

Physiological determinants of urine and plasma myomiRNAs in recreational, middle-age athletes

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Background: The clinical use of micro RNAs (miRNAs) in the diagnostics of muscle injury should take into account the possible influence of many variables such as genetic factors, age, sex, ethnic origin, body and tissue composition as well as physical fitness. Therefore, the present study was designed to identify potential physiological determinants of miRNA 133-a and miRNA 206 in a population of recreational, middle-aged athletes.

Methods: The study group consisted of 28 healthy, middle-aged recreational athletes (11 women and 17 men) regularly engaged in endurance running. Resting urine and blood samples were collected for assessing plasma and urine miRNA 133-a and miRNA 206, and for serum measurement of creatine kinase (CK) and cardiac troponin T (cTnT).

Results: In univariate analysis, no physiological or laboratory parameter was associated with the plasma concentration of both miRNA 133-a and miRNA 206. The urine concentration of miRNA 133-a was associated with sex and body mass index (BMI), whereas the urine concentration of miRNA 206 was associated with sex and serum cTnT. In multivariate logistic regression analysis, the urine concentration of both miRNAs remained associated only with sex. The urine miRNA133-a and miRNA 206 values were found to be 3.0 and 1.9 folds higher in female than in male athletes, respectively. The percent overlap between the female and male populations of athletes was 27% for urine miRNA 133-a and 41% for urine miRNA 206.

Conclusions: The results of this study demonstrate that the plasma concentration of miRNA 133-a and 206 is virtually independent from the most common physiological variables, whereas the urine reference values of these two myomiRNAs should be partitioned by sex.

Keywords: Running; exercise; sport; microRNA; miRNA; epigenetics

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Introduction

Micro RNAs (miRNAs), conventionally abbreviated as miRs or miRNAs, are short endogenous RNA molecules involved in a kaleidoscope of biological pathways (1). There is now consolidated evidence that miRNAs play a substantial role in many regulatory mechanisms, including cell development, differentiation, proliferation and apoptosis (2). A definite role of these short RNA molecules has hence been elucidated in relationship with several human pathologies, namely cancer and cardiovascular disease (2).

Growing interest is focusing on the potential diagnostic and prognostic applications of some highly muscle-specific miRNAs, typically called myomiRNAs (3), in the setting of skeletal muscle (4) and cardiac (5-7) injury. The former condition entails a variety of congenital disorders (e.g., muscular or myotonic dystrophies), along with acute muscle injuries due to direct muscular trauma, infections, toxic damage and exertional rhabdomyolysis. The potential clinical applications in the diagnostics of cardiomyocyte injuries principally include the diagnosis of myocardial ischemia and infarction, as well as the prediction or follow-up of cardiac hypertrophy and heart failure. The subset of muscle-specific miRNAs comprises a discrete number of molecules such as miRNA 1, miRNA 29-b, miRNA 133, miRNA 181-a, miRNA 206, miRNA 208 and miRNA 451. Among these, miRNA 133-a and miRNA 206 were found to be the most promising for understanding the biological response to physical activity and for the potential use for diagnosing muscle injury and in anti-doping testing (4,8).

Personalized or precision medicine is currently defined as an approach for disease treatment and prevention based on individual variability in genes, environment and lifestyle (9). The clinical use of whatever biomarkers should hence take into account the putative influence of many variables belonging to these domains, which most frequently include genetic factors, age, sex, ethnic origin, body composition, dietary habits, physical fitness and comorbidities (10). Therefore, the present study was aimed to identify the potential physiological determinants of miRNA 133-a and miRNA 206 in a population of recreational, middle-aged athletes.

Methods

Study population

The study group consisted of 28 healthy, middle-aged recreational athletes [11 females and 17 males; mean age: 46 years, age range 30–63 years; mean body mass index

(BMI): 23.2 kg/m², range: 18.5–27.9 kg/m²], belonging to a local team of amateur runners. Maximal oxygen consumption (VO_{2max}) was measured by a running test on a treadmill through breath ergospirometric system (Quark B2, Cosmed, Rome, Italy). After appropriate familiarization, the athletes underwent a progressive incremental test, starting from habitual running pace and increasing the speed of 0.5 km/h every min until reaching volitional exhaustion.

All athletes were asked to stop training and to abstain from taking medications 48 hours before blood collection. The blood and urine for laboratory testing were collected early in the morning (i.e., between 8:30 and 9:30 AM) from subjects who had fasted for not less than 8 hours. Blood was drawn into evacuated blood collection tubes containing either K₂ ethylenediaminetetraacetic acid (EDTA) or no additives (Terumo Europe N.V., Leuven, Belgium). A morning urine sample was also collected immediately after blood drawing. The blood samples were centrifuged at 1,300 ×g for 10 min at room temperature; serum and plasma EDTA were then separated and stored in aliquots at –70 °C until measurement. The urine samples were also centrifuged at 1,300 ×g for 10 min at room temperature and the supernatant was then divided in aliquots and stored at –70 °C until measurement.

Laboratory measurements

Total creatine kinase (CK) and cardiac troponin T (cTnT) were both measured in serum using a Roche Cobas 6,000 integrated analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The concentration of cTnT was measured with a high-sensitivity (HS) immunoassay, displaying a limit of blank of 3.0 ng/L, a limit of detection of 5.0 ng/L and a 99th percentile upper reference limit (URL) of 14.0 ng/L (11). Total serum CK was also measured on Cobas 6,000, using the N-acetylcysteine-activated reference IFCC method.

The isolation of miRNA 133-a and miRNA 206 from plasma EDTA and urine samples was performed with the miRNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A standard volume of each plasma sample (200 µL) was supplemented with 5 mol of *Caenorhabditis elegans* miR-39 (Qiagen, Valencia, CA, USA) to normalize the results. miRNAs were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Australia) and the complementary DNA (cDNA) was used as template for the Real Time PCR. The reactions were performed on

a 7,500 Real-Time PCR System (Applied Biosystems, CA, USA) using TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA). Briefly, qRT-PCR was carried out in a total volume of 20 μ L containing 1.33 μ L cDNA, 1 \times Universal PCR Master Mix and 1 μ L gene-specific primers and probes. PCR parameters were as follows: 95 $^{\circ}$ C for 10 min, followed by 40 cycles of 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 1 min. The relative expression levels of miRNA 133-a and miRNA 206 were calculated by using the $2^{-\Delta\Delta C_t}$ method (12).

Statistical analysis

Physiological and laboratory data were reported as median and interquartile range (IQR). The potential associations among the various parameters were evaluated using both univariate (Spearman's correlation) and multivariate regression analyses. In multivariate regression analysis, the serum or urine concentration of myomiRNAs was entered as the dependent variable, whereas significant parameters in univariate analysis were entered as independent variables. Differences between groups were then tested with Mann Whitney U test. The statistical analysis was performed with Analyse-it (Analyse-it Software Ltd., Leeds, UK).

The study was part of an event called "Run For Science", held in Verona (Italy) (the full information of the event can be retrieved from the Verona University Website, at: <http://www.dsnm.univr.it/?ent=iniziativa&id=5382>, Last accessed, 8 March 2017). The study was cleared by the local Institutional Review Board (Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona), and was performed in accordance with the Declaration of Helsinki.

Results

The results of this study are shown in *Tables 1* and *2*. In univariate analysis, no single physiological or laboratory parameter was correlated with plasma miRNA 133-a concentration, whereas the urine concentration of miRNA 133-a was associated with both sex and BMI. Likewise, no single physiological or laboratory parameter was correlated with plasma miRNA 206 concentration, whereas the urine concentration of miRNA 206 was associated with both sex and serum cTnT. In multivariate logistic regression analysis, a modest association remained between urine miRNA 133-a and sex (beta coefficient, -9.9×10^{-6} ; $P=0.046$) but not with BMI (beta coefficient, -1.8×10^{-6} ; $P=0.148$). Similarly, urine miRNA 206 remained correlated with sex (beta coefficient,

2.4×10^{-5} ; $P=0.037$) but not with serum cTnT (beta coefficient, -1.4×10^{-6} ; $P=0.510$).

The value distribution, differentiated between females and males of serum CK, serum cTnT, plasma and urine miRNA133-a and plasma and urine miRNA 206, is shown in *Figure 1*. In agreement with results of regression analyses, the urine values of miRNA133-a and miRNA 206 were found to be 3.0 and 1.9 folds higher in female than in male athletes. The Cohen's d and the effect-size were 1.60 and 0.624 for miRNA 133-a, and 1.09 and 0.478 for miRNA 206, respectively. This implies that the percent overlap between the female and male populations of athletes was 27% for urine miRNA 133-a and 41% for urine miRNA 206, respectively. Notably, the Cohen's d of the value distribution between female and male athletes was 0.035 for plasma miRNA 133-a and 0.356 for plasma miRNA 206, so highlighting a very large percent overlap of values between sexes (i.e., ~97% for miRNA 133-a and ~76% for miRNA 206, respectively).

A significant difference was also observed for CK and cTnT, with the serum values of both biomarkers being nearly 40% higher in male than in female athletes. In no case did the concentration of serum cTnT exceeded the diagnostic cut-off of cardiac injury (i.e., the 99th percentile URL; 14.0 ng/L). The Cohen's d and the effect-size were 0.87 and 0.399 for serum CK, and 1.22 and 0.521 for serum cTnT, respectively. This implies that the percent overlap between the female and male populations of athletes was 48% for CK and 38% for cTnT, respectively.

Discussion

Several lines of evidence now attest that miRNAs are active players in health and disease (13-15). Taken together, our results suggest that the baseline plasma and urine concentration of the two main myomiRNA measured in this study is virtually independent from age, BMI, physical fitness, (i.e., VO_{2max}), as well as from skeletal (i.e., serum CK) and cardiac (i.e., serum cTnT) muscle health. Although the plasma values of both miRNA 133-a and miRNA 206 were also found to be independent from sex, the urine concentration of these two myomiRNA was considerably higher in female than in male athletes. This is a rather unexpected finding, since the serum values of the two more conventional biomarkers of skeletal muscle (i.e., CK) and myocardiocyte (i.e., cTnT) injury were instead increased in male compared to female athletes (*Figure 1*).

The evidence that the biological pathways underlying the

Table 1 Significant determinants of miRNA 133-a values in plasma and urine (univariate analysis)

Variable	miRNA 133-a	
	Plasma	Urine
Age (years)	R= -0.23; P=0.230	R= -0.14; P=0.483
Sex	R= -0.03; P=0.873	R= -0.59; P<0.001
BMI (kg/m ²)	R= -0.26; P=0.173	R= -0.039; P=0.039
VO _{2max} (mL/min/kg)	R= 0.09; P=0.722	R= 0.06; P=0.815
Plasma miRNA 133-a	-	R= 0.05; P=0.784
Urine miRNA 133-a	R= 0.05; P=0.784	-
Plasma miRNA206	R= 0.05; P=0.789	R= 0.21; P=0.277
Urine miRNA 206	R= 0.12; P=0.545	R= 0.05; P=0.784
Serum CK (U/L)	R= 0.21; P=0.279	R= -0.12; P=0.536
Serum cTnT (ng/L)	R= -0.05; P=0.795	R= -0.17; P=0.374

BMI, body mass index; VO_{2max}, maximal oxygen consumption; CK, creatine kinase; cTnT, cardiac troponin T.

Table 2 Significant determinants of miRNA 206 values in plasma and urine (univariate analysis)

Variable	miRNA 206	
	Plasma	Urine
Age (years)	R= -0.23; P=0.244	R= -0.08; P=0.694
Sex	R= -0.23; P=0.237	R= 0.51; P=0.005
BMI (kg/m ²)	R= -0.15; P=0.446	R= -0.24; P=0.217
VO _{2max} (mL/min/kg)	R= 0.02; P=0.940	R= 0.11; P=0.673
Plasma miRNA 133-a	R= 0.05; P=0.789	R= 0.12; P=0.545
Urine miRNA 133-a	R= 0.21; P=0.277	-
Plasma miRNA206	-	R= 0.30; P=0.124
Urine miRNA 206	R= 0.30; P=0.124	R= 0.05; P=0.784
Serum CK (U/L)	R= 0.09; P=0.656	R= 0.16; P=0.408
Serum cTnT (ng/L)	R= -0.28; P=0.148	R= -0.38; P=0.046

BMI, body mass index; VO_{2max}, maximal oxygen consumption; CK, creatine kinase; cTnT, cardiac troponin T.

metabolism of myomiRNA and muscle injury biomarkers in resting subjects is quite divergent, and is also totally independent from the overall BMI, paves the way to some important reflections. First, the lack of correlation between plasma and urine concentration of both miRNA 133-a and 206 implies that the measurement of these myomiRNAs in different body fluids is not interchangeable. No previous studies have assessed miRNA 133-a or miRNA 206 in urine samples of middle aged healthy subjects to the best of our knowledge, so this finding is highly innovative and

underscores that increased renal clearance, and/or reduced reabsorption, may play a significant role in myomiRNAs metabolism. Then, the strong and independent association that we have observed between sex and urine concentration of both miRNA 133-a and miRNA 206 also implies that the urine reference ranges of these biomarkers should be partitioned for the sex. This is especially true for miRNA133-a, since not only was its urine concentration was 3-fold higher in women than in men, but also the percent overlap between the values of male and female athletes

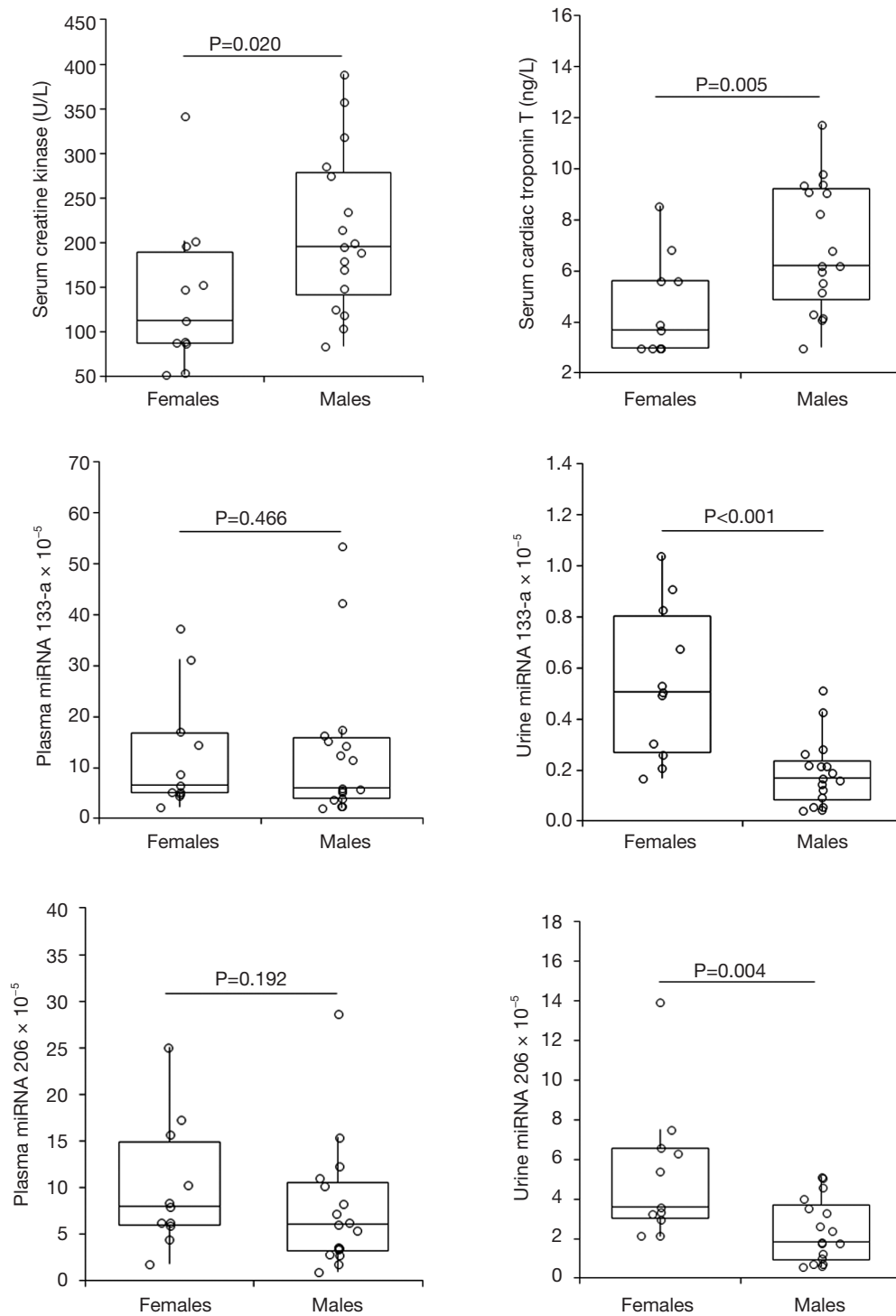


Figure 1 Serum concentration of CK and cTnT, plasma and urine concentration of miRNA 133-a and miRNA 206 in 28 athletes (11 women and 17 men) at rest. CK, creatine kinase; cTnT, cardiac troponin T.

was extremely modest (i.e., 27%). Despite the overlap between values in men and women was less accentuated for miRNA 206 (i.e., 41%), the 1.9-fold increase of urine value of this myomiRNA suggests that its reference range should also be partitioned for the sex. Another substantial aspect highlighted by our data is that case-control studies involving the measurement of myomiRNAs in urine should be planned to exactly match the different study populations for the sex. This is especially important if one considers that miRNAs have a good stability in urine and under many storage conditions, so supporting their increasing use in urine as diagnostic biomarkers for several pathologies (16).

Regardless of these general considerations, it remains unclear why the urine concentration of myomiRNAs should be higher in women than in men, especially if one considers that their plasma concentration is virtually identical between sexes (*Figure 1*). The potential influence of renal function (i.e., glomerular filtration rate) on the clearance of plasma miRNA 133a has been earlier shown (17), but this is not helpful to explain the different urine concentration between women and men observed in our investigation. This aspect should probably be targeted by future studies focusing on clearance of myomiRNAs in humans.

Conclusions

In conclusion the results of this study demonstrate that the plasma concentration of miRNA 133-a and 206 is virtually independent from the most common physiological variables, whereas the urine reference values of these two myomiRNAs should be partitioned for the sex. As regards the future perspectives, the results of our study lead the way to an exciting scenario, where myomiRNA measurement may be used for creating individual training plans for athletes (i.e., personalized training).

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was cleared by the local Institutional Review Board (Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona, Verona, Italy).

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