2016),…

However, how could exercise coordinate every single process to create a perfect pill? Whitham et al. proved how exosomes creates a crosstalk between tissues in exercise response(Whitham et al., 2018). Moreover, they had analysed completely the proteomic of this exosomes to fulfil all markers based on proteins and where they go to do their roles(Whitham et al., 2018). Taking into account another molecules which are

presented in exosomes(Hu et al., 2012), we have to considered microRNAs (miRNAs) as it was purpose by Baggish et al. for first time in 2011 in response to exercise(Baggish et al., 2011). This short chain RNAs are presented in circulation of all human fluids(Weber et al., 2010) and they are negative controllers of gene expression at translation level(Krol et al., 2010). In the field of exercise, they were purposed as better cardiac biomarkers than classical ones in exercise acute conditions(de Gonzalo-Calvo et al., 2018) and also as exercise biomarkers in acute and training adaptations in healthy people and patients(Fernandez-Sanjurjo et al., 2018; Polakovicova et al., 2016). Taking all those things into account, our aims were to determine how circulating miRNA were related with sedentary lifestyle, if miRNAs are conserved between species, where they develop their control roles and how could they determined exercise adaptations and performance.

MATERIALS AND METHODS

Human study

Experimental design

A total of 48 young men divided in three groups were analysed. Firstly, we had a group of 16 young men which completely sedentary (HCON) (less than one hour per month of training). The other two groups were athletes from Spanish national teams of different disciplines. One group of them were 16 athletes specialized in endurance sports, in this case were long distance and triathlon athletes (HEND). The other group were 16 athletes specialized in resistance sports as weightlifting, gymnastics and throwing (HRES). All participants were informed and signed an informed consent.

Blood samples collection

Human blood samples were collected in fasted condition in EDTA-treated vacutainers (EDTA K2 (BD), Becton Dickinson, Franklin Lakes, NJ). When they were completely mixed, they were centrifuged to extract plasma samples. Plasma samples were divided in two groups of eight samples in HCON, HEND and HRES. Eight samples were going to be analysed by NGS sequencing and the other eight plus the same eight samples of NGS were used to internal and external validation by qPCR.

Animal study

Experimental design

A total of 26 C57BL6N male mice (8 weeks old) were randomly divided into three different groups: sedentary control (MCON, n=6), resistance training (MRES, n=8), and endurance training (MEND, n=12). Mice were maintained on a 12 h light/dark cycle (onset at 8:00 AM) and under controlled temperature (22±2 °C) at the Animal Facilities of the University of Oviedo, Spain (authorized facility No. ES330440003591). All procedures were conducted during the early light portion of the cycle and performed in accordance with the institutional guidelines approved by The Research Ethics Committee of the University of Oviedo, Spain (PROAE 10/2016). Mice were fed a pellet rodent diet (Teklad Irradiated Global 18% Protein Rodent Diet, Envigo, Spain) and water *ad libitum*. The food intake and the animals' weight were measured weekly.

Training devices

Endurance training was performed on a treadmill without any aversive stimuli. We used a four-lane commercial treadmill (TSE Systems, Germany), with adjustable speed and slope.

Resistance training was carried out in an own-manufactured ladder. The ladder was built with 25 steel wire steps of 1.5 mm of diameter separated by 15 mm. A resting area of 20x20 cm was placed on top of the ladder. The slope of the ladder was modifiable by the researcher, ranging between 90 and 80° with the horizontal plane.

Training protocols

The same researcher handled and trained the mice during the different stages of training: acclimation period, physical performance tests (pre- and post-training), and training protocols.

Acclimation period

Before starting the training program, mice were acclimatized to the training devices for 2 weeks, 5 sessions per week, and 15 min per training session (Kregel KC, 2006). This period was designed so that training load was kept to a minimum, avoiding training adaptations that could interfere with pre-training maximal performance tests (Kregel KC, 2006).

During the first week, mice were placed on the treadmill without movement and in the resting area in the top of the ladder. The following week, animals walked on the moving treadmill belt (10 cm/s) and they were trained how to climb the ladder, from the 5th top step to the resting area, increasing gradually the number of rungs to 10.

Mice were adapted to run on the treadmill without any aversive stimuli throughout all stages of the training protocol. Aversive stimuli used to encourage treadmill running constitutes a confounder factor and diminishes repeatability of maximal tests (Allen et al., 2015; Conner et al., 2014; Knab et al., 2009; Kregel KC, 2006). At the backside of the treadmill, a static brush was used to keep the animals running on the lane during the whole adaptation and training periods.

During resistance training, animals carry variable weights attached to their tails with clinical tape. In order to make the animals used to it, a piece of clinical tape was attached to their tails while climbing the ladder. After a few days, a lightweight load (5 g) was attached to the animals' tails with clinical tape.

This acclimation protocol allowed us to train all the animals without refusals (Conner et al., 2014).

Maximal performance tests

Forty-eight hours after the end of the acclimation period, mice were randomly distributed in the above-mentioned groups. Then, those at the END and RES groups performed a maximal endurance or resistance test, respectively.

Maximal endurance capacity was determined by an incremental test in the treadmill, adapted from other studies (Ayachi et al., 2016; Lira et al., 2013). After a 10-min warm-up at 15 cm/s with 10° slope, the incremental test started at 20 cm/s. Every 3 min speed increased by 5 cm/s, until exhaustion. Maximum speed (cm/s) and total time (min) were recorded, and total distance (m) calculated as measurements of endurance capacity.

Maximal resistance capacity was tested in the vertical ladder, following a protocol adapted from previous studies (de Deus et al., 2012). Mice performed a warm-up consisting of 3 series of 10 repetitions, 10 steps/repetition, at 90° of slope, without external load. The animals rested for 60 s between series. Then, the slope was set at 85° and the animals performed successive series of 10 steps with increasing external loads until exhaustion. The starting external load was 10 g, increasing 5 g in each series. The animals rested for 120 s in the resting area after each series. If the animals failed to climb 10 steps with a particular weight load, they were allowed for another try with the same load after 120 s of rest. If they failed again, the weight load of the last complete

series was recorded as their maximal weight load. The maximal resistance capacity was then expressed as the maximal weight load relative to body weight (%).

Both tests were repeated at the end of the training period, following the same protocols.

Training protocols

All MEND and MRES animals trained for 4 weeks, 5 days/week (Monday to Friday). Training protocols were adapted from previous works (Codina-Martinez et al., 2020; Kregel KC, 2006) in terms of intensity and duration of sessions. To reduce animal anxiety, mice were trained in groups of four animals sharing the same cage.

Endurance training sessions started with identical warm-up as for the maximal endurance performance test. Then, all sessions of continuous running had a mean duration of 60 min and the distance covered every day was 1000 m, as a fixed volume. However, the intensity in terms of maximal speed, number of stages, as well as the speed and duration of each stage, varied along the week according to this structure: 2 days at high intensity (Tuesday and Friday), 2 days at moderate intensity (Monday and Thursday), and 1 day at low intensity (Wednesday). Speed ranged from 20 to 40 cm/s, which corresponded to 40-80% of mean maximal speed at the pre-training test (Kemi et al., 2002). The duration of each stage varied inversely with speed, between 15 and 5 min (Kemi et al., 2002). The slope was fixed at 10°. Maximal intensity increased throughout the training period, although maintaining the weekly schedule as well as the duration and the distance covered in training sessions.

Resistance training sessions started with an identical warm-up as for the maximal resistance performance test. Then, all sessions were designed to achieve the same exercise volume by means of a combination of number of steps climbed (or distance against gravity) and weight load (Figueiredo et al., 2018). Considering the combination

of these parameters, an accumulated work of 260 mJ ($g \cdot m^2/s^2$), was achieved daily. The number of steps per training session varied between 400-2000 depending on the maximal weight load, which ranged between 20-50 g or 25-65% of the maximal weight load at the pre-training test. We selected these maximum weight ranges because it has been described that below 75% of 1 repetition maximum there is no velocity loss, which is important for standardizing intensity of submaximal efforts (Gentil et al., 2018). Week planning was: 2 days with high weight load and low number of steps (Tuesday and Friday), 2 days of intermediate weight load and number of steps (Monday and Thursday), and 1 day without weight load but a high number of steps (Wednesday). The number of steps and the maximum weight loads increased throughout the training period, although maintaining the weekly schedule, as well as the accumulated work and the percentages of maximal weight load.

Control mice remained in a cage, in the same room, while MEND and MRES animals were training.

Blood Sampling protocol

Animal blood was extracted from Cava vein as the first step of the sacrifice protocol. The extracting procedure was done 24 hours from the last training session. Plasma samples at the same time of extracting were centrifuged 15 min at 2000 g. When the plasma was isolated, they were inmediately freezed with liquid nitrogen and kept at -80 °C.

Sequencing and validation of human and mice plasma samples

The analysis of plasma samples was firstly done by exosome miRNA Next Generation Sequencing (NGS) (Exiqon Exosomes miRNA sequencing Services project) from 500

µl of plasma. As it was said, human samples were divided in two groups and NGS analysis was done in 8 of 16 samples.

Secondly, all plasma samples were treated with thrombin to create serum like samples. Exoquick (SeraMiR) product was used to facilitated exosomes precipitation and extraction. miRCURY RNA isolation kit (Qiagen) was used to extract miRNA from 500 µl in human and 200 µl in mice following the manufacturer's instructions. For ulterior normalization, synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p), lacking sequence homology to human and mice miRNAs, was added as an external reference. The RNA Spike-in kit with synthetic RNA spike-in templates (UniSp2, UniSp4, UniSp5) (Exiqon) was also used in all extractions to monitor RNA isolation efficiency. RNA was eluted in 30 µl RNase-free H_2O, samples were measured by NanoDrop (ThermoFisher) to determine if there were RNA sufficient quantity to next steps and stored in a -80°C freezer.

To validate sequencing data, we had used qPCR as the gold standard technique for this task. cDNA was synthesized using the miRCURY LNA RT kit (Qiagen). This LNA RT miRNA PCR System offers a high sensitivity, specificity and reproducibility (Mestdagh et al., 2014). Briefly, 10 µl RNA samples were reverse transcribed in 50 µl reactions. Additional spike-in (UniSp6) (Qiagen) was added to the cDNA synthesis reaction to check for RT and PCR inhibitors. RT reaction was performed with the following conditions: incubation for 60 min at 42°C, heat-inactivation for 5 min at 95°C, immediately cool to 4°C. Then, cDNA was stored at -80°C. For qPCR, cDNA was diluted 80x and 4 µl used in 10 µl qPCR reactions with ExiLENT SYBR Green master mix (Exiqon) on a 7900HT fast Real-Time PCR System (Applied Biosystems) with the following cycling 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.

Tissue analysis

All the animals were perfused with 20 ml of cold PBS from the same blood extraction point, Cava vein. Quadriceps and liver were completely extracted from mice. Both tissues were immediately frozen at -80°C until analysis. In dry ice conditions, a piece of 50 mg of quadriceps and a piece of 25 mg of liver were cut to make extraction process. The extraction method was mortar and pestle with 700µl of Qiazol(Qiagen). miRNeasy mini kit (Qiagen) was used to extract miRNA following the manufacturer's instructions. All samples were measured by NanoDrop (ThermoFisher) and determined equal quantities of RNA to next step. cDNA was synthesized using the miRCURY LNA RT kit (Qiagen). spike-in (UniSp6) (Qiagen) was added to the cDNA synthesis reaction to check for RT and PCR inhibitors. RT reaction was performed with the following conditions: incubation for 60 min at 42°C, heat-inactivation for 5 min at 95°C, immediately cool to 4°C. Then, cDNA was stored at -80°C. For qPCR, cDNA was diluted 80x and 4 µl used in 10 µl qPCR reactions with ExiLENT SYBR Green master mix (Exiqon) on a 7900HT fast Real-Time PCR System (Applied Biosystems) with the following cycling 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.

miR-29a/b1−/− knock-out mice performance analysis

A total of 16 C57BL6N 14-week-old mice, divided in wild-type control (WT) and miR-29a/b1−/− knock-out mice (KO), were used to determined performance differences. As it was done with training mice two weeks of adaptation was done. Then, both performance tests were performed as it was described in training protocol. There was a difference of two days between test, not to influence one on the other.

RESULTS

Sedentary people had an increased exosomal presence of miR-16-5p, miR-451a and miR-19a-3p compared to athletes.

We have determined a specific exosomal of circulating miRNA of athletes in comparison with sedentary people. Starting with a global approach with RNAseq we can identified 404 miRNA presented in exosomes in plasma (Supplementary Table 1). 241 of them were presented in all of the samples with a minimum concentration of 1 TPM and with 10 TPM 132 miRNAs were obtained. With qPCR validation we have obtained 4 significantly changed miRNA, hsa-miR-16-5p, hsa-miR-451a, hsa-miR-19a-3p and hsa-miR-25-3p (Figure 1A). Three of them had lower circulating expression in athletes of resistance and endurance compared with sedentary people. However, only one miRNA hsa-miR-25-3p had specifically lower levels in endurance athletes. There are another three which had a clear tendency. But one of them had an important role because of the unique response, hsa-let-7f-5p had higher levels in a unique group compared with the others which was resistance athletes.

Decreased in specific exosomal miRNAs determined and increasement in key adaptative exercise-response pathways

The significantly changed miRNA was all of them in lower level in both groups, so their response is common to exercise stimuli. Going deeper in targets analysis with Tarbase.v7 we had obtained, on one hand, a clear relationship with TGFβ pathway, fatty acid biosynthesis, central carbon metabolism, mTOR signaling pathway and AMPK signaling pathway in these three miRNAs (Figure 1C). All of these pathway responses are increased by exercise, due to this fact the repression of miRNAs is completely related with these activations. In Cytoscape image (Figure 2A) there were described

1985 nodes and 3125 edges. These results defined that 57% of edges were interactions between miRNA targets as could be observed in the middle circle of Figure 2A.

On the other hand, we had obtain one specific miRNA of endurance exercise, hsa-miR-25-3p, and one specific resistance exercise miRNA, hsa-let-7f-5p, both of them had targets on cell cycle pathway, but the important thing was that the case of endurance, an activation was produced because of the repression of the miRNA, and in the case of resistance a repression of the pathway could be purposed because of the increased expression (Figure 1C-E).

let-7 miRNA family emerges as a key in mice exercise metabolism

None of the mice was excluded due to refusal of training. Regarding performance in maximal tests, mice in the MEND group significantly increased their maximal endurance capacity by 25.9% after the training period. Mice in the MRES group significantly increased their maximal resistance capacity by 34.3% after the training period.

Mice RNAseq results identified 400 miRNAs presented in exosomes in plasma (Supplementary Table 2). 224 of them were presented in all of the samples with a minimum concentration of 1 TPM and with 10 TPM 99 miRNAs were obtained. qPCR validated significant changes in 11 c-miRNAs which had described an increasement in the expression caused by exercise training comparing to sedentary mice group (mmu-let-7a-5p, mmu-let-7i-5p, mmu-miR-10b-5p, mmu-125b-5p, mmu-miR-126a-3p, mmu-miR-126a-5p, mmu-miR-142-5p, mmu-miR-143-3p, mmu-miR-145a-5p, mmu-miR-29a-3p and mmu-miR-30a-5p) (Figure 1B). As in human samples, there were some of them which were slightly close to change significantly. In this case, mmu-let-7f-5p appears again with an increment in resistance group compared to control. Another two of these cases were mmu-let-7f-5p and mmu-miR-26a-5p.

Let-7f as a common biomarker in human and mice exercise response

The c-miRNAs profiles defined in human and mice exosomes were different in who were the miRNAs significantly modified. However, miRNAs as negative regulators of gene expression must be considered the targets analysis as a better indicator of correlation. Not only considered the names, but also their regulatory function as the main determinant of correlation.

The common point of the expression was again a let-7 family miRNA, let-7f-5p was in relation with resistance adaptation both in humans and in mice.

About target analysis, the common points were clearly stablished. To establish a murine and human correlation we used predicted targets (TargetScan) as gold standard, due to validated targets had bias of pathology and other scientific focus (Figure2 A-B). With this analysis we had obtained that the miRNAs changed target fatty acid degradation in mice and fatty acid biosynthesis in humans, so they determined the inverse pathway, nevertheless, the c-miRNA response was exactly the same, an opposite response: higher in mice and lower in human. They had also determined extracellular matrix and glycans in both species.

We had used human validated targets of them to create a Cytoscape figure in order to determined correlation between species. We had obtained 4812 nodes and 10030 edges. 5218 edges were established between targets of different miRNAs, as can be observed in (Figure 2 C-E) the relationship was really strong with an only circle of common targets.

No changes in exosomal exercise-responsive miRNAs in liver and muscle

We have identified no differences in miRNAs expression neither in liver nor in muscle. All miRNAs, which had been changed in circulation in response to exercise, did have not changed at tissue level (Figure 3A-B).

However, the analysis of those miRNAs in tissue did not end here. We have analyzed two correlations. We analyzed tissue as origin or destination of exosomal c-miRNAs. Let-7c-5p had a positive strong correlation with muscle expression, so it might be a target or excretion tissue of this miRNA. In the case of liver, there were more miRNAs correlated: positively mmu-let-7i-5p, mmu-miR-10b-5p, mmu-miR-142-5p and mmu-miR-29a-3p; negatively mmu-miR-126-3p (Figure 3C).

Exosomal exercise-responsive miRNAs tissue abundance as determinants of exercise performance

We analyzed tissue miRNA expression correlated with global, endurance and resistance performance measured by improvements on performance between pre- and post-training test. In liver tissue, there were common correlation between both models of training Figure 3D, specific miRNAs profile of endurance Figure 3E and specific resistance Figure 3F. In muscle tissue, global performance improvements were correlated positively with mmu-let-7i-5p, mmu-miR-29a-3p (Figure 4B) and mmu-miR-29c-3p.

MiR-29a-3p as an exercise adaptative biomarker and mice performance correlate

MiR-29a-3p was changed in response to resistance exercise in mice plasma (Figure 4A). Correlation between muscle and performance was clearly stablish as could be observed in Figure 4B.

Mice miR-29a/b1−/− had a 30% less endurance performance than wild type mice and a 50% less resistance performance as can be observed in Figure 4C-D.

Discussion

As the first time a complete sedentary-athlete profile was performed in basal conditions. This fact gives an objective measurement, determined and categorized apparently healthy subjects. According to that, disease perspectives and exercise performance could be changed. From this global perspective in humans, three exosomal miRNA must be emphasized as they described the same lower response in both athletes group compared with sedentary people. hsa-miR-451a was demonstrated as a key regulator of mTOR/Akt pathway and also it plays a key role in erythropoiesis, in exercise roles increased muscle adaptations and erytorpoietin adaptations, respectively(Minna et al., 2016; Rasmussen et al., 2010). Hsa-miR-16-5p and hsa-miR-19a-3p both related with increased expression in response to cancer pathologies(Munson et al., 2019; Zhang et al., 2019). However, also in exosomes and in brain hsa-miR-19a-3p is related with ischemia and improvement on brain physiology in repressed conditions as it was observed in our results(Ge et al., 2019; Zhou et al., 2019). Brain was one of the systemic improvements validated in the field of exercise (Mattson, 2012), so this relationship opens a new field of investigation on that topic. As a common point of interaction was hippo signalling pathway which was clearly analysed and related with muscle mass adaptation and size (Csibi and Blenis, 2012; Gnimassou et al., 2017).

MiRNA conservation between species was completely proved it (Friedman et al., 2009), however, this the first time were a whole exosomal miRNA was done in the field of exercise. With this approach the conclusion is clear, there were relationship on targets such as MAPK pathway but there was an only representative miRNA which had the same response in human and in mice. Let-7f-5p was related with resistance exercise response with an increasement both in humans and in animals. Let-7f-5p had as

validated target TGFBR1(Shen et al., 2019) and ECM receptor pathway, both facts were related to resistance exercise adaptation(Gumucio et al., 2015).

Despite of the fact that a specific profile was defined in mice exosomes, the basal expression of these miRNAs at tissue level were not modified by exercise training. However, they established relationships between tissue expression and exercise performance.

On one hand, as it was observed in humans, in our mice, training increased mir-126-3p in circulation(Barber et al., 2019). Besides, this is not the only result about this miRNA because at liver level is the one that it was correlated with resistance and endurance performance improvements in a positive way. Another interesting result was that the presence in exosomes was negatively correlated with liver levels, so at this point it could be purpose as a receptor or a secretory tissue. Taking into account Human MiRNA Tissue Atlas, liver was one of lowest miR-126-3p tissue expression in human body (Ludwig et al., 2016), so the animals which can absorb more exosomes in the liver could be the ones that had a better performance improvements.

On the other hand, at muscle level one miRNA appears as key of performance improvements, mir-29a-3p. Moreover, our results indicated that mir-29a-3p changed in exosomes in response to resistance training. So, going deeper in the analysis, we have analysed exercise performance in KO mice where they were significantly worse than WT. Based on data from Caravia et al. and matched with mir-29a-3p validated targets(Tarbase), fatty acid metabolism pathway is clearly modified in KO: target genes were overexpressed and body fat was significantly lower than in WT (Caravia et al., 2018; Massart et al., 2017). Taking into account these facts, exercise performance could be modified due to the fact that fat was not the main fuel for peak performance.

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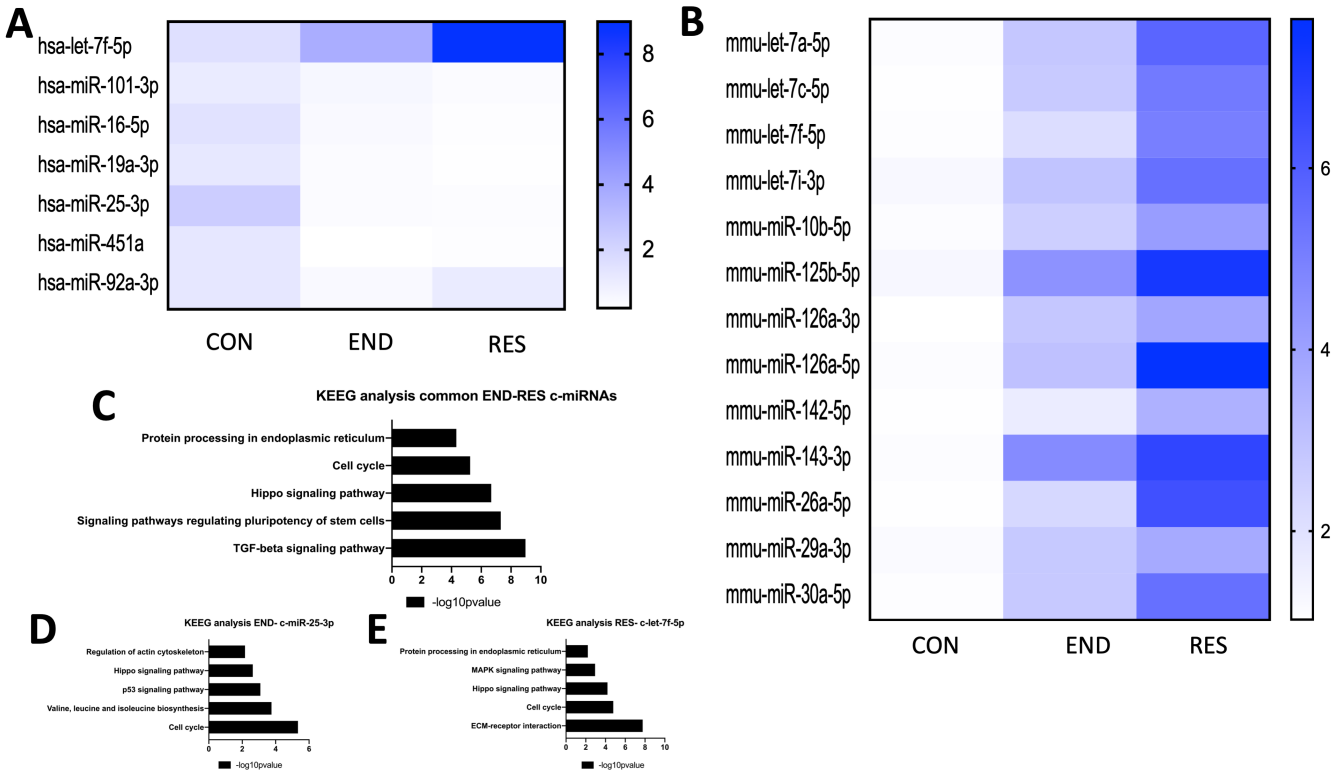
FIGURES

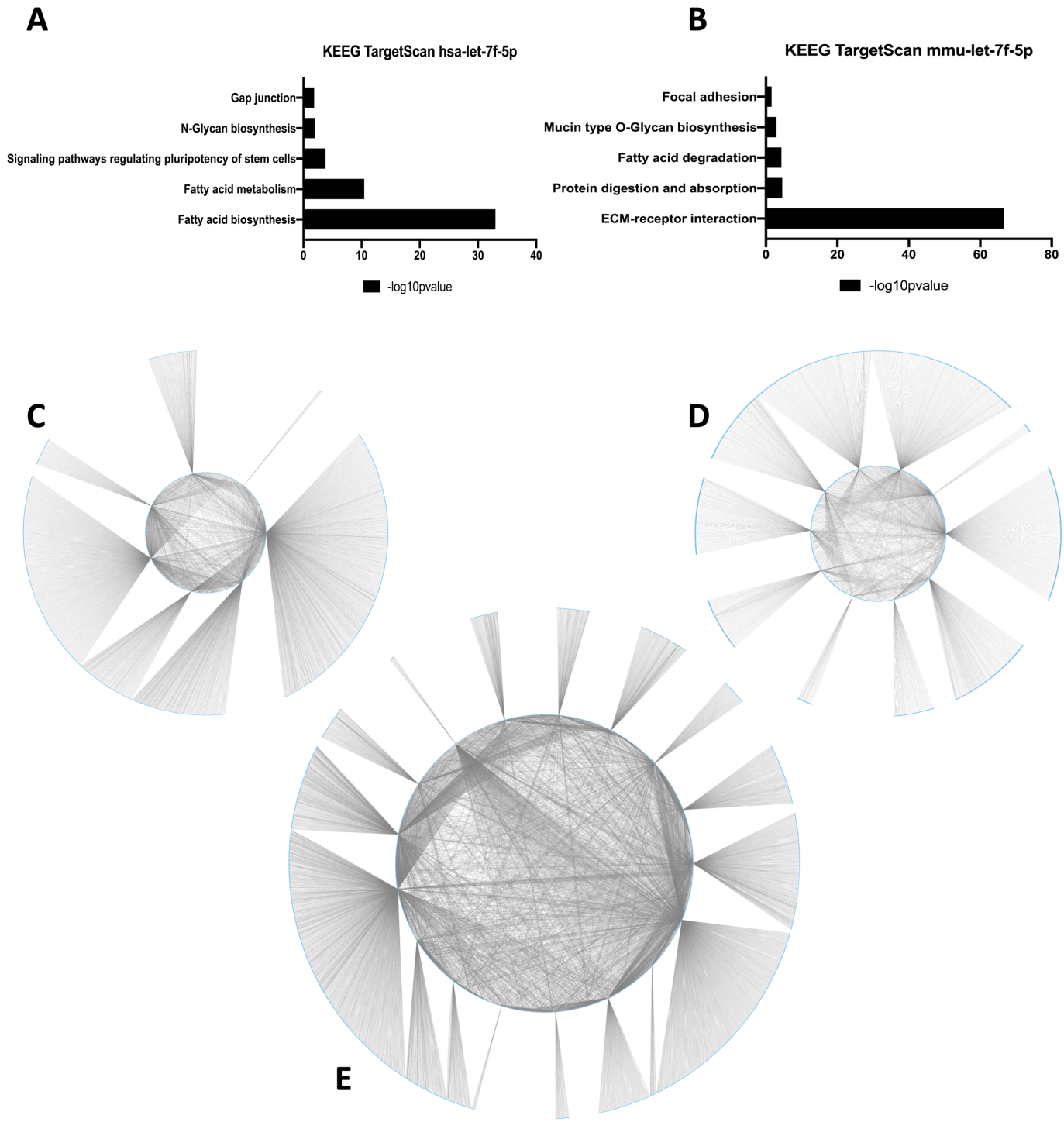
Figure 1. A, B. Exosomal responsive miRNAs in human(**A**) and mice (**B**) with a colour representation of significant difference between control and training groups, endurance and resistance (p-value<0.1). **C-E.** Human keeg analysis of different profiles with mirTarbase observed: **C**. Human miRNAs with the same response in endurance and resistance training. **D.** Human specific endurance training microRNA. **E.** Human specific resistance training microRNA.

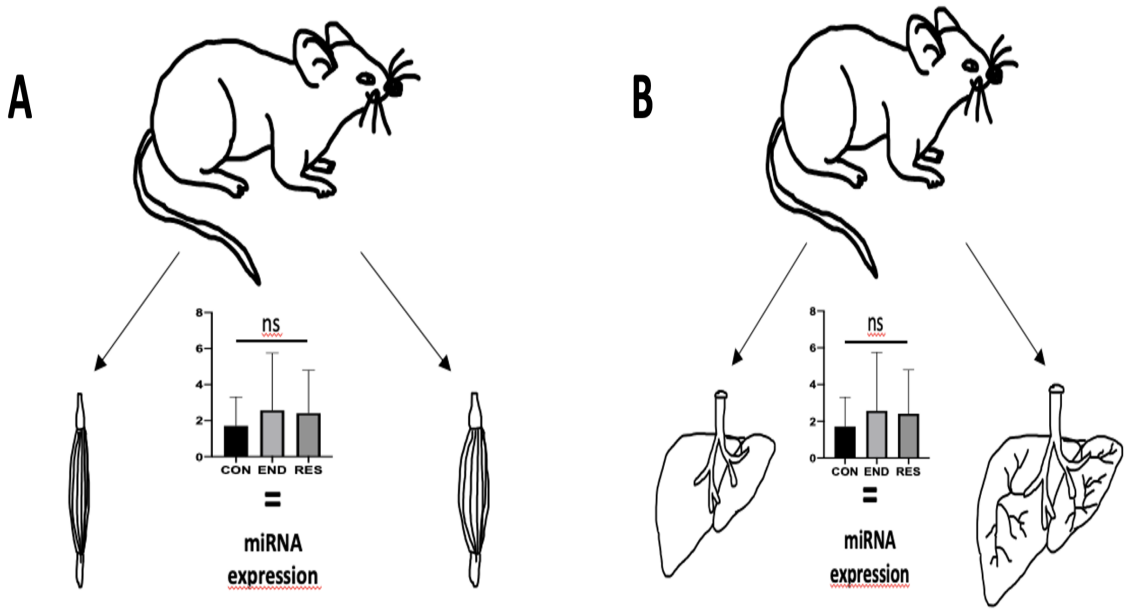
Figure 2. A. Target analysis of let-7f-5p in human with the prediction database TargetScan. **B.** Target analysis of let-7f-5p in mice with the prediction database TargetScan. **C.** Cytoscape representation with nodes and edges of mirWalk analysis of miRTarbase miRNA targets from the whole exercise exosome responsive miRNAs in human samples. **D.** Cytoscape representation with nodes and edges of mirWalk analysis of miRTarbase miRNA targets from the whole exercise exosome responsive miRNAs in mice samples. **E.** Cytoscape representation with nodes and edges of mirWalk analysis of miRTarbase miRNA targets from the whole exercise exosome responsive miRNAs in both species, an interrelation perspective.

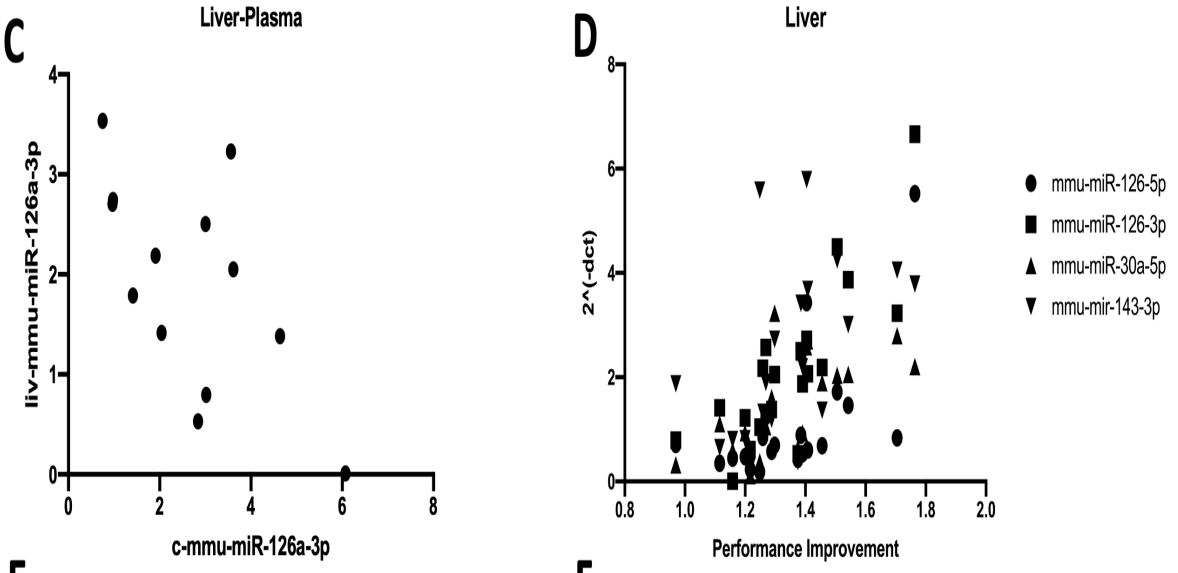
Figure 3. A-B. Picture representation of no significant changes in exosome responsive miRNAs in muscle(A) and liver(B) between trained mice and sedentary ones. **C.** Significant correlation between liver and exosome levels of mmu-miR-126a-3p in mice. **D.** Liver levels of exosome responsive miRNAs significantly correlated with animal performance improvements after training period both resistance and endurance. **E.** Liver levels of exosome responsive miRNAs significantly correlated with animal performance improvements after endurance training period. **F.** Liver levels of exosome responsive miRNAs significantly correlated with animal performance improvements after resistance training period.

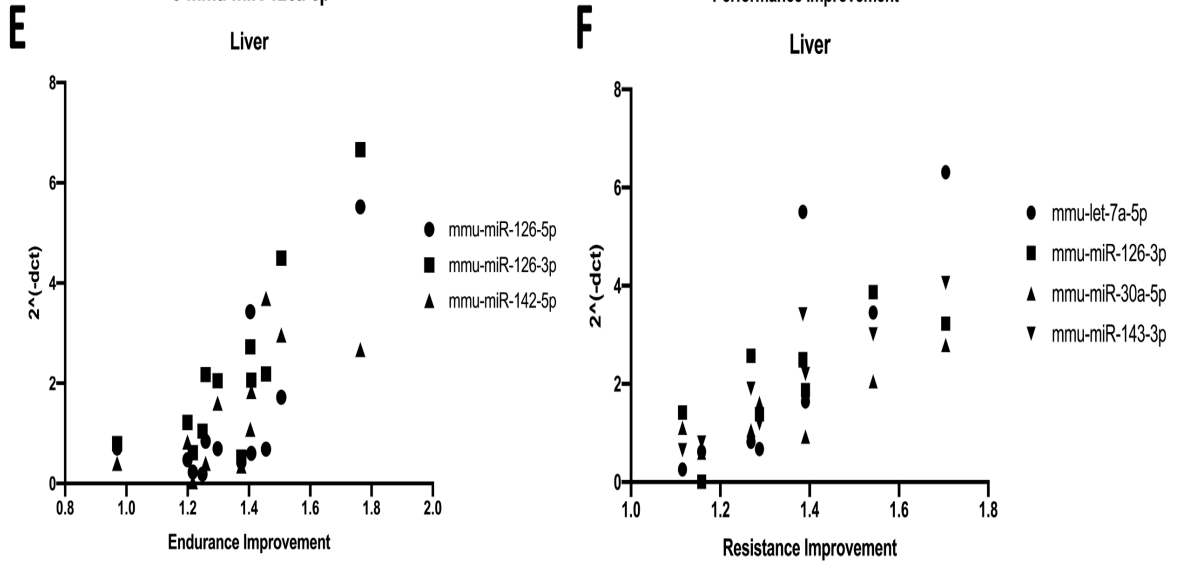
Figure 4. A. Exosome levels of mmu-miR-29a-3p in sedentary mice, endurance trained mice and resistance trained mice (*: p-value<0.05) **B.** Muscle levels of mmu-miR-29a-3p significantly correlated with animal performance improvements after training period both resistance and endurance. **C.** Endurance performance comparative representation between wild-type control mice (WT) and miR-29a/b1−/− knock-out mice (KO) (*: p-value<0.05). **D.** Resistance performance comparative representation between wild-type control mice (WT) and miR-29a/b1−/− knock-out mice (KO) (*: p-value<0.05).

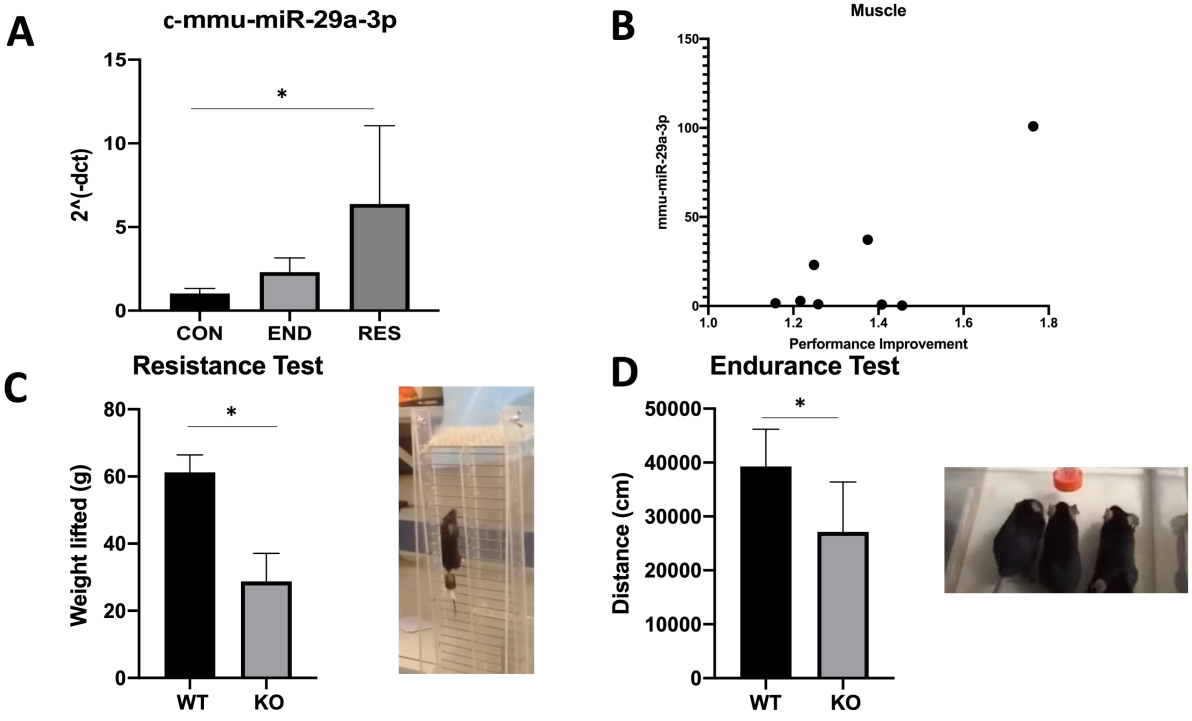

A
hsa-let-7f-5p
hsa-miR-101-3p
hsa-miR-16-5p
hsa-miR-19a-3p
hsa-miR-25-3p
hsa-miR-451a
hsa-miR-92a-3p
CON
END
RES
8
6
4
2
B
mmu-let-7a-5p
mmu-let-7c-5p
mmu-let-7f-5p
mmu-let-7i-3p
mmu-miR-10b-5p
mmu-miR-125b-5p
mmu-miR-126a-3p
mmu-miR-126a-5p
mmu-miR-142-5p
mmu-miR-143-3p
mmu-miR-26a-5p
mmu-miR-29a-3p
mmu-miR-30a-5p
CON
END
RES
6
4
2
C
KEEG analysis common END-RES c-miRNAs
Protein processing in endoplasmic reticulum
Cell cycle
Hippo signaling pathway
Signaling pathways regulating pluripotency of stem cells
TGF-beta signaling pathway
0 2 4 6 8 10
-log10pvalue
D
KEEG analysis END- c-miR-25-3p
Regulation of actin cytoskeleton
Hippo signaling pathway
p53 signaling pathway
Valine, leucine and isoleucine biosynthesis
Cell cycle
0 2 4 6
-log10pvalue
E
KEEG analysis RES- c-let-7f-5p
Protein processing in endoplasmic reticulum
MAPK signaling pathway
Hippo signaling pathway
Cell cycle
ECM-receptor interaction
0 2 4 6 8 10
-log10pvalue


A
KEEG TargetScan hsa-let-7f-5p
Gap junction
N-Glycan biosynthesis
Signaling pathways regulating pluripotency of stem cells
Fatty acid metabolism
Fatty acid biosynthesis
0
10
20
30
40
-log10pvalue
B
KEEG TargetScan mmu-let-7f-5p
Focal adhesion
Mucin type O-Glycan biosynthesis
Fatty acid degradation
Protein digestion and absorption
ECM-receptor interaction
0
20
40
60
80
-log10pvalue
C
D
E


A
ns
CON END RES
=
miRNA
expression
B
ns
CON END RES
=
miRNA
expression


C
Liver-Plasma
liv-mmu-miR-126a-3p
c-mmu-miR-126a-3p
D
Liver
2^(-dct)
Performance Improvement
mmu-miR-126-5p
mmu-miR-126-3p
mmu-miR-30a-5p
mmu-mir-143-3p


E
Liver
2^(-dct)
Endurance Improvement
mmu-miR-126-5p
mmu-miR-126-3p
mmu-miR-142-5p
F
Liver
2^(-dct)
Resistance Improvement
mmu-let-7a-5p
mmu-miR-126-3p
mmu-miR-30a-5p
mmu-miR-143-3p


A
c-mmu-miR-29a-3p
2^(-dct)
15
10
5
0
*
CON
END
RES
B
Muscle
mmu-miR-29a-3p
150
100
50
0
1.0
1.2
1.4
1.6
1.8
Performance Improvement
C
Resistance Test
Weight lifted (g)
80
60
40
20
0
*
WT
KO
D
Endurance Test
Distance (cm)
50000
40000
30000
20000
10000
0
*
WT
KO

Supplementary Table 1. Human RNAseq and qPCR validation data. Results 2^(-dct)

RNASeq	qPCR	HCON	HEND	HRES	Significance
hsa-let-7a-3p					
hsa-let-7a-5p	hsa-let-7a-5p	0,00097858	0,01257951	0,00510173	>0.1
hsa-let-7b-3p					
hsa-let-7b-5p	hsa-let-7b-5p	0,00282114	0,00044773	0,00038481	>0.1
hsa-let-7c-5p	hsa-let-7c-5p	0,00021997	0,00107559	0,00360515	>0.1
hsa-let-7d-3p					
hsa-let-7d-5p					
hsa-let-7e-5p					
hsa-let-7f-1-3p					
hsa-let-7f-5p	hsa-let-7f-5p	0,0003892	0,00091421	0,0022275	<0.1 (0.077)
hsa-let-7g-5p					
hsa-let-7i-3p					
hsa-let-7i-5p	hsa-let-7i-5p	0,00140092	0,00070679	0,00162834	>0.1
hsa-miR-1					
hsa-miR-100-5p					
hsa-miR-101-3p	hsa-miR-101-3p	0,00124234	0,00063347	0,00042469	<0.1 (0.064)
hsa-miR-101-5p					
hsa-miR-103a-3p					
hsa-miR-106b-3p					
hsa-miR-106b-5p					
hsa-miR-107					
hsa-miR-10a-3p					
hsa-miR-10a-5p					
hsa-miR-10b-3p					
hsa-miR-10b-5p	hsa-miR-10b-5p	0,01626139	0,0003708	#¡DIV/0!	>0.1
hsa-miR-1180-3p					
hsa-miR-122-5p					
hsa-miR-1224-5p					
hsa-miR-1226-3p					
hsa-miR-124-3p					
hsa-miR-1246					
hsa-miR-1255a					
hsa-miR-1255b-5p					
hsa-miR-125a-3p					
hsa-miR-125a-5p	hsa-miR-125a-5p	0,06167705	0,01034344	0,04037803	>0.1
hsa-miR-125b-5p	hsa-miR-125b-5p	0,00032825	0,00046249	#¡DIV/0!	>0.1
hsa-miR-126-3p	hsa-miR-126-3p	0,07143553	0,00377239	0,00241965	>0.1
hsa-miR-126-5p					
hsa-miR-1260b					

hsa-miR-1268a					
hsa-miR-1268b					
hsa-miR-127-3p					
hsa-miR-1270					
hsa-miR-1273h-3p					
hsa-miR-1277-3p					
hsa-miR-1277-5p					
hsa-miR-128-3p					
hsa-miR-1284					
hsa-miR-1291					
hsa-miR-1294					
hsa-miR-1296-5p					
hsa-miR-1299					
hsa-miR-1301-3p					
hsa-miR-1304-3p					
hsa-miR-1306-5p					
hsa-miR-1307-3p					
hsa-miR-1307-5p					
hsa-miR-130a-3p					
hsa-miR-130b-3p					
hsa-miR-130b-5p					
hsa-miR-132-3p					
hsa-miR-133a-3p					
hsa-miR-134-5p					
hsa-miR-135a-5p					
hsa-miR-136-3p					
hsa-miR-136-5p					
hsa-miR-139-3p					
hsa-miR-139-5p					
hsa-miR-140-3p					
hsa-miR-140-5p					
hsa-miR-141-3p					
hsa-miR-142-3p	hsa-miR-142-3p	0,00315489	0,00255245	0,00468978	>0.1
hsa-miR-142-5p					
hsa-miR-143-3p					
hsa-miR-143-5p					
hsa-miR-144-3p					
hsa-miR-144-5p					
hsa-miR-145-5p					
hsa-miR-1468-5p					
hsa-miR-146a-3p					
hsa-miR-146a-5p	hsa-miR-146a-5p	0,00119219	0,00084927	0,00181544	>0.1
hsa-miR-146b-3p					

hsa-miR-146b-5p					
hsa-miR-148a-3p					
hsa-miR-148b-3p					
hsa-miR-148b-5p					
hsa-miR-149-5p					
hsa-miR-150-3p					
hsa-miR-150-5p	hsa-miR-150-5p	0,00869422	0,00179029	0,00254674	>0.1
hsa-miR-151a-3p	hsa-miR-151a-3p	4,5579E-05	0,00019013	#¡DIV/0!	>0.1
hsa-miR-151a-5p					
hsa-miR-152-3p					
hsa-miR-154-5p					
hsa-miR-155-5p					
hsa-miR-15a-5p					
hsa-miR-15b-3p					
hsa-miR-15b-5p					
hsa-miR-16-2-3p	hsa-miR-16-2-3p	0,00198516	0,00035311	#¡DIV/0!	>0.1
hsa-miR-16-5p	hsa-miR-16-5p	0,22838487	0,07345021	0,04082892	<0.01
hsa-miR-17-3p					
hsa-miR-17-5p					
hsa-miR-181a-2-3p					
hsa-miR-181a-3p					
hsa-miR-181a-5p					
hsa-miR-181b-5p					
hsa-miR-181c-3p					
hsa-miR-181c-5p					
hsa-miR-182-5p					
hsa-miR-183-5p					
hsa-miR-184					
hsa-miR-185-3p					
hsa-miR-185-5p					
hsa-miR-186-5p					
hsa-miR-18a-5p					
hsa-miR-1908-5p					
hsa-miR-190a-5p					
hsa-miR-190b					
hsa-miR-191-3p					
hsa-miR-191-5p	hsa-miR-191-5p	0,00137533	0,00086079	0,0022254	>0.1
hsa-miR-192-5p					
hsa-miR-193a-5p					
hsa-miR-193b-5p					
hsa-miR-194-5p					
hsa-miR-195-5p					
hsa-miR-196a-5p					

hsa-miR-196b-5p					
hsa-miR-197-3p					
hsa-miR-197-5p					
hsa-miR-199a-3p	hsa-miR-199a-3p	0,00101183	0,00050359	0,00072762	>0.1
hsa-miR-199a-5p					
hsa-miR-199b-3p					
hsa-miR-199b-5p					
hsa-miR-19a-3p	hsa-miR-19a-3p	0,01296426	0,00381417	0,00234627	<0.01
hsa-miR-19b-3p					
hsa-miR-200a-3p					
hsa-miR-200b-3p					
hsa-miR-200c-3p					
hsa-miR-203a					
hsa-miR-204-5p					
hsa-miR-205-5p					
hsa-miR-206					
hsa-miR-20a-5p					
hsa-miR-20b-3p					
hsa-miR-20b-5p					
hsa-miR-21-3p					
hsa-miR-21-5p					
hsa-miR-210-3p					
hsa-miR-2110					
hsa-miR-215-5p					
hsa-miR-22-3p					
hsa-miR-22-5p					
hsa-miR-221-3p					
hsa-miR-221-5p					
hsa-miR-222-3p	hsa-miR-222-3p	0,00052333	0,00078708	#¡DIV/0!	>0.1
hsa-miR-222-5p					
hsa-miR-223-3p					
hsa-miR-223-5p					
hsa-miR-224-5p					
hsa-miR-2277-3p					
hsa-miR-2355-3p					
hsa-miR-23a-3p					
hsa-miR-23a-5p					
hsa-miR-23b-3p					
hsa-miR-23b-5p					
hsa-miR-24-3p					
hsa-miR-25-3p	hsa-miR-25-3p	0,06864934	0,01077911	0,00998553	<0.05
hsa-miR-25-5p					
hsa-miR-26a-1-3p					

hsa-miR-26a-2-3p					
hsa-miR-26a-5p					
hsa-miR-26b-3p					
hsa-miR-26b-5p					
hsa-miR-27a-3p					
hsa-miR-27a-5p	hsa-miR-27a-5p	0,22879773	0,03846838	0,19109257	>0.1
hsa-miR-27b-3p					
hsa-miR-27b-5p					
hsa-miR-28-3p					
hsa-miR-28-5p					
hsa-miR-296-5p					
hsa-miR-299-5p					
hsa-miR-29a-3p					
hsa-miR-29b-1-5p					
hsa-miR-29b-3p					
hsa-miR-29c-3p					
hsa-miR-29c-5p					
hsa-miR-301a-3p	hsa-miR-301a-3p	5,9416E-05	0,00025332	#¡DIV/0!	>0.1
hsa-miR-3074-5p					
hsa-miR-30a-3p					
hsa-miR-30a-5p					
hsa-miR-30b-5p					
hsa-miR-30c-1-3p					
hsa-miR-30c-5p					
hsa-miR-30d-3p					
hsa-miR-30d-5p	hsa-miR-30d-5p	14060781,1	0,00092492	0,00084244	>0.1
hsa-miR-30e-3p					
hsa-miR-30e-5p					
hsa-miR-31-5p					
hsa-miR-3124-5p					
hsa-miR-3131					
hsa-miR-3138					
hsa-miR-3143					
hsa-miR-3158-3p					
hsa-miR-3168					
hsa-miR-3173-5p					
hsa-miR-3195					
hsa-miR-32-3p					
hsa-miR-32-5p					
hsa-miR-320a					
hsa-miR-320b	hsa-miR-320b	0,00080938	6,232E-05	#¡DIV/0!	>0.1
hsa-miR-320c					
hsa-miR-320d					

hsa-miR-323a-3p					
hsa-miR-323b-3p					
hsa-miR-324-3p					
hsa-miR-324-5p					
hsa-miR-326					
hsa-miR-328-3p					
hsa-miR-329-3p					
hsa-miR-330-5p					
hsa-miR-331-3p					
hsa-miR-335-3p	hsa-miR-335-3p	0,00082986	0,01341555	#¡DIV/0!	>0.1
hsa-miR-335-5p					
hsa-miR-337-3p					
hsa-miR-337-5p					
hsa-miR-338-3p					
hsa-miR-339-3p					
hsa-miR-339-5p					
hsa-miR-33a-5p					
hsa-miR-340-3p					
hsa-miR-340-5p					
hsa-miR-342-3p	hsa-miR-342-3p	0,00073729	0,00058731	7,3522E-05	>0.1
hsa-miR-342-5p					
hsa-miR-345-5p					
hsa-miR-34a-5p					
hsa-miR-34c-5p					
hsa-miR-3605-3p					
hsa-miR-3605-5p					
hsa-miR-361-3p					
hsa-miR-361-5p					
hsa-miR-3613-3p					
hsa-miR-3613-5p					
hsa-miR-3614-5p					
hsa-miR-3615					
hsa-miR-363-3p					
hsa-miR-3653					
hsa-miR-3679-5p					
hsa-miR-369-3p					
hsa-miR-369-5p					
hsa-miR-370-3p					
hsa-miR-373-3p					
hsa-miR-374a-3p					
hsa-miR-374a-5p					
hsa-miR-374b-3p					
hsa-miR-374b-5p					

hsa-miR-375					
hsa-miR-376a-3p					
hsa-miR-376b-3p					
hsa-miR-376b-5p					
hsa-miR-376c-3p					
hsa-miR-377-3p					
hsa-miR-378a-3p	hsa-miR-378a-3p	0,01712169	0,00012684	0,00107996	>0.1
hsa-miR-379-5p					
hsa-miR-381-3p					
hsa-miR-382-3p					
hsa-miR-382-5p					
hsa-miR-3913-5p					
hsa-miR-409-3p					
hsa-miR-409-5p					
hsa-miR-411-5p					
hsa-miR-412-5p					
hsa-miR-421					
hsa-miR-423-3p					
hsa-miR-423-5p					
hsa-miR-424-5p					
hsa-miR-425-3p					
hsa-miR-425-5p					
hsa-miR-429					
hsa-miR-4306					
hsa-miR-431-5p					
hsa-miR-432-5p					
hsa-miR-4433-3p					
hsa-miR-4433b-3p					
hsa-miR-4433b-5p					
hsa-miR-4435					
hsa-miR-4446-3p					
hsa-miR-450a-5p					
hsa-miR-4511					
hsa-miR-451a	hsa-miR-451a	0,12567661	0,02106744	0,02554043	<0.01
hsa-miR-4532					
hsa-miR-454-3p					
hsa-miR-454-5p					
hsa-miR-4668-5p					
hsa-miR-4714-3p					
hsa-miR-4732-3p					
hsa-miR-4732-5p					
hsa-miR-4755-3p					
hsa-miR-4772-3p					

hsa-miR-4792					
hsa-miR-483-3p					
hsa-miR-483-5p					
hsa-miR-484					
hsa-miR-485-3p	hsa-miR-485-3p				
hsa-miR-485-5p					
hsa-miR-486-3p	hsa-miR-486-3p				
hsa-miR-486-5p	hsa-miR-486-5p	0,0154023	0,01123061	0,00682534	>0.1
hsa-miR-487a-5p					
hsa-miR-487b-3p					
hsa-miR-487b-5p					
hsa-miR-491-5p					
hsa-miR-493-3p					
hsa-miR-493-5p					
hsa-miR-494-3p					
hsa-miR-496					
hsa-miR-499a-5p					
hsa-miR-500a-3p					
hsa-miR-501-3p					
hsa-miR-5010-5p					
hsa-miR-502-3p					
hsa-miR-503-5p					
hsa-miR-504-3p					
hsa-miR-504-5p					
hsa-miR-505-3p					
hsa-miR-505-5p					
hsa-miR-508-3p					
hsa-miR-5090					
hsa-miR-5187-5p					
hsa-miR-518b					
hsa-miR-532-3p					
hsa-miR-532-5p					
hsa-miR-542-3p					
hsa-miR-542-5p					
hsa-miR-543					
hsa-miR-548a-3p					
hsa-miR-548am-5p					
hsa-miR-548ay-5p					
hsa-miR-548j-5p					
hsa-miR-548o-5p					
hsa-miR-551b-3p					
hsa-miR-552-5p					
hsa-miR-5586-5p					

hsa-miR-574-3p					
hsa-miR-574-5p					
hsa-miR-576-5p					
hsa-miR-582-3p					
hsa-miR-582-5p					
hsa-miR-584-5p	hsa-miR-584-5p				
hsa-miR-589-5p					
hsa-miR-590-3p					
hsa-miR-598-3p					
hsa-miR-625-3p					
hsa-miR-625-5p					
hsa-miR-628-3p					
hsa-miR-628-5p					
hsa-miR-629-5p					
hsa-miR-641					
hsa-miR-642a-5p					
hsa-miR-6511a-3p					
hsa-miR-6514-5p					
hsa-miR-652-3p					
hsa-miR-652-5p					
hsa-miR-654-3p					
hsa-miR-660-5p					
hsa-miR-664a-3p	hsa-miR-664a-3p	4,0487E-05	8,9544E-05	0,00068792	<0.1 (0.076)
hsa-miR-664a-5p					
hsa-miR-664b-5p					
hsa-miR-671-3p					
hsa-miR-671-5p					
hsa-miR-6721-5p					
hsa-miR-6740-5p					
hsa-miR-6741-3p					
hsa-miR-6741-5p					
hsa-miR-6759-5p					
hsa-miR-676-3p					
hsa-miR-6793-5p					
hsa-miR-6805-5p					
hsa-miR-6851-3p					
hsa-miR-6852-5p					
hsa-miR-6858-5p					
hsa-miR-6859-5p					
hsa-miR-6866-3p					
hsa-miR-6893-3p					
hsa-miR-7-5p					

hsa-miR-744-3p					
hsa-miR-744-5p					
hsa-miR-760					
hsa-miR-769-5p					
hsa-miR-7976					
hsa-miR-874-3p					
hsa-miR-874-5p					
hsa-miR-875-5p					
hsa-miR-889-3p					
hsa-miR-9-5p					
hsa-miR-92a-3p	hsa-miR-92a-3p	0,02290708	0,00856341	0,01964535	<0.1 (0.09)
hsa-miR-92b-3p					
hsa-miR-93-3p					
hsa-miR-93-5p					
hsa-miR-941					
hsa-miR-942-5p					
hsa-miR-95-3p					
hsa-miR-96-5p					
hsa-miR-98-5p					
hsa-miR-99a-5p					
hsa-miR-99b-3p					
hsa-miR-99b-5p	hsa-miR-99b-5p				

Supplementary Table 2. Mouse RNAseq and qPCR validation data. Results 2^(-dct)

RNAseq	qPCR	Control	Endurance	Resistance	Significance
mmu-let-7a-1-3p	mmu-let-7a-1-3p	1,06373584	1,380071	1,10455686	>0.1
mmu-let-7a-5p	mmu-let-7a-5p	1,12610207	2,85290746	5,77662921	<0.05
mmu-let-7b-5p					
mmu-let-7c-1-3p					
mmu-let-7c-2-3p					
mmu-let-7c-5p	mmu-let-7c-5p	1,04017152	2,75535502	5,18955685	<0.1 (0.053)
mmu-let-7d-3p					
mmu-let-7d-5p					
mmu-let-7e-3p					
mmu-let-7e-5p					
mmu-let-7f-1-3p					
mmu-let-7f-2-3p					
mmu-let-7f-5p	mmu-let-7f-5p	1,08937162	2,1222359	5,03538219	<0.1 (0.054)
mmu-let-7g-3p					
mmu-let-7g-5p					
mmu-let-7i-3p	mmu-let-7i-3p	1,24268027	2,96392752	5,47888353	<0.05
mmu-let-7i-5p					
mmu-let-7j					
mmu-miR-100-5p					
mmu-miR-101a-3p					
mmu-miR-101a-5p					
mmu-miR-101b-3p					
mmu-miR-101b-5p					
mmu-miR-103-3p					
mmu-miR-106a-5p					
mmu-miR-106b-3p					
mmu-miR-106b-5p	mmu-miR-106b-5p	1,11200811	1,16410571	1,51762225	>0.1
mmu-miR-107-3p					
mmu-miR-10a-3p					
mmu-miR-10a-5p	mmu-miR-10a-5p	1,08129988	0,56726194	0,61226225	>0.1
mmu-miR-10b-3p	mmu-miR-10b-3p	1,01621648	0,4307777	0,6017256	>0.1
mmu-miR-10b-5p	mmu-miR-10b-5p	1,10627093	2,58712615	4,15279019	<0.05
mmu-miR-1198-5p					
mmu-miR-122-5p	mmu-miR-122-5p	1,64757162	1,22360956	0,57973344	>0.1
mmu-miR-1247-3p					
mmu-miR-1249-3p					
mmu-miR-125a-3p					
mmu-miR-125a-5p					
mmu-miR-125b-1-3p					
mmu-miR-125b-2-3p					

mmu-miR-125b-5p	mmu-miR-125b-5p	1,27892061	4,4742661	7,20396112	<0.05
mmu-miR-126a-3p	mmu-miR-126a-3p	1,02652228	2,84616243	3,8629683	<0.05
mmu-miR-126a-5p	mmu-miR-126a-5p	1,11156077	3,03064824	7,63857822	<0.05
mmu-miR-127-3p					
mmu-miR-127-5p					
mmu-miR-128-3p	mmu-miR-128-3p	1,04755609	0,91531073	0,82768387	>0.1
mmu-miR-129-5p					
mmu-miR-1306-3p					
mmu-miR-1306-5p					
mmu-miR-130a-3p					
mmu-miR-130a-5p					
mmu-miR-130b-3p					
mmu-miR-130b-5p					
mmu-miR-132-3p					
mmu-miR-133a-3p					
mmu-miR-133b-3p					
mmu-miR-134-5p	mmu-miR-134-5p	1,06171636	1,3155736	0,85066713	>0.1
mmu-miR-135b-5p					
mmu-miR-136-3p					
mmu-miR-136-5p					
mmu-miR-138-2-3p					
mmu-miR-138-5p					
mmu-miR-139-3p					
mmu-miR-139-5p					
mmu-miR-140-3p					
mmu-miR-140-5p					
mmu-miR-141-3p					
mmu-miR-141-5p	mmu-miR-141-5p	1,09804896	2,04414951	2,31940916	>0.1
mmu-miR-142-3p	mmu-miR-142-3p	1	0,36251717	1,15591412	>0.1
mmu-miR-142-5p	mmu-miR-142-5p	1,05029241	1,65765535	3,52475122	<0.05
mmu-miR-143-3p	mmu-miR-143-3p	1,12021536	4,67536187	6,71397267	<0.05
mmu-miR-143-5p					
mmu-miR-144-3p					
mmu-miR-144-5p					
mmu-miR-145a-3p					
mmu-miR-145a-5p	mmu-miR-145a-5p	1,18486442	4,91994483	3,51622937	>0.1
mmu-miR-145b					
mmu-miR-146a-5p	mmu-miR-146a-5p	1,15508447	1,55514054	1,1900739	>0.1
mmu-miR-146b-5p					
mmu-miR-147-3p					
mmu-miR-148a-3p	mmu-miR-148a-3p	1,12854316	1,20207612	0,72476761	>0.1
mmu-miR-148a-5p					
mmu-miR-148b-3p					

mmu-miR-148b-5p					
mmu-miR-149-5p					
mmu-miR-150-3p					
mmu-miR-150-5p	mmu-miR-150-5p	1,01066088	1,43075112	1,79425105	>0.1
mmu-miR-151-3p					
mmu-miR-151-5p					
mmu-miR-152-3p					
mmu-miR-154-5p					
mmu-miR-155-5p					
mmu-miR-15a-3p					
mmu-miR-15a-5p					
mmu-miR-15b-3p					
mmu-miR-15b-5p	mmu-miR-15b-5p	1,11499912	1,25936355	1,91725044	>0.1
mmu-miR-16-1-3p					
mmu-miR-16-5p	mmu-miR-16-5p	1,15229729	1,85237919	4,13248247	>0.1
mmu-miR-17-3p					
mmu-miR-17-5p					
mmu-miR-181a-1-3p					
mmu-miR-181a-5p					
mmu-miR-181b-5p					
mmu-miR-181c-3p					
mmu-miR-181c-5p					
mmu-miR-181d-5p					
mmu-miR-182-5p					
mmu-miR-183-5p					
mmu-miR-1839-5p					
mmu-miR-184-3p	mmu-miR-184-3p	1,18542957	1,13593778	1,494691	>0.1
mmu-miR-1843a-3p					
mmu-miR-1843a-5p					
mmu-miR-1843b-5p					
mmu-miR-185-3p					
mmu-miR-185-5p					
mmu-miR-186-3p					
mmu-miR-186-5p					
mmu-miR-187-3p					
mmu-miR-187-5p					
mmu-miR-18a-3p					
mmu-miR-18a-5p					
mmu-miR-18b-5p					
mmu-miR-190a-5p					
mmu-miR-190b-5p					
mmu-miR-191-3p					
mmu-miR-191-5p	mmu-miR-191-5p	1,01921311	1,28479643	1,67160556	>0.1

mmu-miR-192-3p					
mmu-miR-192-5p					
mmu-miR-1927					
mmu-miR-1934-5p					
mmu-miR-1938					
mmu-miR-193a-5p					
mmu-miR-193b-5p					
mmu-miR-194-5p					
mmu-miR-1943-3p					
mmu-miR-1943-5p					
mmu-miR-195a-3p					
mmu-miR-195a-5p					
mmu-miR-1964-3p					
mmu-miR-1968-5p					
mmu-miR-196a-5p					
mmu-miR-196b-5p					
mmu-miR-1981-5p					
mmu-miR-199a-3p					
mmu-miR-199a-5p					
mmu-miR-199b-3p					
mmu-miR-199b-5p	mmu-miR-199b-5p	1,21770223	1,06460235	1,36110571	>0.1
mmu-miR-19a-3p					
mmu-miR-19b-3p					
mmu-miR-1a-3p	mmu-miR-1a-3p	2,82000699	0,79459539	0,52275605	>0.1
mmu-miR-200a-3p	mmu-miR-200a-3p	1,13758973	1,28859595	1,16283772	>0.1
mmu-miR-200a-5p	mmu-miR-200a-5p	1,0502432	1,3156955	0,86937243	>0.1
mmu-miR-200b-3p					
mmu-miR-200c-3p					
mmu-miR-203-3p	mmu-miR-203-3p	1,23696078	3,88964882	2,73698047	>0.1
mmu-miR-204-5p					
mmu-miR-205-3p					
mmu-miR-205-5p	mmu-miR-205-5p	1,36733663	5,76950186	4,69690199	>0.1
mmu-miR-206-3p					
mmu-miR-20a-5p					
mmu-miR-20b-5p					
mmu-miR-210-3p					
mmu-miR-211-3p					
mmu-miR-2137	mmu-miR-2137	1,45595914	1,08794305	3,29544443	>0.1
mmu-miR-214-3p					
mmu-miR-214-5p					
mmu-miR-215-3p					
mmu-miR-215-5p					
mmu-miR-216a-5p					

mmu-miR-217-5p					
mmu-miR-218-5p					
mmu-miR-219a-5p					
mmu-miR-21a-3p					
mmu-miR-21a-5p	mmu-miR-21a-5p	1,04887838	1,14250129	1,11183316	>0.1
mmu-miR-22-3p					
mmu-miR-22-5p					
mmu-miR-221-3p					
mmu-miR-221-5p					
mmu-miR-222-3p					
mmu-miR-222-5p					
mmu-miR-223-3p					
mmu-miR-223-5p					
mmu-miR-224-5p					
mmu-miR-23a-3p					
mmu-miR-23a-5p					
mmu-miR-23b-3p					
mmu-miR-24-3p					
mmu-miR-25-3p					
mmu-miR-25-5p					
mmu-miR-26a-2-3p	mmu-miR-26a-2-3p	1,12264591	3,03880168	0,33833737	>0.1
mmu-miR-26a-5p	mmu-miR-26a-5p	1,0312857	2,30004431	6,38561697	<0.1 (0.053)
mmu-miR-26b-3p					
mmu-miR-26b-5p					
mmu-miR-27a-3p					
mmu-miR-27a-5p					
mmu-miR-27b-3p					
mmu-miR-28a-3p					
mmu-miR-28a-5p					
mmu-miR-296-3p					
mmu-miR-296-5p					
mmu-miR-298-5p					
mmu-miR-299a-3p					
mmu-miR-299a-5p					
mmu-miR-29a-3p	mmu-miR-29a-3p	1,1624775	2,81175937	3,7537344	<0.05
mmu-miR-29a-5p					
mmu-miR-29b-1-5p					
mmu-miR-29b-2-5p					
mmu-miR-29b-3p					
mmu-miR-29c-3p					
mmu-miR-29c-5p					
mmu-miR-300-3p					
mmu-miR-301a-3p					

mmu-miR-301b-3p					
mmu-miR-3057-5p					
mmu-miR-3068-5p					
mmu-miR-3072-5p					
mmu-miR-3074-5p					
mmu-miR-30a-3p					
mmu-miR-30a-5p	mmu-miR-30a-5p	1,08803656	2,76862218	5,46553783	<0.05
mmu-miR-30b-3p					
mmu-miR-30b-5p					
mmu-miR-30c-2-3p					
mmu-miR-30c-5p					
mmu-miR-30d-3p					
mmu-miR-30d-5p					
mmu-miR-30e-3p					
mmu-miR-30e-5p					
mmu-miR-31-5p					
mmu-miR-3107-3p					
mmu-miR-3107-5p					
mmu-miR-32-5p					
mmu-miR-320-3p					
mmu-miR-322-3p	mmu-miR-322-3p	1,02806091	1,52108929	1,78586343	>0.1
mmu-miR-322-5p					
mmu-miR-324-3p					
mmu-miR-324-5p					
mmu-miR-326-3p					
mmu-miR-328-3p	mmu-miR-328-3p	1,06763205	1,59212322	1,52239326	>0.1
mmu-miR-329-5p					
mmu-miR-33-5p					
mmu-miR-330-3p					
mmu-miR-330-5p					
mmu-miR-335-3p					
mmu-miR-335-5p					
mmu-miR-337-5p					
mmu-miR-338-3p					
mmu-miR-339-3p					
mmu-miR-339-5p					
mmu-miR-340-5p					
mmu-miR-341-3p					
mmu-miR-341-5p					
mmu-miR-342-3p	mmu-miR-342-3p	1,03517212	1,63328293	1,81385269	>0.1
mmu-miR-345-3p					
mmu-miR-345-5p	mmu-miR-345-5p	1,81291578	1,06588232	1,77473543	>0.1
mmu-miR-3473b					

mmu-miR-3473d	mmu-miR-3473d	1,1209719	0,76474745	0,38885028	>0.1

mmu-miR-3473e
mmu-miR-34a-5p
mmu-miR-34b-3p
mmu-miR-34c-5p
mmu-miR-350-3p
mmu-miR-350-5p
mmu-miR-351-3p
mmu-miR-351-5p
mmu-miR-3535
mmu-miR-361-3p
mmu-miR-361-5p
mmu-miR-362-5p
mmu-miR-363-3p
mmu-miR-365-1-5p
mmu-miR-365-2-5p
mmu-miR-365-3p
mmu-miR-369-3p
mmu-miR-369-5p
mmu-miR-370-5p
mmu-miR-374b-5p
mmu-miR-375-3p
mmu-miR-376b-5p
mmu-miR-376c-3p
mmu-miR-378a-3p
mmu-miR-378a-5p
mmu-miR-378c
mmu-miR-378d
mmu-miR-379-5p
mmu-miR-381-3p
mmu-miR-382-3p
mmu-miR-382-5p
mmu-miR-409-3p
mmu-miR-411-5p
mmu-miR-421-3p
mmu-miR-423-3p
mmu-miR-423-5p
mmu-miR-425-3p
mmu-miR-425-5p
mmu-miR-429-3p
mmu-miR-431-5p
mmu-miR-434-3p
mmu-miR-434-5p

mmu-miR-450a-5p					
mmu-miR-450b-3p					
mmu-miR-450b-5p					
mmu-miR-451a	mmu-miR-451a	1,20896901	0,97063619	0,86391993	>0.1
mmu-miR-455-3p					
mmu-miR-455-5p					
mmu-miR-466a-3p					
mmu-miR-466b-3p					
mmu-miR-466c-3p					
mmu-miR-466d-3p	mmu-miR-466d-3p	1	2,72273313	1,54712445	>0.1
mmu-miR-466e-3p					
mmu-miR-467c-5p					
mmu-miR-467d-5p					
mmu-miR-467e-5p					
mmu-miR-484					
mmu-miR-485-3p					
mmu-miR-485-5p					
mmu-miR-486-3p	mmu-miR-486-3p	1,2509707	1,19095164	2,4798718	>0.1
mmu-miR-486-5p	mmu-miR-486-5p	1,18276277	2,08697663	5,08368817	>0.1
mmu-miR-491-5p					
mmu-miR-497-5p					
mmu-miR-500-3p					
mmu-miR-501-3p					
mmu-miR-503-3p					
mmu-miR-503-5p					
mmu-miR-505-5p					
mmu-miR-5106					
mmu-miR-5107-5p					
mmu-miR-5108					
mmu-miR-5113					
mmu-miR-5114					
mmu-miR-5119					
mmu-miR-5121					
mmu-miR-5123					
mmu-miR-5124a					
mmu-miR-5126					
mmu-miR-5128					
mmu-miR-5130					
mmu-miR-532-3p					
mmu-miR-532-5p					
mmu-miR-540-3p					
mmu-miR-541-5p					
mmu-miR-542-3p					

mmu-miR-542-5p					
mmu-miR-5627-3p					
mmu-miR-574-3p					
mmu-miR-574-5p					
mmu-miR-598-3p					
mmu-miR-615-3p					
mmu-miR-6236					
mmu-miR-652-3p					
mmu-miR-6538					
mmu-miR-664-3p					
mmu-miR-664-5p					
mmu-miR-669a-3p	mmu-miR-669c-3p	1,26509286	2,20353315	4,31313683	>0.1
mmu-miR-669c-5p					
mmu-miR-669f-3p					
mmu-miR-669o-3p					
mmu-miR-671-3p					
mmu-miR-671-5p					
mmu-miR-672-5p					
mmu-miR-674-3p					
mmu-miR-674-5p					
mmu-miR-676-3p					
mmu-miR-677-5p					
mmu-miR-690	mmu-miR-690	1,19652347	1,16109283	1,13578279	>0.1
mmu-miR-6978-5p					
mmu-miR-700-3p					
mmu-miR-700-5p					
mmu-miR-7033-5p					
mmu-miR-706					
mmu-miR-708-5p	mmu-miR-708-5p	1,30477522	2,93749682	2,75572893	>0.1
mmu-miR-709					
mmu-miR-744-5p					
mmu-miR-760-3p					
mmu-miR-7646-3p					
mmu-miR-7688-5p					
mmu-miR-7a-5p					
mmu-miR-7b-5p					
mmu-miR-802-5p					
mmu-miR-8109					
mmu-miR-8114	mmu-miR-8114	1,22668941	1,3323542	1,77388063	>0.1
mmu-miR-8116					
mmu-miR-8117					
mmu-miR-872-3p					
mmu-miR-872-5p					

mmu-miR-874-3p
mmu-miR-874-5p
mmu-miR-877-3p
mmu-miR-879-3p
mmu-miR-9-5p
mmu-miR-92a-1-5p
mmu-miR-92a-3p
mmu-miR-92b-5p
mmu-miR-93-3p
mmu-miR-93-5p
mmu-miR-98-5p
mmu-miR-99a-3p
mmu-miR-99a-5p
mmu-miR-99b-3p
mmu-miR-99b-5p

*Supplementary Table 3. **A**. KEEG analysis of validated targets (Tarbase v7) of hsa-miR-19a-3p, hsa-miR-16-5p and hsa-miR-451a. **B**. KEEG analysis of validated targets (Tarbase v7) of hsa-miR-25-3p. **C**. KEEG analysis of validated targets (Tarbase v7) of hsa-let-7f-5.p*

KEGG pathway	p-value	Genes	miRNAs
Proteoglycans in cancer	1,63E-18	88	3
Prion diseases	1,48E-11	13	2
Viral carcinogenesis	4,65E-10	82	3
TGF-beta signaling pathway	1,08E-09	40	3
Prostate cancer	2,25E-08	50	3
Signaling pathways regulating pluripotency of stem cells	4,91E-08	63	3
Fatty acid biosynthesis	8,57E-08	4	2
Hepatitis B	8,57E-08	63	3
Adherens junction	1,81E-07	38	3
Hippo signaling pathway	2,14E-07	56	3
Glioma	4,19E-07	33	3
Cell cycle	5,60E-06	57	3
Colorectal cancer	6,87E-06	33	3
Pancreatic cancer	6,87E-06	35	3
Chronic myeloid leukemia	7,75E-06	37	3
Oocyte meiosis	1,04E-05	48	2
RNA transport	2,25E-05	71	2
Endometrial cancer	2,25E-05	28	3
Bacterial invasion of epithelial cells	2,64E-05	35	3
Estrogen signaling pathway	3,11E-05	43	3
Protein processing in endoplasmic reticulum	4,65E-05	70	2
p53 signaling pathway	6,95E-05	36	2
FoxO signaling pathway	7,53E-05	58	3
Shigellosis	0,000124162	32	3
Glycosaminoglycan biosynthesis - keratan sulfate	0,000189301	6	2
Melanoma	0,00019353	33	3
Non-small cell lung cancer	0,000275343	27	3
Sphingolipid signaling pathway	0,00034239	51	3
Prolactin signaling pathway	0,00034239	34	3
Bladder cancer	0,000351011	22	3
Pathways in cancer	0,00041622	137	3
Central carbon metabolism in cancer	0,000588651	32	3
Small cell lung cancer	0,000725539	39	3
mTOR signaling pathway	0,0016319	29	3
Neurotrophin signaling pathway	0,0016319	51	3
Progesterone-mediated oocyte maturation	0,0016319	40	3

Renal cell carcinoma	0,0016319	32	3
Thyroid cancer	0,002118072	14	3
Ubiquitin mediated proteolysis	0,002416532	54	3
Other types of O-glycan biosynthesis	0,002900794	11	2
Insulin signaling pathway	0,003537148	56	3
Acute myeloid leukemia	0,004955279	27	3
AMPK signaling pathway	0,007676192	50	3
Dorso-ventral axis formation	0,00882073	15	2
Thyroid hormone signaling pathway	0,009658482	48	3
Apoptosis	0,009930241	35	3
Ribosome biogenesis in eukaryotes	0,012270945	36	2
Type II diabetes mellitus	0,015730768	21	3
Adrenergic signaling in cardiomyocytes	0,015770149	48	3
Citrate cycle (TCA cycle)	0,022554076	13	2
TNF signaling pathway	0,022554076	44	3
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	0,026763341	7	2
Fatty acid metabolism	0,028305822	14	2
ErbB signaling pathway	0,030370317	32	3
Hepatitis C	0,044397994	49	3
Focal adhesion	0,048375962	71	3

KEGG pathway	p-value	Genes	miRNAs
Prion diseases	1,08E-23	3	1
Lysine degradation	4,51E-07	10	1
Cell cycle	4,58E-06	30	1
Viral carcinogenesis	4,58E-06	30	1
Valine, leucine and isoleucine biosynthesis	0,000177058	2	1
Adherens junction	0,000177058	13	1
Chronic myeloid leukemia	0,000232027	17	1
p53 signaling pathway	0,000815916	15	1
Proteoglycans in cancer	0,00087798	24	1
FoxO signaling pathway	0,001106241	24	1
Endometrial cancer	0,001106241	11	1
Pathways in cancer	0,001498555	42	1
Colorectal cancer	0,001571138	11	1
Thyroid cancer	0,001696283	7	1
Hippo signaling pathway	0,002328827	20	1
Prostate cancer	0,002328827	17	1
Melanoma	0,002340632	12	1
Hepatitis B	0,003135443	23	1
2-Oxocarboxylic acid metabolism	0,004218726	4	1
Regulation of actin cytoskeleton	0,006786458	26	1

Bladder cancer	0,006786458	10	1
HTLV-I infection	0,011958331	34	1
MAPK signaling pathway	0,014001276	28	1
Protein processing in endoplasmic reticulum	0,014001276	22	1
Estrogen signaling pathway	0,014001276	12	1
Glioma	0,014001276	11	1
Non-small cell lung cancer	0,017503177	9	1
Oocyte meiosis	0,021253461	15	1
Dorso-ventral axis formation	0,025665577	7	1
Thyroid hormone signaling pathway	0,025665577	19	1
TGF-beta signaling pathway	0,033266756	9	1
Small cell lung cancer	0,039796579	13	1

KEGG pathway	p-value	Genes	miRNAs
ECM-receptor interaction	1,71E-08	13	1
Cell cycle	1,64E-05	32	1
Viral carcinogenesis	1,64E-05	39	1
Proteoglycans in cancer	1,64E-05	44	1
Lysine degradation	1,97E-05	11	1
Hippo signaling pathway	6,22E-05	33	1
Hepatitis B	6,22E-05	35	1
Thyroid hormone signaling pathway	0,000155076	28	1
Chronic myeloid leukemia	0,000267548	20	1
Glioma	0,00027404	18	1
Pathways in cancer	0,000700639	70	1
MAPK signaling pathway	0,00112707	47	1
Oocyte meiosis	0,00112707	27	1
Small cell lung cancer	0,001946856	23	1
Adherens junction	0,002164727	22	1
Thyroid cancer	0,002314312	9	1
Bladder cancer	0,0029723	14	1
Melanoma	0,004744591	16	1
Transcriptional misregulation in cancer	0,005514293	35	1
Protein processing in endoplasmic reticulum	0,006217953	34	1
p53 signaling pathway	0,006350706	19	1
FoxO signaling pathway	0,007117349	31	1
Huntington's disease	0,012219132	28	1
Bacterial invasion of epithelial cells	0,012219132	15	1
Epstein-Barr virus infection	0,020922994	42	1
Endocytosis	0,02114467	39	1
Colorectal cancer	0,028369251	14	1
PI3K-Akt signaling pathway	0,028818203	62	1

Thyroid hormone synthesis	0,028818203	10	1
Prostate cancer	0,032815647	21	1

Discusión

La base teórica de partida fue la dificultad para la determinación como biomarcadores de los c-miRNA en el ámbito del ejercicio. Esta primera conclusión se alcanza ante las diferentes metodologías utilizadas para el análisis de estos y la gran heterogeneidad de protocolos seguidos en los diferentes estudios del tema(25). El poco control sobre los sujetos de estudio partiendo de un background diferente, de edades distintas y de factores externos incontrolados como la nutrición hacen que la mayoría de los estudios sean incomparables. Además, la metodología de análisis y la normalización de datos modifican en gran medida los resultados, por lo que crean una segunda deslegitimación de dichas investigaciones a la hora de sacar conclusiones conjuntas. Respecto a la metodología de análisis son muy pocos los estudios que hayan realizado un screening completo de los c-miRNA en respuesta al ejercicio, lo que crea respuestas sesgadas hacia lo previamente descrito o limitado al foco de estudio.

Con este primer objetivo completado, el abordaje de los siguientes trató de paliar los déficits detectados en esta aproximación teórica de los estudios del tema. Por otro lado, se analizaron no solo los déficits sino las preguntas sin respuesta que hasta el momento quedan, ¿qué rol tienen los c-miRNA en los tejidos para las adaptaciones al ejercicio? Y, ¿cómo es la interrelación circulante-tejido?

La primera pregunta planteada para paliar los déficits detectados fue, ¿cómo los mismos sujetos responden ante diferentes estímulos de ejercicio? Al analizar los mismos sujetos ante tres diferentes dosis de ejercicio de carrera, 10 km (10K), media maratón (MM) y maratón (M) pudimos definir distintos perfiles de c-miRNA en respuesta aguda para cada una de ellas, es decir, realizando una comparativa entre la expresión previa

antes de la competición frente a la expresión justo después de terminar obtuvimos respuestas especificas en cada una de las dosis de ejercicio(64).

La metodología de medidas repetidas nos permitió obtener conclusiones eliminando uno de los factores de heterogeneidad, las diferencias interindividuales de bagaje de entrenamiento y de factores externos que puedan modificar la respuesta.

El control de la nutrición previa a cada una de las competiciones, realizando siempre exactamente el mismo protocolo alimentario en cada una de ellas, hace que nuestros resultados tengan una mayor relevancia y reproducibilidad.

El tercer punto de eliminación de heterogeneidad de cara a obtener resultados comparables fue el tratamiento de datos. La utilización del método de $2^{(-ddct)}$(65) aplicado a cada una de las dosis de ejercicio tomando el valor previo a la carrera como normalizador, hace que el *background* personal sea eliminado y los cambios se deban única y exclusivamente a la carrera.

El cuarto punto que hace de este estudio un avance en análisis de c-miRNA en ejercicio es el abordaje global de la respuesta plasmática. El análisis del panel completo de c-miRNA nos da una perspectiva total de cómo distintos volúmenes de ejercicio definen perfiles específicos de c-miRNAs produciendo diferentes respuestas. La conclusión es clara, el volumen de ejercicio en busca del máximo rendimiento, “dosis”, produce respuestas no solo únicas sino modulaciones distintas durante ejercicio, atribuyéndole un papel regulatorio a los c-miRNA durante el desarrollo de las pruebas.

No obstante, el control de todas estas variables nos hace tener resultados comparables en respuesta a la maratón con los obtenidos por Baggish *et al.* 2014 y Mooren *et al.* 2014 en los que se describió la sobreexpressión circulante del miR-1-3p

post-maratón al igual que en nuestro estudio, pero en los que se obtuvo una respuesta muy limitada al utilizar una selección de miRNA musculares en sus análisis(48, 49).

Los resultados de las distintas dosis no solo determinaron diferentes respuestas en función de la prueba realizada, sino que también sirvieron para determinar las variaciones a lo largo de una misma temporada del perfil basal de miRNA en respuesta al entrenamiento(64). Estas diferencias basales se asocian al nivel de entrenamiento realizado durante esa temporada por los deportistas y nos puede aportar mucha información en los siguientes análisis. Teniendo en cuenta el rendimiento de los corredores en cada una de las pruebas, pudimos observar un mayor acercamiento a sus mejores marcas en los 10K. Mediante el perfil de c-miRNA en respuesta basal podemos obtener un perfil de respuesta en la mayoría de ellos en los que la progresión de la temporada hace que bajen sus niveles en los 10K respecto a la MM pero retorna a niveles similares previo a la M. Este hecho es comparable con lo descrito por Nielsen *et al.* en 2014 donde se describe que la mayoría de c-miRNA en respuesta al entrenamiento producen una represión a diferencia de su respuesta aguda donde la mayoría ascienden a nivel circulante(55).

Profundizando y enfocando nuestra mirada hacia el rendimiento deportivo se realizó el análisis de la respuesta aguda de los c-miRNA a un esfuerzo máximo y su correlación con diferentes parámetros relacionados con el metabolismo y la capacidad de ejercicio. En este punto describimos por primera vez la caracterización de subperfiles de c-miRNA en el ámbito del ejercicio, es decir, la caracterización de ciertos c-miRNA relacionados con las diferentes adaptaciones fisiológicas que el ejercicio conlleva. De este modo, se establece el miR-106b-5p como predictor de la velocidad aeróbica máxima y de la generación de lactato en el deportista. Por otra parte, en relación con la carga cardiaca, se establece un subperfil con miR-21-5p, miR-183-5p y miR-29b-3p correlacionado con

la frecuencia cardiaca máxima de cada sujeto. Por último, se establece un tercer subperfil de carga muscular o de daño muscular con la correlación de la CK total con los valores post prueba de miR-425-3p, miR-629-5p, let-7c-5p y miR-340-5p.

A este respecto y, teniendo en cuenta en el estudio previo, se pueden obtener conclusiones más allá de las meras correlaciones de lo ya publicado, dado que estableciendo que los mismos sujetos son los que han realizado el estudio de dosis y el de la prueba de esfuerzo se puede determinar el mir-106b-5p no solo como predictor de la velocidad aeróbica máxima sino como posible identificador final de la velocidad de competición dado que a mayores niveles del miRNA antes de la competición menor velocidad del deportista ante un mismo volumen de carrera.

Una vez definidos los perfiles de respuesta aguda a las pruebas, cabe entrar en la valoración de los miRNA como biomarcadores de una respuesta aguda al ejercicio de resistencia ya estudiada, la carga cardiaca (66). Para este análisis se compararon no solo la respuesta aguda a las tres dosis, sino también la respuesta en recuperación, analizándose el perfil 24 horas después del esfuerzo. Todo ello fue comparado con los biomarcadores proteicos de daño cardiaco utilizados hasta el momento para el análisis de patologías cardiacas y por ende del ejercicio. La respuesta aguda al ejercicio de los marcadores proteicos de daño cardiaco es mayor a mayor volumen de ejercicio. La respuesta mas elevada se observa en la maratón, sin embargo, carecen de especificidad ya que no existe diferencia con la insuficiencia cardiaca. Ha de ser el proceso recuperativo de los niveles de dichos marcadores proteicos, 24-72 horas después, quien determine si ha sido un fallo cardiaco o una respuesta controlada al ejercicio. Sin embargo, los c-miRNA tienen una respuesta especifica a cada dosis de ejercicio y una respuesta específica en aquellos miRNA determinados previamente como marcadores cardiacos. Marcadores agudos de infarto como el miR-423-5p se mantienen estables en respuesta al

ejercicio con lo que los miRNA caracterizan la respuesta cardiaca con una mayor definición que los marcadores utilizados hasta el momento(67). Otros, como el miR-27a-3p y el miR-16-5p, describen respuestas contrarias a las observadas en patologías cardiacas, donde, en el primero de ellos, en infarto se produce una bajada y en el caso del maratón un aumento de los niveles y, en el segundo caso, se produce un descenso en la recuperación post-ejercicio, sin embargo, en infarto se observa una bajada aguda y una subida en recuperación(68).

Al respecto del subperfil cardiaco generado con los datos de la prueba de esfuerzo, obtenemos un cambio significativo de los miRNA: miR-21-5p y miR-29b-3p en respuesta aguda a la maratón observándose esto asociado a una elevada carga cardiaca en respuesta a esta dosis de ejercicio.

Retomando el tema de las adaptaciones al entrenamiento, se abordó el objetivo de analizar el perfil basal de c-miRNA realizándose medidas de los perfiles basales de sujetos entrenados de manera crónica en ejercicio de fuerza y resistencia comparados con sujetos sedentarios de la misma edad. Se obtuvieron perfiles distintos en los que destaca la represión de cuatro miRNA que determinan un perfil especifico de personas entrenadas frente a personas sedentarias. De este modo se pueden establecer cuatro biomarcadores de ejercicio físico (mir-16-5p, miR-451a, miR-19a-3p y miR-25-3p) sin especificidad de modalidad de deporte ni tipo de entrenamiento. Con la utilización de estos biomarcadores se podría establecer un gradiente poblacional para la prevención de patologías asociadas con el estilo de vida. Abriendo este hecho la posibilidad de una valoración biológica objetiva de el nivel poblacional de sedentarismo.

Por otra parte, al realizar una comparativa cualitativa de los microRNAs que nos varían entre entrenados y sedentarios podemos comprobar como el perfil observado durante la temporada de los corredores amateurs concuerda con lo dicho anteriormente.

La represión del miR-25-3p como consecuencia al entrenamiento ya fue descrita por Nielsen *et al.* en 2014 y ratificado en nuestro estudio sobre dosis de ejercicio en 2020(55, 64). Los demás c-miRNA identificados en este perfil basal, que tienen una represión comparando entrenados y sedentarios, también describen la misma respuesta en el estudio de dosis de ejercicio, un descenso previo a la prueba de 10K, en la que obtienen sus mejores marcas, y un retorno a niveles más altos para la maratón donde el rendimiento fue peor de lo esperado, en algunos casos los miRNA vuelven a nivel inicial en otros a un nivel intermedio entre inicial y 10K.

Una vez realizado el análisis completo a nivel circulante en humano, observando la respuesta aguda y la crónica en diferentes situaciones y sujetos, con el fin de profundizar en el estudio de los miRNA en tejidos se realizó una comparativa interespecie utilizando como animal modelo los ratones. Además de esta profundización hacia tejido, la utilización del modelo murino nos permitió la obtención de datos y conclusiones a partir de animales modificados genéticamente. En este estudio se describen perfiles específicos de miRNA de exosomas mediante RNAseq en personas entrenadas y de ratones entrenados comparados con personas y ratones sedentarios, respectivamente. Alcanzado este punto, se realizó un análisis de dianas génicas de dichos perfiles de cara a esclarecer las funciones regulatorias de los mismos y poder determinar el modelo murino como un buen modelo en las adaptaciones al ejercicio. La conclusión a nivel funcional parece clara al obtenerse un 52% de dianas compartidas entre el perfil humano y el murino, generando un solo núcleo de interacción mediante el análisis de miRWalk.

Al quedar determinada la idoneidad del modelo en este ámbito, el primer punto de la respuesta a nivel murino fue la comprobación de la correlación entre aquellos miRNA que cambiaban significativamente en respuesta al ejercicio en exosomas y su presencia en tejidos. Este hecho permite comprobar que existe una comunicación extracelular-

intracelular mediante miRNA en la respuesta adaptativa al ejercicio. La correlación existe entre miRNA en tejido y circulante tanto en musculo con let-7c-5p como en hígado con el let-7i-5p, miR-10b-5p, miR-126a-3p, miR-142-5p y miR-29a-3p. Al igual de lo observado por D'souza et al. observamos una correlacion entre la presencia en músculo y en exosomas de algunos miRNA, específicamente el miR-126-3p que observan su correlación entre exosomas y plasma total, nosotros obtenemos una correlación entre exosomas e hígado(57). Sin embargo, el punto que quedaría por dilucidar es si el hígado o el músculo son los receptores o los emisores de estos miRNA. La única respuesta que podemos dar con nuestros datos es que existen dos tipos de correlaciones, negativas y positivas. Ambas respuestas determinan una interacción y una regulación, sin embargo, dos explicaciones podrían ser plausibles de ser ciertas bien se podría identificar la negativa como emisor y la positiva como receptor, o viceversa. De este modo, la comprobación in vitro se hace necesaria como futuro a seguir en el esclarecimiento de esta relación.

Al respecto de las adaptaciones al ejercicio a nivel de tejido, el papel de los miRNA en los tejidos parece claro en la capacidad adaptativa del animal al entrenamiento. La mejora del rendimiento en fuerza y resistencia se correlaciona con cuatro miRNA en hígado (miR-126a-3p, miR-126a-5p, miR-143-3p y miR-30a-5p) y en el caso del músculo son 3 (let-7i-5p, miR-29a-3p y miR-29c-3p).

Hasta el momento todos los estudios que comparaban la imagen representada por los c-miRNA en comparación con tejido concluían que no eran comparables o que no cambiaban del mismo modo(57, 69, 70), esto nos hace volver a nuestro punto de partida teórico en el que una de las limitaciones de los estudios era su sesgo hacia miRNA previamente descritos. Cuando el abordaje fue realizado desde un punto de vista global de una secuenciación de miRNA de exosomas, una validación mediante qPCR, una

determinación de los que cambiaban significativamente y un análisis posterior de los mismos en los tejidos obtenemos correlaciones con el rendimiento y con la presencia en exosomas, let-7i-5p, miR-126a-3p y miR-29a-3p.

Realizando un análisis basado en nuestros datos, el miR-126a-3p obtenemos un cambio en respuesta al entrenamiento de fuerza y resistencia, una correlación negativa de su presencia en exosomas con sus niveles en hígado y sus niveles en hígado una correlación positiva con la mejora en resistencia y fuerza en respuesta al entrenamiento.

Al realizar un análisis práctico, mediante la utilización de un ratón knockout miR-29a/b1−/− previamente descrito(71), pudimos obtener la conclusión del papel determinante del miR-29 en el rendimiento físico tanto en fuerza como en resistencia. Analizando los resultados en global de nuestro estudio obtenemos que el miR-29a-3p varía a consecuencia del entrenamiento de fuerza a nivel circulante, se correlaciona a nivel de músculo con la mejora en respuesta al entrenamiento tanto en fuerza como en resistencia, pero no cambia su expresión en respuesta al ejercicio, y se correlacionan sus niveles en hígado con el nivel circulante. Esto último nos hace pensar en el papel regulador del miR-29a a nivel hepático en la adaptación al ejercicio, los estudios realizados a este respecto correlacionan su sobreexpresion con una menor probabilidad de desarrollo de enfermedad hepática en respuesta a una dieta alta en grasas(72), pero también una menor biogénesis mitocondrial a nivel hepático lo que concuerda con la hiperplasia mitocondrial descrita por Caravia *et al.* en ratones knockout a nivel cardiaco(71). Por otra parte, se ha descrito una represión circulante de miR-29a-3p en respuesta a enfermedad de hígado graso no alcohólico(73), lo que nos permitiría destacar el papel del ejercicio de fuerza en la prevención de patologías.

Por último, realizando una retrospectiva de los resultados obtenidos en este miRNA en el global de estudios no solo queda circunscrito a este último de análisis ratón-humano

sino que ya en la prueba de esfuerzo en humanos se produce un sobreexpresión del miR-29b-3p en respuesta aguda, en respuesta al análisis de los miRNA cardiaco es el miR-29a-3p quien sube en respuesta aguda a la maratón y el miR-29b-3p el que 24 horas después de la misma se observa una represión. Con lo que nos hace pensar que dicho locus 29a/b1 tiene una clara influencia en las respuestas adaptativas al ejercicio y su consecuente rendimiento.

Conclusiones

La realidad molecular subyacente al ejercicio físico analizada en esta tesis nos hace concluir la importancia regulatoria tanto intracelular como intertisular de los miRNA. A nivel circulatorio permitiendo su utilización como biomarcadores de ejercicio a distintos niveles y en el ámbito celular como determinantes tanto del rendimiento como de la salud.

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