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ORIGINAL ARTICLE

The temporal expression of circulating microRNAs after acute experimental pain in humans

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Abstract

Background: MicroRNAs (miRNAs) can modulate several biological systems, including the pain system. This study aimed to evaluate the temporal expression of circulating miRNAs in the plasma of healthy volunteers as a marker for epigenetic changes before and after an acute, experimental, pain provocation by intramuscular hypertonic saline injection.

Methods: Twenty volunteers were randomly allocated into two groups and received either hypertonic (pain) or isotonic (control) saline injection in the first dorsal interosseous muscle of their dominant hand. Pain intensity was continuously recorded for 20 minutes after injection on a VAS scale from 0 to 100 (0 indicates no pain and 100 the worst imaginable pain). Blood samples were taken at baseline, 30 minutes, 3 hours, and 24 hours post-injection, and plasma was separated. MiRNA extracts were used for RNA sequencing with the Illumina NextSeq platform. MiRNA transcripts were compared between the pain and the no-pain, control group at every time point. Significant differences were considered when folds were >2 and the False Discovery Rate was $p < 0.05$.

Results: After 30 minutes, 4 miRNAs were significantly altered in the pain group compared to controls, which increased to 24 after 3 hours and to 42 after 24 hours from baseline ($p < 0.0001$). Two miRNAs were consistently upregulated throughout the experiment. Enrichment analysis showed significant miRNAs involved in brain perception of pain, brain signalling and response to stimuli.

Conclusions: This exploratory study is the first to report on the temporal expression of circulating miRNAs after an acute, human experimental muscle pain model.

Significance: This exploratory study evaluated the temporal profile of circulating miRNAs in the plasma of healthy subjects after acute experimental pain. Several miRNAs were altered in subjects at the times of follow-up after the acute pain model when compared to controls. MiRNAs previously associated with pain processes were altered in the pain group. Our results, by showing the fast

and prolonged modifications of miRNA elicited by the acute experimental pain model, add new perspectives to the topic of epigenetics and pain.

1 | INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA with the ability to modulate a large proportion of the genome post-transcriptionally and are part of the epigenetic modifications (Elramah et al., 2014). The main function of miRNAs is to bind multiple RNA messenger (mRNA) targets, in either imperfect or perfect complementarity (Gu et al., 2009), and enable the translation inhibition of the targeted mRNA (O'Brien et al., 2018). Considering that 60% of the mammalian gene products are miRNA targets, it is inherent that miRNAs might contribute to pain sensation through translational regulation of pain-related mRNA crucial in pain processing pathways (Friedman et al., 2009). Several preclinical studies, using translational pain models, have reported the dysregulation of miRNAs in association with the function of key regulators of pain process such as γ-aminobutyric acid-α1 (GABAα1), cyclooxygenase 2, transient receptor potential cation channel subfamily V member 1 (TRPV1), and multiple Na^+ and Ca^{2+}-channels (Favereaux et al., 2011; Li et al., 2011; Sengupta et al., 2013). MiRNAs involvement have have also been proven for the modulation of endogenous analgesic mechanisms, with miR-23b playing a key role in the expression regulation of μ-opioid receptor (MOR) (Andersen et al., 2014). Several in vivo models of inflammation have been used to investigate the role of miRNAs in inflammatory pain processing (Ghorpade et al., 2013; Li et al., 2013; Xie et al., 2013). Kusuda and coworkers found that miR-1, −16, and −206 are differentially regulated in dorsal root ganglia (DRG) and dorsal horn in different animal models of pain, under inflammatory and neuropathic pain conditions as well as following acute noxious stimulation (Kusuda et al., 2011), suggesting that miRNAs might change expression over time, but these data need to be confirmed in human studies. Emerging evidence has demonstrated links between pre-treatment miRNAs and response to standard pain treatments, highlighting miRNAs as potential prediction pain biomarkers (Giordano et al., 2020). The discovery of miRNAs, not only in tissues but also in body fluids including blood, urine, and cerebrospinal fluid, sheds light on the possibilities of miRNA as biomarkers to aid in diagnosis and pain research (Blondal et al., 2013; Giordano et al., 2020; Moldovan et al., 2014) as largely utilized in other areas such as e.g. cancer (Cheng, 2015). The above-mentioned studies highlight the importance of understanding the mechanisms behind acute physiological pain and how miRNA-induced transcriptional repression of mRNA targets is involved in pain pathophysiology. To investigate this, the use of intramuscular injection of hypertonic saline is a great tool to induce muscle hyperalgesia. Injection of hypertonic saline into a muscle induces pain that is described using descriptors like those used for clinical pain (Hodges et al., 2003). This technique has been used since the 1930 s (Kellgren, 1938) to study the sensory properties of muscle pain and its effect on sensorimotor function (Arendt-Nielsen et al., 1996; Svensson et al., 2000). The temporal effect of epigenetic modifications concerning pain is still unclear, and the effects on circulating miRNAs expression to an acute painful event are yet to be demonstrated in humans (Møller Johansen et al., 2021). For these reasons, the aim of this exploratory, randomized, controlled study was to evaluate the temporal profile of circulating microRNAs in the plasma of healthy subjects after acute experimental muscle pain stimulation by hypertonic saline injection.

2 | MATERIALS AND METHODS

2.1 | Participants

Twenty subjects were recruited to participate in the study. Exclusion criteria included pregnancy or breastfeeding, smoking, use of medications (e.g. painkillers and anti-inflammatory drugs), medical history or current neurological, musculoskeletal or mental illnesses and acute or chronic pain. Following the Helsinki Declaration, all subjects signed an informed consent form, and the Regional Ethics Committee approved the study protocol (N-20200015). The protocol was registered on clinicaltrials.gov (submitted on 28 October 2020, trial number: NCT04439994).

2.2 | Study design

The participants were randomly allocated into a pain group receiving painful muscle stimulation and a control group receiving isotonic saline. A bolus injection of 0.2 ml hypertonic saline (7% NaCl) was used for the "pain" group and 0.2 ml isotonic saline (0.9%) for the "control" group. Injections were done in the first dorsal interosseous (FDI) muscle of the dominant hand, as previously described (le Pera et al., 2001). The site of injections was determined by palpation of the contracted FDI muscle and boluses were administered to it using a 1 ml syringe with a disposable needle (27G), after skin cleansing with

alcohol. Pain intensities following hypertonic and isotonic injections were monitored for 20 minutes using an electronic 100 mm visual analogue scale (eVAS V1.0 software Aalborg University, Denmark) with zero (0) representing no pain, and the end at one hundred (100) representing the worst pain imaginable, installed on a tablet. Participants were asked to evaluate their pain in the area of injection and were not asked to report any other pain coming from other body sites. Whole blood samples (5 ml) were collected through venipuncture into EDTA tubes. Plasma separation was conducted following standard operating procedure and transferred and divided into 1 ml aliquots and placed in a −80°C freezer. Blood samples were collected at baseline for every participant at 09:00 to avoid normal fluctuations of blood composition molecules. Additional blood samples were collected 30 minutes, 3 hours and 24 hours after the injections, to make sure to avoid a "plateau" effect of the miRNA expressions (Santoro et al., 2020). Samples were not obtained directly after saline injection, to avoid alteration of the pain perception derived from the intervention. Moreover, the blood sample was not performed during the painful period to allow the participants to report the pain sensation derived only from intervention. Venipunctures for blood sampling was performed alternately, starting from the non-dominant hand (contralateral to the injection site) in order to avoid persistent stress at the site of needle insertion The study design is outlined in Figure 1. The analyses of next-generation sequencing and bioinformatics were performed by DNASense Aps.

2.3 | Library preparation

MiRNAs were extracted using the miRNeasy Serum/Plasma Advanced Kit (Qiagen), and sequencing libraries were prepared using the NEXTFLEX Small RNA-Seq Kit v3 (PerkinElmer). A companion kit for tRNA and γ-RNA blocking during library preparation was applied, NEXTFLEX tRNA/YRNA Blockers for Small RNA-seq Library Prep. In addition, the optional PAGE gel clean-up of sequencing libraries was applied to remove adapter-dimer products (5% Criterion TBE Precast Gels, BioRad). The final cDNA sequencing libraries were pooled in equimolar concentrations and sequenced (1 × 75 bp, SE) on a NextSeq 550 platform (Illumina).

2.4 | Transcriptome mapping

Forward cDNA reads from the raw fastq files were filtered for PhiX using Bowtie2 (v2.4.1) by aligning the reads against a database comprised of the Coliphage phi-X174 genome (Refseq accession NC_001422.1) (Langmead &

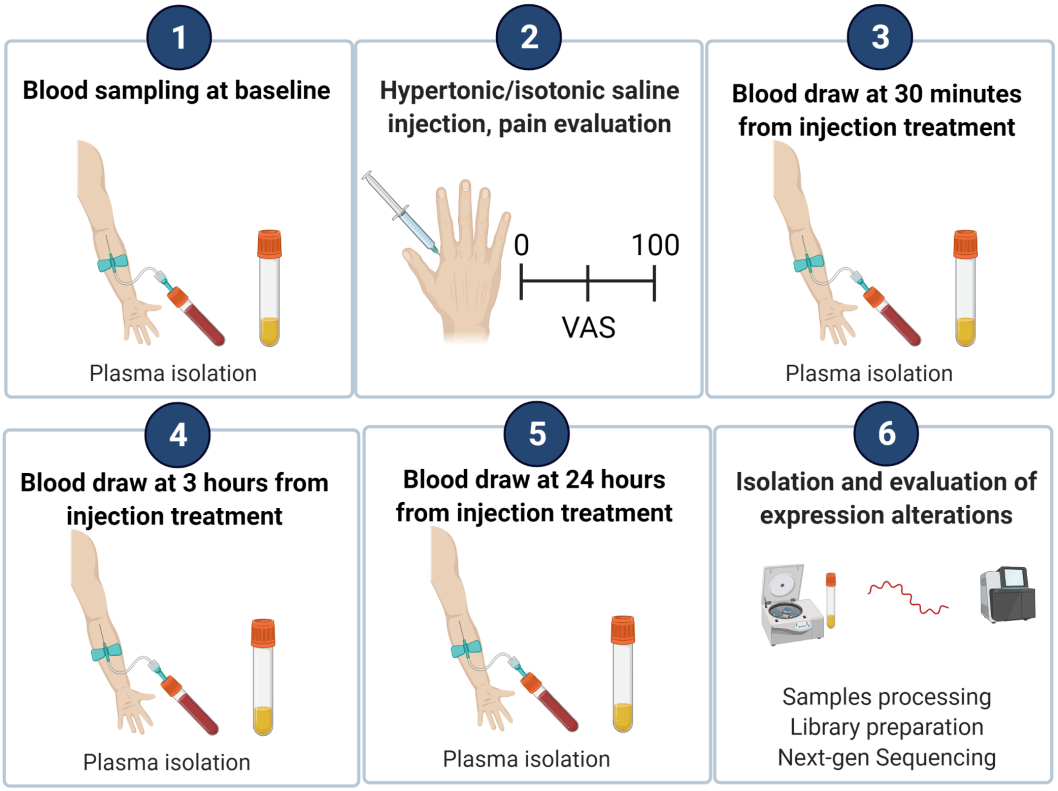

FIGURE 1 Study design and sample processing. © Biorender.

Salzberg, 2012). Reads passing the PhiX-filtering were then trimmed twice with cutdapt (Martin, 2011), first removing sequencing adaptors and secondly trimming four bases of the beginning, and 1 base of the end of each read, as per the bioinformatic requirements for the NEXTFLEX kit. Lastly, reads were cropped to 22 bases using Trimmomatic (v0.39), quality filtered (Q-score > 30) and sequences shorter than 16 nucleotides were discarded. The quality filtered cDNA reads were aligned against the miRBase (release 22.1) (Kozomara et al., 2019) mature miRNA sequences from Homo sapiens with the Bowtie2—very-sensitive option. All alignments were ported to unsorted .sam files and these were then sorted by miRBase accession number and converted to .bam files using samtools (v1.13) (Li et al., 2009). Finally, transcript/gene count tables were made using samtools idxstats.

2.5 | Differential gene expression analysis

Further bioinformatic processing of cDNA read mapping was done via RStudio IDE (1.4.1717) running R version 4.1.0 (2021-05-18) and using the R packages: tidyverse (1.3.1), seqinr (4.2.8), ShortRead (1.50.0) and DESeq2 (1.32.0) (Wickham, 2009). Transcript/gene count tables were augmented with experiment metadata and used for input in the default DESeq2 workflow. The DESeq2 workflow was used to normalize read counts by the geometric mean count and identify differentially expressed genes. Power calculation was not performed due to the exploratory nature of the study, but the DESeq2 tool was used to reduce false positive results and normalize raw data (Schurch et al., 2016). The statistical analysis was designed to specifically identify differentially expressed genes concerning the pain group at 30 minutes, 3 hours, and 24 hours when compared to the control group therefore the samples from the pain group were compared against those from the control group at each time point after stimulation (30 minutes, 3 hours, and 24 hours) whilst also correcting for differences between experimental pain model groups before stimulation (i.e., the baseline assessment). Changes in gene transcript counts were considered statistically significant with the dual criteria of the observed change in expression >2-fold and the Benjamini-Hochberg corrected p-value < 0.05 (Benjamini & Hochberg, 1995) comparing the two groups.

2.6 | Gene ontology

Gene Ontology annotations for RNA gene products from Homo sapiens (taxon identifier 9606) were retrieved from QuickGO (Binns et al., 2009) and exported as a tab-delimited file. Database mappings between QuickGO and miRBase were retrieved from RNAcentral FTP Archive's current release (23 June 2021 03:16 PM).

2.7 | Target prediction analysis

Target prediction analysis of altered miRNAs was performed, using mirPath v. 3.0 (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath) software a web-based tool that predicted miRNA-gene product interactions by scanning miRNA sequences overlapping with gene sequence product (Vlachos et al., 2015). Moreover, the evaluation process, in which the altered miRNAs regulate the targeted genes, was run on mirPath v. 3.0, and the genes predicted were enriched for biological processes (Vlachos et al., 2015). Only significantly differentially expressed miRNA ($p \leq 0.05$) were included in the enrichment analysis of the biological processes. Target prediction was performed on each group of altered miRNAs (30 minutes, 3 hours, 24 hours) and a subset of miRNAs previously associated with pain processes or pathological conditions characterized by a pain component (Gazerani, 2019; Leinders et al., 2016; Park et al., 2014; Wen et al., 2019; Xu et al., 2014; Zhang et al., 2019).

2.8 | Statistical analysis of pain assessment

Differences in peak pain intensities were assessed through an independent sample t-test in SPSS statistics (V.27, IBM Corporation).

3 | RESULTS

3.1 | Demographic and pain assessment

Participants were distributed equally between the two groups (age average of 25.9 years; 50% females). Significantly higher pain intensities were found for the hypertonic saline compared to the isotonic saline injections ($p < 0.001$) (average peak VAS 58.8 and 5.8, respectively). After the injection of hypertonic saline, the pain intensity reached the peak after approximately 90 seconds and the pain lasted for approximately 10 minutes (Figure 2), whereas the control group reported a minimal pain intensity right after injection, which lasted for a few seconds and plateaued to 0 for the rest of the assessment (Figure 2).

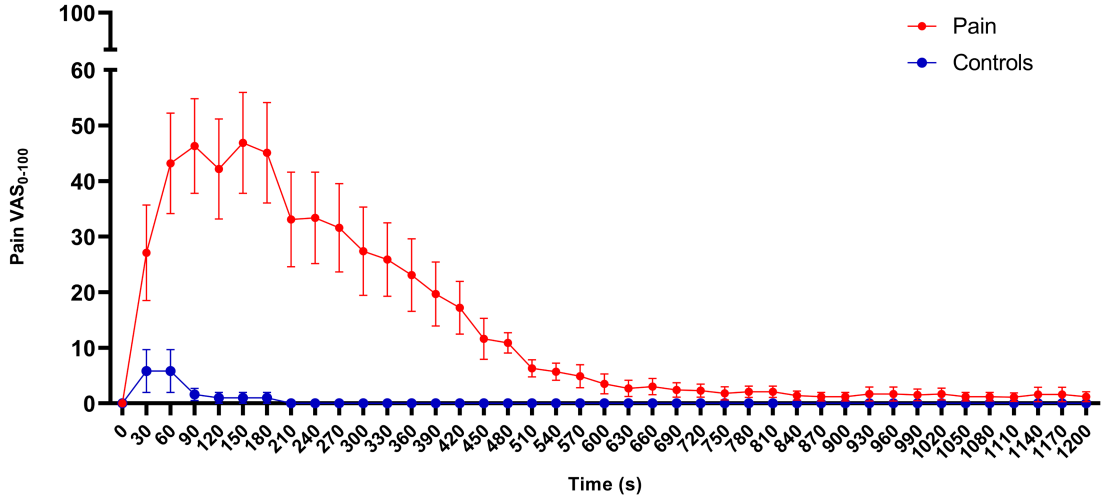

FIGURE 2 Pain temporal profile. The average pain intensity reported by participants included in the pain group (red) and control group (blue) for 20 minutes of recordings. Error bars depict the standard error of the mean (SEM).

3.2 | Differential temporal microRNA expression

Analysis of microRNA expression identified a total of 843 miRNA transcripts for each time point hereof a total of 66 microRNAs were significantly altered at one of the three different time points when comparing the pain and control groups (Figure 3).

Two transcripts (miR-144-3p and miR-377-3p) were consistently significantly upregulated in the pain group compared to the control group throughout the entire study observation period (Figure 4).

The number of altered miRNAs was 4 microRNAs after 30 minutes (Table 1), 24 after 3 hours (Table 2) and 42 after 24 hours (Table 3) when comparing the hypertonic saline group to the isotonic saline group. Within the pain and control group, the miRNA expression levels showed no statistical differences when evaluated between the four time points.

3.3 | Enrichment analysis of the biological process of differentially expressed microRNAs

Enrichment analyses of the biological process were performed to highlight which gene's product is regulated by the significantly altered miRNAs. MirPath V.3 software (Vlachos et al., 2015) identified 52 biological processes in which the 4 miRNAs altered after 30 minutes, in the pain group, were involved. Moreover, 201 biological processes were enriched for the 24 significant miRNAs altered after 3 hours from injection, and 110 for the miRNAs altered after 24 hours. Interestingly, an enrichment for a sub-set of miRNAs previously involved in pain conditions showed how biological processes involving brain perception, signalling, response to stimuli, and axon guidance were significant (Figure 5).

4 | DISCUSSION

This study is the first human study to describe the temporal profile of circulating miRNAs expression after acute, experimental muscle pain stimulation. Data showed that 4 miRNAs were dysregulated at 30 minutes, 24 miRNAs were dysregulated at 3 hours, and 42 miRNAs were dysregulated at 24 hours in the pain group compared with the control group. Additionally, a consistent upregulation of miR-144-3p and miR-377-3p in the hypertonic saline group was detectable throughout the study period.

This indicates that an acute painful event is able to trigger long-lasting changes in the functioning of the genetic apparatus via epigenetic changes.

4.1 | The temporal profile of miRNAs alteration

Amongst epigenetic modifications, miRNAs have been shown to be involved in key protein regulations of preclinical and clinical importance and are defined as “master switches” for the regulations of developmental, physiological, and pathological processes (Kalpachidou et al., 2020; Leinders et al., 2017). Although many studies have been conducted to demonstrate an association of miRNAs in acute and chronic pain (Polli et al., 2020; Tavares-Ferreira et al., 2019), no current evidence are present in relation to the temporal profiling of changes and alterations in the expression of miRNAs in humans after an acute,

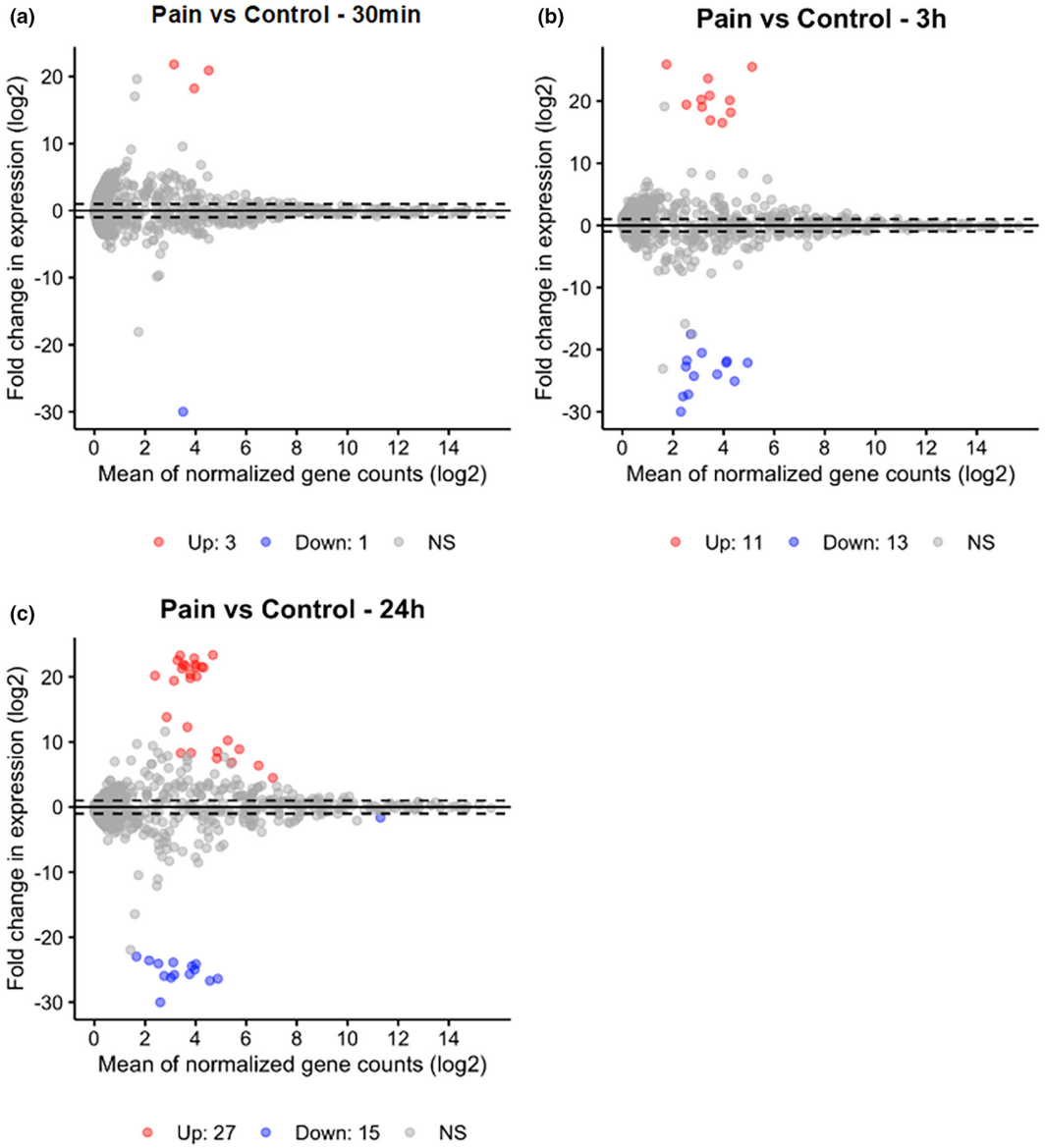

FIGURE 3 MicroRNAs expression. The MA plots show the fold change in gene expression between experiment sampling points as a function of the averaged, normalized transcript count of each gene. Each point corresponds to one gene with statistically significant up (red) and downregulation (blue) indicated for (a) 30 minutes, (b) 3 hours and (c) 24 hours. Non-significant (NS) changes are in grey. Dashed lines indicate the levels corresponding to a twofold change in gene expression.

well-controlled, experimental pain stimulus. A preclinical study has investigated the progressive miRNAs alteration in a rat model of bone pain and found that miRNAs like the let-7 family and miR-21 were significantly upregulated in plasma and splenocytes at day 14 relative to day 3 after bone injury, but not in sham-operated animals (Silva et al., 2018). Both these miRNAs have been previously shown to be directly associated with pain mechanism and pain perception (Chen et al., 2020; Leinders et al., 2016). In the specific miR-21, expressed by primary sensory neurons, has been shown to be released through exosomes and to be involved in the regulation of macrophages activation, such as the pro-inflammatory phenotype M1, and has been demonstrated how the deletion of it can reduce neuropathic pain induced by the inflammatory response (Chen et al., 2020; Simeoli et al., 2017).

The present results with detected miRNA modifications 24 hours after an acute pain insult suggest an alteration not only due to the experimental pain but may also point toward an effect of miRNAs on self-regulation (Jiao & Slack, 2012; Zisoulis et al., 2012) and homeostatic plasticity (Dubes et al., 2019). It is currently unclear whether the changes caused by experimental muscle pain can be identified only in the alteration in a short time (30 minutes) or the effect could include the changes continuing to develop up to 24 hours after the insult. Even though

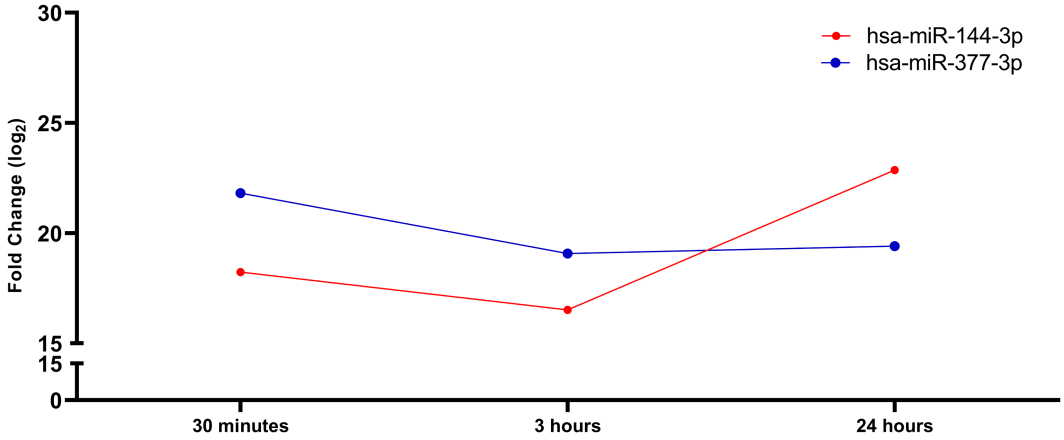

FIGURE 4 Overtime expression of miRNA. The dot plot shows fold changes of two miRNAs transcript over-expressed throughout the whole experiment, fold change represents the difference between the pain and control group.

Accession	Gene symbol	Foldchange (log_2)	*p*-Value
MIMAT0027416	hsa-miR-6758-5p	−29.998	1.12^{-06}
MIMAT0000436	hsa-miR-144-3p	18.236	3.33^{-08}
MIMAT0000730	hsa-miR-377-3p	21.818	5.06^{-07}
MIMAT0018443	hsa-miR-374c-5p	20.912	7.05^{-10}

TABLE 1 List of identified microRNAs 30 minutes from the injection, in the pain versus control group

TABLE 2 List of identified microRNAs 3 hours from the injection, in pain versus control group

Accession	Gene symbol	Foldchange (log_2)	*p*-Value	Accession	Gene symbol	Foldchange (log_2)	*p*-Value
MIMAT0004509	hsa-miR-93-3p	−22.117	3.82^{-16}	MIMAT0021120	hsa-miR-5189-5p	−21.840	8.16^{-07}
MIMAT0000094	hsa-miR-95-3p	−25.083	2.38^{-12}	MIMAT0000436	hsa-miR-144-3p	16.516	3.33^{-05}
MIMAT0000428	hsa-miR-135a-5p	−22.735	0.0004	MIMAT0000453	hsa-miR-154-3p	23.639	2.48^{-08}
MIMAT0000682	hsa-miR-200a-3p	−27.540	1.93^{-07}	MIMAT0001621	hsa-miR-369-5p	18.179	$2.84–^{05}$
MIMAT0004681	hsa-miR-26a-2-3p	−24.271	8.16^{-07}	MIMAT0000730	hsa-miR-377-3p	19.078	0.0004
MIMAT0004683	hsa-miR-362-3p	−30.000	9.00^{-09}	MIMAT0004693	hsa-miR-330-5p	16.928	0.0002
MIMAT0004690	hsa-miR-379-3p	−23.978	7.98^{-11}	MIMAT0001536	hsa-miR-429	19.438	0.00044
MIMAT0001545	hsa-miR-450a-5p	−22.126	5.40^{-10}	MIMAT0004793	hsa-miR-556-3p	20.134	3.57^{-05}
MIMAT0002172	hsa-miR-376b-3p	−20.521	4.32^{-07}	MIMAT0004797	hsa-miR-582-3p	25.925	0.0012
MIMAT0005876	hsa-miR-1285-3p	−27.215	7.07^{-05}	MIMAT0003311	hsa-miR-641	20.280	0.0004
MIMAT0017997	hsa-mir-3617-5p	−17.522	0.014	MIMAT0025848	hsa-miR-6511b-3p	20.906	2.62^{-06}
MIMAT0019855	hsa-miR-4732-5p	−21.749	0.028	MIMAT0027507	hsa-miR-6803-3p	25.534	2.80^{-12}

the alteration of miRNA has not been thoroughly investigated in relation to acute pain, a study has evaluated changes in other circulating non-coding RNA after laser stimulation showing an alteration of these molecules in the serum of healthy participants (Santoro et al., 2020). A recent study has highlighted how alteration of circulating miR-1 in acute chest pain improves the diagnosis of acute myocardial infarction (AMI) when evaluated at 3 hours from chest pain onset (Su et al., 2020). Moreover, miRNAs have been associated with muscle pain in previous studies, upregulation of miR-124, miR-150, and miR-155 has for example been found in patients with low back pain (Luchting et al., 2017; Sabina et al., 2022). MiR-124 is involved in the regulation of neurogenesis, neuronal differentiation, and stress response (Uchida et al., 2010), and has been demonstrated to be altered in the serum of AMI patients at 24 hours from chest pain onset when compared to controls (Guo et al., 2017). In

TABLE 3 List of identified microRNAs 24 hours from the injection, in pain versus control group

Accession	Gene symbol	Foldchange (log_2)	*p*-Value	Accession	Gene symbol	Foldchange (log_2)	*p*-Value
MIMAT0004484	hsa-let-7d-3p	−1.620	0.003	MIMAT0001618	hsa-miR-191-3p	23.365	1.54^{-16}
MIMAT0000095	hsa-miR-96-5p	−25.941	1.12^{-05}	MIMAT0000441	hsa-miR-9-5p	21.419	6.75^{-10}
MIMAT0004553	hsa-miR-7-1-3p	−26.223	1.16^{-12}	MIMAT0004606	hsa-miR-136-3p	21.715	5.15^{-14}
MIMAT0000255	hsa-miR-34a-5p	−24.126	1.59^{-13}	MIMAT0000453	hsa-miR-154-3p	23.284	2.20^{-09}
MIMAT0000448	hsa-miR-136-5p	−23.575	0.0004	MIMAT0000458	hsa-miR-190a-5p	20.402	3.47^{-09}
MIMAT0000462	hsa-miR-206	−25.775	1.08^{-06}	MIMAT0000682	hsa-miR-200a-3p	20.184	2.55^{-05}
MIMAT0001536	hsa-miR-429	−24.044	1.64^{-07}	MIMAT0000730	hsa-miR-377-3p	19.405	1.45^{-05}
MIMAT0004796	hsa-miR-576-3p	−24.436	2.75^{-13}	MIMAT0002175	hsa-miR-485-5p	20.073	9.57^{-15}
MIMAT0004809	hsa-miR-628-5p	−24.971	8.39^{-13}	MIMAT0004766	hsa-miR-146b-3p	13.817	0.0003
MIMAT0003311	hsa-miR-641	−23.864	7.39^{-07}	MIMAT0002871	hsa-miR-500a-3p	7.476	0.0013
MIMAT0004984	hsa-miR-941	−26.689	2.98^{-13}	MIMAT0002876	hsa-miR-505-3p	4.486	0.003
MIMAT0005876	hsa-miR-1285-3p	−29.997	3.52^{-07}	MIMAT0004792	hsa-miR-92b-5p	12.279	0.005
MIMAT0022720	hsa-miR-1304-3p	−26.371	2.22^{-23}	MIMAT0003241	hsa-miR-576-5p	21.274	1.39^{-10}
MIMAT0005924	hsa-miR-1270	−25.678	7.25^{-09}	MIMAT0003322	hsa-miR-652-3p	8.529	0.0007
MIMAT0015012	hsa-miR-3143	−22.973	0.0003	MIMAT0022714	hsa-miR-766-5p	19.814	1.31^{-07}
MIMAT0004482	hsa-let-7b-3p	6.850	0.0003	MIMAT0004947	hsa-miR-885-5p	10.227	0.0004
MIMAT0022842	hsa-miR-98-3p	8.322	0.0013	MIMAT0005933	hsa-miR-1277-3p	21.487	6.98^{-13}
MIMAT0000098	hsa-miR-100-5p	6.374	0.0001	MIMAT0018961	hsa-miR-4443	8.289	0.004
MIMAT0004549	hsa-miR-148a-5p	22.540	6.48^{-11}	MIMAT0021043	hsa-miR-5010-5p	21.555	2.00^{-16}
MIMAT0004587	hsa-miR-23b-5p	21.875	1.51^{-07}	MIMAT0025479	hsa-miR-6511a-3p	8.880	0.0039
MIMAT0000436	hsa-miR-144-3p	22.860	4.03^{-11}	MIMAT0027416	hsa-miR-6758-5p	21.882	0.0006

this study, the miRNA alterations found 24 hours after stimulation, could then be explained by a regulatory neuronal pathway in which discovered miRNA are potentially involved. It is important to point out the high variability in the expressed transcripts, which might indicate a self-regulation mechanism of miRNAs but also suggest a canonical pathway of RNA messenger regulation is driven by these molecules (Jiao & Slack, 2012; O'Brien et al., 2018). In the present study, only two miRNAs showed a time-stable altered expression in the pain group when compared with controls. These miRNAs, miR-144-3p and miR-377-3p, have been largely investigated in the literature, with a focus on cancer research and their protective effect on tumour growth (Kooshkaki et al., 2020; Yuan et al., 2019). Regarding pain, only a few preclinical studies have shown miR-144-3p as being involved in the regulation of proinflammatory cytokines and neuropathic pain(Hu et al., 2014; Zhang et al., 2020).

4.2 | Biological implications of altered miRNAs

Studies have found that biological and physiological effects of circulating miRNAs are dysregulated in patients with different painful conditions such as painful knee osteoarthritis, complex regional pain syndrome, migraine, peripheral neuropathy, and fibromyalgia when compared to healthy controls (Birklein et al., 2018; Gallelli et al., 2019; Giordano et al., 2020; Leinders et al., 2016). The current study adds to the previous findings by reporting some of the miRNAs associated with pain pathways and highlights new markers yet to be associated with pain in humans (Gazerani, 2019; Leinders et al., 2016; Park et al., 2014; Wen et al., 2019; Xu et al., 2014; Zhang et al., 2019). Within the 42 dysregulated transcripts, miRNA such as let-7b and let-7d were differentially expressed in the pain group, confirming their potential involvement in pain regulation mechanisms (Leinders et al., 2016; Park et al., 2014). MiRNAs of this family have been shown to be expressed in stem cells involved in neuronal differentiation at the developmental level, to induce apoptosis of cortical neurons, and to be expressed in dorsal root ganglion regulating pain and the expression of several ion channels(Park et al., 2014; Zhao, Lee, et al., 2010; Zhao, Sun, et al., 2010). These two miRNAs have been associated with painful behaviour in rats through TLR7 and TRPA1 receptors activation (Park et al., 2014), but also with reduced small nerve

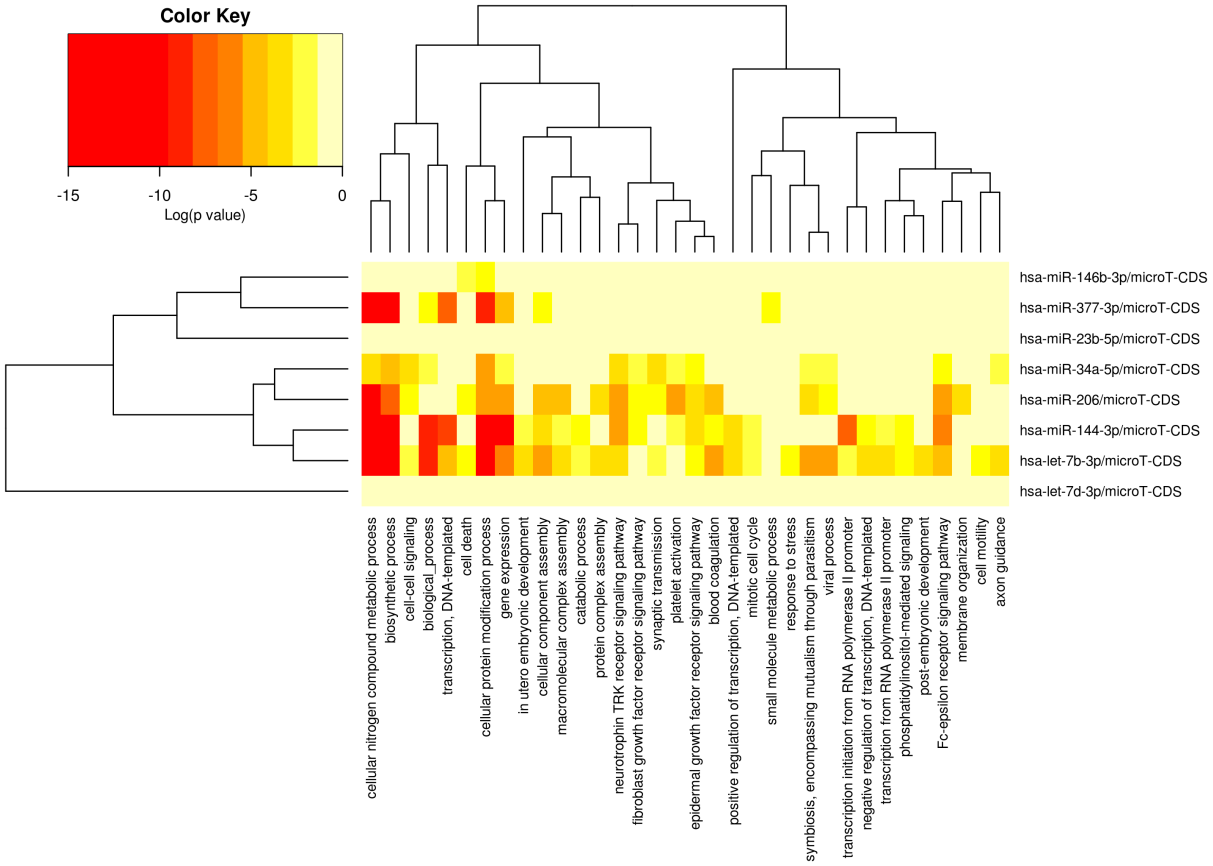

FIGURE 5 miRNA versus GO biological process heatmap. The heatmap depicts the level of enrichment in the GO biological process of a sub-set of miRNAs altered in the pain group versus control group, enabling the identification of miRNA subclasses or biological processes that characterize similar miRNAs, when clustered together. Significance ($p < 0.05$) is represented in a colourimetric scale and as –log (*p*-value).

fibre density in patients with fibromyalgia (Leinders et al., 2016). In the present study upregulation of miR-let7b and downregulation of miR-let-7d were shown in subjects that received a painful hypertonic injection, and the enrichment analysis confirmed the significant association of miR-let7b whit neuronal process and response to stress.

MiRNA such as miR-34a-5p has been previously studied and an upregulation is found following painful migraine episodes and a decreased level is found following treatment for migraine (Andersen et al., 2016; Brandenburger et al., 2019; Gallelli et al., 2019). The current study, reports a downregulation of miR-34a-5p 24 hours after a painful injection and this result can be explained as a regulatory function of this miRNA in pain sensation, confirmed also by its involvement in synaptic transmission and neuronal processes shown by the enrichment analysis. In addition, it has been shown that miR-23b, is involved in the regulation of expression of μ-Opioid Receptor (MOR1) and that an upregulation of miR-23b is associated with opioid tolerance and thus potentially regulating endogenous analgesic mechanisms (Wu et al., 2008, 2009). The upregulation of the same miRNA has recently been associated with acute pain in animal models for laminitis (Lecchi et al., 2018). The results present in this manuscript, show an upregulation of miR-23b that can be detected after 24 hours in subjects who receives an injection of hypertonic saline. Moreover, in the current study, downregulation of miR-206 was shown 24 hours after a hypertonic saline injection, which is in line with a previous preclinical study, where downregulation of miR-206 was associated with a reduction of neuropathic pain in a rat model (Wen et al., 2019).

4.3 | Study limitations

This exploratory study is limited by the small sample size and future studies should investigate bigger cohorts in order to validate the alteration of highlighted miRNAs. Variable expression by genetic factors within subjects of the same group could be pointed out as a limitation, although the high variance of personal expression of miRNAs in humans depends on many confounding factors

(Wu et al., 2018). These factors were partly controlled for by the inclusion and exclusion criteria.

A sample size calculation for the present study was not performed due to the exploratory nature of the study, to account for it a validated pipeline that avoids statistical errors was run (Schurch et al., 2016).

The traumatic usage of a needle for injection and blood drawings can be included in the potential confounding factor for miRNAs dysregulation, and to avoid misinterpretation of results, the expression levels at each time point were normalized for baseline and miRNAs altered by technical procedures. The pain and the control group received the same number of needle trauma, which should normalize potential expression modifications of the discovered miRNAs due to the stressful intervention but this should be further explored in future research. The present study cannot define the exact recipient cells of the presented dysregulated miRNAs and their direct involvement in specific pain processes and pathways.

5 | CONCLUSIONS

This is the first human exploratory study to investigate the temporal profile expression of circulating miRNAs in plasma for 24 hours after an acute, experimental, painful muscle stimulation. This study showed an alteration of 4 circulating miRNAs after 30 minutes from injection, 24 transcripts after 3 hours, and 42 miRNAs after 24 hours. Several miRNAs (including miR-let7, miR-34a, and miR-206) have previously been associated with pain pathways for migraines, neuropathic pain, and inflammatory pain. The current study suggests that the alteration of these miRNAs is not only expressed in chronic painful conditions but can be also induced by an acute painful event. The implications of this for both the prevention and management of chronic pain remain to be studied. More research will be needed to validate the alteration found in the circulating miRNAs, confirm their association with pain mechanisms and explore the duration of the epigenetic modifications.

AUTHOR CONTRIBUTIONS

All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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