Circulating inflammatory miRNA signature in response to different doses of aerobic exercise

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miRs are small noncoding RNAs that control gene expression at the posttranscriptional level by degrading or inhibiting mRNA translation. miRs play a critical role in normal development, homeostasis, and the cellular response to physiologic and pathophysiologic stress (17, 39). Indeed, they have been described as fine-tuning regulators of many biological processes in both health and disease, including exercise and inflammation. Both acute and chronic exercise alters the intracellular profile of miRs in skeletal muscle and in the immune and cardiovascular systems (14, 38, 69). Previous studies have highlighted the role of miRs in exercise-induced adaptation, including skeletal muscle hypertrophy, remodeling and metabolism (15, 66), angiogenesis (34) and cardiac structural alterations (16). A reciprocal association between inflammation and miRs has been reported. In vitro and in vivo investigations have demonstrated that inflammatory stimuli activate miRNA biogenesis (73). Genetic ablation of miRNA biogenesis induces inflammatory perturbations (65). Given their dynamic nature, miRs have been proposed to participate in the optimization of the inflammatory response and the maintenance of homeostasis (36). The cellular pathways altered by inflamma-

circulating microRNAs; exercise; inflammation
tion-related miRs range from cytokine synthesis to leukocyte activation (48). Interestingly, evidence indicates a miR-mediated link between exercise and inflammation; brief bouts of intense exercise alter the miR profile of mononuclear leukocytes, many of which are involved in inflammatory mechanisms (59).

Relatively recent studies have demonstrated that miRs can be detected in body fluids (41). miRs are actively secreted in extracellular vesicles (81) or associated with RNA-binding proteins (3) or lipoproteins (76). Importantly, recently reported data point to extracellular miRs as key regulatory signals in cell-cell communication (25). Cells selectively release specific miRs, which, like other soluble factors such as cytokines or hormones, function as autocrine, paracrine, or endocrine factors by mediating intercellular gene regulation and phenotypic control (9).

Altered c-miR profiles have been reported after both acute endurance and resistance exercise and chronic exercise interventions (4, 67). However, most studies have analyzed a limited selection of miRs previously described as mediators of physiological processes linked to exercise adaptations in skeletal muscle, heart, or vasculature (2, 4–6, 21, 43, 75). Given the physiological importance of the exercise-induced inflammatory response in health and training adaptation, the roles of miRs as intercellular communicators and regulators of physiological responses, and their potential as biomarkers, more detailed investigation is warranted. To date no studies have specifically examined the global response of circulating inflammation-related miRs (c-inflammamiRs) to acute exercise. Moreover, any analysis of exercise-induced changes in c-inflammamiRs should consider the magnitude of the inflammatory response to acute exercise. The goal of the present study was to analyze the response of a panel of c-inflammamiRs to different doses of acute aerobic exercise to further our understanding of the role of c-inflammamiRs in exercise-induced inflammation.

MATERIALS AND METHODS

Ethics statement. All experimental procedures were approved by the respective Research Ethics Committees of the Centro de Estudios Universitarios San Pablo University and the University of Oviedo, in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Experimental design. An observational experimental design was used. Participants completed three races: a 10-km race (10-km), a half-marathon (HM), and a marathon (M), each separated by 1 mo. Although all trials involved the same type of exercise (endurance running), they differed in terms of duration, intensity, and energy demands and thus represent distinct exercise doses (8, 12, 40, 68).

Participants. Volunteers were recruited from the members of MAPOMA Sports Association, a sports club open to amateur and professional runners who wish to participate in the popular Madrid marathon. Figure 1 shows a schematic overview of participant recruitment. All runners in the amateur training group (n = 35) were invited to an informative briefing. A member of the research team presented the aims and methodology of the study and answered the questions of the potential participants, of whom 18 (51%) agreed to participate. The remainder (n = 17) showed interest in the study but were unable to participate for various reasons (family and professional commitments, travel, or injuries). Before participation, each volunteer underwent a thorough medical screening to determine eligibility. A number of inclusion and exclusion criteria were established. The inclusion criteria were as follows: 1) men over 18 yr (only men were selected for their more stable hormonal status); 2) no smokers or frequent passive smokers; 3) officially registered for the Madrid marathon; 4) regular training (at least 50 km/wk); 5) previous participation in at least two marathons; and 6) written informed consent. The exclusion criteria were as follows: 1) any chronic disease; 2) body mass index (BMI) >30 kg/m²; and 3) any dietary or pharmacological treatment during the course of the study. Although 14 volunteers fulfilled these criteria and initially participated in the study, only 9 participants completed the study. The remaining five participants were unable to finish at least one of the races or missed one of the blood extractions and were excluded.

Assessment of body composition and aerobic capacity. Two weeks before the first race, the participants had their body composition and maximal oxygen uptake (V\text{O}_2\text{max}) assessed at the Physiology of Effort Unit. Two ISAK Level III-certified anthropometrists measured height and body mass using a combined medical scale (model 778; Seca, Hamburg, Germany; precision: 0.1 cm for height and 0.1 kg for weight). BMI was then calculated from these measurements. The equation of Kyle et al. (32) was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance device (Total Body Scan; Bio-Logic). This equation is considered the most appropriate by the Spanish Group of Kinanthropometry (1).

V\text{O}_2\text{max} was determined by indirect calorimetry (Oxycon Pro; Jaeger) using an incremental protocol on a treadmill (LE- 600 C; Jaeger-HP Cosmos).

Training schedule and training history. During their visit to the laboratory, the volunteers were interviewed about their training volume over the previous 3 mo and their training history. Participants were asked not to alter their usual training schedule, and all performed low-intensity training the day before each race.
**Blood sampling.** Four blood samples were collected for each race: one before and three at various stages after the end of the exercise bout. Identical sampling time points were used for all races. Samples were collected by experienced technical staff, using standardized techniques and materials. The first blood sample (Basal) was collected from fasting participants ~1.5 h before the race before warm-up. Participants then ate their breakfast, performed their warm-up exercises, and began the race. A second blood sample (Post0) was drawn within 10 min of completing the race. In both cases, samples were collected and processed in a field laboratory near the race start and finish lines.

The following morning (Post24) and 3 days after the race (Post72), participants reported to the Clinical Laboratory at the Center for Sports Medicine after overnight fasting for collection of additional samples. These samples were taken at the same time of day as basal sample. The total volume of blood taken per race was <50 ml.

For complete blood counts (total leukocytes, neutrophils, lymphocytes, monocytes, and eosinophils) and lymphocyte subsets analysis, blood was collected in EDTA vacuum blood tubes (Becton Dickinson, Franklin Lakes, NY), stored at room temperature (20–25°C), and analyzed within 4 h. For serum separation, blood samples were collected in vacutainers [No Additive (Z); Becton Dickinson, Franklin Lakes, NJ]. Samples were stored at room temperature for at least 15 min to allow clot formation and then immediately centrifuged for 15 min at 4,000 rpm and 10°C. Serum samples were then aliquoted and stored at −80°C for later analysis.

**Dietary control.** Participants were asked to keep a food diary for 5 consecutive days: 2 days before the first race, the race day, and the 2 following days. They were specifically asked not to alter their usual dietary pattern during the recording periods but were asked to abstain from caffeine and alcohol consumption for at least 24 h before race. No limitations on the type or amount of food or beverages consumed were imposed at any time during these 5 days of food recording. All foods and beverages consumed were recorded using standard culinary measures (44), and food labels were collected for information on food supplements, snacks, and packaged foods. No participants reported using nutritional supplements. Volunteers received specific oral guidelines and detailed written instructions on how to put this method into practice. A telephone number was made available to answer any queries about the dietary recording process.

To minimize the impact of food intake on results, volunteers were asked to apply the same dietary regimen on the days before and after the races and to keep a food diary, following the same instructions given for the first race.

Food records were carefully reviewed immediately after completion, and participants were contacted to clarify any ambiguities. Dietary records were analyzed using nutrient analysis software (DIAL: Alce Ingenieria).

**Leukocyte counts and biochemical determinations.** Complete blood counts were determined by standardized clinical laboratory procedures using an automated hematology analyzer (ADVIA 120; Siemens Healthcare Diagnostics).

Whole blood samples (100 μl) were incubated in darkness at room temperature (20–25°C) for 15 min with 10 μl of specific fluorescent-conjugated monoclonal antibodies (Becton Dickinson Biosciences) for subsequent evaluation of the CD16+CD56+ natural killer (NK) subset by flow cytometry. Next, 2 ml of lysing solution (Becton Dickinson Biosciences) were added to lyse red blood cells and the samples were mixed gently and incubated for 20 min in darkness at room temperature. Immediately after incubation, tubes were centrifuged for 5 min at 330 g and 4°C and the supernatant was removed. Samples were washed twice with phosphate-buffered saline (PBS) and centrifuged for 5 min (300 g), and the supernatant was removed at the end of each step. Finally, 500 μl of PBS were added to each tube. The samples were also incubated with the corresponding control. Fluorescence was analyzed using a FACSCALIBUR cytometer (Becton Dickinson).

High-sensitivity C-reactive protein (hs-CRP) and creatine kinase (CK) were analyzed in serum samples using an AU400 clinical analyzer (Beckman Coulter) and Beckman reagents. Cytokines (IL-1β, IL-6, IL-8, IL-10, and TNF-α) were assayed using an IMMULITE ONE analyzer (Siemens Healthcare Diagnostics) and proprietary cytokine kits.

**RNA isolation and quantitative RT-PCR.** Total circulating RNA was isolated from 200 μl of serum using the miRCURY RNA isolation kit (Exiqon), following the manufacturer’s instructions. RNA spike-in kit (Exiqon) was used in all extractions. For RNA extraction, samples were supplemented with 5 fmol of Caenorhabditis elegans miR-39-3p (cel-miR-39-3p) for normalization of the data. For miRNA quantification, cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon) and miRNAs were quantified by quantitative real-time PCR (qRT-PCR) using the ExiLINTE SYBR green master mix (Exiqon) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). To rule out hemolysis or the presence of nucleases or inhibitors, the miRCURY miRNA Quality Control PCR Panel (Exiqon) was used before miRNA analysis. This panel (Exiqon) includes specific primers to analyze miR-23a and miR-451, which are normally found in plasma and serum and serve as markers of hemolysis.

**Quantification of circulating miRNAs.** miRNA quantitative analysis was restricted to a panel of 106 circulating miRs whose roles in inflammatory processes have been previously demonstrated. Those miRs were chosen after an extensive review of the literature. Specifically, we chose miRs implicated in the regulation of cytokine synthesis and inflammatory pathways and in leukocyte proliferation, differentiation, and activation (36–38, 48, 49, 79). Candidates were also selected based on previous studies of extracellular inflammation-related miRs in pathological conditions (9, 19, 22, 26, 30, 50). Some of those miRs (miR-126, -143, -146a, -155, -21, -221, and -223) have also been previously studied in exercise-related studies (4–6, 43, 46, 67, 75). To avoid potential bias due to controversy in miR nomenclature, we evaluated -3p and -5p chains. Expression of circulating miRNA was normalized to that of cel-miR-39-3p and relative expression analysis was performed using GenEx software (MultiD Analyses AB).

**Functional in silico analysis.** In silico identification of molecular pathways potentially affected by the observed alterations in c-inflammation levels was performed using the web-based computational tool DIANA-miRPath v2.0 (77). DIANA-miRPath v2.0 utilizes experimentally verified miRNA targets from TarBase v6 and predicted miRNA targets from the DIANA-microT-CDS algorithm and combines the results with the pathway tool KEGG (Kyoto Encyclopedia of Genes and Genomes) to identify possible targets. The level of significance was set at P < 0.05.

**Statistical analysis.** Descriptive statistics were used to characterize study populations and to analyze the studied parameters. Normality was determined using the Kolmogorov-Smirnov test. One-way ANOVA for repeated measures with Bonferroni correction was used to compare inflammatory parameters between races (10-km, HM, and M) and sampling time points (Basal, Post0, Post24, and Post72). Fold change with respect to the Basal sample was determined to quantify the magnitude of the inflammatory response. Data are presented as the means ± SE. Correlations between variables were analyzed using Pearson’s correlation analysis, and the results are presented using Pearson’s correlation coefficient and 95% confidence interval [p (95% CI)]. Some samples showed concentrations that were below the limit of detection (cytokines IL-6, IL-8, IL-10, and TNF-α in Basal, Post24, and Post72). The limit of detection was used for statistical purposes. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Chicago, IL). Differences were considered statistically significant at P < 0.05.

For miRNA analysis, each detected miRNA was analyzed using the following test: H_0: \( \mu_{i} = \mu_{24h,i} \) and H_1: \( \mu_{i} < \mu_{24h,i} \), where \( \mu_{i} \) and \( \mu_{24h,i} \) are the means of the i-th miRNA (1 ≤ i ≤ 81) at Post0 and Post24. Data were analyzed using repeated measures
Table 1. Physical characteristics and training profile of participants

<table>
<thead>
<tr>
<th>Anthropometric/Training Profile</th>
<th>Physical characteristics</th>
<th>Training habits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>39.1 ± 2.2</td>
<td>Training history, yr: 6.6 ± 1.3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>175.2 ± 1.6</td>
<td>Training volume, km/wk: 69.7 ± 5.0</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>76.6 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>%BF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.3 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;O2max&lt;/sub&gt;, ml·kg&lt;sup&gt;–1&lt;/sup&gt;·min&lt;sup&gt;–1&lt;/sup&gt;</td>
<td>59.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Training habits</td>
<td></td>
<td></td>
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<tr>
<td>Training history, yr</td>
<td></td>
<td></td>
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<tr>
<td>Training volume, km/wk</td>
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</tbody>
</table>

Data represent means ± SE. BMI, body mass index; %BF, percent body fat; V<sub>O2max</sub>, maximal oxygen uptake. *The equation of Kyle et al. (32) was used to estimate percent body fat (%BF) based on the information obtained using a multifrequency bioimpedance device.

ANOVA and P values were adjusted using the false discovery rate (FDR) criterion, applying the Benjamini-Hochberg method. For miRNAs with a FDR < 0.05, we analyzed the difference between each pair; these differences were considered relevant if they fulfilled the following criteria: 1) P < 0.050 (paired Student’s t-test) and 2) a difference between means of >1.5. A customized R (www.r-project.org) function was used for all analyses.

RESULTS

Physical characteristics, training habits, and dietary control. Anthropometric data and the training practices and training history of the participants are presented in Table 1. The overall profile of the participants is one of middle-aged, slightly overweight, experienced amateur runners with moderately high workloads. No differences in the daily energy and macronutrient intake of participants were observed over 5 consecutive days before, during, and after each race (data not shown).

Classical inflammatory response. Based on their demonstrated role in exercise-induced inflammatory responses, a panel of cellular and soluble inflammatory markers was chosen for comparative analysis. Total leukocyte and neutrophil counts increased immediately after all races, as expected (Fig. 2, A and B). Twenty-four hours after the 10-km and HM races, leukocyte and neutrophil counts in samples had returned to preexercise levels. After the marathon, leukocyte and neutrophil counts returned to basal levels in less than 24 and 72 h, respectively. Lymphocyte and monocyte counts increased significantly immediately after 10-km and M races, respectively. Both parameters returned to basal levels in less than 24 h (Fig. 2, C and D). Significant perturbations in eosinophil [Basal vs. Post0, 10-km: 160 ± 3 vs. 110 ± 3 (P < 0.010); HM: 190 ± 3 vs. 100 ± 3 (P < 0.050); M: 120 ± 2 vs. 60 ± 1 (P < 0.050)] and NK counts [Basal vs. Post0, 10-km: 433.62 ± 59.30 vs. 801.15 ± 78.18 (P < 0.010)] were also observed.

Levels of the soluble inflammatory mediators IL-6, IL-8, and IL-10 increased immediately after the M race (Fig. 2, E–G). IL-6 and IL-10 levels returned to basal levels within 24 h. By contrast, IL-8 levels remained elevated at 24 h and had returned to basal levels by 72 h. In HM samples levels of IL-6 and IL-10, but not IL-8, were increased immediately after the race, and returned to preexercise levels within 24 h (Fig. 2, E and G). hs-CRP levels were increased in HM and M samples at 24 and 72 h (Fig. 2H). Soluble inflammatory mediators showed no significant changes at any time point after the 10-km race. No differences in circulating TNF-α levels were observed for any race (data not shown). IL-1β levels were below the limit of detection for all races and time points.

Based on the kinetics observed and to further characterize the magnitude of the inflammatory response for each race, we compared the Post0-Basal fold change between the three races. Significantly greater fold changes in leukocyte, neutrophil, and monocyte counts and IL-8 levels were observed in M samples compared with both 10-km and HM samples (Table 2). Significant differences were also observed for IL-6 and IL-10 in M vs. 10-km samples; IL-6, IL-8, and IL-10 in HM vs. 10-km samples; and lymphocyte count in 10-km vs. M samples (Table 2). As expected, hs-CRP showed delayed kinetics with respect to other inflammatory parameters. Analysis of the Post24-Basal fold change for this parameter revealed a significant increase in M samples compared with both 10-km and HM samples and in HM vs. 10-km samples.

Previous studies have demonstrated a close and direct association between muscle damage and exacerbated exercise-induced inflammatory responses (31, 53). We thus analyzed levels of the muscular damage marker CK in all three races. For HM and M races, circulating CK levels were increased at Post0, Post24, and Post72 with respect to basal levels (Table 3). CK increases were also observed at Post0 and Post24 after the 10-km race (Table 3). For all races the greatest increases were detected at Post24: significant increases were observed for M vs. both 10-km and HM and for HM vs. 10-km.

Circulating inflamma-miR response. Once we characterized the inflammatory response for each of the different races we evaluated the profile of a panel of circulating inflammation-related miRs. To avoid the confounding influence of erythrocyte miRNAs released due to hemolysis we first analyzed miR-23a and miR-451. In all samples, the ΔCt values for these two miRNAs were far below 7 (data not shown), ruling out the possibility of hemolysis. c-inflammamiR profiles were determined for the 10-km (low inflammatory response) and M (high inflammatory response) races by analyzing Basal, Post0, and Post24 samples. Due to the possible confounding effects of factors such as diet, lifestyle, training, and recovery on c-miR levels we did not analyze Post72 samples. c-miR-150 levels were significantly increased (2.7-fold) after the 10-km race (Fig. 3A). Significant increases in the levels of c-let-7d-3p, -7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -223-5p, -29a-3p, 34a-5p, -424-3p, and -424-5p were observed after the M race (Fig. 3B). c-miR-223-5p showed the greatest increase (4.7-fold). All c-miRs returned to basal levels by 24 h postrace. Significant correlations were detected between c-inflammamiRs whose expression was significantly increased after the M race, except for c-miR-424-3p (data not shown).

A total of 80 and 69 c-inflammamiRs showed no differences in expression after the 10-km and M races, respectively, with respect to corresponding basal levels (Table 4). Twenty-five c-inflammamiRs with Ct values >35 or whose replicates showed inconsistent results were excluded from further analysis (Table 4).

Based on our findings of increased muscle damage in response to exercise and to further characterize the c-inflammamiR response to exercise, we analyzed levels of four circulating myomiRs: c-miR-1, -133a, -133b, and -206. Results re-
Correlations between inflammatory parameters and c-inflammamiRs. To further explore the association between classical inflammatory response induced by exercise and the observed c-inflammamiR alterations, we correlated alterations in the levels of inflammatory variables with those in c-inflammamiR expression. Increases in miR-150-5p expression immediately after the 10-km race were directly correlated with increased leukocyte [0.715 (0.100 – 0.930)] and neutrophil [0.747 (0.170 – 0.940)] counts during the race. Increased let-7f-2-3p expression immediately after the M race was directly correlated with increased hs-CRP levels during Post24-Post0 [0.704 (0.080 – 0.930)]. The observed increase in miR-29-3p expression after the M race was inversely correlated with the fold change in IL-10 during the Post24-Post0 recovery period [-0.682 (−0.930; −0.030)]. A weak association was observed between the increases in miR-125b-5p and miR-34-5p expression during the M race and the decreases in hs-CRP [0.661 (−0.010; 0.920)] and IL-6 [0.676 (−0.060; 0.930)], respectively, during the recovery phase.

Pathways targeted by c-inflammamiRs. In silico analysis was performed to identify the biological mechanisms, pathways, and functions most closely linked with the observed c-inflammamiR profile. The DIANA-miRPath v.2.0 computational tool was used to analyze verified and predicted molecular pathways that may have been altered in response to the c-inflammamiR profile observed after the M race (77). Let-7d-3p, let-7f-2-3p, and miR-223-5p were not experimentally verified to target any gene, while miR-424-3p was verified to target...
Characterization of the inflammatory response in each of the different races (10-km, HM, and M) showed that, as expected (78), aerobic exercise of sufficient duration and intensity induces an inflammatory response, followed by a recovery period over the next 24 h during which most inflammatory parameters return to basal levels. The magnitude of the inflammatory response differed between races. We observed significant alterations in leukocyte subsets and in the levels of soluble inflammatory mediators (IL-6 IL-8, IL-10, and hs-CRP) after the M race, in line with previous studies that have analyzed the inflammatory response after prolonged or exacerbated acute exercise (7, 31, 51). These increases were significantly higher than those seen after 10-km and HM races. Samples taken after the HM race revealed a greater inflammatory response than seen after the 10-km race, particularly in the levels of soluble inflammatory mediators. Taken together, our results indicate a clear dose-dependent effect of aerobic exercise on systemic inflammation.

After analyzing the exercise-induced inflammatory response, we next characterized the c-inflammamiR profile after the races with lowest (10-km) and highest (M) inflammatory responses. Interestingly, we detected specific exercise dose-dependent c-inflammamiR responses. miR-150-5p was the only miR from the c-inflammamiR panel for which significant changes in expression were found after the 10-km race. This response differed substantially to that observed after the M race, which induced alterations in 12 c-inflammamiRs (let-7d-3p, -7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -29a-3p, -34a-5p, -424-3p, -424-5p) (P < 0.050). Most pathways were related to cancer, immune system disease, and inflammation. The let-7d-3p was predicted to target three genes and thus was not considered for further analysis. Fifty-six KEGG pathways were enriched with the predicted targets of c-inflammamiRs let-7f-2-3p, miR-125b-5p, -132-3p, -143-3p, -148a-3p, -223-3p, -29a-3p, -34a-5p, -424-3p, and -424-5p (P < 0.050). A number of inflammation-related pathways were identified, including the PI3K-Akt signaling pathway, the TGF-β signaling pathway, the Wnt signaling pathway, the MAPK signaling pathway, the adipokinecytokine signaling pathway, the mammalian target of rapamycin signaling pathway, the B-cell receptor signaling pathway, the p53 signaling pathway, the hypoxia-inducible factor-1 signaling pathway, the T-cell receptor signaling pathway, the VEGF signaling pathway, and the Hedgehog signaling pathway.

**DISCUSSION**

The close association between inflammation and physical activity has important consequences for training adaptations and health. However, limited information is currently available regarding the c-inflammamiR response to acute exercise. Given the key role proposed for c-miRs in cell-cell communication and their potential as biomarkers, we analyzed the effects of different doses of acute aerobic exercise on a panel of c-inflammamiRs. We compared the profiles of c-inflammamiRs before, immediately after, and 24 h after participants ran 10-km and M races. Our finding revealed that acute aerobic exercise induces a c-inflammamiR response and that this response varies with exercise dose.

**Table 3. Circulating creatine kinase levels in 10-km, half-marathon, and marathon races**

<table>
<thead>
<tr>
<th>Variable</th>
<th>10-km</th>
<th>HM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK, U/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>181.67 ± 41.39</td>
<td>182.67 ± 50.27</td>
<td>136.22 ± 23.55</td>
</tr>
<tr>
<td>Post0</td>
<td>246.22 ± 57.35*</td>
<td>273.89 ± 66.06*</td>
<td>298.89 ± 42.92*</td>
</tr>
<tr>
<td>Post24</td>
<td>264.00 ± 48.66*</td>
<td>416.89 ± 113.59*</td>
<td>578.67 ± 67.66*</td>
</tr>
<tr>
<td>Post72</td>
<td>178.78 ± 36.09</td>
<td>232.67 ± 53.72*</td>
<td>261.00 ± 41.32*</td>
</tr>
</tbody>
</table>

Data represent means ± SE. CK, creatine kinase. Post72, 72 h after race. Basal vs. Post0, Post24, and Post72: *P < 0.050, ‡P < 0.010, †P < 0.001. Post24-Post0 fold change: 10-km vs. HM: ‡P < 0.050; 10-km vs. M: *P < 0.001; HM vs. M: †P < 0.001. /H11021 P 0.001. /H11021 P 0.050; 10-km vs. M:a P 0.050; 10-km vs. M:b P 0.050; 10-km vs. M:c P 0.050, d P 0.050, g P 0.050, e P 0.010; 10-km vs. M:e P 0.001.
ing status and intensity, the type and duration of exercise interventions, methodology and experimental design, and differences in the age and genetic and environmental backgrounds of the participants (38). Indeed, the observed increase in the circulating levels of miR-21 and -221 induced by exhaustive exercise is blunted after a period of exercise training in healthy competitive athletes, suggesting a maximal level of c-miRs in exercise is blunted after a period of exercise training in healthy competitive athletes, suggesting a maximal level of c-miRs in circulation in more active participants (4). The complex biology of miRs may also affect study outcomes. A different circulation in more active participants (4). The complex biology of miRs may also affect study outcomes. A different criterion. Results represent the mean relative expression with respect to Basal sample. AU, arbitrary units. *P < 0.050 and difference between means >1.5.

Table 4. c-inflammamiRs that were undetectable or showed no significant differences after 10-km and marathon races

| Not detected                  | hsa-let-7e-3p, hsa-let-7i-3p, hsa-miR-105-5p, hsa-miR-106a-3p, hsa-miR-124-5p, hsa-miR-125a-3p, hsa-miR-125b-1-3p, hsa-miR-125b-2-3p, hsa-miR-132-5p, hsa-miR-143-5p, hsa-miR-147a, hsa-miR-155-3p, hsa-miR-187-3p, hsa-miR-195-3p, hsa-miR-214-5p, hsa-miR-27b-5p, hsa-miR-30c-1-3p, hsa-miR-30c-2-3p, hsa-miR-517a-3p, hsa-miR-517c-3p, hsa-miR-579-3p, hsa-miR-641, hsa-miR-9-3p, hsa-miR-9-5p, hsa-miR-99b-3p, hsa-miR-99b-5p, hsa-miR-106b-3p, hsa-miR-106b-5p, hsa-miR-124-3p, hsa-miR-125a-5p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-126-5p, hsa-miR-132-3p, hsa-miR-132-5p, hsa-miR-143-3p, hsa-miR-143-5p, hsa-miR-145-3p, hsa-miR-145-5p, hsa-miR-146a-5p, hsa-miR-146b-5p, hsa-miR-148a-3p, hsa-miR-148b-3p, hsa-miR-155-3p, hsa-miR-16-1-3p, hsa-miR-16-2-3p, hsa-miR-16-3p, hsa-miR-16-4p, hsa-miR-16-5p, hsa-miR-16-6p, hsa-miR-17-3p, hsa-miR-17-5p, hsa-miR-181a-3p, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-182-5p, hsa-miR-18a-3p, hsa-miR-18a-5p, hsa-miR-195-3p, hsa-miR-195-5p, hsa-miR-199a-3p, hsa-miR-199a-5p, hsa-miR-199b-3p, hsa-miR-199b-5p, hsa-miR-19a-3p, hsa-miR-19a-5p, hsa-miR-19b-3p, hsa-miR-19b-5p, hsa-miR-20a-3p, hsa-miR-20a-5p, hsa-miR-21-3p, hsa-miR-21-5p, hsa-miR-223-3p, hsa-miR-223-5p, hsa-miR-27a-3p, hsa-miR-27a-5p, hsa-miR-27b-3p, hsa-miR-29a-3p, hsa-miR-29a-5p, hsa-miR-30c-3p, hsa-miR-30c-5p, hsa-miR-301a-3p, hsa-miR-326, hsa-miR-342-3p, hsa-miR-342-5p, hsa-miR-346, hsa-miR-34a-3p, hsa-miR-34a-5p, hsa-miR-349-3p, hsa-miR-349-5p, hsa-miR-369-3p, hsa-miR-369-5p, hsa-miR-424-3p, hsa-miR-424-5p, hsa-miR-473a-5p, hsa-miR-473b-5p, hsa-miR-517a-3p, hsa-miR-517c-3p, hsa-miR-579-3p, hsa-miR-641, hsa-miR-9-3p, hsa-miR-9-5p, hsa-miR-99b-3p, hsa-miR-99b-5p, hsa-miR-106a-5p, hsa-miR-106b-3p, hsa-miR-106b-5p, hsa-miR-124-3p, hsa-miR-125a-5p, hsa-miR-125b-5p, hsa-miR-125b-5p, hsa-miR-126-3p, hsa-miR-126-5p, hsa-miR-132-3p, hsa-miR-132-5p, hsa-miR-143-3p, hsa-miR-143-5p, hsa-miR-145-3p, hsa-miR-145-5p, hsa-miR-146a-5p, hsa-miR-146b-5p, hsa-miR-148a-3p, hsa-miR-148b-3p, hsa-miR-155-3p, hsa-miR-16-1-3p, hsa-miR-16-2-3p, hsa-miR-16-3p, hsa-miR-16-4p, hsa-miR-16-5p, hsa-miR-17-3p, hsa-miR-17-5p, hsa-miR-181a-3p, hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-182-5p, hsa-miR-18a-3p, hsa-miR-18a-5p, hsa-miR-195-3p, hsa-miR-195-5p, hsa-miR-199a-3p, hsa-miR-199a-5p, hsa-miR-199b-3p, hsa-miR-199b-5p, hsa-miR-19a-3p, hsa-miR-19a-5p, hsa-miR-19b-3p, hsa-miR-19b-5p, hsa-miR-20a-3p, hsa-miR-20a-5p, hsa-miR-21-3p, hsa-miR-21-5p, hsa-miR-223-3p, hsa-miR-223-5p, hsa-miR-27a-3p, hsa-miR-27a-5p, hsa-miR-27b-3p, hsa-miR-29a-3p, hsa-miR-29a-5p, hsa-miR-30c-3p, hsa-miR-30c-5p, hsa-miR-301a-3p, hsa-miR-326, hsa-miR-342-3p, hsa-miR-342-5p, hsa-miR-346, hsa-miR-34a-3p, hsa-miR-34a-5p, hsa-miR-349-3p, hsa-miR-349-5p, hsa-miR-369-3p, hsa-miR-369-5p, hsa-miR-424-3p, hsa-miR-424-5p, hsa-miR-663a, hsa-miR-92a-3p, hsa-miR-92b-3p, hsa-miR-92b-5p, hsa-miR-92b-5p. |

Fig. 3. A: circulating inflammatory microRNAs (c-inflammamiR) response for 10-km race. miR-150-5p was upregulated immediately after exercise (Post0), returning to basal levels in <24 h (Post24). B: c-inflammamiR response for marathon race. Twelve miRNAs were upregulated immediately after exercise (Post0), returning to basal levels in <24 h (Post24). Data were analyzed by one-way ANOVA for repeated measures and P values adjusted using the false discovery rate criterion. Results represent the mean relative expression with respect to Basal sample. AU, arbitrary units. *P < 0.050 and difference between means >1.5.
expression and cellular pathways intimately involved in the inflammatory response. In silico analysis corroborates this notion. We observed a close correlation between neutrophil and monocyte proliferation and activation (29, 64). The cytokine IL-6 (27, 35). miR-223 and -424 are fine-tuners of inflammatory processes, including cytokine synthesis (42). miR-150 secreted from monocyte/macrophage has been described as a class of inflammatory factor (82). A recent study reported that HDL-miR-223 delivery to endothelial cells induces the translational repression of the intercellular adhesion molecule (ICAM-1), which is implicated in the recruitment of inflammatory leukocytes (72). The coordinated alteration in the c-inflammamiR profile, in concert with other mechanisms, may thus modulate the inflammatory cascade during acute exercise.

Our results point to a role of c-miRs as regulators of the body’s response to acute exercise, in line with previous findings (4, 6, 43, 67).

The contribution of c-inflammamiRs to the exercise-induced inflammatory cascade and the role of extracellular miRs in cell-cell communication, including inflammatory processes (26), opens the door to speculation as to their pro- or anti-inflammatory functions. Strenuous exercise, such as marathon running and ultrarunning, has been associated with a deleterious proinflammatory response mainly related to cell damage (13, 78). It is thus reasonable to hypothesize that the c-inflammamiR profile observed after running the M race is associated with a proinflammatory response. Nevertheless, as stated above, the c-inflammamiR pattern observed after the M race has predominantly anti-inflammatory effects. These results support previous findings linking alterations in the levels of inflammatory-related miRs during pathological processes with the appropriate inhibition and termination of the proinflammatory response (49). The c-inflammamiR response should be interpreted as a compensatory anti-inflammatory mechanism to control the development, magnitude, and resolution of the response rather than a proinflammatory response to acute exercise. This situation is highly related to the anti-inflammatory nature of the classical inflammatory response to acute exercise.

In conclusion, the c-inflammamiR profile observed after the M race is associated with a proinflammatory response. Nevertheless, as stated above, the c-inflammamiR pattern observed after the M race has predominantly anti-inflammatory effects. These results support previous findings linking alterations in the levels of inflammatory-related miRs during pathological processes with the appropriate inhibition and termination of the proinflammatory response (49). The c-inflammamiR response should be interpreted as a compensatory anti-inflammatory mechanism to control the development, magnitude, and resolution of the response rather than a proinflammatory response to acute exercise. This situation is highly related to the anti-inflammatory nature of the classical inflammatory response induced by exercise. IL-6 is the first cytokine released into circulation during exercise, mainly from skeletal muscle. The circulating concentration of this inflammatory mediator increases exponentially during exercise and decreases after exercise (54). IL-6 is traditionally described as a proinflammatory cytokine. However, in the context of physical activity, IL-6 has a strong anti-inflammatory component (56). The infusion of recombinant IL-6 in young healthy volunteers, simulating acute exercise, induces an anti-inflammatory rather than a proinflammatory response (71). Indeed, secretion of this cytokine during acute exercise stimulates the secretion of anti-inflammatory mediators such as IL-10, IL-1 receptor antagonist (IL-1ra), and the soluble TNF receptors (sTNF-R) (18, 51, 71). Furthermore, the release of IL-6 in response to muscle contraction appears to regulate the biological activity of the proinflammatory cytokines TNF-α and IL-1β (70).

As anticipated based on previous findings (31, 53), we observed an increase in CK levels 24 h after the 10-km and M races, with the greatest increase detected after the M race. We thus investigated whether exercise-induced damage to skeletal muscle may trigger the nonspecific release of c-inflammamiRs into circulation. Although the intracellular muscle-specific miRs miR-1 and miR-133a are upregulated by endurance

| Table 5. Circulating myomiR levels; fold change with respect to Basal sample for 10-km and marathon races |
|-----------------|-----------------|-----------------|
|                 | Post0           | Post24          |
| hsa-miR-1       |                 |                 |
| 10-km           | 1.2 ± 0.5       | 0.7 ± 0.2       |
| M               | 2.4 ± 0.3       | 0.4 ± 0.5       |
| hsa-miR-133a    |                 |                 |
| 10-km           | 1.9 ± 0.4       | 1.0 ± 0.5       |
| M               | ND              | ND              |
| hsa-miR-133b    |                 |                 |
| 10-km           | 2.3 ± 0.6       | 1.0 ± 0.8       |
| M               | 2.5 ± 0.3       | 0.5 ± 0.5       |
| hsa-miR-206     |                 |                 |
| 10-km           | 1.1 ± 0.2       | 0.4 ± 0.6       |
| M               | ND              | ND              |

Data represent means ± SE. ND, nondetected values.
exercise (47), circulating levels of all myomiRs evaluated remained stable after both the 10-km and M races. In addition, alterations in c-inflammamiR profiles seen after each race differed substantially, suggesting a specific release pattern. Thus inflammation-related miRs may be selectively released during aerobic exercise and may form part of the acute response to different doses of physiological stress, rather than being released into circulation in response to nonspecific tissue damage.

Our study has several limitations. First, a larger sample size (>9) would have been desirable. The strict inclusion criteria and the invasive nature of the study limited the number of volunteers. Second, the cellular sources of the c-miRs of interest remain unclear. The c-inflammamiRs for which significant alterations were observed are highly expressed in a variety of cells and tissues. Moreover, the data on c-miR origin and delivery are conflicting. Some authors have reported a similar pattern of both c-miRs and parent cells (10), while others have proposed that c-miRs are unrelated to intracellular miRs or related only to a subset thereof (9,58). Third, we could not determine the extent to which the observed changes in c-inflammamiR expression translate into control of the gene expression and function of the recipient cell. Mechanistic in vitro and in vivo studies are necessary to elucidate the release mechanisms and the physiological role of c-inflammamiRs in cell-cell communication. Fourth, it remains unclear whether changes in c-inflammamiR levels are a cause or consequence of the proinflammatory signature described here. Fifth, it needs to be explored the effect of local inflammation induced by repeated venipuncture on the profile of inflammatory circulating miRs. Finally, no samples were taken during exercise. As such, we cannot rule out the possibility of nonlinear exercise-induced changes in miR levels; our findings may be masking a more complex response observed over a longer time frame. It is also worth noting that the time window between the Basal and Post0 time points differed for each race.

In conclusion, after extensively evaluating the exercise-induced inflammatory response and analyzing a broad range of c-inflammamiRs, we provide the first evidence of a c-inflammamiR response that parallels the classical inflammatory cascade. This c-inflammamiR response has an anti-inflammatory component, which may be involved in the control of the exercise-induced inflammatory response. Moreover, the c-inflammamiR profiles observed after low and high doses of acute exercise differ substantially, pointing to c-inflammamiRs as candidate biomarkers of the magnitude of the exercise-induced inflammatory cascade and, therefore, exercise dose. Our findings provide a better understanding of the inflammatory adaptations to acute aerobic exercise and the physiological adaptations associated with a safe and healthy exercise. Additional observational studies in larger samples and functional approaches will be required to validate these findings and provide further insight into the role of c-inflammamiRs in the exercise-induced inflammatory response.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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