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# Implication of microRNAs as messengers of exercise adaptation in junior female triathlonists

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**While expression profile of muscle-specific miRNAs following endurance training is well-characterized, information about exercise-induced changes of metabolism-regulating miRNAs is limited, especially in female and junior athletes. Major aim of this study was to examine a set of miRNAs related to mitochondrial function and metabolism in highly professional junior female athletes. The Hungarian National Junior Triathlon Team (n = 4), completed standardized running and cycling sessions. Expression levels of miR-133a, miR-210, miR-494 and miR-127-3p were determined by RT-qPCR in whole blood and serum samples, withdrawn directly before, and after the exercise, and 24 and 48 h later. The expression of miR-494, miR-127-3p and miR-210 showed strong correlation with each other. In serum, nearly significant increment of miR-127-3p levels was detected, that may be a novel biomarker of exercise adaptation. Its expression was significantly higher than that of miR-210. In whole blood, significantly higher miR-210 than miR-494 and miR-127-3p levels were observed. MiRNA expression profile of the youngest athlete was markedly different compared to others. Our results suggest that miRNAs related to mitochondrial function and metabolism are involved in exercise adaptation. The present study may facilitate further research with larger potential participant pools, contributing to improved prevention and treatment of chronic diseases of civilization.**

MicroRNAs (miRNAs) are 19–22 nucleotide long, evolutionarily conserved RNA molecules, that are responsible for post-transcriptional regulation of gene expression[1]. Besides having an essential role in the development and function of skeletal muscle[2], miRNAs are also involved in exercise-induced adaptation processes[3], and are increasingly applied as novel biomarkers of cardiorespiratory fitness[4].

*MiR-133a* is a member of the family of muscle-specific miRNAs, referred to as myomiRs, that are involved in myogenic differentiation programmes and muscle fiber type specification[5]. Moreover, the role of miR-133a in the biosynthesis of mitochondria in skeletal muscle has also been confirmed[1]. In accordance with the up-regulation of miR-133a in consequence of endurance training[2], increased expression level of miR-133a, miR-1 and miR-206 following chronic swimming in a rat model was shown[6]. The correlation between the expression levels of these miRNAs and maximum oxygen uptake ($VO_2$ max) indicated the potential role of them as biomarkers of aerobic capacity[7].

Since being the major member of hypoxia-induced miRNAs (hypoxamiRs), *miR-210* is considered as a central driver of cellular response to hypoxia[8], promoting the shift from oxidative phosphorylation (OXPHOS) to lactate production[9], inhibiting apoptosis[10,11] and inducing angiogenesis[12]. *miR-210* also targets mitochondrial glycerol-3-phosphate dehydrogenase (GPD2) enzyme, that is an integral component of the mammalian respiratory chain and glycerophosphate shuttle connecting mitochondrial and cytosolic metabolic processes[13]. The level of circulating miR-210 was reduced at post-acute exercise in basketball athletes, though no robust correlation was identified to the markers of exercise capacity[14].

The term 'mitomiRs' summarizes a group of miRNAs detected in mitochondrial specimens, among which *miR-494* is so far the most frequently identified one[15]. MiR-494 has an important effect on mitochondrial biogenesis in skeletal muscle[16,17], furthermore, it negatively regulates the expression of SIRT3 enzyme[18], that has a central role in fine-tuning mitochondrial energy metabolism in response to both exercise and nutritional signals[19,20]. In C57BL/6 mice, chronic voluntary wheel exercise resulted in the significant decrement of miR-494 expression in the gastrocnemius muscle[21].

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A further member of the mitomiR group, *miR-127-3p*[15] is a key regulator of the bioenergetic activity of mitochondria through targeting the β subunit of the ATP-driven rotary motor enzyme, the mitochondrial $F_1$-ATPase[22]. Since SEPT7 gene was identified as a further target of this miRNA[23], the enhancement of myogenic cell differentiation by miR-127-3p[24] is consonant with the proposed role of septins in muscle regeneration and development following injuries[25]. To our best knowledge, changes of miR-127-3p expression following physical exercise were evaluated here for the first time.

In the present study, the cornerstone of the selection of miRNAs to be examined was their implication in the regulation of mitochondrial function and metabolic pathways. Regulation of metabolism by miRNAs is important not only for elite athletes but for the wider population too. As a result of exercise, there are often opposite changes in the expression level of miRNAs than those previously observed in chronic civilization diseases. miR-210 has a role in the development of ischemic cardiovascular disease[26], and contributes to obesity-induced adipose tissue inflammation and insulin resistance[27]. Via the regulation of PI3K/AKT/mTOR signalling pathway, miR-494 is involved in the regulation of cardiomyocyte damage after high blood glucose levels[28]. miR-494 was also found to protect the function of pancreatic β-cells by targeting PTEN in gestational diabetes mellitus[29]. miR-127 has been described as a crucial regulator of insulin secretion[30]. Although growing amount of data are available upon the expression profile of muscle-specific miRNAs following endurance training, information about mitochondria-related and metabolism-regulating miRNAs is very limited. In addition, vast majority of miRNA expression data following exercise sessions was obtained from adult male athletes[31,32]. Therefore, exercise-induced expression changes of miRNAs in junior female athletes are largely unknown. In the present study, we hypothesized that the expression of the above miRNAs is regulated by aerobic exercise training, and time-dependent expression patterns can be applied as biomarkers of exercise adaptation. Thus, the expression of miR-133a, miR-210, miR-494 and miR-127-3p was evaluated in whole blood and serum samples of junior female members of the Hungarian National Junior Triathlon Team, consisting of highly professional word-class athletes.

## Results

### Patterns of miRNA expression levels and markers of exercise capacity

In the Hungarian National Junior Triathlon Team (n = 4), expression levels of miR-133a, miR-210, miR-494 and miR-127-3p were determined by RT-qPCR in whole blood and serum samples, withdrawn directly before, and after standardized running and cycling exercise sessions, and 24 and 48 h later (Fig. 1).

Principal components analysis (PCA) and clustering of normalized miRNA expression levels in whole blood and serum obtained at the above detailed four sampling time points resulted in a markedly different expression pattern in case of the youngest athlete (#4) compared to the remaining three participants (Fig. 2a,b). Comparing miRNA expression levels normalized to the results of the first sampling time point (before the exercise) (Fig. 2c–i), major contribution of miR-210 (Fig. 2c), miR-127-3p (Fig. 2d) and miR-133a (Fig. 2e) in whole blood to the detached miRNA expression pattern of Athlete #4 is suggested. Analysis of anthropometric parameters and exercise markers listed in Table 1 also showed a less obvious distinctive pattern in the same participant (Athlete #4) (Fig S1).

### Evaluation of miRNA expression changes following exercise sessions

Time dependence of the expression of miR-210, miR127-3p, miR-494 and miR-133a was examined with RT-qPCR. Comparing samples taken right before, right after the exercise, 24 h and 48 h later, no statistically significant changes of normalized miRNA expession levels were confirmed, neither in whole blood nor in serum specimens (Fig. 3a–g). The increment of miR-127 expression levels in serum samples following exercise sessions was nearly significant ($P = 0.054$) (Fig. 3f). On the other hand, miR-127 expression showed a remarkable decrement in whole blood (Fig. 3b), accompanied with an increasing tendency of miR-210 levels. To identify whether specific miRNA expression patterns characterize the individual time points, multivariate analyses were performed. However, the data points of the four time points overlapped (Fig. 3h), and between the time points no significant difference could be detected with ANOSIM (all $P = 1$, $-0.156 \leq R \leq 0.083$).

Since in Athlete #4 a markedly different miRNA expression pattern was detected (Fig. 2a), changes of miRNA expression levels following exercise sessions were also evaluated based on the expression data of only Athletes #1–3, but no significant changes of the expression levels were confirmed (Fig S2).

### Correlations between miRNA expression levels and markers of exercise capacity

To examine the possible associations between the expression levels of different miRNAs, independently of the time point, correlation analyses were performed. Spearman Rank Correlation on pooled data of all time points revealed a number of statisticaly significant correlations between the expression levels of different miRNAs both in whole blood and serum specimens (Fig. 4a). In whole blood, the expression of miR-210 showed strong correlation with both miR-127-3p ($r = 0.750$, $P = 0.001$) (Fig. 4b) and miR-494 ($r = 0.688$, $P = 0.004$) (Fig. 4c), while strong correlation was also confirmed between the levels of miR-494 and miR-127-3p ($r = 0.750$, $P = 0.001$) (Fig. 4d). Expression of miR-133a showed moderate correlation with miR-127-3p ($r = 0.509$, $P = 0.046$) (Fig. 4e). In serum samples, the expression of miR-127-3p strongly correlated with miR-210 ($r = 0.679$, $P = 0.005$) (Fig. 4f) and miR-494 ($r = 0.679$, $P = 0.005$) (Fig. 4g). Moderate correlation was confirmed between the expression of miR-210 and miR-494 ($r = 0.529$ and $P = 0.037$) (Fig. 4h). These data indicate that the expression of miRNAs strongly correlate within the blood and in the serum.

Correlations were also evaluated between miRNA expression levels and anthropometric data, and markers of exercise capacity. Despite no further statistically significant correlations were confirmed, due to the small number of participants (n = 4), we mention here some nearly significant results (Fig. 4i, Fig S3).

Normalized $VO_2$ showed nearly significant correlation with the expression of miR-494, miR-127-3p and miR-210 in right before, right after the exercise and 24 h later obtained whole blood samples, respectively ($r = 1.000$,

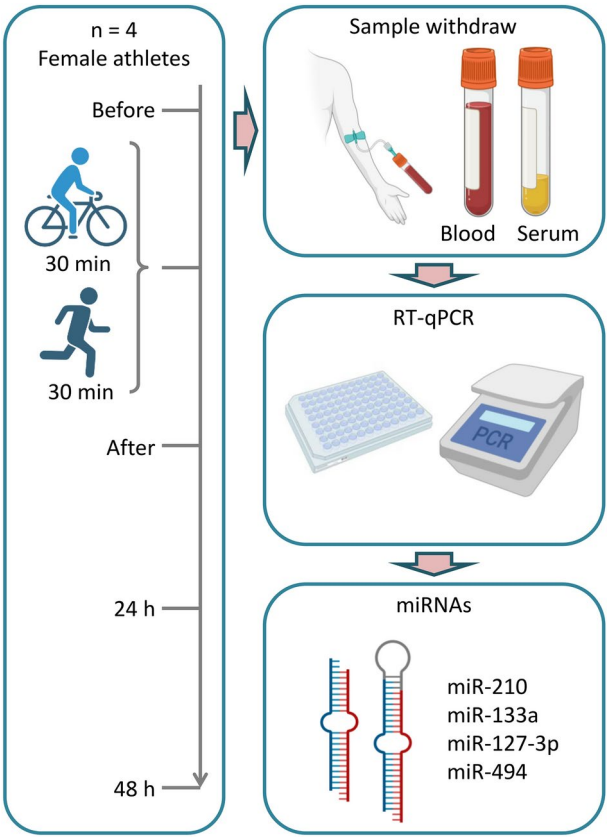


**Fig. 1**. Study design. Female athletes of the Hungarian National Junior Triathlon Team (n = 4) performed physical exercise including 30 min cycling and 30 min treadmill running. Whole blood and serum samples were taken right before, right after the exercise, and then 24 h and 48 h later. Expression levels of miRNAs (miR-133a, miR-210, miR-494, miR-127-3p) were determined with RT-qPCR. Images were created with BioRender.com. Abbreviations: qPCR, quantitative polymerase chain reaction; RT, reverse transcription.

$P = 0.083$). Age of athletes and the expression levels of both miR-127-3p and miR-210 showed nearly significant correlation in whole blood samples obtained 48 h after the exercise ($r = 1.000$, $P = 0.083$). BMI showed nearly significant correlation with the expression of miR-494 in whole blood specimens taken right after and 24 h after the exercise sessions ($r = 1.000$, $P = 0.083$). In serum samples taken right before and 48 h after the exercise, expression of miR-494 nearly significantly correlated with age of athletes ($r = 1.000$, $P = 0.083$), while expression of miR-210 in serum samples taken right before the exercise showed nearly significant correlation with age of athletes ($r = 1.000$, $P = 0.083$). BMI values showed nearly significant correlation with the expression of miR-494 and miR-210 in serum specimens obtained 24 h after the exercise sessions ($r = 1.000$, $P = 0.083$).

## Comparisons of miRNA expression levels at different sampling time points

To determine the most abundant miRNAs in the whole blood and serum samples, expression levels of miR-210, miR-127-3p, miR-494, and miR-133a at each individual time point were compared.

Data of Fig. 3 were rearranged and re-evaluated to compare the relative expressions of the individual miRs to each other. The expression level of miR-133a was significantly higher than miR-494 in whole blood taken right before, right after the exercise, and then 24 h and 48 h later (all $P \leq 0.037$) (Fig. 5a–d). When comparing datasets of different miRNAs that contain the expression results of all sampling time points, the expression level of miR-133a was confirmed to be significantly higher than miR-210 ($P = 0.004$), miR-127-3p ($P = 0.001$) and miR-494 ($P = 0.002$) too (Fig. 5i).

Comparison of miR-210 with the levels of both miR-127-3p and miR-494 revealed opposing relationships in whole blood and serum samples. The expression level of miR-210 in whole blood was higher than both miR-127-3p and miR-494 at all time points, but these differences were not statistically significant (all $P \geq 0.171$) (Fig. 5a–d). However, when comparing datasets of different miRNAs that contain the expression results of all sampling time points, both differences were statistically significant (Fig. 5i). On the other hand, in serum specimens, the expression level of miR-127-3p was higher than miR-210 at all sampling time points (Fig. 5e–h), which difference was statistically significant right before the exercise, and then 24 h and 48 h later ($P \leq 0.024$) (Fig. 5e,g,h). Also, when comparing datasets of different miRNAs containing the expression results of all sampling time points, the expression level of miR-127-3p was significantly higher than the level of miR-210 ($P = 0.014$) (Fig. 5j). Similarly to miR-127-3p, the expression level of miR-494 was also higher than miR-210 right before, right after

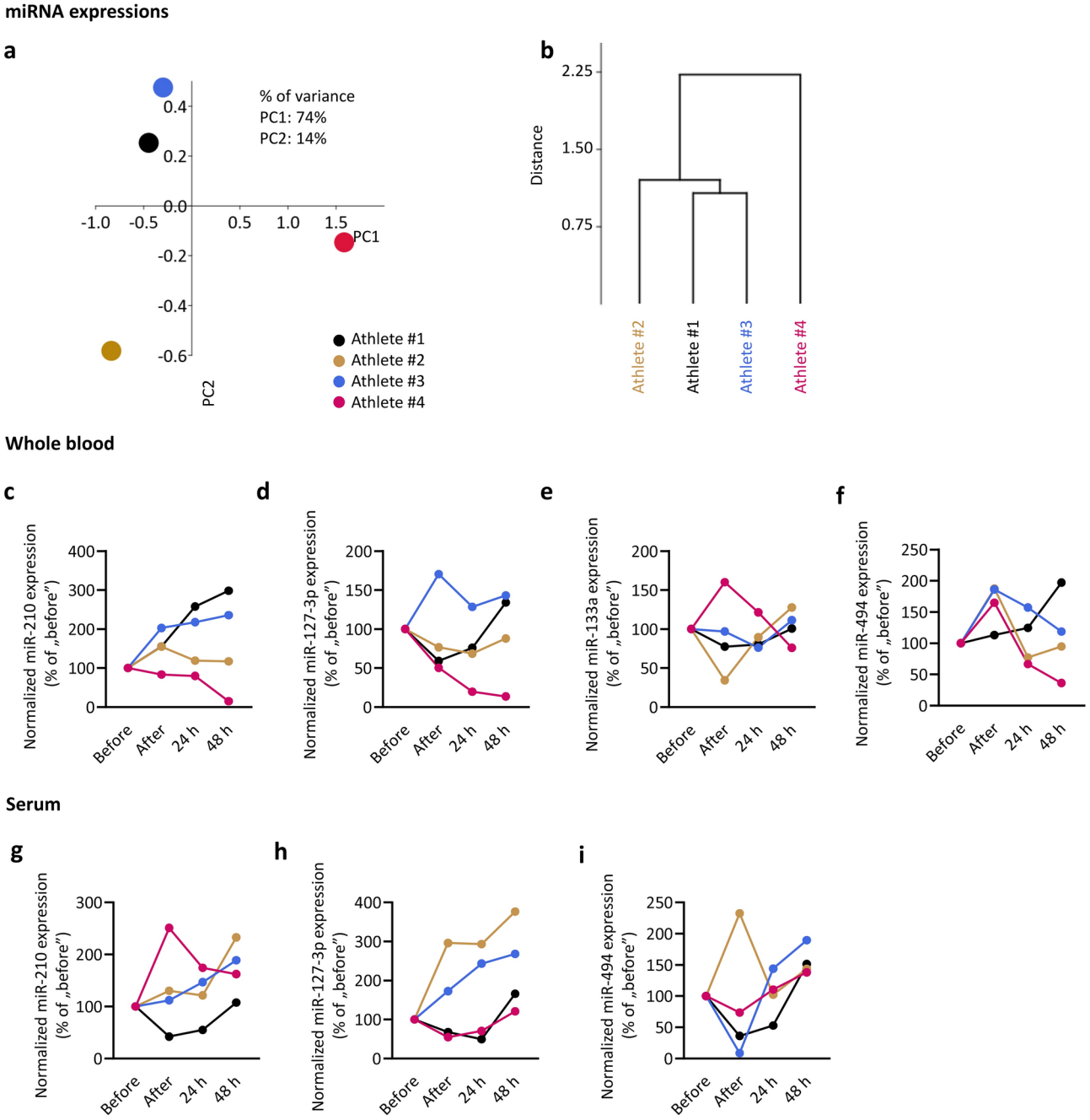


**Fig. 2**. Multivariate analyses. (**a**) Principal components analysis and (**b**) cluster analysis considering the expression of all examined miRNAs at all time points. Time dependent expression of (**c**) miR-210, (**d**) miR-127-3p, (**e**) miR-133a and (**f**) miR-494 in the whole blood, (**g**) miR-210, (**h**) miR-127-3p and (**i**) miR-494 in serum, individually normalized to the reference gene and to the "Before" time point of each athlete.

| | Age (years) | Body weight (kg) | Height (m) | BMI | $VO_2$ max (ml/min) | $VO_2$ norm (ml/min/kg) | $VO_2$ peak for cycling | $VO_2$ peak for running |
|---|---|---|---|---|---|---|---|---|
| Athlete #1 | 16 | 53.7 | 1.73 | 17.94 | 3003 | 55.92 | 2100 | 2200 |
| Athlete #2 | 15 | 57 | 1.72 | 19.27 | 3206 | 56.25 | 1950 | 2590 |
| Athlete #3 | 17 | 47 | 1.53 | 20.08 | 2886 | 61.40 | 1850 | 2400 |
| Athlete #4 | 14 | 50.9 | 1.67 | 18.25 | 3411 | 67.01 | 1625 | 2100 |

**Table 1**. Anthropometric parameters and markers of exercise capacity of the four athletes of the Hungarian National Junior Triathlon Team. BMI, body mass index; $VO_2$ max, maximum oxygen uptake.

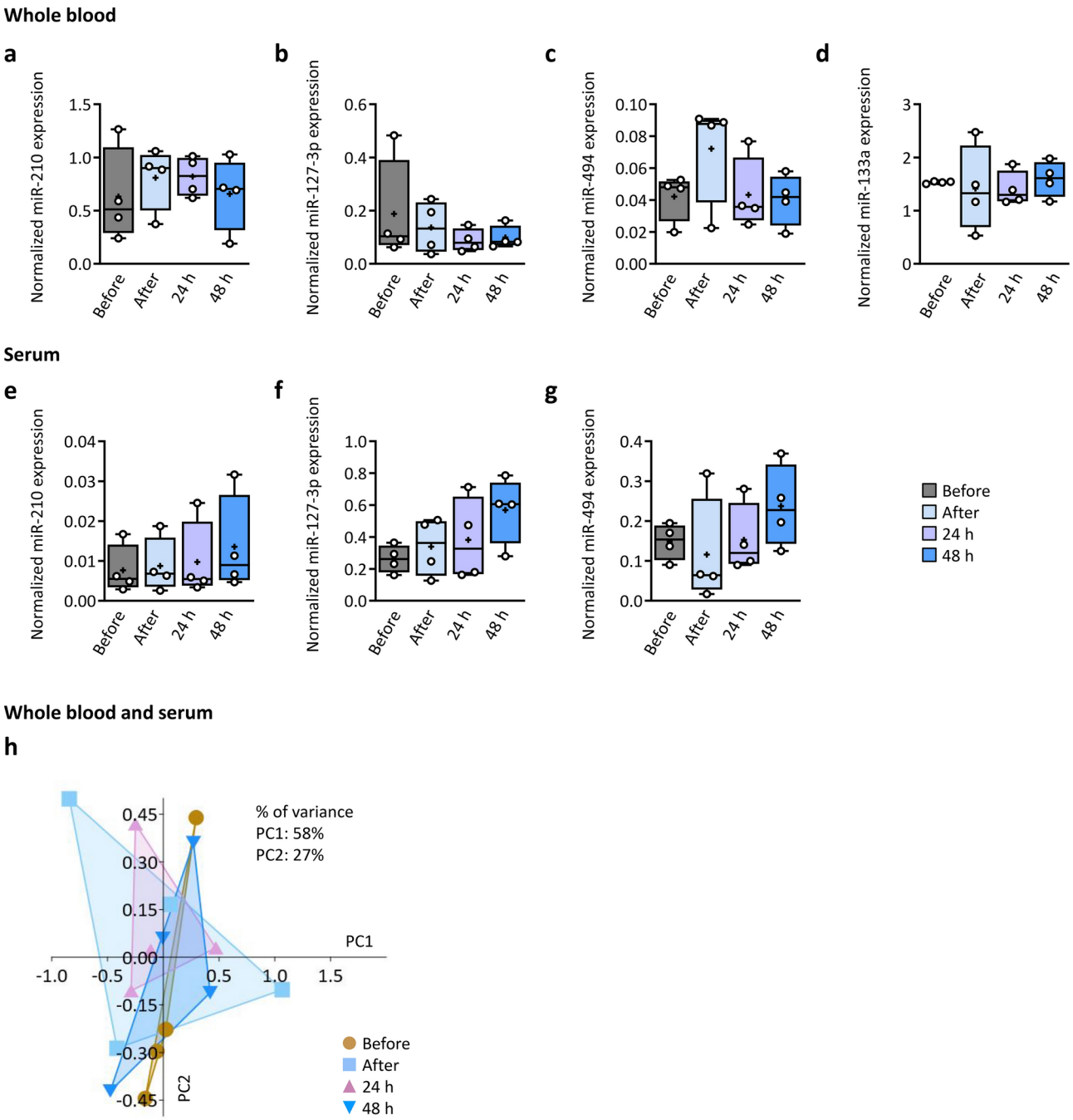


**Fig. 3**. Time dependence of miRNA expression levels. Normalized expression of (**a**) miR-210, (**b**) miR-127-3p, (**c**) miR-494, (**d**) miR-133a in whole blood, and (**e**) miR-210, (**f**) miR-127-3p, and (**g**) miR-494 in serum, determined with RT-qPCR, right before, right after the exercise, and then 24 h and 48 h later (boxes: 75th–25th percentiles, whiskers: minimum and maximum, middle line: median, +: mean, dots: individual data points). n = 4 per time point. No significant ($P < 0.05$) differences were detected with Repeated Measures One Way Analysis of Variance or Friedman test. (**h**) Principal components analysis considering the expression of all examined miRNAs in whole blood and serum at the four time points. Data points represent individual athletes. n = 4 per time point.

the exercise, and then 24 h and 48 h later (Fig. 5e–h), which difference was statistically significant right before the exercise and 48 h later (both $P \leq 0.035$) (Fig. 5e,h).

The expression level of miR-127-3p was significantly higher than the level of miR-494 in serum samples obtained 48 h after the exercise ($P = 0.025$) (Fig. 5h).

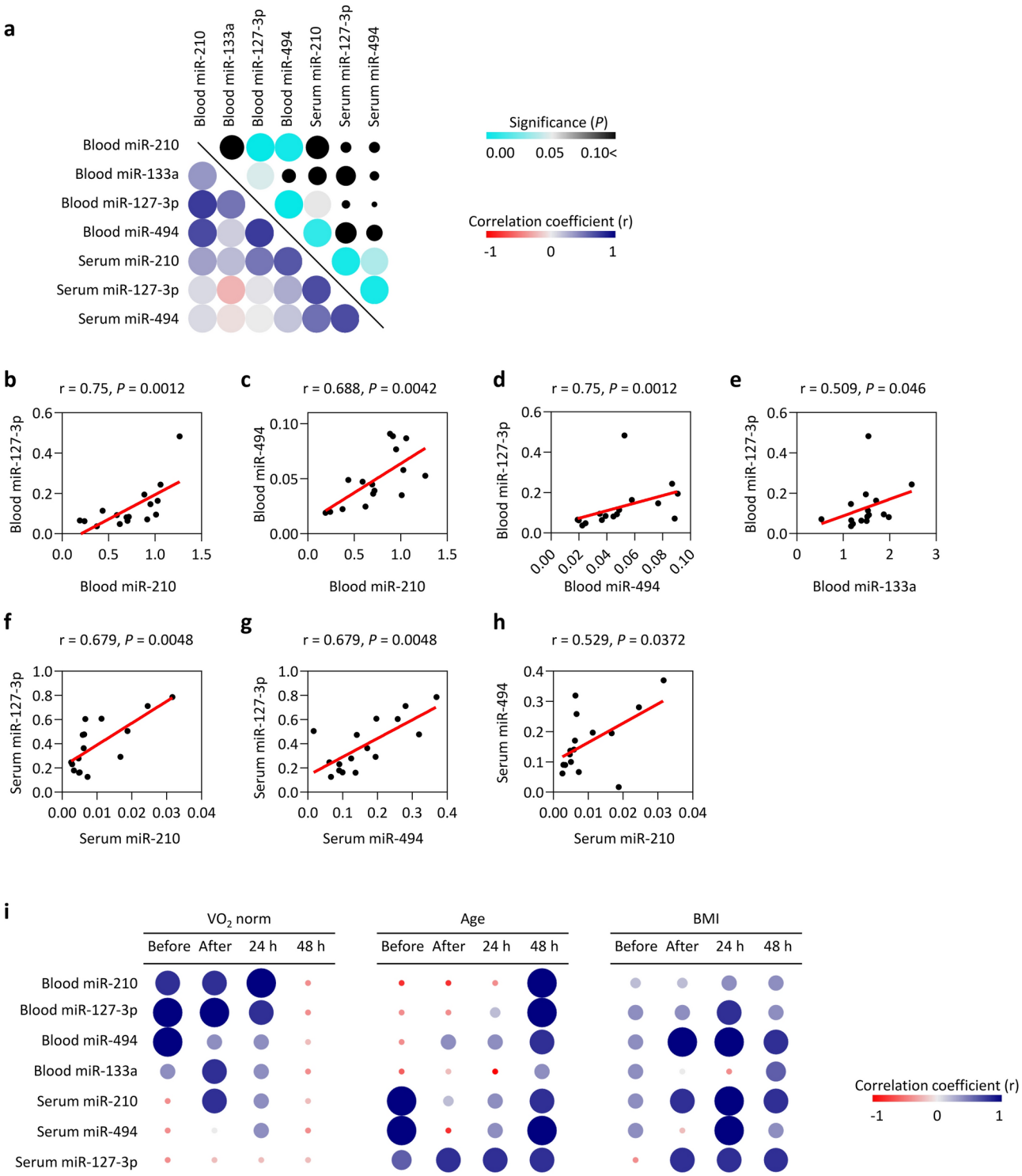


**Fig. 4**. Correlation analyses. (**a**) Spearman correlation matrix of the *P* values [decreasing size of dots indicate less significant *P* values] and the correlation coefficients (*r*) of normalized miRNA expression levels in whole blood and serum considering all time points in all athletes. Scatter plot and linear regression of the Spearman correlation between (**b**) miR-127-3p and miR-210 in whole blood, (**c**) miR-494 and miR-210 in whole blood, (**d**) miR-127-3p and miR-494 in whole blood, (**e**) miR-127-3p and miR-133a in whole blood, (**f**) miR-127-3p and miR-210 in serum, (**g**) miR-127-3p and miR-494 in serum, and (**h**) miR-494 and miR-210 in serum. Red regression lines indicate significant ($P < 0.05$) correlations. n = 16 (4 time points of 4 athletes pooled). (**i**) Correlation coefficients (*r*) of Spearman correlations between normalized expression levels of miRNAs, anthropometric parameters and markers of exercise. For *P* values see Figure S2.

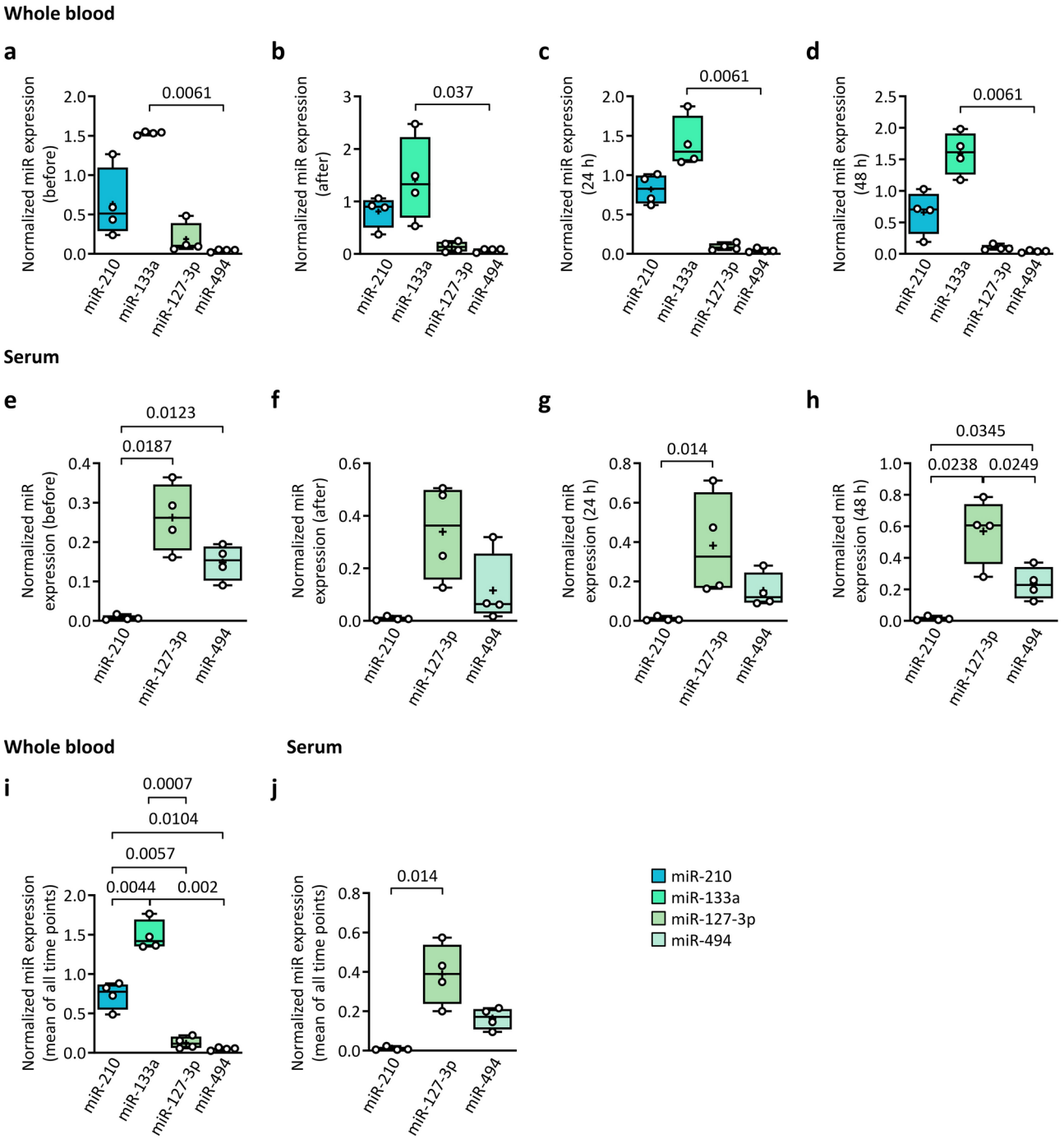


**Fig. 5**. Comparison of the expression of different miRNAs per time point. Normalized expression of miR-210, miR-133a, miR-127-3p and miR-494 in whole blood, determined with RT-qPCR, (**a**) right before and (**b**) right after the exercise, and then (**c**) 24 h and (**d**) 48 h later. Normalized expression of miR-210, miR-127-3p and miR-494 in serum, determined with RT-qPCR, (**e**) right before and (**f**) right after the exercise, and then (**g**) 24 h and (**h**) 48 h later. Expression of miRNAs in (**i**) whole blood and (**j**) serum, using the mean of the four time points. *P* values above the graphs were determined with Repeated Measures One Way Analysis of Variance or Friedman test.

## Discussion

While the vast majority of previously published data on exercise-induced miRNA expression changes were obtained from adult male athletes, present study was performed with whole blood and serum samples of junior female triathlon athletes, evaluating a miRNA profile composed of myomiRs (miR-133a), hypoxamiRs (miR-210) and miRNAs that regulate mitochondrial function and metabolic pathways (mitomiRs: miR-494, miR-127-3p).

Our major findings include (1) significantly higher miR-210 than miR-494 and miR-127-3p expression levels in whole blood, and the opposite relationship in serum samples, (2) increasing tendency of miR-210 and decrement of miR-127-3p in whole blood following exercise sessions, (3) nearly significant increment of miR-127-3p levels in serum, (4) significant strong correlation between the expression of miR-494, miR-127-3p and miR-210 in both whole blood and serum samples, (5) markedly different miRNA expression pattern in the youngest athlete.

Since the first definition of *epigenetics* was established by Conrad Hal Waddington in 1942[33], the rapid acceleration of this field revealed reversible and heritable patterns of epigenetic modifications, featured by a remarkable degree of plasticity[34]. Besides DNA methylation and histone modifications, increasing amount of evidence supports the role of miRNAs in health and disease. In order to improve morbidity and mortality rates of chronic diseases of civilization, it is essential to get deeper insights into exercise-induced processes of epigenetic adaptation, including miRNA expression changes that is the central focus of this study. The elite athletes of the Hungarian National Junior Triathlon Team had a very strict daily routine with standardized diet and physical exercise regime. Our measurements were performed during the offseason period of athletes (not during their regular seasons), as this is when the fluctuation in their endurance parameters is the smallest and when their diet is the most controlled.

In accordance with its well-established role in cellular responses to hypoxia including a transient metabolic shift to anaerobic glycolysis and lactate production[9,10], the observed increment of miR-210 expression in whole blood samples of athletes suggests an *acute impact* of miR-210 on metabolism following exercise sessions. In addition, miR-210 was recently shown to increase the stability of HIF-1α transcription factor due to the suppression of the enzyme glycerol-3-phosphate dehydrogenase 1-like (GPD1L)[35]. Opposite to the decrement of circulating miR-210 expression confirmed in basketball athletes[14], serum samples of triathlon athletes showed increased miR-210 levels following exercise sessions, which may be related to the different nature of basketball and triathlon in terms of being mixed aerobic-anaerobic versus fully aerobic training, respectively.

In case of miR-127-3p, a decrement of expression levels was confirmed in whole blood following exercise sessions, that consequentially results in the up-regulation of its targets including SIRT3 and β-$F_1$-ATPase enzymes, leading to enhanced mitochondrial function and increased OXPHOS capacity as part of a supposed *chronic impact* of endurance training on metabolic homeostasis. On the other hand, serum samples taken after exercise showed a nearly significant increment of miR-127-3p levels ($P=0.053$). The opposite observations regarding miR-127-3p expression in whole blood and serum specimens following exercise sessions may be explained by decreasing transcription of miR-127-3p and its transport from cellular blood components into plasma by exosomes[36]. According to recent data, miRNAs may also be released from blood cells into serum during coagulation process[37].

Determination of the most abundant miRNAs in whole blood and serum samples can help the assessment of their applicability to monitorize exercise adaptation. In serum samples, expression of miR-210 was significantly lower than miR-127-3p right before the exercise, and then 24 and 48 h later. In whole blood, miR-210 expression was higher than miR-127-3p, but these differences were not statistically significant in any time points. MiR-494 expression was significantly higher than miR-210 in serum right before the exercise and 48 h later. On the other hand, the level of miR-210 was higher than miR-494 in whole blood, but these differences were not statistically significant in any time points.

Correlation analyses of data obtained from both whole blood and serum samples revealed significant positive coexpression of SIRT3- and β-$F_1$-ATPase-regulating miR-494 and miR-127-3p, respectively. Also significant strong positive correlations have been established between the two mitomiRs and the expression of miR-210 in whole blood and serum as well. Further novel associations between miRNA expression levels and markers of exercise capacity have also been confirmed. Normalized maximum oxygen uptake showed nearly significant correlation with the expression of miR-494, miR-127-3p and miR-210 in whole blood at different sampling time points. In accordance with previously published findings[7], nearly significant correlation was confirmed between $VO_2$ and miR-133a expression in whole blood obtained 24 h after the exercise sessions. All in all, examining correlation of different miRNAs with each other may reveal fundamental interactions in their regulation. The correlations revealed between miRNAs and exercise markers suggests the role of these miRNAs in exercise adaptation. By confirming significant correlations, the panel of genes to be tested can be narrowed, which is especially important in the case of limited amount of test samples.

In case of the youngest triathlon team member, Athlete 4# (14 years), patterns of both miRNA expression levels and exercise capacity markers were markedly different as compared to Athletes #1–3, that possibly resulted from younger age, hormonal conditions (lack of menses), yet unrevealed genetic polymorphisms and epigenetic factors.

The limitations of the study include the low number of members of the Hungarian National Junior Triathlon Team. This may have sensitized the statistics to outlying datapoints, decreasing the probability of finding significant differences. Due to the lack of similarly preconditioned test subjects, the number of cases was not feasible to increase, since the involvement of differentially preconditioned subjects would have distorted results. Additionally, it would not have been safe to subject individuals who do not have sufficient endurance and fitness to this very intensive training course. Instead of creating a control group, the study was self-controlled, i.e. anthropometric values and miRNA expression levels of the athletes were compared to the values measured in control samples taken right before the training program. The amount of samples, especially serum, was also limited, hence the expression levels of only a few miRNAs have been evaluated. Expression levels of miRNAs were not measured in skeletal muscle biopsies due to ethical issues. The lack of direct evaluation of metabolic parameters and mitochondrial function are further limitations. The different genes that were used for normalization in whole blood and serum limited the comparability of results. On the other hand, divergent miRNA fingerprints in whole blood and serum have also been established by Unger et al.[38] emphasizing both the different RNA content

of samples and the non-identical influence of pre-analytical variables on them. Though further research is absolutely needed to decide whether the miRNAs examined are appropriate candidates for monitorizing exercise adaptation, standardization of miRNA profiling methods is essential to provide comparability of results obtained by different studies. Despite these limitations, our results provide novel molecular details about metabolism-regulating miRNAs and exercise adaptation of a highly standardized group of world-class junior female athletes. Although the results of this study are primarily relevant for elite athletes, they may still be of widespread interest from several points of view. Very few data are available on exercise-induced miRNA expression changes among junior female athletes. Furthermore, based on our results, the miRNA pattern can help to distinguish those individuals among athletes, who are expected to perform particularly well. In addition, as a result of exercise, there are often opposite changes in the expression level of metabolism-regulating miRNAs than those observed in chronic civilization diseases.

In summary, our results suggest that exercise-induced miRNA expression changes play a role in metabolic adaptation to endurance training. In whole blood, increasing tendency of miR-210, regulator of anaerobic glycolysis, and decreasing tendency of OXPHOS-regulating miR-127-3p was detected following standardized triathlon exercise sessions. Up to our knowledge, expression of miR-127-3p following endurance training was evaluated here for the first time. Though no statistically significant changes have been confirmed, the increment of miR-127 expression levels in serum samples following exercise sessions was nearly significant ($P=0.054$). Rather than miR-127-3p itself, the different miRNA expression pattern (including different expression of miR-127-3p) of Athlete #4 can be applied as a biomarker, since it was associated with outstanding performance. Since her markers of exercise capacity were also outstanding, the different miRNA profile obtained in case of Athlete #4, including miR-127-3p expression, may be a suitable biomarker for recognizing athletes predestined for outstanding results. However, factors that influence inter-individual differences in adaptation processes have yet to be identified. Here we revealed age related inter-individual differences in miRNA patterns. Since physical exercise is a protective factor against the widespread dysregulation of miRNAs in obesity, diabetes and hypertension[39,40], increasing awareness about the importance of exercise training in childhood and adolescence is essential. The present study may facilitate further research on metabolism-regulating miRNAs with larger potential participant pools in order to make achievements in both the prevention and treatment of chronic diseases of civilization.

## Methods

### Participants and samples

The study was performed on whole blood and serum samples of the Hungarian National Junior Triathlon Team, that consists of five female members (Athletes #1–5), aged 14–17 years, among whom Athlete #5 was excluded due to participating at a school swimming regular season during the offseason period. Therefore, miRNA expression levels were measured in the remaining four athletes (Athletes #1–4) (Fig. 1). The athletes performed standardized amounts and types of training during their offseason period. Their diet was tailored to their body weight and physical activity, with strictly controlled calorie and protein intake. Athletes had their last meals 2 h before the execution of the training course included in this study, in the form of exactly the same meals. Blood samples were taken right before, right after the exercise, and then 24 h and 48 h later, by a professional healthcare nurse[41]. The study was approved by the Medical Research Council (No. 3890-8/2018/EÜIG) and complied with the Declaration of Helsinki. All participants provided a written informed consent.

### Exercise sessions

Exercise sessions have been described in details in a previous publication of our team[41]. As official regular season events are defined as biathlon in junior athletes, swimming was not included in current exercise sessions. Briefly, cycling and running disciplines of a junior triathlon race were mimicked with 30 min cycling and then for an additional 30 min running on a treadmill. The running platform had an inclination of 5 degrees to simulate air resistance. Rate of exercise was adjusted to prior results of $VO_2$ measurements. All exercise protocols were started at 9:00 AM, 2 h after breakfast[41]. Schedule of exercise sessions was also in accordance with the hormonal condition of team members, since it was performed in the follicular phase of the menstruation cycle in Athletes #1–3. The youngest athlete (Athlete #4) has not menstruated until performing present study. Anthropometric parameters and markers of exercise capacity are summarized in Table 1.

### Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA fraction was isolated from whole blood and serum samples with Trizol reagent. Concentration of isolated RNA was measured with NanoDrop ND-1000 spectrophotometer at 260 nm wavelength. Isolated RNA was stored at − 80 °C temperature. Reverse transcription into cDNA was performed with Omniscript RT kit (Qiagen, cat. no.: 205113) in 20 µl reaction volumes that contained RNase inhibitor reagent (Promega, N261A). The qPCR reactions were performed with SYBR Green Mastermix (Roche, cat. no.: 04887352001), in a Light Cycler 480 Master instrument (Roche). Thermal profile was the following: initiation for 3 min at 95 °C (1×); amplification: 10 s at 95 °C, 30 s at 58 °C, 1 s at 72 °C (45×); cooling: 10 min at 40 °C (1×)[6]. Due to the limited amounts of specimens, expression of miR-133 was measured only in whole blood samples, and all qPCR reactions were performed in duplicates. $C_p$ values were determined with the Light Cycler 480 SW 1.5.0 software (Roche). Relative copy numbers were calculated via the $\Delta C_p$ method.

Based on previously published data[42], spliceosomal U6 RNA was successfully used in our prior human miRNA projects as a normalization gene[43]. However, while the stability index of U6 RNA was excellent in whole blood samples (1.51), its value in serum samples was not acceptable (> 5), necessitating the administration of a different normalization gene for serum samples[44]. For purposes of miRNA profiling in human serum samples,

| Type | miRNA | Sequence |
|---|---|---|
| Stem-loop | hsa-miR-133a-3p | GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC CAGCTG |
| Stem-loop | hsa-miR-210-3p | GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC TCAGCC |
| Stem-loop | hsa-miR-494-3p | GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GAGGTT |
| Stem-loop | hsa-miR-127-3p | GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AGCCAA |
| Stem-loop | hsa-let-7a-5p | GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC AACTAT |
| Stem-loop | U6 | GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA |
| Forward | hsa-miR-133a-3p | GTGTTTGGTCCCCTTCAAC |
| Forward | hsa-miR-210-3p | GTTTCTGTGCGTGTGACAG |
| forward | hsa-miR-494-3p | GGGTGAAACATACACGGGA |
| Forward | hsa-miR-127-3p | GTTTTCGGATCCGTCTGAG |
| Forward | hsa-let7a-5p | GGGTGAGGTAGTAGGTTGT |
| Universal reverse primer for qPCR | | CTGCAGGGTCCGAGGT |
| Upstream | U6 | CTCGCTTCGGCAGCACATA |
| Downstream | U6 | GTGCAGGGTCCGAGGT |

**Table 2**. Sequences of primers for reverse transcription and quantitative polymerase chain reactions.

let-7a has been confirmed to be a suitable reference gene[45], the stability of which value was also acceptable in our serum samples (2.46). Therefore, U6 was used in whole blood samples and let-7a in serum specimens for normalization. Sequences of primers are listed below (Table 2).

## Statistical analysis

To test normal distribution, the Shapiro–Wilk normality test was used. Depending on a normal distribution, statistical significance between the time points or miRNA expression levels was tested by Repeated Measures One Way Analysis of Variance (RM-ANOVA) followed by Tukey test for all pairwise multiple comparisons, or non-parametric Friedman test followed by Dunn's test for all pairwise multiple comparisons, and multiplicity adjusted $P$ values were reported. To test correlations, Spearman correlation coefficient (r) was calculated. All correlation coefficients were interpreted according to Evans[46] (0.00–0.19 very weak; 0.20–0.39 weak; 0.40–0.59 moderate; 0.60–0.79 strong; 0.80–1.00 very strong). Heat map was created with Morpheus (https://software.broadinstitute.org/morpheus). Multivariate analyses were performed considering all time points[47,48]. Principal components analysis (PCA) with a variance–covariance matrix (in case of evaluating miRNA expressions) or correlation matrix (in case of evaluating vital parameters) routine, hierarchical cluster analysis with the unweighted pair-group average (UPGMA) algorithm and the Euclidean similarity index, and non-parametric one-way analysis of similarities (ANOSIM) with Euclidean similarity index were computed[47,49]. Considering multiplicity issues, the Bonferroni-corrected $P$ values were reported. All calculations were performed with Prism v. 9.5.0 (GraphPad Software), or Past v. 4.04; $P < 0.05$ was considered statistically significant. Data was expressed as mean ± SD. Box plot diagrams always show the 75th and 25th percentiles (upper and lower borders of the boxes), the minimum and maximum (whiskers), the mean value (+), the median (middle line), and the individual data points (dots).

## Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Author contributions

Z.G. designed analysis of miRNA expression levels, interpretation of results, data analyzation, writing original draft of the manuscript; J.F. designed and performed miRNA expression analysis, data analyzation, interpretation of results, revised manuscript; T.O. data analyzation and figure preparation, figure legends, revised manuscript; I.G.S. performed experiments; I.B. conceived and designed research, revised manuscript; L.C. conceived and designed research, revised manuscript. All authors read and approved the final version of the manuscript.

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## Declarations

## Competing interests

The authors declare that they have no competing interests.

## Ethics approval

The study was approved by the appropriate institutional and/or national research ethics committee (Medical Research Council, No. 3890-8/2018/EÜIG) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-73670-8.

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