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Title: **microRNAs as biomarkers of musculoskeletal pain in long distance runners: the MiMuS study protocol**

Authors: Maria Rosaria Tumolo, Tiziana Grassi, Carlo Giacomo Leo, Pierpaolo Mincarone, Francesco Bagordo, Antonella Bodini, Egeria Scoditti, Alessandra Panico, Antonella De Donno, Elisabetta De Matteis, Vincenzo Canali, Saverio Sabina

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microRNAs as biomarkers of musculoskeletal pain in long distance runners: the MiMuS study protocol

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- **Abstract:** MiRNAs are involved in the generation and progression of musculoskeletal pain, a condition that causes significant clinical, economic, and social burden. In runners, the presence of musculoskeletal pain related to inflammatory state or ongoing underlying tissue damage may result in poor training ability and performance. This study aims to evaluate the association between circulating and salivary miRNAs and pain in runners with and without musculoskeletal pain, and to observe whether dysregulated miRNAs can distinguish between responders and non-responders to a kinesiological intervention. The possible correlation between these miRNAs and inflammatory molecules, stress parameters and individual or behavioral characteristics will be evaluated. Finally, *in silico* analysis will be used to characterize miRNAs function. Ethics approval has been obtained.

- **Keywords:** microRNA; biomarker; musculoskeletal pain; inflammation; runners.

- **Main body of text:**

INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNA molecules, approximately 20 to 22 nucleotides in length, playing critical roles in several biological processes including cell proliferation, differentiation, metabolism, inflammation, and apoptosis [1]. These molecules regulate genes at the post-transcriptional level leading to translational repression or degradation of their messenger RNA (mRNA) expression [2].

MiRNAs have been detected not only inside cells, but also in extracellular body fluids, such as blood, serum, plasma and saliva [3–5]. Extracellular miRNAs can be packaged into exosomes or microvesicles and transferred to non-phagocytic cells, where they can modulate gene expression and function [4].

Several authors attribute to miRNAs not only a role in the physiological context, but also as potential biomarkers for the evaluation of the progression and treatment efficacy of disease states (liver disease, coronary heart ischemic disease, cancer, etc) [6–8]. Noteworthy, the expression of miRNAs can be also influenced by lifestyle factors [9], exposure to several environmental contaminants [10], and these molecules also appear to function in the epigenetic regulation of the stress conditions [11]. Furthermore, there is evidence that miRNAs are involved in the generation and progression of inflammatory painful processes, including musculoskeletal pain,

as they modulate the nociceptive signal by intervening in several pathways regulated by factors involved in the genesis of the symptom such as intracellular and extracellular molecules, and inflammatory factors (cytokines, peptides, lipids) [12–14]. A systematic review on miRNA expression changes in people with pain concluded that a number of miRNAs (miR-124a, miR-146a, miR-155, etc.) were found dysregulated in subjects with musculoskeletal pain [13].

Musculoskeletal pain is a persistent or recurrent pain affecting muscles, tendons, ligaments and bones [15]. It is present in approximately 1.71 billion people globally, causing a significant clinical, economic, and social burden [16]. In particular, subjects who participate in track and field training and competitions, as well as in road, trail and cross-country foot races, often experience musculoskeletal pain related to inflammatory state or ongoing underlying tissue damage, which occur mainly at the lower extremities [17–20]. In presence of musculoskeletal pain, athletes can continue training, especially if there are no physical signs of injury [20]. However, continuing training despite the presence of pain can lead to excessive stress on the musculoskeletal system, increasing the risk of serious injury [19,21,22]. In this regard, the prevention of musculoskeletal pain requires special consideration because it may incur high economic costs, as well as lead to poor training ability and performance, even loss of competition and adversely affect quality of life [21,23]. Given the different complexity of pain to be managed, different intervention strategies are currently available to relieve pain and prevent future injuries, including pharmacological treatment, physiotherapy, kinesiology, etc. [19,24,25]. Kinesiology, in particular, represents an emerging, non-invasive approach, in which the kinesiologist can operate through gymnastics exercises to prevent compensatory postural arrangements and promote functional recovery [26]. In athletes experiencing musculoskeletal pain, exercise-based approaches, among which kinesiology, may relieve pain and prevent recurrence of symptoms, but overall, they are poorly described [27–29]. Therefore, in absence of a "gold standard", further studies in this area are recommended.

In general, as individual response to interventions is variable, potential predictors of treatment efficacy could help identify subjects who may benefit from kinesiological approach. In this context, there is evidence that in addition to miRNAs, long non-coding RNAs (lncRNAs) are also involved in pathological pain processes, such as

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inflammation [30]. LncRNAs, i.e. transcripts characterized by a length of over 200 nucleotides [31], are able to associate with miRNAs and have the function of miRNA "sponge". This means that, when miRNAs are kidnapped by their respective lncRNAs, they are blocked in this state and cannot perform their function, i.e. to mediate RNA interference phenomena towards certain target mRNAs, by modulating their expression [32]. Therefore, the cross-talk between miRNA and lncRNA can regulate mRNA expression in the pathophysiological process of pain [33].

In the light of above, the University of Salento, in collaboration with the National Research Council and the "Vito Fazzi" Hospital of Lecce, promoted the "microRNAs as biomarkers of musculoskeletal pain in long distance runners" (MiMuS) study. The aims of this study are: i) to evaluate whether a panel of pain-related miRNAs are differentially expressed in blood and saliva of long distance runners with musculoskeletal pain (cases) when compared to a similar subjects without musculoskeletal pain (controls); ii) to assess if the dysregulated miRNAs in the cases may distinguish between responders and non-responders to a kinesiological intervention; iii) to detect any association between the dysregulated miRNAs and inflammatory molecules involved in the pain processes, some stress parameters in athletes and other individual or behavioral characteristics; iv) to characterize the function of miRNAs and their pathophysiological implications.

METHODS

Study Design

The MiMuS study is a pilot study coordinated by the Department of Biological and Environmental Sciences and Technology at University of Salento, which involves the Institute of Clinical Physiology of the National Research Council of Lecce and the Cancer Screening Operating Unit at "Vito Fazzi" Hospital of Lecce. It runs two years and consists of four phases: 1) a case-control study to evaluate the association between pain and miRNAs (circulating and salivary) in long distance runners with (cases) and without (controls) musculoskeletal pain; 2) a pre-post study, on cases, to evaluate possible significant changes of miRNAs expression after kinesiological intervention; 3) a correlation analysis between the dysregulated miRNAs and inflammatory molecules (cytokines) involved in pain processes, some stress parameters (salivary cortisol and perceived stress questionnaire) that may be

present in athletes and individual or behavioral characteristics; 4) *in silico* analyses to search for both target genes and lncRNAs associated with dysregulated miRNAs in order to characterize the function of miRNAs and their pathophysiological implications.

All data, which are circulating and salivary miRNAs, cytokines, pain, salivary cortisol and perceived stress questionnaire will be collected at 3 time points of the study: baseline (time point 0) for case and control groups, and in the group of cases after the kinesiological intervention (time point 1) and at a 3-month follow-up (time point 2).

The study flowchart (figure 1) shows all study procedures schematically.

[Figure 1 near here]

Recruitment and questionnaire administration

The subjects recruited in the study will be selected among long distance runners registered with Brindisi and Lecce sections of the Italian Federation of Athletics (FIDAL), aged at least 35 years (amateur runners), in possession of the medical certificate for competitive sport activity, who will have voluntarily accepted the invitation to participate by signing the informed consent and completing a self-administered questionnaire. The exclusion criteria will be as follows: doctor's indication not to carry out physical activity during the investigation period; presence of chronic diseases; ongoing pregnancy or less than 6 months from the childbirth; less than 6 months from weaning.

The questionnaire consists of 66 questions divided into 7 sections including both validated questionnaires and a part specifically developed by the researchers: 1) sociodemographic data (e.g. age, gender, ethnicity, marital status, educational level); 2) information about individual variables (e.g. weight, height); 3) information on lifestyle (e.g. smoking, alcohol and water consumption, dietary habits); 4) information on occupational and environmental exposure (use of pesticides, work in a rural zone, etc.); 5) information on hereditary-familial risk profile for cancer; 6) level of physical activity using the short form of the International Physical Activity Questionnaire (IPAQ) [34]; 7) information on the possible presence of musculoskeletal pain with indication of

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the intensity of pain [35], time from which the pain is occurred, the anatomical region involved and the type of injury (only if confirmed by doctor).

Eligible participants will be divided into two groups based on their answers to questions related to musculoskeletal pain: subjects with pain will be included in the group of cases, subjects without pain for at least one month will be included in the control group (useful for the first phase of the study). The choice of the type of pain to treat focuses on the one with the best ratio between sample size and pain level, suitably measured.

Sample size

In this study, according to the recommendations for pilot studies ~~[30,31]~~, the enrollment of a number of cases not less than 30 subjects is assumed [36–39]. In addition, each subject with musculoskeletal pain will be matched to a control by sex and age for a total of at least 60 subjects recruited (this number will be increased in case of greater adherence to the study).

Intervention

The group of cases will undergo a kinesiological intervention according to the Canali Postural Method® (CPM), a method that intervenes on a subject to prevent, reduce or eliminate compensatory postural arrangements. The CPM uses individualized gymnastic exercises and does not intervene directly on any painful region, but only on possible peripheral causes of a muscular nature (resistance and/or dominance) which may have elicited the pain [40].

The intervention will last for 6 weeks with three weekly sessions. One weekly session will be carried out at the "Posture and Movement Laboratory (MP-LAB)" of the Institute of Clinical Physiology assisted by an operator in possession of the CPM certifications and the other two weekly sessions self-managed by the subjects in their own training environment. The correct execution of the exercises will be shown to the subjects weekly and verified during the session held at the MP-LAB. Subjects will be asked to keep a diary at home in which to note their exercise sessions, while any changes in pain intensity noted during the weekly pain assessment at the MP-LAB will be used as a proxy for exercise success at home.

Biological sample collection

Fasting venous blood samples will be obtained in the morning (from 8 to 10 a.m.) from all subjects to measure miRNA expression and cytokine levels using special vacutainer type test tubes with EDTA as anticoagulant. Plasma will be separated, aliquoted and frozen at −80°C until RNA extraction.

Before saliva collection, all subjects will be required to avoid eating or smoking for at least 2 h. The saliva sample will be collected in Falcon tubes, then centrifuged and the supernatants transferred into Eppendorf tubes and stored at −80°C until analysis.

Outcome assessment

Pain assessment

Pain will be assessed using the Numerical Rating Scale (NRS), a validated 11-point unidimensional measure of pain intensity [41]. Higher scores indicate greater pain intensity [35]. The assessment will be performed at baseline, after the kinesiological intervention and at a 3-month follow-up. Furthermore, to get more information on the pain progression, at each assisted intervention session (weekly), subjects will be asked if the intensity of the pain has changed compared to the previous week ("How would you rate your average musculoskeletal pain intensity over the last week?") [41]. Depending on the effectiveness of the intervention, subjects will be divided in responders and non-responders; response to intervention will be defined as a reduction of pain greater than or equal to 50% (NRS) [39] at the end of the intervention program. A sensitivity analysis will evaluate the effect of this threshold on the results obtained.

Evaluation of miRNAs and cytokines

On the basis of the current literature, the following miRNAs involved in musculoskeletal pain pathogenesis and inflammation will be investigated: hsa-miR-124-3p, hsa-miR-150-5p, hsa-miR-155-5p, hsa-miR-146a-5p [12,13]. Moreover, we will select hsa-miR-133b and hsa-miR-206 due to their muscle-specific expression and their response to muscle damage [36].

~~Whole blood will be obtained by venipuncture using special vacutainer type test tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. A saliva sample will be collected in special Falcon tubes at the time of enrollment. In particular,~~ MmiRNA will be extracted and purified using the Quick-cfRNA

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Serum & Plasma ~~Quick-RNATM MiniPrep Plus~~ kit (Zymo Research) following the manufacturer's instructions. Total RNA concentration and purity will be evaluated by NanoDrop spectrophotometer at the absorbance 230, 260 and 280 nm. The expression of the selected miRNAs will be determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR), considered the gold standard since it offers a good balance between cost, precision, and sample size along with a large functional dynamic range [42] ~~[30]~~. The miRNAs will be assayed individually in each sample using TaqMan MicroRNA Assays (Applied Biosystems, CA) according to the manufacturer's protocol. For synthesis of each miRNA-specific cDNA, 15 ng of total RNA will be reverse transcribed using TaqMan miRNA reverse transcription kit in a 15 µl reaction volume containing 1.50 of 10X RT buffer, 0.15 µl of 100 mM dNTPs, 0.19 µl of RNase inhibitor (20 units/ml), 1 µl of MultiScribeTM Reverse Transcriptase (50 units/ml), 4.16 µl of Nuclease-free water, and 3 µl of each of the miRNA specific prime. The RT mixture will be incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. qRT-PCR will be performed using a 7500 Real Time PCR system with Taqman 2X PCR Universal Master Mix (Applied Biosystems, CA) for miRNA detections in a 20 µl total reaction volume, consisted of 2.5 ml of the RT product, 10 ml TaqMan 2X Universal PCR Master Mix No AmpErase UNG, 1 ml TaqMan MicroRNA Assay (20X) containing the TaqMan primer-probe mixture. The instrument will be set for 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec with detection of FAM fluorescence. An endogenous control will be used to normalize the miRNAs expression. All PCRs will be performed in triplicates for each analyzed sample.

With regard to inflammation, the plasma~~blood~~ levels of pro-inflammatory cytokines, including interleukin-1, interleukin-6, and tumor necrosis factors will be analyzed using the enzyme-linked immunosorbent assay (ELISA) [43]. ELISA is a well-recognized tool for quantitative, antibody-based analysis of soluble proteins in a sample. The ELISA indeed take advantages of the specificity of antibodies to capture and quantify an analyte of interest from a given volume of sample with remarkable sensitivity. Notably, the four basic steps involved in an indirect sandwich ELISA will be 1) capturing analyte from sample with capture antibody, 2) detecting captured analyte with detection antibody that is labeled with biotin, 3) detection amplification with streptavidin that has been conjugated with a detector enzyme; finally, 4) substrate is added and signal measured via optical density using

a microplate reader. The analyte concentration in the sample will be then determined by retrieving the concentration from a given absorbance of the sample in the range of a standard curve.

Both qRT-PCR and ELISA will be performed at baseline in both groups, and only in the case group also after the kinesiological intervention and at a 3-month follow-up.

Analysis of miRNA function

To understand the biological and functional role of miRNAs involved in pain, an *in silico* analysis of target genes and lncRNAs associated with the dysregulated miRNAs will be performed.

In particular, popular databases including TargetScan [44], miRDB [45], and miRNet [46] will be used to select miRNA target genes; miRNet [46] and Starbase [47] (V3.0) databases will be used to research the lncRNAs. InteractiVenn [48] will be used as a tool to select target genes and lncRNAs in common for the selected databases, which will be used for pathway enrichment analyses. The miRNA-target and the miRNA-lncRNA network will be visualized with Cytoscape software [49] (version 3.7.1). Pathway enrichment analyses will be conducted to better understand the functional role of dysregulated miRNAs; in this regard, GeneTrail2 will be used (version 1.6) [50] as a platform to access the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Stress levels assessment

Salivary cortisol has been widely used as an objective biomarker of physiological stress [51]. For the analysis, subjects will be requested to refrain from eating and drinking during the 2 h before sampling. The samples will be frozen at −20° until the ELISA, that will be performed according to the manufacturer's instructions [52]. In particular, approximately 50 µl of untreated saliva sample will be dispensed into each well followed by the anti-cortisol antibody and the labeled antigen. An incubation time of 20 hours will be required to optimize the immunoreaction. Subsequently, the plate will be washed three times with phosphate-buffered saline, and the enzyme substrate will be dispensed at 150 µl/well. another incubation will follow for 30 min at room temperature in the shade and after the reaction will be stopped by adding 150 µl of 1 N H_2SO_4. Finally, the optical density will be measured.

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Psychological stress will be measured by perceived stress questionnaire, a scale that measure the degree to which situations in one's life are appraised as stressful. The questionnaire consists of 10 items related to the last 30 days using a scale from 0 (never) to 4 (very often), in which the higher the score, the greater the stress (a score of 0–13 is considered a low perceived stress level, 14–26 as moderate perceived stress level, and 27–40 as high perceived stress level) [53,54]. Both salivary cortisol test and the perceived stress scale will be performed at baseline in all subjects and, only in the case group, after the intervention and at a 3-month follow-up.

Statistical analysis

Testing for differences between groups will be accomplished by the *t*-test for all data with normal distribution (the Gaussianity of the data will be evaluated with the Shapiro-Wilk test) and the nonparametric Mann-Whitney Test for all data without normal distribution. In the case group, a logistic regression analysis will be performed to identify predictors of response to treatment, and the predictive accuracy of the model will be evaluated through the receiver operating characteristics (ROC) curve and the calculation of the area under the curve (AUC). In addition, a preliminary correlation analysis and a regression analysis will be performed to evaluate the relationship between miRNA expression, cytokine levels and stress levels measured by salivary cortisol, while to evaluate the association in the case group between treatment response and miRNA expression, cytokine and stress levels measured by salivary cortisol, test for differences of location parameters will be considered again. As far as the stress level measured by the perceived stress scale, an association analysis will be performed in a completely analogous way. Values will be expressed as mean and standard deviation (SD) in the case of Gaussianity or, otherwise, through the median and the interquartile interval. The significance level α=0.05 will be considered. Statistical analysis will be performed using the software open source R [55].

Ethics and dissemination

The study has been approved by the Ethical Committee of the Lecce Local Health Authority (ASL/LE) on 02 February 2023 with deliberation n. 0000108. All data will be collected and analyzed confidentially in accordance with Italian laws (General Data Protection Regulation UE 2016/679; Legislative Decree n. 196 of 30 June 2003 and subsequent additions) for research purposes.

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Each participant will be asked to sign an informed consent after an adequate detailed explanation of the study, including the processing and handling of their samples, the anonymization of their data and their right to withdraw their voluntary consent at any time. An alphanumeric code will be generated to identify both biological samples and questionnaires.

All data, analyzed in an aggregate and anonymous way, will be used for the organization of scientific meetings and workshops to show the preliminary and final results. Moreover, the results will be presented at national and international conferences and then published in peer-reviewed journals. The results will also form part of a doctorate thesis registered at the University of Salento.

CONCLUSION

Firstly, this pilot study will allow to identify the potential miRNAs associated with the type of musculoskeletal pain that will be considered, and to evaluate the relationship between circulating and salivary miRNAs in order to contribute to the development of non-invasive biomarkers that are easier to collect and manage than blood collection. Secondly, as shown in other pathophysiological settings [56–59], the analysis of miRNAs at the end of the intervention and at follow-up could aid in the search for potential predictive biomarkers of intervention efficacy and, consequently, of intervention strategies for pain management. Thirdly, the cytokines analysis in concert with miRNA pattern will provide a better understanding of the pathophysiological mechanisms underlying pain. *In silico* analysis will allow to observe if dysregulated miRNAs during pain inflammatory states could also be involved in the molecular pathways leading from inflammation to the potential development of pathologies [59,60].

Finally, data on individual, medical, lifestyle, environmental exposure, and stress parameters which will be used to investigated the possible correlation among these variables, their relationship with pain and miRNA profile, helping in a potential phenotypic characterization of pain responses in athletes that could be used in future larger studies. The obtained results will be further validated in an independent cohort of runners.

From a public health point of view, as already highlighted above, given that inflammatory pain in the athletic world represents a critical issue, being able to identify potential biomarkers to be used to relieve pain and

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prevent future injuries would also be useful to initiate an appropriate intervention for the prevention of musculoskeletal pain.

- **Summary Points:**

1) MiRNAs are involved in the generation and progression of musculoskeletal pain.

2) In runners, the presence of musculoskeletal pain related to inflammatory state or ongoing underlying tissue damage may result in poor training ability and performance.

3) This study will involve long distance runners with and without musculoskeletal pain.

4) The study will provide insight into which miRNAs are potentially involved in the musculoskeletal pain that will be considered.

5) This work will potentially provide information on the use of non-invasive biomarkers (salivary miRNAs) in the evaluation of musculoskeletal pain.

6) This study will provide insight into ~~allow~~ if the dysregulated miRNAs could be associated with response to kinesiological intervention, an emerging, non-invasive approach.

7) The *in silico* analysis will provide a better understanding of the pathophysiology of musculoskeletal pain.

8) The study will contribute to investigate the possible associations between miRNAs and pain, stress parameters and individual or behavioral characteristics.

- **Figure legends:** *miRNA (plasma~~blood~~ and salivary samples); cytokines (plasma~~blood~~ sample); pain (Numerical Rating Scale); stress (salivary samples and perceived stress scale).

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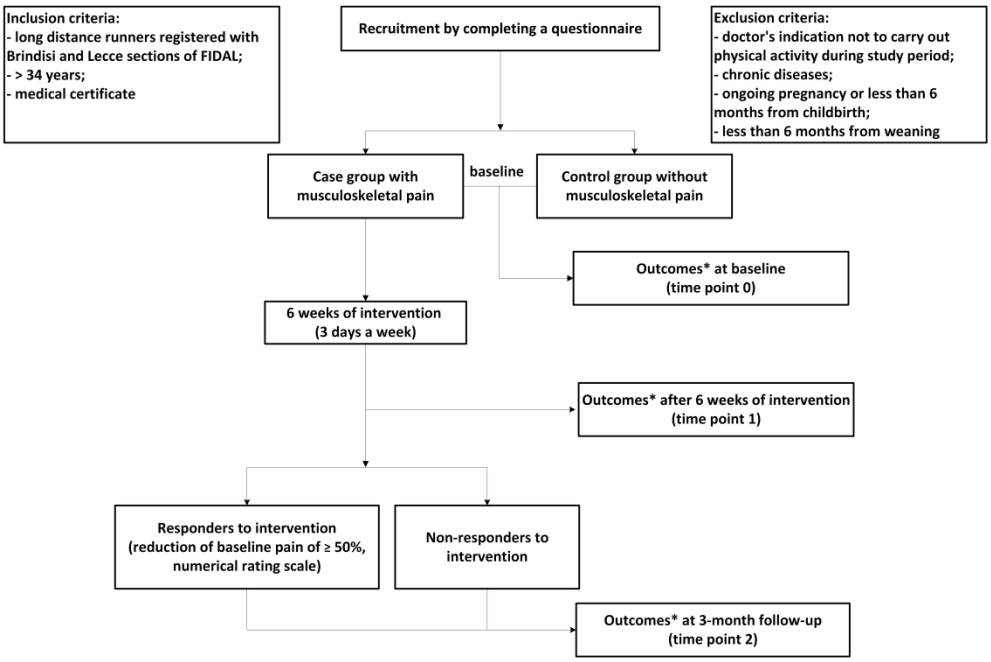
Highlights how miRNAs during inflammatory states could also be involved in the molecular pathways leading from inflammation to the potential development of pathologies.



Caption: Flow chart of study procedures.
Legend: *miRNA (plasma and salivary samples); cytokines (plasma sample); pain (Numerical Rating Scale); stress (salivary samples and perceived stress scale).

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