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Decrease in Plasma miR-27a and miR-221 After Concussion in Australian Football Players

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ABSTRACT

INTRODUCTION: Sports-related concussion (SRC) is a common form of brain injury that lacks reliable methods to guide clinical decisions. MicroRNAs (miRNAs) can influence biological processes involved in SRC, and measurement of miRNAs in biological fluids may provide objective diagnostic and return to play/recovery biomarkers. Therefore, this prospective study investigated the temporal profile of circulating miRNA levels in concussed male and female athletes.

METHODS: Pre-season baseline blood samples were collected from amateur Australian rules football players (82 males, 45 females). Of these, 20 males and 8 females sustained an SRC during the subsequent season and underwent blood sampling at 2-, 6- and 13-days post-injury. A miRNA discovery Open Array was conducted on plasma to assess the expression of 754 known/validated miRNAs. miRNA target identified were further investigated with quantitative real-time PCR (qRT-PCR) in a validation study. Data pertaining to SRC symptoms, demographics, sporting history, education history and concussion history were also collected.

RESULTS: Discovery analysis identified 18 candidate miRNA. The consequent validation study found that plasma miR-221-3p levels were decreased at 6d and 13d, and that miR-27a-3p levels were decreased at 6d, when compared to baseline. Moreover, miR-27a and miR-221-3p levels were inversely correlated with SRC symptom severity.

CONCLUSION: Circulating levels of miR-27a-3p and miR-221-3p were decreased in the sub-acute stages after SRC, and were inversely correlated with SRC symptom severity. Although further studies are required, these analyses have identified miRNA biomarker candidates of SRC severity and recovery that may one day assist in its clinical management.

KEYWORDS: Traumatic brain injury, mild traumatic brain injury, biomarker, inflammation, vascular injury, pathophysiology

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Introduction

Sports-related concussion (SRC) is a common form of mild traumatic brain injury (mTBI) that can result in debilitating symptoms that are disruptive to daily life.[1,2] These symptoms often resolve within weeks,[1,3] however, there is growing evidence that neurobiological abnormalities persist beyond the resolution of symptoms.[4-6] Furthermore, 10% to 30% of SRC patients go on to suffer from persistent post-concussion symptoms (PPCS),[2] and SRC has been linked to the later development of neurodegenerative conditions, such as chronic traumatic encephalopathy.[7] Notably, there appears to be an increased risk of long-term neurological consequences in those who experience repetitive mTBIs,[8,9] with evidence suggesting this may be due to the repetitive injuries occurring within a period of vulnerability after the initial SRC.[10]

Although we now have a better understanding of the risks associated with SRC, the methods to assist with the clinical diagnosis and subsequent treatment/management of SRC are still lacking.[1,3] Diagnosis relies heavily on subjective, unreliable signs and symptoms, as do decisions pertaining to recovery and when it is safe for athletes to return to play (RTP).[11] Consequently, many SRC cases go undiagnosed, and athletes may be cleared to RTP before their brains have fully recovered. It is therefore imperative that an objective set of diagnostic and recovery/RTP biomarkers are developed.

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Table 1. Baseline demographic results for male and female Australian rules footballers featured in the miRNA validation study.

	MALE FOOTBALLERS			FEMALE FOOTBALLERS		
	SRC	CONTROL	*P*-VALUE	SRC	CONTROL	*P*-VALUE
N	20	19	-	8	8	–
Age	23.8 ± 3.5	23.9 ± 3.1	.85	24.6 ± 4.1	23.5 ± 3.4	.56
Years of education	16.6 ± 2.2	16.9 ± 1.4	.54	15.9 ± 2.2	15.5 ± 2.1	.73
Years of sport	16.1 ± 5.0	17.5 ± 3.5	.33	18.4 ± 5.4	16.1 ± 5.9	.44
Years of collision sport	13.9 ± 4.6	14.4 ± 4.6	.78	8.4 ± 5.7	7.4 ± 5.2	.72
No. of previous concussions	2.1 ± 1.8	2.3 ± 1.7	.78	0.1 ± 0.4	1.3 ± 1.8	.13

Players who sustained a concussion during the collection period were allocated into the SRC group, with non-concussed footballers serving as controls for each sex. Results are presented as mean ± SD.

Circulating biomarkers provide a potential window into the pathophysiology and recovery of the brain following SRC.[12-14] MicroRNAs (miRNA) are a class of short single-stranded non-coding RNAs that modulate the translation of messenger RNAs (mRNA) and proteins.[15,16] As such, miRNA can influence biological processes involved in the pathophysiological response to brain injury including apoptosis, neuronal repair, angiogenesis and immune/inflammatory responses.[17-20] Recent studies have therefore begun to investigate circulating miRNA as biomarkers for mTBI.[17,19-22] For example, mTBI patients recruited from an emergency department had increased plasma levels of miR-92a and miR-16, which are involved in inflammatory and apoptotic pathways, within 10 hours of injury compared to healthy controls.[18] Another study involving emergency department patients found that those with mTBI had upregulation of 10 miRNAs (miR-151, miR-20a, miR-362, miR-486, miR-505, miR-499, miR-625, miR-638, miR-381, miR-142), and downregulation of 4 miRNAs (miR-30d, miR-328, miR-27b, miR-92) in serum within 1 hours of injury compared to healthy controls.[21] Das Gupta et al[19] found that a subpopulation of mTBI patients had increased levels of miR-9a (implicated in angiogenesis and neurogenesis) and miR-136 (implicated in inflammation) within 2 days of injury compared to age-matched controls. In a study investigating mTBI patients with extracranial injury, serum miR-425 (implicated in necroptosis) and miR-502 (implicated in autophagy) were downregulated within 12 hours of injury.[22] Specific to SRC, one study found that a proportion of concussed athletes had upregulation of circulating miR-153 (implicated in angiogenesis), miR-223 (implicated in inflammation) and miR-let-7a (implicated in apoptosis) acutely (ie, ~8 hours) after SRC compared to their offseason baseline samples.[20]

Although these studies are promising in the context of mTBI diagnosis, they focus primarily on acute changes and do not capture the dynamic variations in miRNAs during SRC recovery, which is highly relevant in the context of RTP decisions. Therefore, in this prospective study we investigated the temporal profile of circulating miRNA levels in male and female Australian footballers (ie, Australia's most participated collision sport[23]) at 2-, 6- and 13-days post-SRC in comparison to their pre-injury baseline and control Australian footballers without SRC.

Methods

Study population and study design

A total of 127 (82 males, 45 females) amateur Australian rules football players were recruited for this study (male collection period 2016-2019, female 2018-2019). The Melbourne Health Human Ethics committee approved study procedures (#2015.012), with all participants providing informed written consent. A matched prospective cohort study was conducted, featuring the same cohort and study design as previously described in our recent protein biomarker study.[4] Briefly, baseline samples were collected at each pre-season. Players who self-reported a diagnosis of concussion in the preceding 6 months, had a history of neurosurgery or severe psychiatric disturbances were excluded. Twenty-eight players (20 males, 8 females) diagnosed by a team physician with an SRC (ie, GCS 13-15) during the subsequent season with blood sampling at 2-, 6- and 13-days post-SRC (Table 1). Of these 28 participants, 2 male footballers were diagnosed as sustaining a concussion in 2 separate seasons. As these players met the inclusion criteria of the study, and had participated in 2 separate baseline collections, they were included in analysis. Additionally, 2 players (1 male, 1 female) were diagnosed as concussed twice in the one season, with only their first concussion samples included. All within-subject comparisons were made relative to baseline samples from the corresponding pre-season. The miRNA discovery study featured sets of samples from 9 Australian footballers sustaining SRC in the 2018 season (5 males, 4 females), except one baseline, one 2-day sample, three 6-day samples and two 13-day samples not collected due to logistic reasons (eg, participant unavailability); therefore, the sample size for the

OpenArray was 6 to 8 individuals per time-point. For the validation study on the 28 participants, a small number of baseline and post-SRC collections were missed due to logistical reasons, resulting in a dataset of 23 to 26 samples per time-point for validation studies. An additional 27 baseline samples (19 males, 8 females) from closely matched players that did not sustain a SRC were selected by researchers blinded to biomarker data for use as controls in the validation study (Table 1). Sample sizes were based on our previous mTBI blood biomarker studies.[4,24]

Clinical interview and symptom evaluation

A questionnaire was administered pertaining to factors including demographics (ie, age, gender, height, weight), sporting history (ie, sports played, age they commenced collision sports, age they commenced all sport, years participating in collision sport, years participating in all sports), years of education and history of concussion (number and dates of previous concussions).[4,24-29] The 'sport concussion assessment tool' (SCAT; SCAT3 for 2017/18 players, SCAT5 for 2019 players) assessed symptom severity (maximum 132) at 2-, 6- and 13-days after SRC.

Blood collection

Using standard phlebotomy procedures, whole blood was collected into BD Vacutainer® K2 EDTA tubes for plasma separation. Samples were briefly stored at 4°C (<2 hours), centrifuged at 1100*g* for 10 minutes at room temperature, and plasma transferred into aliquots and stored at −80°C.

miRNA discovery

The expression of 754 known and validated miRNAs was assessed using an OpenArray™ platform.[30] RNA was isolated from 100 µL of plasma using QIAcube HT (Qiagen) robotic workflow. Briefly, 10 µg of glycogen and 0.5 mL Trizol (Thermo Fisher Scientific) were added to 100 µL of plasma and the mixture was thoroughly vortexed. This was followed by the addition of 0.1 mL chloroform and vigorously shaken. Aqueous and organic phases were separated by centrifugation at 3100*g* at 4°C for 25 minutes. RNA precipitation and elution were carried out on the QIAcube-HT automated robotic system using the RNeasy 96 QIAcube HT kit in a final elution volume of 75 µL of nuclease-free water. RNA concentration was determined using the Eon Microplate Spectrophotometer (Biotek, USA) on the day of miRNA processing. Discovery panels of 754 known and validated human miRNAs were assessed by qRT-PCR on Thermo Fisher's TaqMan-based qRT-PCR on a high-throughput OpenArray platform as described in detail previously.[31,32] Briefly, 50 ng total RNA underwent reverse transcription before 12 cycles of preamplification. Each sample was diluted 1:40 in 0.1xTE (pH 8.0) combined with TaqMan OpenArray PCR Master Mix and loaded onto TaqMan OpenArray™ panels using the AccuFill™ system. The qRT-PCR was completed using the QuantStudio® 12K Flex (Thermo Fisher).

Analyses of OpenArray™ data was performed using GenEx 7.0 qPCR data analysis software (MultiD). Filtering of low-quality amplification of miRNA was performed by removing data points where the quality control flags 'AmpScore' or 'CqConf' were <1.24 or <0.8 respectively. Individual miRNAs were also removed from analysis where less than 80% of samples contained amplification values. Missing data points of remaining samples were imputed as the maximum cycle threshold (Ct) + 2. Normalisation was performed using the ΔCt method. The most stable miRNA for normalisation, miR-24-3p and miR-484, were identified by GeNorm and confirmed by manufacturers recommendations. Fold changes were calculated using the ΔΔCt method and log2-transformed for statistical analysis.

miRNA validation

Following completion of discovery data set analysis, 18 miRNA candidates were chosen for further investigation with qRT-PCR in a validation study of all samples from 28 players diagnosed with SRC (ie, baseline, 2-, 6- and 13-day samples) and 27 controls. miR-24-3p and miR-484 were used as endogenous controls (as identified in discovery study), along with ath-miR-159a as an exogenous spike-in control.

Plasma samples (100 µL) were thawed at room temperature, with 10 µg glycogen (RNA grade, Thermo Fisher Scientific) added to each sample, followed by 500 µL of Trizol containing the spike-in control (ath-miR-159a). The final concentration of ath-miR-159a in the sample was 3.2×10^8 copies/mL. Samples were vortexed for 40 seconds, followed by a 10-minute incubation at room temperature. Samples were then briefly centrifuged at 4°C, 100 µL of chloroform was added, vortexed for 40 seconds, and then incubated at room temperature for 15 minutes. After centrifugation (12 000*g*, 15 minutes, 4°C), 300 µL of the aqueous phase was transferred into a new 1.5 mL tube. One volume of 70% ethanol was added to each tube and mixed immediately by pipetting. Total RNA, including the miRNA, was isolated from the sample using spin technology with the RNeasy® Mini Kit and wash buffer RWT (Qiagen). The obtained RNA sample was then processed with TaqMan Advanced miRNA cDNA Synthesis Kits (Life Technologies), and a miR-Amp reaction was performed. The final cDNA product was diluted 1 in 5 for qRT- PCR with the TaqMan Advanced miRNA Assays (Thermo Fisher Scientific). A QIAgility (Qiagen) was used for plate set up, and the reactions were performed by QuantStudio™ (ThermoFisher). Cycle threshold values were determined. Spike in control ath-159a was consistently expressed in all samples (cycle threshold coefficient of variation (CV) = 2.09%), as were endogenous controls miR-24-3p and miR-484 (CVs 5.38% and 4.83%, respectively). Quantification of each miRNA was performed using normalisation to the geometric mean of endogenous controls

(ΔCt), with fold changes calculated using the ΔΔCt method and log2-transformed for statistical analysis.

Pathway analysis

Two microRNAs (miR-27a-3p and miR-221-3p) are further selected for Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis using a web tool (PMID: 22870325) (miRSystem, ver. 20160513; http://mirsystem.cgm.ntu.edu.tw/) as described earlier (PMID 33755745). Only the pathways with empirical *P*-values (≤.05) that are targetted by these microRNAs are presented.

Statistics

Demographic variables were compared between SRC and non-SRC footballers of each sex with an unpaired *t*-test (Table 1). Open Array miRNA (log2) data were analysed by one-way ANOVA (parametric data) or Kruskal-Wallis (non-parametric data), and validation study results (log2) were analysed with a within subjects mixed effects model, with factors of sex and time and Dunnett's multiple comparisons test conducted where appropriate. Non-SRC controls were not included in statistical analyses. SCAT 5 symptom severity scores at 2-, 6- and 13-days were analysed for correlation with validation study miRNA fold-changes (2-, 6- and 13-day). Correlation analysis was performed using R (R Core Team, 2020) with the pair.panels function the psych package to calculate Spearman correlation coefficients and generate bivariate scatter plot matrices. Statistical significance was set at $P < .05$. All statistical tests were performed using GraphPad Prism version 8.1.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Demographics

When comparing footballers in the SRC and non-SRC groups, there were no significant within-sex differences for self-reported age, total years of education, total years of sport and collision sport participation, nor history of concussion (Table 1).

miRNA discovery

Of the 754 miRNAs assessed, 186 miRNAs were consistently detected in plasma in the Open Array discovery study. Of these, 18 miRNAs were found to be significantly altered between time-points in the discovery study with miR-19b-1-5p, miR-20b-5p, miR-21-5p, miR-27a-3p, miR-28-5p, miR-103a-3p, miR-106a-5p, miR-125-5p, miR-142-3p, miR-194-5p, miR-221-3p, miR-223-3p, miR-301b, miR-338-5p, miR-643, miR-769-5p, miR-1260a and miR-1290 all found to have $P < .05$ with ANOVA or Kruskal-Wallis test analyses (test chosen based on data distribution). GeNorm analyses revealed that miR-24-3p and miR-484 were most suitable for use as reference miRNA for the validation study.

miRNA validation: Temporal profile

Of the 18 miRNAs, 2 (miRs 338-5p and 1290) were not detected in the majority of plasma samples, 3 (miRs 19b-1-5p, 643, 769-5p) were detected in less than 95% of samples and 1 (miR-1260a) demonstrated amplification in no template control. As such, these 6 miRNAs were excluded from analysis. Two miRNAs (miRs 194-5p and 301b) did not reach cycle threshold for one sample and were allocated a cycle threshold value of 40 to enable data analysis. As such, a total of 12 miRNA targets were analysed. To reduce data skew induced by outliers to allow for a linear mixed effects model to be applied to log2 relative miRNA expression data, a conservative outlier test (ROUT, Q = 1%) was applied, resulting in 18 values (out of a total of 1846) removed (ie, miR-21-5p × 1; miR-194-5p × 1; miR-106a-5p × 3; miR-125b-5p × 1; miR-223-3p × 10; miR-301-3p × 2).

A mixed effects model was used to compare plasma miRNA levels over time and between sex for individuals at baseline, 2-, 6- and 13-days post-concussion, with this data, along with non-SRC controls (not used in statistical analysis), presented in Figure 1. Analysis of miR-27a-3p (Figure 1C), revealed a main effect of time ($F(2.516, 52.84) = 3.656$, $P = .024$), but no effect of sex ($F(1, 26) = 2.066$, $P = .163$) or a sex by time interaction ($F(3, 63) = 0.625$, $P = .602$). Post-hoc analyses of miR-27a-3p levels found that when compared to baseline, levels were decreased at 6d ($P = .003$), but not altered at 2d ($P = .820$) or 13d ($P = .067$). Analysis of miR-221-3p (Figure 1J) revealed a main effect of time ($F(1.827, 38.37) = 4.456$, $P = .021$), but no effect of sex ($F(1, 26) = 0.872$, $P = .359$) or a sex by time interaction ($F(3, 63) = 0.307$, $P = .821$). Post-hoc analyses of miR-221-3p levels found that when compared to baseline, levels were decreased at 6d ($P = .004$) and 13d ($P = .007$), but not 2d ($P = .168$). No other significant effects of time nor interactions were found, although a main effect of sex was found for miR-21-3p ($F(1, 26) = 4.882$, $P = .0361$) and miR-223-3p ($F(1, 25) = 8.519$, $P = .007$), with plasma levels of both miRNAs higher in females than males.

miRNA validation: Symptom and miRNA correlation

Our previous study featuring analysis of SCAT symptoms in this cohort at 2-, 6- and 13-days revealed that when compared to baseline, SCAT symptoms were only statistically elevated above baseline levels at 2-day.[4] Nonetheless, as some players reported symptoms at 6- and/or 13-days post-injury, correlation analyses were performed to determine if symptoms aligned with miRNA levels, both within and across time-points. Firstly, as shown in Figure 2, plasma miRNA levels at 2 days were compared with SCAT symptom severity scores at 2-, 6- and 13-days. When comparing 2-day plasma miRNA levels and 2-day symptoms, Spearman correlation analyses revealed a significant negative correlation only for miR-106a-5p ($r = -.48$, $P = .026$). No correlations were found between 2-day miRNA and 6d symptoms.

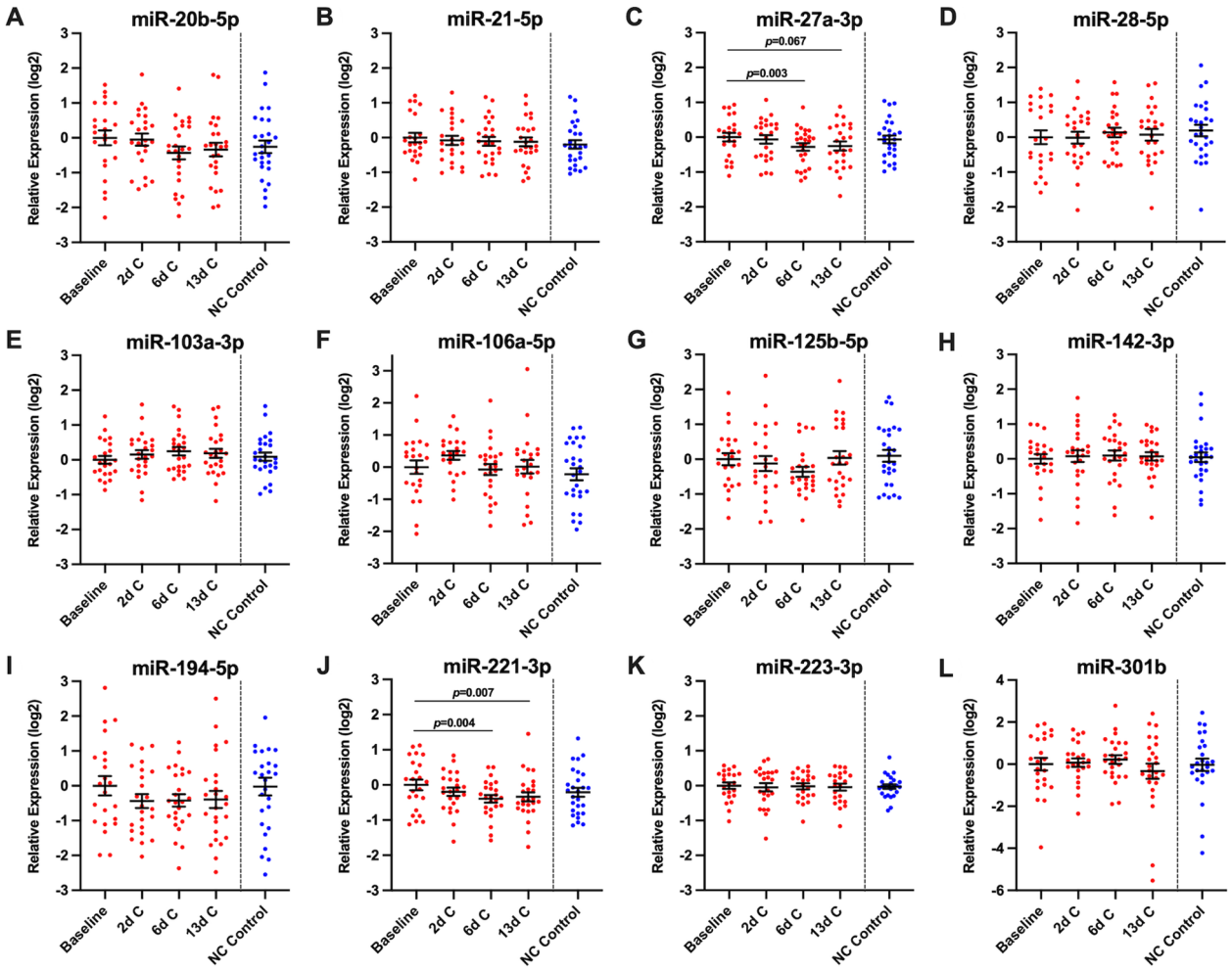


Figure 1. Temporal profile of plasma miRNAs after SRC. Levels are compared between pre-season (ie, baseline), and post-concussion at 2-days (2d C), 6-days (6d C), and 13-days (13d C). Non-concussed controls are included as point of reference, and were not included in this statistical analysis. When compared to baseline, plasma levels of miR-27a-3p were significantly decreased at 6-days (C), and miR-221-3p levels were reduced at 2- and 6-days (J). No significant differences from baseline were observed for miR-20b-5p (A), miR-21-5p (B), miR-28-5p (D), miR-103a-3p (E), miR-106a-5p (F), miR-125b-5p (G), miR-142-3p (H), miR-194-5p ()), miR-223-3p (K), miR-301b (L). Individual participant results are plotted, along the group mean ± SEM.

Symptoms at 13-day were negatively correlated with 2-day miRNA levels of miR-106a ($r=-.57$, $P=.011$), miR-27a ($r=-.44$, $P=.048$) and miR-223 ($r=-.51$, $P=.021$). Plasma miRNA levels at 6-day were also compared to symptoms at 2-, 6- and 13-days (Figure 3). A negative correlation of 6-day miRNA levels was observed with symptoms at 2-day for miR-27a ($r=-.49$, $P=.017$), miR-20b ($r=-.53$, $P=.010$), miR-223 ($r=-.434$, $P=.049$) and with symptoms at 13-days for miR-27a ($r=-.45$, $P=.033$) and miR-223 ($r=-.47$, $P=.036$). For miRNA levels at 13-days (Figure 4), plasma miRNA negatively correlated with symptoms at 2-day for miR-103a ($r=-.49$, $P=.021$), miR-27a ($r=-.53$, $P=.012$) and miR-20b ($r=-.660$, $P=.001$), and symptoms at 6-days for miR-221 ($r=-.43$, $P=.038$). Numerous positive correlations were found between miRNAs at all 3 time-points, along with 1 negative correlation between at 13-days. Unsurprisingly, positive correlations were found between symptoms at each time-point. Last, correlations between plasma miRNAs and relevant clinical characteristics (ie, age and number of previous concussions) were investigated. At baseline there was a positive correlation between age and miR-223 ($r=.536$, $P=.010$); at 2-days there was a positive correlation between age and miR-194 ($r=.445$, $P=.029$) and a negative correlation between concussion history and miR-28 ($r=-.513$, $P=.012$); and at 13-days there was a negative correlation between concussion history and miR-221 ($r=-.422$, $P=.036$).

miRNA validation: KEGG pathway analysis

Eight KEGG pathways targetted by the 2 significantly altered miRNA in the validation study were found to have empirical P-values $<.05$ (Figure 5). These included alterations in protein processing in the endoplasmic reticulum, T cell receptor signalling, axon guidance, mitogen-activated protein kinase (MAPK) signalling and neuroactive ligand-receptor binding.

Discussion

miRNAs are increasingly recognised as having great potential as circulating biomarkers of injury and disease. In the context

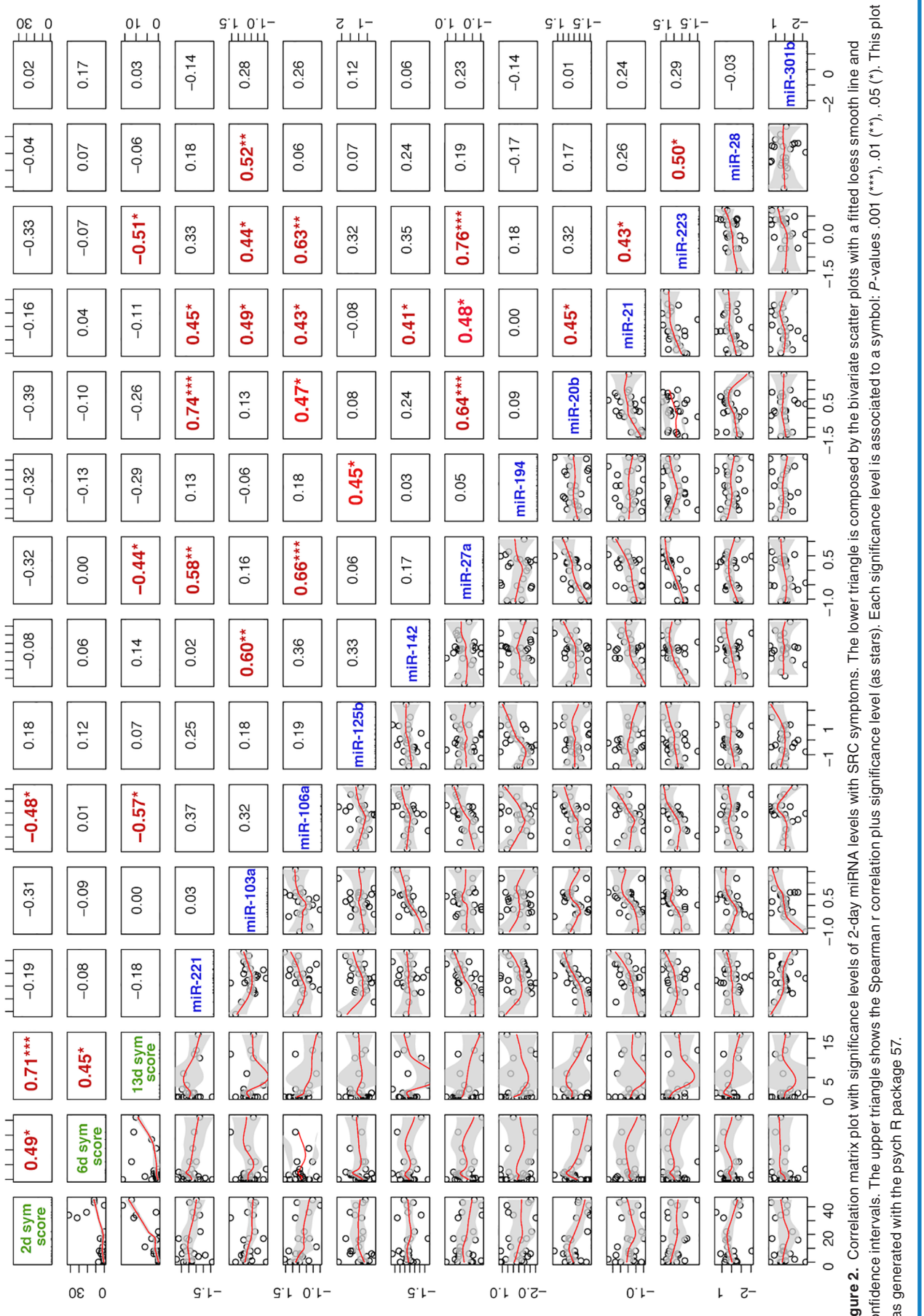


Figure 2. Correlation matrix plot with significance levels of 2-day miRNA levels with SRC symptoms. The lower triangle is composed by the bivariate scatter plots with a fitted loess smooth line and confidence intervals. The upper triangle shows the Spearman r correlation plus significance level (as stars). Each significance level is associated to a symbol: *P*-values .001 (***), .01 (**), .05 (*). This plot was generated with the psych R package 57.

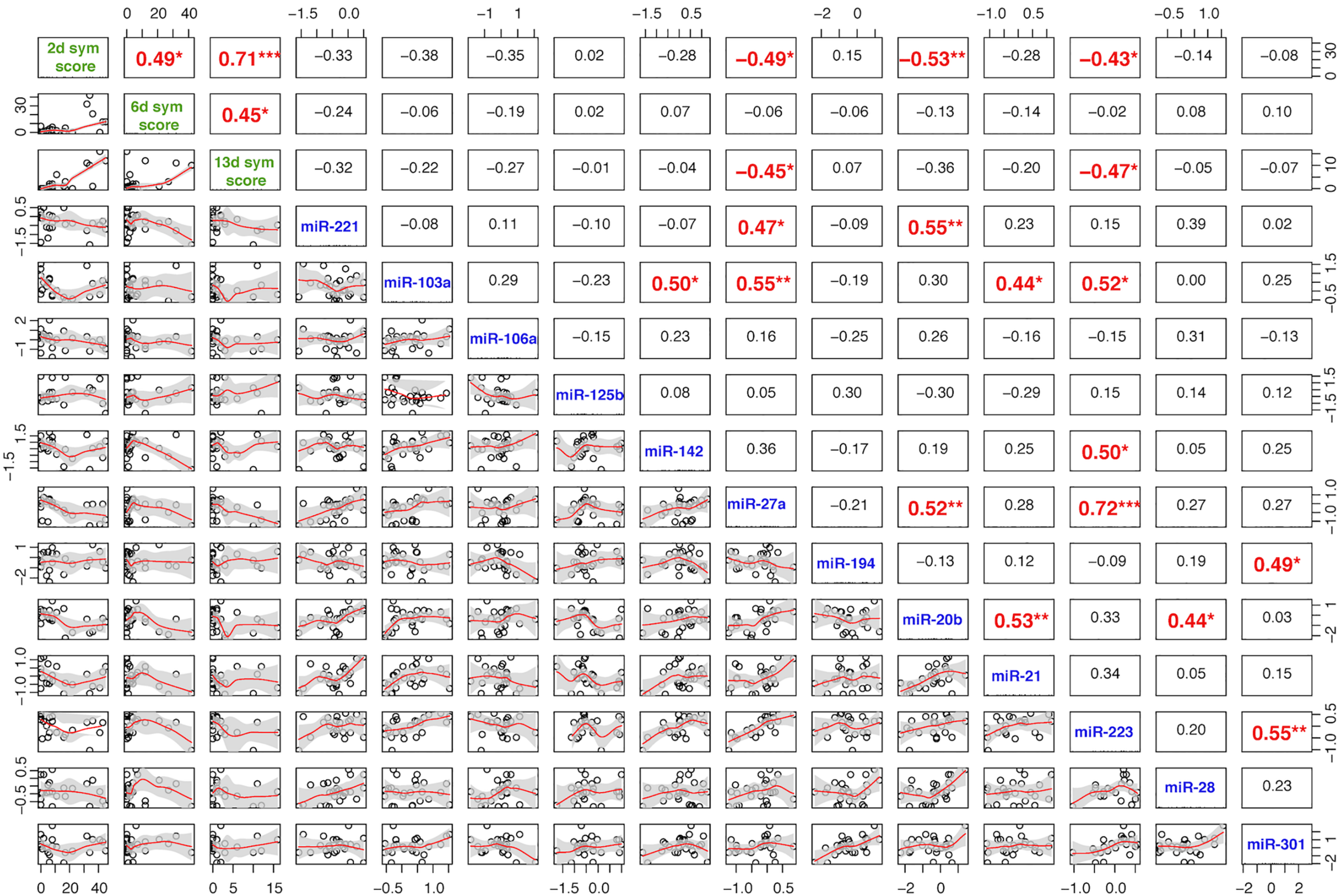


Figure 3. Correlation matrix plot with significance levels of 6-day miRNA levels with SRC symptoms. The lower triangle is composed by the bivariate scatter plots with a fitted loess smooth line and confidence intervals. The upper triangle shows the Spearman r correlation plus significance level (as stars). Each significance level is associated to a symbol: *P*-values .001 (***), .01 (**), .05 (*). This plot was generated with the psych R package 57.

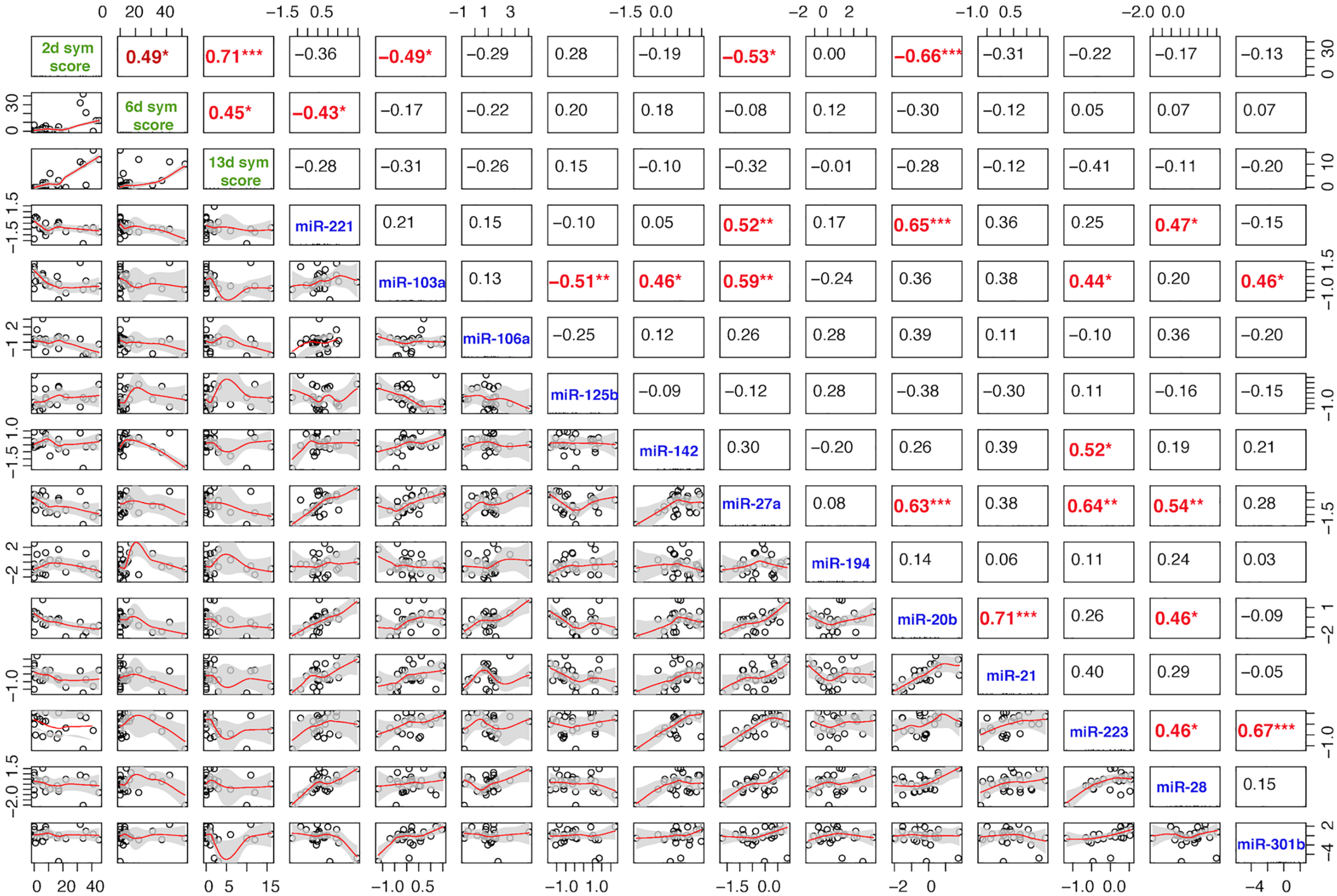


Figure 4. Correlation matrix plot with significance levels of 6-day miRNA levels with SRC symptoms. The lower triangle is composed by the bivariate scatter plots with a fitted loess smooth line and confidence intervals. The upper triangle shows the Spearman r correlation plus significance level (as stars). Each significance level is associated to a symbol: *P*-values .001 (***), .01 (**), .05 (*). This plot was generated with the psych R package in R 57.

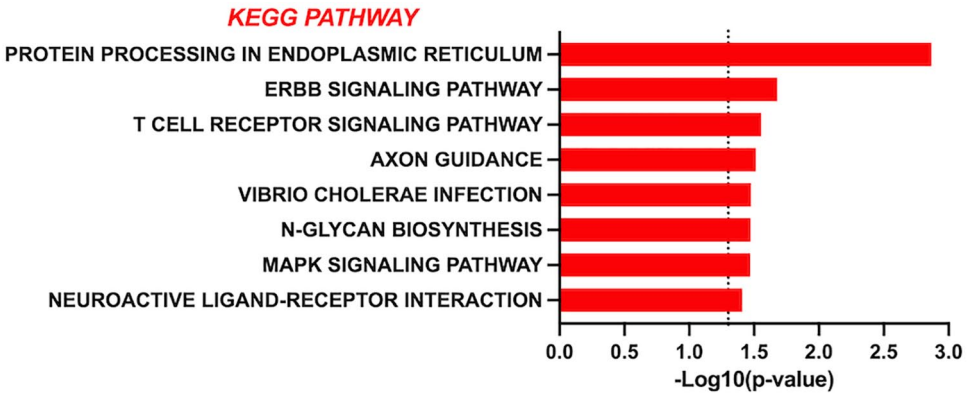


Figure 5. Bar graph of KEGG pathways targetted by the 2 microRNAs (miR-27a-3p and miR-221-3p). Significance converted to –log10(*P* value), dotted line indicates cut-off value of $P = .05$. Eight significant pathways targetted by these 2 microRNAs are presented.

of mTBI, initial studies have shown that levels of certain circulating miRNAs may be altered acutely after injury; however, the evolution of miRNA changes after mTBI is poorly understood. In this prospective study of male and female Australian footballers, we present evidence that plasma levels of both miR-27a and miR-221 are decreased in the sub-acute stages after SRC. Moreover, we found that plasma levels of these miRNAs were inversely correlated with SRC symptom severity. Although requiring further investigation and validation in other larger cohorts, these promising findings indicate that the validated miRNA candidates may have utility in assisting SRC management. Herein, we discuss the nature of our findings, their potential pathophysiological and clinical relevance, limitations of the study and areas for follow-up research.

Potential clinical significance of subacute decrease of miR-27a and miR-221 after SRC

Although self-reporting of symptoms is a widely acknowledged limitation for both SRC management and research, the independence of our SCAT tests from RTP decisions provides some confidence that such evaluations may have less bias susceptibility. As such, although requiring validation, the miRNA correlations with symptoms observed provides further evidence of an association with SRC. Most notably, we found that plasma miR-27a were inversely correlated with SCAT symptom severity across multiple time-points. Specifically, we found that symptoms at 2 days post-SRC (ie, the overall peak of symptom reporting of the time-points in this study) were inversely correlated with miR-27a levels in plasma at 6- and 13-days, and that symptoms at 13-days correlated with plasma miR-27a levels at 2- and 6-days. For miR-221, levels at 13-days correlated with 6-day symptom severity only.

In addition to providing further evidence of SRC specificity, these miRNA findings may indicate potential of miRNAs as objective biomarkers to assist SRC management. For example, that 2- and 6-day levels of miR-27a were inversely correlated with 13-day symptoms may suggest use for predicting and monitoring symptom recovery. Notably, although our previous protein biomarker studies in the males of this cohort found elevated serum glial fibrillary acidic (GFAP; 2-days), neurofilament light (NfL; 6- and 13-days) and tau (13-days) after SRC, we did not observe such correlations with symptoms.[4] Nonetheless, it is important to acknowledge that resolution of symptoms may not coincide with pathophysiological recovery, and therefore it is possible that biomarkers may have different uses for indicating each aspect of 'recovery' after SRC.

Potential neurobiological significance of miR-27a and miR-221 decrease after SRC

Studies of other neurological conditions indicate that miR-221 may have important neurobiological roles, and that decreased circulating levels of miR-221 occur in brain injury or disease. miR-221 is one of the most abundant miRNAs in the human brain,[33] with in vitro studies suggesting a critical role of miR-221 in neuronal cell differentiation and survival.[34,35] miR-221 is also thought to have important roles in vascular homoeostasis via inhibitory effects on endothelial cell proliferation and motility,[36] therefore alterations in circulating levels may reflect response to neurovascular injury. Moreover, serum miR-221 levels have been shown to be decreased within the first week after ischaemic stroke,[37,38] and pre-treatment with a miR-221 mimic reduced neuroinflammation and infarct size after ischaemic stroke in mice.[39] There is also clinical evidence that serum miR-221 is decreased in Parkinson's disease patients,[40,41] and in vitro findings that Parkinson's disease-associated loss-of-function mutations of *DJ-1* resulted in reduced miR-221 levels.[42]

We also found a decrease in plasma miR-27a levels at 6-days after SRC. miR-27a appears to have several functions related to TBI pathophysiology, including negative regulation of neuroinflammation,[43] and protection from neuronal apoptosis,[44] and blood brain barrier disruption.[45] Experimental TBI studies have revealed a down-regulation of miR-27a in the injured brain tissue within the first 24 hours of injury in both mice[44] and rats,[46] with intracerebroventricular administration of either a miR-27a mimic,[44] or lentivirus encoding miR-27a,[46]

reducing lesion volume and neuronal cell death. These studies provide some evidence that the reduced plasma miR-27a observed in the current study may reflect changes in the brain following trauma. To the best of our knowledge, altered miR-27a has not yet been reported in fluids after TBI, possibly attributed to the acute sampling time-points in previous studies. Nevertheless, multiple clinical studies in other forms of brain injury and disease have revealed similar decreases in circulating miR-27a. For example, in patients with intracerebral haemorrhage, decreased miR-27a was observed in serum collected within 48 hours of admission.[45] In 2 populations of AD patients,[47] CSF levels of miR-27a were decreased when compared to controls, with this decrease found to correlate with AD pathology (ie, high CSF tau and phosphorylated-tau, and low β-amyloid 42).

Although requiring validation using luciferase assays, our analysis of KEGG pathways targetted by miR-27a and miR-221 also revealed significant pathways that may be a part of mTBI pathophysiology. Considered alongside the aforementioned literature on these miRNA in brain injury and disease, we hypothesise that the decreased plasma miR-221 and miR-27a after SRC may indeed be associated with the pathophysiological aftermath of mTBI.

Study Strengths and Limitations

This study uses a high sensitivity TaqMan™ qRT-PCR chemistry for assessing mature microRNAs that are biologically relevant. The study is one of the first demonstrating miRNA expression profiles over multiple time-points in 2 weeks from SRC. Identified candidates target several key pathways associated with neuronal health and pathophysiology (discussed above). Since microRNAs can be more stable biomarkers, which can be reliably measured in fresh and frozen samples, they offer the potential to be validated in other SRC studies. As miRNAs are not only biomarkers, but regulators of gene expression, they may identify new regulatory pathways associated with SRC recovery as targets for future therapy. With several technological advances on the horizon that use nanotechnological platforms for point-of-care miRNA detection, the study findings offer an important advance in the identification of candidate molecules for future test advising on RTP for SRC.

With that said, this study had several limitations the warrant further discussion. Our discovery cohort was relatively small (9 athletes), and it is likely that some miRNAs that are altered in plasma after SRC were not revealed in this study. Furthermore, although corrections were made for multiple comparisons, our study was not sufficiently powered to implement a false discovery rate between miRNAs. As such, it is possible that multiple type I statistical errors were made. However, the inverse correlations with SRC symptom severity for the 2 miRNAs found to be significantly decreased in plasma after concussion (ie, miR-221 and miR-27a), along with the similar findings in other neurological conditions for these 2 miRNAs, provides some assurance of the validity of these findings. Finally, although our discovery and validation cohorts included males and females, our relatively small number of females in the validation study (ie, 8/28 SRC subjects) may have precluded discovery of miRNAs differentially altered by SRC between sexes.

Future Directions

Although this study has identified 2 blood-based miRNA candidates that may be useful biomarkers of SRC, there remains several important areas for future research. Firstly, these miRNAs should be investigated in larger cohorts of male and female athletes and non-athletes to validate these findings. Moreover, additional time-points and objective clinical outcomes (eg, cognitive testing) will enable further insights, for example, whether early plasma miRNA levels are prognostic for cognitive deficits and PPCS. Finally, given the recent promise of saliva-based miRNA as acute biomarkers of SRC,[48] it is likely that saliva miRNA may also have utility in the sub-acute and chronic stages after SRC. Studies comparing miRNA levels between paired blood and saliva samples would be of interest, as would studies that investigate how saliva and blood miRNA levels relate to those in the brain. Although these studies may be challenging to conduct in mTBI patients, pre-clinical mTBI models and more severe TBI patient studies would be informative.

Conclusions

In a prospective study of Australian footballers, we found that miR-27a and miR-221 levels were significantly decreased in plasma in the sub-acute stages after SRC. Negative correlations of these miRNAs with SRC symptom severity provide further evidence of an association with the pathophysiology of mTBI. Future studies are required to determine how these miRNAs, and others, may be used as biomarkers to assist in SRC management.

Authors' Note

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Author Contributions

S.J.M., C.J.T., S.R.S. and T.J.O. conceptualised the study. S.J.M., C.J.T., S.R.S., R.A., W.T.O., M.S., A.V.C., G.N., A.A.H., M.V.J. and R.N. each made significant contributions to data collection and/or analysis. S.R.S., C.J.T. and S.J.M. drafted the manuscript. All authors contributed to final manuscript editing.

Data Availability

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

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