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# Effects of endurance racing on horse plasma extracellular particle miRNA

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**Keywords:** horse; endurance racing; extracellular particles (EP); microRNA (miRNA).
**Running title:** Endurance racing on horse miRNA

**Summary**

**Background:** Physical exercise is an essential factor in preventing and treating metabolic diseases by promoting systemic benefits throughout the body. The molecular factors involved in this process are poorly understood. Micro RNAs (miRNAs) are small non-coding RNAs that inhibit mRNA transcription. MiRNAs, which can participate in the benefits of exercise to health, circulate in plasma in extracellular particles (EP). Horses that undergo endurance racing are an excellent model to study the impact of long-duration/low intensity exercise in plasma EP miRNAs.
**Objectives:** To evaluate the effects of 160 km endurance racing on horse plasma extracellular particles and their miRNA population.
**Study design:** Cohort study.
**Methods:** We collected plasma from 5 Arabian horses during five time-points of an endurance ride. Extracellular particles were purified from plasma and characterised by electron microscopy, resistive pulse sensing (qNano), and western blotting. Small RNAs were purified from horse plasma EP, and sequencing was performed.
**Results:** Endurance racing increased EP concentration and average diameter compared to before the race. Western blotting showed a high concentration of extracellular vesicles proteins 2 h after the race, which returned to baseline 15 h after the race. MicroRNA differential expression analysis revealed increasing levels of eca-miR-486-5p during and after the race, and decreasing levels of eca-miR-9083 after the end.
**Conclusions:** This study adds new data about the variation in plasma EP concentrations after long-distance exercise and brings new insights about the roles of exercise-derived EP miRNAs during low-intensity endurance exercise.

## Introduction

Endurance racing is classified as a competition of low intensity and long duration [1]. However, in the last decade, speeds advanced to the point of being considered moderate-

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intensity [2]. During the competition, horses complete a test of typically 160 km (100 miles) in a day, passing through veterinary checkpoints during the race[3]. Haematological determinations have been used over the years in order to compare the responses of horses to various types of exercise [4]. Several studies document the differences in parameters before and after exercise, relating them to athletic performance and fluid losses undergone by animals [5] [6] [7]. Significant changes in plasma and serum biochemical parameters occur during endurance racing, reflecting the metabolic state of the animal and helping veterinarians to assess negative consequences on performance and health [8] [9]. In recent years, small molecules, such as microRNAs (miRNAs), were discovered circulating in plasma, serum, and other biological fluids, making them promising biomarkers for diseases [10] [11].

MicroRNAs are short noncoding RNA molecules that negatively regulate messenger RNA and play essential roles in the general biological process [12]. In an RNAse-rich environment, such as plasma, miRNAs are protected from degradation by being transported associated with extracellular vesicles (EVs) [13], lipoprotein particles [14], and argonaute-2 protein complexes [15]. Extracellular vesicles are lipid bilayered membrane vesicles with a size range of 40 to 1000nm in diameter, which can be classified into exosomes and microvesicles [16]. Extracellular vesicles, lipoprotein particles, and protein complexes can be purified from cell culture supernatants or biologic fluids using polymer-based precipitation kits [17]. The International Society for Extracellular Vesicles (ISEV) recommends using the term Extracellular Particles (EPs) for samples purified by polymer-based precipitation kits and that may include EVs, lipoprotein particles and argonaute-proteins [18].

Circulating miRNAs are altered in response to different protocols of acute and chronic exercise in both healthy and diseased populations [19], raising questions of the importance of EVs delivering miRNAs between skeletal muscle and muscle-organ crosstalk during exercise [20]. This crosstalk may contribute to the systemic biological effects of exercise [21]. Equines subjected to endurance racing represent an excellent model for understanding the molecular mechanisms involved in adaptation and success in this category of exercise. This work aimed to evaluate the effects of 160 km endurance racing on horse plasma extracellular particles and their miRNA population.

## Material and Methods

### Horse plasma samples

We selected Arabian horses participating in a 160 km international endurance race, regulated by the International Equestrian Federation (FEI). Each animal had gone through

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conditioning program, finishing the required official races of shorter distances for at least four years before being allowed to compete. The race started with 18 horses, but 9 were eliminated (3 due to orthopaedic problems, and 6 due to metabolic disorders). We excluded the horses with haemolysed plasma due to the inhibitory effect of haemoglobin on the PCR reaction. Thus at the end, we were left with 5 horses. The horses examined by the official veterinary staff and were considered fit to perform the test. During the ride, horses were also evaluated (every 30 km) to ascertain if any metabolic or orthopaedic alteration could prevent them from continuing and completing the ride (vet check).

Samples were taken pre-race the night before (T0), at the second veterinarian-gate (66 km, T1), at the end of the race (T2), 2 hours after the race (T3), and 15 hours after the race (T4) (Figure 1a). Venous blood samples (10mL of whole blood) were obtained by antiseptic jugular venipuncture in tubes with EDTA (Vaccuette) and negative pressure. For plasma purification and platelets removal, blood was centrifuged for 2,000 x g for 15min at room temperature, and platelet-poor plasma was stored in -20ºC up to 3 hours after sampling.

**Extracellular particle purification**

The investigators who performed EPs purification and sequencing were blinded of the identity of the samples. Extracellular particle purification was performed using the commercial kit Exoquick™ (Exosome Precipitation Solution, System Biosciences Inc., Mountain View, CA, USA) following manufacturer's recommendations, with the following modifications of the standard protocol. Extracellular particles were purified from 500µL of plasma. Thromboplastin was added into platelet-poor plasma to allow the removal of fibrins and fibrinogens to prevent their precipitation by ExoQuick. Platelet-poor plasma was treated with Thromboplastin 1:1, incubated at room temperature for 5 min, and centrifuged in a standard microcentrifuge at 8,000 x g for 5 min. After that, serum-like samples were incubated with 126µL of Exoquick overnight at 4ºC and then centrifuged by 10,000 x g for 30 min to pellet EPs. The pellet was resuspended in 200µL of PBS 1X. For EP characterisation, samples were pooled, forming five samples (T0, T1, T2, T3, and T4), each one comprising five horses. Extracellular vesicle characterisation was performed by electron microscopy and tunable resistive pulse sensing (qNano).

**Electron microscopy**

Electron microscopy for horse plasma EP visualisation was performed following the previously described protocol [22]. Briefly, a drop containing 10µL of pooled EPs purified from all

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samples was placed on parafilm and processed for negative staining. Grids were washed three times in PBS 1X. The EPs samples were fixed in a drop of 2% paraformaldehyde for 10 min and washed in PBS. Grids were post-fixed with 2.5% glutaraldehyde, incubated for 10 min and washed five times using distilled water. Finally, grids were contrasted with 3% uranyl acetate for 15 min. The images were acquired using 80 KVs in a transmission electron microscope JEOL model 1011, at the University of Brasilia electron microscopy laboratory.

**Estimating the concentration and size distribution of EPs using tunable resistive pulse sensing**

Tunable resistive pulse sensing analysis (TRPS) was performed using the qNano system (Izon, Christchurch, New Zealand). Tunable resistive pulse sensing analysis is a variant of resistive pulse sensing (RPS). It measures nanoparticles suspended in electrolytes on a particle-by-particle basis as they pass through a nanopore. When the particle passes through a nanopore it changes an electric current. The blockage in the current is proportional to the size of the particle. Extracellular particle samples were diluted in PBS. Samples were measured using an NP100 pore with the following settings: Stretch 47.01mm, Voltage 1V, and applied pressure of 20 cm.H2O (full pressure). The NP100 Izon pore can detect particles between 50nm and 330nm. The average current between all samples ranged from 81nA to 90nA. Calibration was performed using beads of known size, diluted in PBS, and measured at identical settings. Analyses of EP concentration and size were performed using Izon Control Suite 2.2 software.

**Identification of EP protein markers by western blotting**

Extracellular particle protein purification was performed using aliquots of 5µL of EPs suspension, diluted in 50µL RIPA 1X protein lysis buffer (150mM NaCl; 1.0% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 25mM Tris-HCl, pH 7.4) plus 0.5µL protease inhibitor cocktail (Sigma-Aldrich) and vortexed for 20 seconds. The total protein quantification was performed using the Protein Assay kit Qubit® (Life Technologies) according to the manufacturer's recommendations. Twenty (20) µg of EP protein were separated by electrophoresis on 12% SDS-PAGE using the Bio-Rad Mini-Protean II cast system. Electrophoresis was performed with 150V and 15mA for 2h in parallel. One gel was stained with Coomassie blue, and the other gel was transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad). The transfer was performed using the Trans-Blot® SD semi-dry transfer cell (Bio-Rad) in transfer buffer containing 48mM Tris, 39mM glycine, 20% (v/v) methanol and 1.3mM SDS for 1h at 15V and 200mA. The

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membranes were blocked for 1h in 3% BSA diluted in TBST 1X buffer (50mM Tris.HCl pH 7.4; 150mM NaCl; 0.1% Tween 20). After blocking, the membranes were incubated overnight with rabbit primary polyclonal antibody anti-CD9, CD81, Alix, Flottilin and Calnexin (Santa Cruz Biotechnology), at a concentration of 1: 500 diluted in 1% BSA in TBST buffer 1X. Anti-IgG secondary antibody (1: 1000; Sigma) conjugated with alkaline phosphatase was incubated for 1 hour in 1% BSA diluted in TBST buffer. Membranes were developed using NBT/BCIP (AP colour development buffer, Bio-Rad) until the appearance of the colour.

**RNA extraction, NGS library preparation, and sequencing**

Small RNAs were purified from 24 samples. As we used a 24-sample library Preparation kit Set, the T3 sample from horse 1 (H1T3) was removed from sequencing. Small RNA purification was performed with 150 µL of EPs resuspended in PBS1x using miRCURY™ RNA Isolation Kit – Biofluids (Exiqon, Denmark) following the manufacturer's instructions. Purified small RNA samples were characterised and quantified using Agilent Bioanalyzer – small RNA chip. Small RNA sequencing libraries were constructed using the TruSeq® Small RNA Library Preparation kit Set A (24 runs) (Illumina Inc.), following the manufacturer's instructions. Briefly, both 3′ and 5′ adaptors were ligated along with the unique index sequence in each sample. The adaptor-ligated RNA fragments were then reverse transcribed and amplified by 15 PCR cycles. Then, enriched small RNA libraries purified from horse plasma EPs were obtained after running a native polyacrylamide gel electrophoresis and cutting the fragments of interest. The libraries were eluted from the gel and quantified using KAPA Library Quantification Kits for NGS (Kapa Biosystems). After that, the 50-base read length sequencing was performed on a MiSeq (Illumina Inc.). To avoid batch effects during sequencing, pooled libraries were sequenced twice. Library preparation and sequencing were performed at the center for High-Performance Genomics of the Federal District (Genomic-DF, Catholic University of Brasilia, Brazil). Demultiplexing (generating individual sample groups) of the reads after sequencing was performed by sorting the different index sequences.

**Small RNA sequencing**

The sequencing data were analysed using the commercial software NEXTGENE® (SoftGenetics). The quality parameters used were: *median score threshold* ≥ 13 and *called a base number of each read* ≥16. At most, two mismatches were allowed for mapping small RNA sequences to the databases. The software converted data files from FastQ to fasta. After filtering

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the low-quality reads and trimming the adapters, *Equus caballus* sequencing databases were used to align the selected reads.

The database alignment procedures were applied in the following order: *Equus caballus* mature miRNA database, available at miRBase.org [EquCab2.0], miRNA unmatched sequences were aligned to *Equus caballus* tRNA sequences (downloaded from GtRNAdb database) which contains tRNA gene predictions made by the tRNAscan-SE. Finally, remaining reads were aligned to *the Equus caballus* ribosomal RNA database (downloaded from Ensembl). Reads that aligned to each database were removed from the following alignment.

### Data analysis

For statistical analysis of the data, GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) was used. Statistical significance for horse plasma EP miRNA yield was performed by two-way ANOVA and Tukey's multiple comparisons. We considered statistical significance of $p<0.05$. Heatmaps for horse plasma EPs small RNA sequencing data were generated with the pheatmap library. For miRNAs differential expression analysis, T0 levels were considered as baseline for comparisons. MicroRNAs were considered differentially expressed if $p<0.05$ in two-way ANOVA and $p<0.05$ after Dunnett's multiple comparisons test.

## Results

### Endurance racing increases horse plasma EP concentration and diameter

The plasma polymer-based precipitation method commercialised as Exoquick™ allowed us to visualise small EVs and other particles from horse plasma, as demonstrated by transmission electron microscopy (TEM) which showed small EV-like particles from 40 to 100 nm **(Figure 1B, red arrow)**. We also detected lipoprotein-like particles below 50nm in diameter **(Figure 1B, yellow arrow).** TRPS showed that EP average diameter between T0 and T1 remained the same at 59.2 nm while it increased at T2 (63.8 nm), T3 (65.2 nm), and remained the same at T4 (65.2) compared to T3 **(Figure 1C)**. Plasma EP concentration increased from $5.6x10^{12}$ per mL at T0 to $1.2x10^{13}$ per mL at T1, $3.6x10^{13}$ per mL at T2, $3.6x10^{13}$ per mL at T3 and $1.1x10^{14}$ per mL at T4 **(Figure 1D)**.

Endurance racing also increased plasma EP protein average concentration, ranging from 3.44 $mg.mL^{-1}$ at T0 time point to 4.39 $mg.mL^{-1}$ at T1 time point ($p < 0.05$), to 3.80 $mg.mL^{-1}$ at T2 time point, 3.67 $mg.mL^{-1}$ at T3 time point and 4.25 $mg.mL^{-1}$ at T4 time point ($p < 0.05$) **(Figure 1E)**. Total horse plasma EP protein profile was accessed by SDS-PAGE, and western blotting

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was performed to identify extracellular vesicles (EV) proteins. Twenty (20) µg of total protein purified from horses' plasma EPs was separated by electrophoresis. The SDS-PAGE visually showed defined bands from around 10 to 200 kDa in Coomassie blue staining. Some gel bands were detected in the size range of albumin and IGG proteins, indicating the presence of non-EVs protein aggregates in our preparation **(Figure 1F)**. Western blotting showed that plasma EPs had no endoplasmic reticulum protein Calnexin, and showed that T3 time point was enriched in EV proteins flotillin-1, CD81 and CD9 compared to the other time points **(Figure 1G)**.

**Endurance racing changes horse plasma EP miRNA content**

After performing biophysical and biochemical characterisation from horse plasma EPs, we purified and characterised EP small RNA, aiming to conduct library construction and sequencing. With an input of 150 µL of EP suspension, we sequenced 24 horses' EP samples. Purified EP small RNA showed enrichment in RNAs between 15 and 150 bp, a representative size of miRNA and other small RNA fragments **(Figure 2A)**. We did not detect ribosomal subunits 18S and 28S RNA peaks in Bioanalyser characterisation. The total yield of EP small RNA ranged from 20.7 ng in the lowest sample to 167 ng in the highest sample. The total yield of small RNA was increased at time point T3 compared to time points T0, T1, T2, and T4 ($p<0.001$ in two-way ANOVA, and $p<0.01$ in Tukey's multiple comparison test) **(Figure 2B).**

Raw read numbers obtained after sequencing, filtering, trimming, and alignment to *Equus caballus* mature miRNA database, available at miRBase.org, are summarised in **Table 1**. Overall, 13.4% of the total reads were considered mappable reads (after filtering and trimming). Reads that did not align with the previous database were aligned to the following one. The percentages of mappable reads aligned to small RNA databases were 3.1% for miRNA, 1.9% for rRNA, and 0.9% for snRNA (**Table 1**).

A principal component analysis (PCA) generated with horse plasma EP miRNA reads showed a separation between horses from T0, T3, and T4 time points. Horses from time points T1 and T2 were localised either with T0 or T4 **(Figure 3A)**. The relative expression of the 30 most abundant *Equus caballus* plasma EP mature miRNAs was shown in the heatmap. Both arms of red blood cell-derived miRNA eca-mir-486 were the most abundant miRNAs before, during, and after exercise (**Figure 3B** and **C**).

As expected for plasma, with low RNA tissue level, we chose the top eight miRNAs (eca-miR-486-5p, 486-3p, 30d, 30e, 25, 92a, 9083, 9092) for comparison. The miRNA read counts were compared between T0, T1, T2, T3, and T4 time points. We detected statistically significant increases in the level of eca-miR-486-5p in T1 and T4 compared to T0, and decreasing levels of

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eca-miR-9083 immediately after the end of the race (T3) and 15 h after the end of the race (T4) ($p<0.05$ two-way ANOVA, $p<0.05$ Dunnett's multiple comparisons test).

## Discussion

Exercise promotes human health [23]. The molecular mechanisms underlying exercise promotion for animal health are not fully understood. Cell communication mediated by miRNAs was shown to be important in several metabolic diseases [24] and cancer [25]. The impacts caused by long-distance exercise on the population of horse plasma EP miRNAs can bring us insight into what metabolic changes are happening in the animal's body and may help us to define better biomarkers for metabolic stress.

For horses submitted to endurance racing, biochemical analyses of metabolites have been used to predict metabolic elimination during the race [8]. After long-distance endurance rides, healthy horses had high heart creatine kinase (CK-MB) activity 12 h after the race compared to pre-race. Other serum analytes like creatine kinase, protein, albumin, bilirubin, y-glutamyltransferase, serum amyloid A, fibrinogen, haptoglobin, iron, aspartate transaminase, and urea remained increased 12 h after the race compared to before the race. [26]

Although there is a lack of statistical power in determining the horse plasma EP concentrations and size between time-points, variations of exercise have been shown to increase EV concentration in biological fluids in other studies. For example, acute exercise increases EV concentration in human plasma after a single bout of cycling [27]. Regular exercise has also been shown to increase EV baseline concentration in a mouse model [28]. Regular exercise also increased concentrations of exosomes from heart and serum in mouse models of type 2 diabetes [29]. Our group has previously reported that treadmill acute aerobic exercise increases EV concentration in rat serum [30]. Exercise-induced increases in plasma EP and EV concentration might be explained at least in part by the decrease in plasma volume caused due to fluid shifts during exercise [31] [32] although previous studies have shown that horses' weight loss (mainly caused by dehydration) does not reach 5% of the animal's total weight following endurance races [33]. In addition, water intake is allowed during the race and there are specific points for this on the trails. At the end of each stage (every 30 km on average) the horses are required to rest for 40 minutes and food, electrolytes and water are provided *ad libitum*.

The concentration of EVs in human plasma under normophysiological states is approximately $10^{10}$ EVs per mL [34]. Extracellular vesicle concentration is highly variable depending on the method used to purify and quantify EVs. We detected $5.6x10^{12}$ EPs per mL in horse blood before the race (baseline), which reached a peak at time point T4 of $1.1x10^{14}$ EPs per mL, but

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this value might be overestimated due to the presence of lipoprotein particles and protein aggregates that co-purified and were detected by TRPS methodology. EP concentration measured by qNano may be taking into account all plasma precipitated vesicles, and particles, including exosomes, microvesicles, protein aggregates, and lipoproteins, released during a period of food intake and high tissue regeneration.

Western blotting revealed that time point T3 was enriched in EV proteins (Flotillin-1, CD81, CD9, and TSG101). These results suggest a peak in EV concentration 2 h after the race (T3), which returns to baseline at T4, 15 h after the race. It was previously shown that exosomes returned to the baseline level 90 minutes after exercise on a bicycle; instead, treadmill exercise causes more sustained EVs to return to baseline and may take a few hours to achieve it [27]. Comparing lipoprotein particles, protein complexes and EVs after exercise, EVs are the most studied, mainly as potential biomarkers for metabolic disorders, such as impaired endothelial function and insulin resistance [35]. After exercise, purified EVs in biological fluids may have therapeutic utility in the treatment of obesity and type 2 diabetes mellitus [36]. During exercise, plasma and serum EV levels change after cycling and running in humans [27] and after acute aerobic exercise in rats [30].

Regarding miRNA expression levels after exercise, the impact of exercise on the miRNA population purified from muscle tissue has been extensively studied [37] [38] [39] and total circulating miRNAs in serum or plasma to be used as a marker for maximal oxygen consumption [40] [41]. Extracellular vesicles were described as being functional carriers of miRNAs in circulation in 2007 [42] , and miRNAs delivered by EVs were well described as having essential functions in cancer [43] and metabolic diseases [44] . However, recently, EV miRNA was suggested as being an essential part of the systemic benefits that exercise causes to human health [13] . *In vivo* and *in vitro* studies of circulating EVs found following exercise described enhanced protection against cardiac Ischaemia/Reperfusion injury compared to endogenous EVs purified from before exercise, suggesting that EVs purified after exercise may have potential as a therapy for myocardial injury in the future [28].

We described here high levels of eca-miR-486-5p and 486-3p across all EP samples. It was described that miRNAs hsa-miR-486-5p, 16-5p, 451a, 106a-5p, 17-5p, 93-5p, 20a-5p, 107, and 20b-5p are enriched in red blood cells and can be significantly affected by hemolysis of ~0.05% erythrocytes [45] [46] . The high levels of miR-486 found in all samples, in part, can be explained by haemolysis during blood sampling, but we did not find high expression levels from the other miRNAs. Also, we found high levels of plasma EP eca-miR-30d, 25, and 92a. These miRNAs cofractionated in the latest fractions of size exclusion chromatography, suggesting their association with ribonucleoprotein complexes, such as Argonaute 2 (Ago2), and not with

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extracellular vesicles [15]. After comparing miRNA levels between race time points, we found statistically a significant increase in eca-miR-486-5p at T1 and T4 compared to T0, and a decreasing level of eca-miR-9083 after the end of the race (T3) and 15 h after the end of the race (T4).

MicroRNA 486-5p is conserved between *Equus caballus* and *Homo sapiens* [47]. Human miRNA 486-5p is one of the most downregulated microRNAs in non-small-cell lung cancer [48 49]. Cell culture experiments demonstrated that miR-486-5p could directly bind to the 3′-UTR region of the gene ARHGAP5, which, in low levels, inhibits lung cancer cell migration and invasion [50]. Circulating miR-486 was shown to significantly decrease after a 60-min single bout of steady-state cycling exercise [41]. Another study compared muscle, plasma and plasma exosomal miRNA levels obtained from 10 young men submitted to a single bout of high-intensity interval cycling exercise and found that 9 exosomal miRNAs increased immediately after and 4 h following exercise (miR-1-3p, -16-5p, -222-3p, -23a-3p, -208a-3p, -150-5p, -486-5p, -126-3p, and -378a-5p) [51].

In a previous study of horse whole blood miRNAs after endurance racing, 167 miRNAs were differentially expressed when compared before and after the 160 km race. Many of these miRNAs regulated genes involved in glucose metabolism, fatty acid oxidation, mitochondrion biogenesis, and immune response pathways [52]. It is difficult to be certain about the sources and target tissues of these plasma exercise-induced EPs. We speculate that blood cells, endothelial cells, muscle tissue, and liver are the primary source and target tissues for exercise EPs. Muscle is known to secrete interleukins [53] and recent reviews point to the potential role of muscle as an EV secretory tissue[13].

The current descriptive study adds essential knowledge to the developing field of plasma extracellular vesicles/particles and exercise. The limitations of this study are the lack of statistical power to demonstrate differences in plasma EP concentrations and sizes and we did not take account of circadian rhythm miRNA variations related to long-lasting exercise. However, we showed that endurance racing increases equine plasma EP concentration, with EVs reaching a concentration peak 2 h after the race and returning to baseline 15 h after the race. We also showed increasing levels of eca-miR-486-5p in T1 and T4 compared to T0, and decreasing levels of eca-miR-9083 immediately after the end of the race (T3) and 15 h after the end of the race (T4). Further studies are needed to address the specific EP concentration recovery periods after long-lasting aerobic exercise. Cell culture experiments could help to understand the role of exercise-delivered EP miRNA in target tissues.

**Authors' declaration of interests.**

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No competing interests have been declared.

**Ethical animal research**

The study was approved by the Animal Use Ethics Committee of the University of Brasília, Brazil, protocol number 47819/2012.

**Owner informed consent**

Owners gave consent for their animals' inclusion in the study.

**Data accessibility**

All sequencing files were deposited in Sequence Read Archive (SRA) under the BioProject (PRJNA574867) at https://www.ncbi.nlm.nih.gov/biosample?LinkName=bioproject_biosample_all&from_uid=574867 and will be available following an embargo from the date of publication to allow for commercialisation of research findings.

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**Authorship**

G. Oliveira Jr performed most of the experiments and prepared the manuscript. W. Porto and C. Palu contributed to small RNA sequencing pipeline analysis. L. Pereira contributed to EPs characterisation (western blotting, electron microscopy and resistive pulse sensing analysis). A. Reis contributed to small RNA extraction, and library construction and sequencing. T. Marçola and A. Teixeira Neto contributed to horse blood sampling and preparation of the manuscript. O. Franco and R. Pereira contributed to the experimental design and preparation of the manuscript.

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**Figure legends**

**Fig 1:** Endurance racing increases the horse plasma EP concentration and diameter. (A) Workflow representing the experimental design used to investigate the impact of endurance racing on horse plasma EP miRNAs. (B) Electron micrographs of horse plasma EP purified by Exoquick. Red arrow indicates small extracellular vesicle-like particles, and yellow arrow indicates lipoprotein-like particles. (C) Pooled samples from each time point were used to measure EP size and concentration by tunable resistive pulse sensing (TRPS, QNano). The analysis showed an increase in Eps' average diameter, (D) and concentration. (E) Endurance racing increased horse plasma EP protein average concentration (F) Horse plasma EP protein profile was analyzed by SDS-PAGE 12%. The SDS-PAGE shows well-defined bands from around 10 kDa to 200 kDa in Coomassie blue staining (G) Western blotting showed that horse plasma EPs had no endoplasmic reticulum protein Calnexin, and showed that time point T3 was enriched in EV markers flotillin-1, CD81 and CD9 compared to the other time points. Peripheral blood mononuclear cells lysate were used as positive control (PC), and PBS mixed with laemmli loading buffer was used as negative control (NC).

**Fig 2:** Horse plasma EP small RNA is enriched in time point T3. (A) The concentration of EP small RNAs from the horse plasma was quantified and characterised by 2100 Bioanalyzer (Agilent) using a small RNA chip. EP samples from all time points showed RNA ranging from 20 to 60nt in size and absence of long RNAs. (B) The total yield of EP small RNA was increased at time point T3 compared to time points T0, T1, T2 and T4 ($p<0.001$ in two-way ANOVA, and $p<0.01$ in Tukey's multiple comparison test) (C) Percentage of mappable reads after small RNA sequencing from horse plasma EPs. The first column represents the general profile of reads. The second column represents only the known reads mapped against *Equus caballus* databases (mature miRNA, rRNA, and snRNA).

**Fig 3:** Endurance racing changes the horse plasma EP miRNA. (A) A principal component analysis (PCA) generated with horse plasma EP miRNA reads showed a strong separation between horses from T0, T3, and T4 time points. Horses from time points T1 and T2 were localized with either T0 or T4. (B) Heatmap representing the relative expression of the 30 most abundant *Equus caballus* plasma EP mature miRNAs.

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**Fig 4**: Differential expression analysis from 8 most present horse plasma EP miRNAs during an endurance race. MicroRNA levels obtained during and after the race were compared to before-race levels, aiming to look at differences in their expression. Two microRNAs were differentially present in horse plasma EPs during the race. Eca-miR-486-5p was found increased during the first veterinarian gate-stop (60km, T1) and 15 h after the end of the race (T4), compared to before the race (T0). Eca-miR-9083 was found decreased two hours after the race (T3) and 15 h after the end of the race (T4) ($p<0.05$ two-way ANOVA, $p<0.05$ Dunnett's multiple comparisons test).

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## References


1 Bergero, D., Assenza, A. and Caola, G. Contribution to our knowledge of the physiology and metabolism of endurance horses. *Livestock Production Science* **92**, 167-176 (2005).

2 Schott, H.C. II, Marlin, D.J., Geor, R.J., Holbrook, T.C., Deaton, C.M., Vincent, T., Dacre, K., Schroter, R.C., Jose-Cunilleras, E and Cornelisse, C. Changes in selected physiological and laboratory measurements in elite horses competing in a 160 km endurance ride. *Equine Veterinary Journal* **38**, 37-42 (2006).

3 Nagy, A., Dyson, S. J. and Murray, J. K. A veterinary review of endurance racing as an international competitive sport. *The Veterinary Journal* **194**, 288-293 (2012).

4 Kingston, J. In: *Equine Sports Medicine and Surgery* pp 1295-1296 (Elsevier, 2004).

5 Carlson, G. P., Ocen, P. O. and Harrold, D. Clinicopathologic alterations in normal and exhausted endurance horses. *Theriogenology* **6**, 93-104 (1976).

6 Ferraz, G. d. C., Teixeira, N., D'Angelis, F. d. F., Lacerda, N. and Queiroz, N. Hematological and cardiac alterations in Arabian horses submitted to incremental effort test in treadmill. *Brazilian Journal of Veterinary Research and Animal Science* **46**, 431-437 (2009).

7 Cywińska, A. *et al.* Acute phase protein concentrations after limited distance and long distance endurance rides in horses. *Research in veterinary science* **93**, 1402-1406 (2012).

8 Trigo, P., Castejon, F., Riber, C. and Muñoz, A. Use of biochemical parameters to predict metabolic elimination in endurance rides. *Equine Veterinary Journal* **42**, 142-146 (2010).

9 Noleto, P. G. *et al.* Effect of a 130-km endurance ride on the serum biochemical profile of Mangalarga Marchador horses. *Journal of Equine Veterinary Science* **39**, 7-11 (2016).

10 Mitchell, P. S. *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences* **105**, 10513-10518 (2008).

11 Cortez, M. A. and Calin, G. A. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. *Expert opinion on biological therapy* **9**, 703-711 (2009).

12 Sohel, M. H. Extracellular/circulating microRNAs: release mechanisms, functions and challenges. *Achievements in the Life Sciences* **10**, 175-186 (2016).

13 Safdar, A. and Tarnopolsky, M. A. Exosomes as Mediators of the Systemic Adaptations to Endurance Exercise. *Cold Spring Harbor Perspectives in Medicine*, a029827 (2017).

14 Vickers, K. C., Palmisano, B. T., Shoucri, B. M., Shamburek, R. D. and Remaley, A. T. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nature cell biology* **13**, 423 (2011).


This article is protected by copyright. All rights reserved

15 Arroyo, J. D. *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences* **108**, 5003-5008 (2011).

16 Raposo, G. and Stoorvogel, W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* **200**, 373-383 (2013).

17 Lobb, R. J. *et al.* Optimized exosome isolation protocol for cell culture supernatant and human plasma. *Journal of extracellular vesicles* **4**, 27031 (2015).

18 Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of extracellular vesicles* **7**, 1535750 (2018).

19 Sapp, R. M., Shill, D. D., Roth, S. M. and Hagberg, J. M. Circulating microRNAs in acute and chronic exercise: more than mere biomarkers. *Journal of Applied Physiology* **122**, 702-717 (2016).

20 Wang, H. and Wang, B. Extracellular vesicle microRNAs mediate skeletal muscle myogenesis and disease. *Biomedical reports* **5**, 296-300 (2016).

21 Whitham, M. *et al.* Extracellular vesicles provide a means for tissue crosstalk during exercise. *Cell metabolism* **27**, 237-251. e234 (2018).

22 Lässer, C., Eldh, M. and Lötvall, J. Isolation and characterization of RNA-containing exosomes. *JoVE (Journal of Visualized Experiments)*, e3037-e3037 (2012).

23 Hawley, J. A. and Holloszy, J. O. Exercise: it's the real thing! *Nutrition reviews* **67**, 172-178 (2009).

24 Fernández-Hernando, C., Ramírez, C. M., Goedeke, L. and Suárez, Y. MicroRNAs in metabolic disease. *Arteriosclerosis, thrombosis, and vascular biology* **33**, 178-185 (2013).

25 Reddy, K. B. MicroRNA (miRNA) in cancer. *Cancer cell international* **15**, 38 (2015).

26 Flethøj, M. *et al.* Changes in heart rate, arrhythmia frequency, and cardiac biomarker values in horses during recovery after a long-distance endurance ride. *Journal of the American Veterinary Medical Association* **248**, 1034-1042 (2016).

27 Frühbeis, C., Helmig, S., Tug, S., Simon, P. and Krämer-Albers, E.-M. Physical exercise induces rapid release of small extracellular vesicles into the circulation. *Journal of extracellular vesicles* **4** (2015).

28 Bei, Y. *et al.* Exercise-induced circulating extracellular vesicles protect against cardiac ischemia–reperfusion injury. *Basic Research in Cardiology* **112**, 38 (2017).

29 Chaturvedi, P., Kalani, A., Medina, I., Familtseva, A. and Tyagi, S. C. Cardiosome mediated regulation of MMP9 in diabetic heart: role of mir29b and mir455 in exercise. *Journal of cellular and molecular medicine* **19**, 2153-2161 (2015).

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This article is protected by copyright. All rights reserved

30 Oliveira Jr, G. P. *et al.* Effects of Acute Aerobic Exercise on Rats Serum Extracellular Vesicles Diameter, Concentration and Small RNAs Content. *Frontiers in Physiology* **9** (2018).

31 Kawabata, T., Suzuki, T. and Miyagawa, T. Effect of blood volume on plasma volume shift during exercise. *Journal of Thermal Biology* **29**, 775-778 (2004).

32 Convertino, V. A. Blood volume response to physical activity and inactivity. *The American journal of the medical sciences* **334**, 72-79 (2007).

33 Conceição, N., Fatima, C., Duarte, P., Manhaes, P. and Teixeira, N. Percentual of body weight loss of horses submitted to endurance exercise in west center of Brazil. *Ars Veterinaria* **30**, 19-22 (2014).

34 Johnsen, K. B., Gudbergsson, J. M., Andresen, T. L. and Simonsen, J. B. What is the blood concentration of extracellular vesicles? Implications for the use of extracellular vesicles as blood-borne biomarkers of cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* (2018).

35 Eichner, N. Z., Erdbrügger, U. and Malin, S. K. Extracellular Vesicles: A Novel Target for Exercise-Mediated Reductions in Type 2 Diabetes and Cardiovascular Disease Risk. *Journal of diabetes research* **2018** (2018).

36 Safdar, A., Saleem, A. and Tarnopolsky, M. A. The potential of endurance exercise-derived exosomes to treat metabolic diseases. *Nature Reviews Endocrinology* (2016).

37 D'Souza, R. F. *et al.* Acute resistance exercise modulates microRNA expression profiles: Combined tissue and circulatory targeted analyses. *PloS one* **12**, e0181594 (2017).

38 McCarthy, J. J. and Esser, K. A. MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy. *Journal of applied physiology* (2007).

39 Camera, D. M., Ong, J. N., Coffey, V. G. and Hawley, J. A. Selective modulation of microRNA expression with protein ingestion following concurrent resistance and endurance exercise in human skeletal muscle. *Frontiers in physiology* **7**, 87 (2016).

40 Baggish, A. L. *et al.* Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. *The Journal of physiology* **589**, 3983-3994 (2011).

41 Aoi, W. *et al.* Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men. *Frontiers in physiology* **4**, 80 (2013).

42 Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology* **9**, 654 (2007).

43 Zhang, X. *et al.* Exosomes in cancer: small particle, big player. *Journal of Hematology & Oncology* **8**, 83 (2015).

This article is protected by copyright. All rights reserved

44 Guay, C. and Regazzi, R. Exosomes as new players in metabolic organ cross-talk. *Diabetes, Obesity and Metabolism* **19**, 137-146 (2017).

45 Pritchard, C. C. *et al.* Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer prevention research* **5**, 492-497 (2012).

46 Shkurnikov, M. Y. *et al.* Analysis of Plasma microRNA Associated with Hemolysis. *Bulletin of experimental biology and medicine* **160**, 748-750 (2016).

47 Kozomara, A., Birgaoanu, M. and Griffiths-Jones, S. miRBase: from microRNA sequences to function. *Nucleic acids research* **47**, D155-D162 (2018).

48 Zhu, J. *et al.* Expression profile analysis of microRNAs and downregulated miR-486-5p and miR-30a-5p in non-small cell lung cancer. *Oncology reports* **34**, 1779-1786 (2015).

49 Tian, F. *et al.* Aberrant miR-181b-5p and miR-486-5p expression in serum and tissue of non-small cell lung cancer. *Gene* **591**, 338-343 (2016).

50 Wang, J. *et al.* Downregulation of miR-486-5p contributes to tumor progression and metastasis by targeting protumorigenic ARHGAP5 in lung cancer. *Oncogene* **33**, 1181 (2014).

51 D'Souza, R. F. *et al.* Circulatory exosomal miRNA following intense exercise is unrelated to muscle and plasma miRNA abundances. *American Journal of Physiology-Endocrinology and Metabolism* **315**, E723-E733 (2018).

52 Mach, N. *et al.* Integrated mRNA and miRNA expression profiling in blood reveals candidate biomarkers associated with endurance exercise in the horse. *Scientific reports* **6** (2016).

53 Trovato, E., Di Felice, V. and Barone, R. Extracellular Vesicles: delivery vehicles of myokines. *Frontiers in Physiology* **10**, 522 (2019).

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| Samples | Total reads | Trimmed and Filtered Reads | Mappable reads % | miRNA Mapped reads | miRNAs % | rRNA mapped reads | rRNA % | snRNA mapped reads | snRNA % |
|---|---|---|---|---|---|---|---|---|---|
| **H1T0** | 1039511 | 68151 | 6.6 | 7129 | 10.5 | 680 | 1 | 2491 | 3.7 |
| **H1T1** | 1423349 | 291957 | 20.5 | 9758 | 3.3 | 1492 | 0.5 | 3676 | 1.3 |
| **H1T2** | 970272 | 79366 | 8.2 | 12112 | 15.3 | 274 | 0.3 | 697 | 0.9 |
| **H1T4** | 1321186 | 259290 | 19.6 | 5638 | 2.2 | 2220 | 0.9 | 255 | 0.1 |
| **H2T0** | 1451794 | 215600 | 14.9 | 4039 | 1.9 | 30653 | 14.2 | 13175 | 6.1 |
| **H2T1** | 1049473 | 222868 | 21.2 | 8152 | 3.7 | 644 | 0.3 | 3228 | 1.5 |
| **H2T2** | 1389314 | 291126 | 21 | 2541 | 0.9 | 4622 | 1.6 | 123 | 0 |
| **H2T3** | 905388 | 119138 | 13.2 | 1617 | 1.4 | 1014 | 0.9 | 155 | 0.1 |
| **H2T4** | 1117307 | 165401 | 14.8 | 5336 | 3.2 | 617 | 0.4 | 70 | 0 |
| **H3T0** | 112438 | 15982 | 14.2 | 588 | 3.7 | 1284 | 9.1 | 651 | 4.8 |
| **H3T1** | 697061 | 129008 | 18.5 | 1473 | 1.1 | 6699 | 5.5 | 2031 | 1.7 |
| **H3T2** | 827697 | 133106 | 16.1 | 4152 | 3.1 | 1917 | 1.5 | 192 | 0.2 |
| **H3T3** | 1765077 | 95847 | 5.4 | 1604 | 1.7 | 105 | 0.1 | 33 | 0 |
| **H3T4** | 932706 | 225712 | 24.2 | 5736 | 2.5 | 685 | 0.3 | 374 | 0.2 |
| **H4T0** | 987823 | 91399 | 9.3 | 1036 | 1.1 | 7008 | 8.4 | 381 | 0.5 |
| **H4T1** | 1362729 | 223276 | 16.4 | 5084 | 2.3 | 2963 | 1.4 | 2265 | 1.1 |
| **H4T2** | 1109392 | 182828 | 16.5 | 1485 | 0.8 | 476 | 0.3 | 28 | 0 |
| **H4T3** | 2028554 | 119152 | 5.9 | 1060 | 0.9 | 584 | 0.5 | 72 | 0.1 |
| **H4T4** | 1563592 | 158658 | 10.1 | 5556 | 3.5 | 592 | 0.4 | 108 | 0.1 |
| **H5T0** | 1431863 | 105843 | 7.4 | 4120 | 3.9 | 2496 | 2.5 | 349 | 0.4 |
| **H5T1** | 1154555 | 149202 | 12.9 | 9877 | 6.6 | 503 | 0.4 | 1518 | 1.1 |
| **H5T2** | 1147142 | 90537 | 7.9 | 1478 | 1.6 | 370 | 0.4 | 32 | 0 |
| **H5T3** | 625885 | 69395 | 11.1 | 6026 | 8.7 | 456 | 0.7 | 117 | 0.2 |
| **H5T4** | 1198794 | 201356 | 16.8 | 8566 | 4.3 | 996 | 0.5 | 203 | 0.1 |
| **Total** | 27612902 | 3704198 | 13.4 | 114163 | 3.1 | 69350 | 1.9 | 32224 | 0.9 |

**Table 1:** Raw read numbers obtained after sequencing, filtering, trimming, and alignment to *Equus caballus* mature miRNA database, available at miRBase.org. Overall, 13.4% of the total reads were considered mappable reads (after filtering and trimming). Reads that did not align with the previous database were aligned to the following one (rRNA and snRNA). The

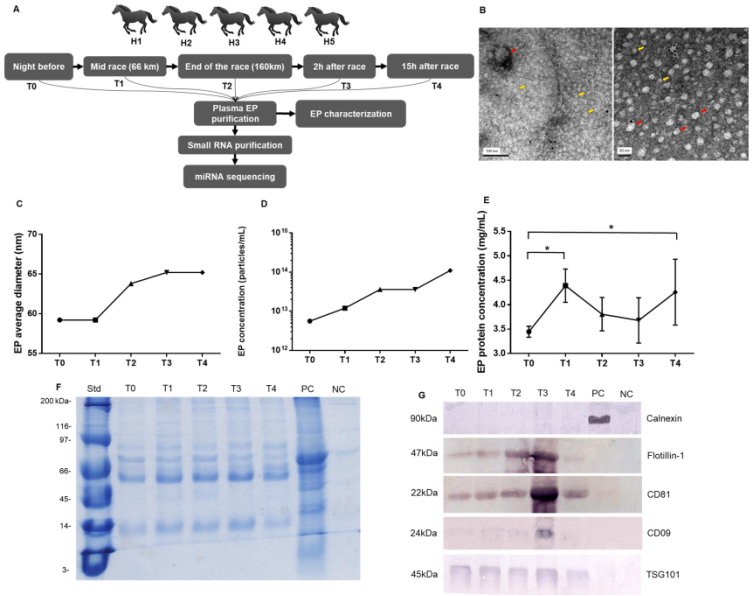
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percentages of mappable reads aligned to microRNAs databases were 3.1%, rRNA 1.9%, and snRNA 0.9%.

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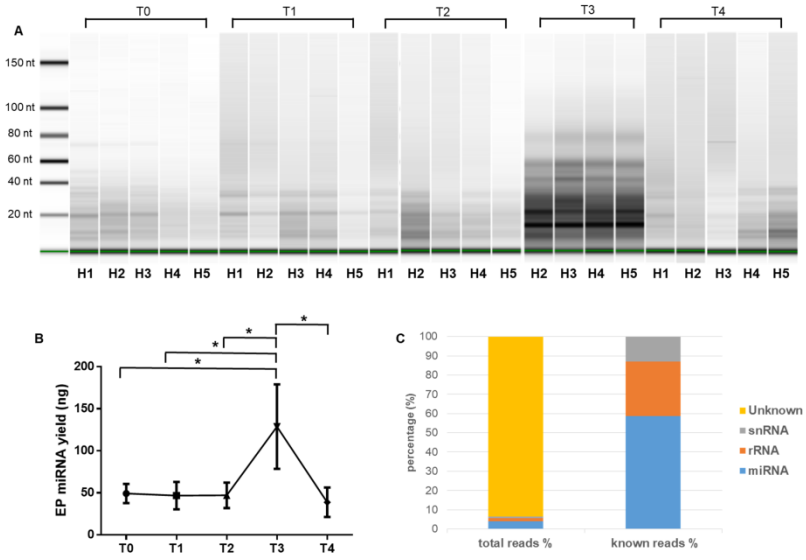
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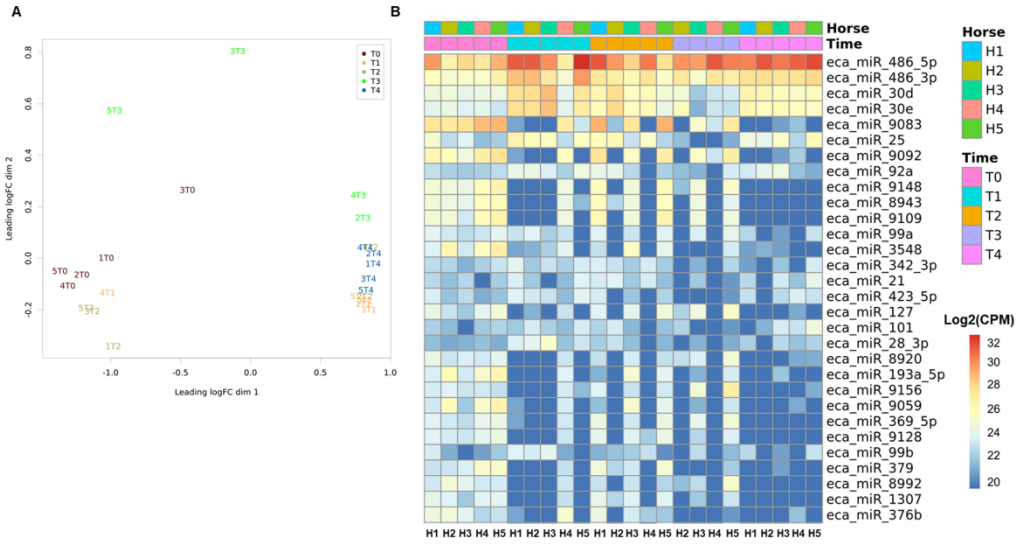
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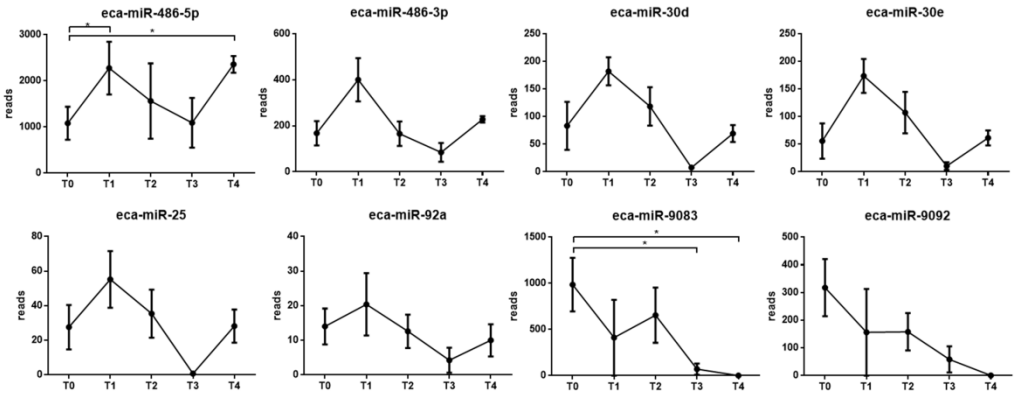
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