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Changes in circulating microRNAs following head impacts in soccer

Aim: To explore the short-term effects of accidental head impacts and repetitive headers on circulating microRNAs, accounting for the effects of high-intensity exercise alone.

Methods: Blood samples were collected from professional soccer players at rest. Repeat samples were drawn 1h and 12h after three conditions: (1) accidental head impacts in a match, (2) repetitive headers during training, and (3) high-intensity exercise. 89 samples were screened to detect microRNAs expressed after each exposure. Identified microRNAs were then validated in 98 samples to determine consistently deregulated microRNAs. Deregulated microRNAs were further explored using bioinformatics to identify target genes and characterize their involvement in biological pathways.

Results: Accidental head impacts led to deregulation of eight microRNAs that were unaffected by high-intensity exercise; target genes were linked to 12 specific signaling pathways, primarily regulating chromatin organization, Hedgehog and Wnt signaling. Repetitive headers led to deregulation of six microRNAs that were unaffected by high-intensity exercise; target genes were linked to one specific signaling pathway (TGF β). High intensity exercise led to deregulation of seven microRNAs; target genes were linked to 31 specific signaling pathways.

Conclusion: We identified microRNAs specific to accidental head impacts and repetitive headers in soccer, potentially being useful as brain injury biomarkers.

Keywords: repetitive head impacts; subconcussive; traumatic brain injury; soccer; football; microRNA; neurodegeneration

Introduction

Soccer involves voluntary heading of the ball as well as a risk for accidental head impacts. Taken together, soccer players are exposed to repetitive head impacts over time. The extent to which such exposure affects brain health, short or long term, is poorly understood (1-3). With millions of people playing soccer worldwide, an improved understanding of the potential effects of repetitive head impacts might ultimately have substantial influence on public health. One key obstacle when evaluating potential brain injury from head impacts is the absence of objective diagnostic and prognostic biomarkers (4). Several blood-based biomarkers have shown potential for detecting mild traumatic brain injury (mTBI), as injury to neuronal tissue can lead to the release of different substances into the blood (5, 6). However, the utility of circulating biomarkers is complicated by factors such as sample timing (7), poor specificity (8), and the ability to cross the blood-brain barrier (5).

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression, and are involved in diverse physiological and pathological processes (9). A review by Atif and Hicks (10) highlighted eight candidate miRNAs in blood that were altered in response to mTBI. While emphasizing that such findings are preliminary, they suggested that miRNAs may identify brain injury across the severity spectrum as well as provide prognostic information (10). Several characteristics make miRNAs suitable for providing insight into the pathophysiology of head injuries. First, they are abundantly expressed in the brain (11). Second, they have an ability to cross the blood-brain barrier (12), allowing for their release into the blood upon direct cellular injury. Third, miRNAs can also be up- or downregulated as a result of adaptive cell processes (10). Fourth, miRNAs are relatively stable when stored in peripheral biofluids, an important prerequisite for reliable clinical use (13, 14). Last, as miRNAs are associated with gene activity and molecular signaling pathways, bioinformatics can be used to characterize the role of implicated miRNAs in higher-level processes such as neuroplasticity, neuroinflammation or neurodegeneration (15).

Although previous studies have identified candidate miRNAs as biomarkers for mTBI (16-20), including in sport-specific settings (21-23), the effects of soccer-related head impacts on miRNAs are still largely unexplored. The specific effects from rigorous physical activity in such settings are also unknown and must be accounted for in order to isolate miRNAs specific to the brain. Indeed, previous studies have highlighted how a lack of specificity may impede the use of TBI biomarkers in sports (8, 24). Thus, the main aim of this exploratory study was to evaluate the effects of accidental head impacts (concussive and non-concussive) and repetitive headers in soccer on circulating miRNA levels, while accounting for the effects of high-intensity exercise alone. As a secondary aim, we used bioinformatics to identify target genes of the implicated miRNAs in order to characterize their roles in biological processes and signaling pathways.

Materials and methods

Study design and participants

This study was based on a previous prospective cohort study, assessing the effects of head impacts in senior elite soccer on neuropsychological tests (25) and serum protein biomarkers (8). Players from the [*anonymized for peer review*] were followed over two consecutive seasons (2004 and 2005). The players were aged 18 to 35 years; their demographic characteristics have been described in detail elsewhere (8). In the original study, blood samples were drawn and analyzed to assess whether head impacts in soccer could cause structural brain injury, detected as an increase in serum levels of protein S100B (8).

The current study was based on a selection (see below) from a subset of blood samples (n=274) available from the 332 players that were followed in the 2005 season.

Samples were selected and reanalyzed for changes in circulating miRNAs in response to the following conditions: 1) accidental head impacts (concussive and non-concussive) in a match, 2) repetitive headers during training, and 3) high-intensity exercise. Specifically, miRNA levels were measured 1 h and 12 h after each of these conditions and compared to baseline values at rest (Figure 1).

[*Anonymized for peer review*] and the [*anonymized for peer review*] approved the original study. Participants provided written, informed consent, which also included the opportunity to store the blood samples for later analyses. The current study was approved by the [*anonymized for peer review*].

[Figure 1 near here]

Accidental head impacts in a match

Before the 2005 season, the players had baseline blood samples taken. For this study, 35 baseline samples were available to serve as reference for accidental head impacts (Figure 1). During the regular season, research personnel attending each match were instructed to draw repeat blood samples 1 and 12 h after head impacts that involved a high risk of head injury; for this study, 35 samples (21 non-concussive and 14 concussive) were available 1 h after and 19 samples (11 non-concussive and 8 concussive) 12 h after (Figure 1). *Head impact incidents* were defined as any situation where 1) a player appeared to be hit in the head (including face or neck); 2) the match was interrupted by the referee; and 3) the player remained lying on the ground for more than 15 s. All three criteria had to be fulfilled for an incident to be included, in accordance with a previously used definition (26, 27).

All *head impact incidents* were subcategorized as either *concussive* or *non-concussive* based on symptoms reported to the research personnel by the player himself or by his team medical staff. This information was cross-referenced with injury surveillance data from the league, which at the time included all time-loss injuries (8). The concussion definition was based on the 2001 Vienna consensus statement (28), briefly summarized as any direct or indirect blow to the head resulting in short-lived neurological impairment.

Repetitive headers and high-intensity exercise

Three of the teams (n=48 players) from the [*anonymized for peer review*] also participated in two organized training sessions, completed on separate days. In cooperation with coaching staff, both sessions were designed to closely mimic drills and activities typically seen in soccer. Specifically, one session consisted of *high-intensity exercise* only, with no heading of the ball allowed. The other training session included repetitive headers with heading characteristics mimicking a typical soccer game, using drills such as corner kicks and other set pieces. Ball speed and distance travelled varied depending on the specific situation that arose, as is typical for game play. Importantly, for this session, the exercise intensity was otherwise low. Using structured video analysis, the average number of headers per player during the repetitive headers session was estimated to 19, ranging from 7 to 33 (8).

On the morning before the first session, players had baseline samples taken at rest; for this study, 47 samples were available, serving as reference for both sessions (Figure 1). Then, repeat blood samples were drawn 1 h and 12 h after each of the two training sessions. For this study, 38 samples were available at 1 h and 30 samples at 12 h for repetitive headers, while 37 samples were available at 1 h and 33 samples at 12 h for high-intensity exercise (Figure 1).

Collection and storage of blood samples

Venous blood samples were drawn from an antecubital vein and collected into a standard gel 7 mL tube (BD Vacutainer blood collection tube; Becton Dickinson, Franklin Lakes, NJ).

Samples were allowed to clot for 30 min before they were centrifuged (3000 *g* for 10 min); the resultant serum from each sample was divided into two 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) and stored in the freezer within two hours (8). One of the tubes was used for the original study, while the other was stored (-80°C) for later analyses.

Identifying deregulated miRNAs

The identification of *deregulated miRNAs* involved two stages, i.e. screening and validation (Figure 1). In the screening stage, samples from each of the 10 groups (i.e. two baseline groups and eight follow-up groups; see Figure 1) were pooled in order to identify *primary miRNA hits*. In the validation stage, *primary miRNA hits* were validated in individual samples from each group to identify *deregulated miRNAs*. The details of these two stages are outlined below.

Selection of samples and screening for primary miRNA hits

From the total of 274 samples, 89 samples were included for the screening stage aiming to identify *primary miRNA hits* – i.e., miRNAs that were deregulated (up- or downregulated) compared to baseline. Specifically, for this stage, nine samples (n=8 samples for *concussions 12 h*) from all 10 groups were included (Figure 1). From the available samples, those with serum tau levels closest to the median in each of the groups were selected for screening (24); this was done to avoid outliers and thereby include representative samples.

Primary miRNA hits were identified according to the following steps. First, one serum aliquot from each sample was thawed on ice and centrifuged. Then, RNA was extracted from 200 µL of serum supernatant, using miRCURY RNA Isolation Kit – Biofluids (Exiqon A/S, Vedbæk, Denmark). Primary analysis of miRNA levels was done using pooled samples within each group. Each equivolumic pool consisted of three randomly selected individual samples, with every group thereby being represented by three pools (Figure 1). The profiles of extracted RNA samples were assessed by 2100 Bioanalyzer using the RNA 6000 Pico Kit (Agilent Technologies, USA; Supplementary figure 1). This sample pooling strategy was used to enable cost-efficient discovery of miRNAs, and has previously been reported as a primary screening approach for discovery of miRNA biomarkers (29). Reverse transcription from RNA to cDNA in each pool was performed using miScript II RT Kit (Qiagen, Hilden, Germany). Prior to primary screening, the samples were assessed for hemolysis evaluating the amplification of miR-23a-3p and miR-451a (30). MiRNA profiling was performed with real-time qPCR, using the Human Serum & Plasma miScript miRNA PCR Arrays and miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) amplified by QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA); this kit includes assays for quantifying the levels of 84 different miRNAs, a panel designed by Qiagen based on their relevance and abundance in serum (Supplement 1). Finally, fold change of miRNA expression in each sample was compared to the average of its respective baseline group, using the $2^{-\Delta\Delta CT}$ method (31). For this, we used miR-9-5p as endogenous control; this was identified as the most stable miRNA across all samples (Supplementary figure 2), evaluated with the Endogenous control pipeline using ExpressionSuite software v. 1.1 (Applied Biosystems, Foster City, USA). Any miRNA that was identified as altered compared to baseline (see below in *Statistical analyses*) at the screening stage was defined as a *primary miRNA hit* and included in the validation stage (Figure 1). The statistical analyses are outlined in detail below.

Selection of samples and validation of primary miRNA hits

Following the screening stage, 98 of the 274 available samples were analyzed to validate (i.e., confirm or reject) the *primary miRNA hits*. Specifically, for the validation stage, 10

samples (n=8 for *concussions 12 h*) were randomly selected from each of the 10 groups (Figure 1). Validation of *primary miRNA hits* was performed using specific assays (primers-probe combinations) in each individual sample; otherwise, the sequential steps were the same as for the screening stage outlined above. Any *primary miRNA hit* that was altered compared to baseline also at the validation stage was defined as a *deregulated miRNA* (see below in *Statistical analyses*). A detailed account of miRNA quantification is included as supplementary material (Supplement 1). The potential tissue origin of any deregulated miRNA was evaluated using the human miRNA tissue atlas (32) and the GeneCards database (33).

Statistical analyses

All data were log-transformed due to non-normal distributions (D'Agostino-Pearson omnibus test). Differences compared to baseline, expressed as log miRNA fold change, were evaluated using Welch's unequal variances t-test. Up- or downregulated miRNA levels with p-values <0.05 were considered statistically significant; miRNAs that were identified as primary hits in the screening stage *and* subsequently validated in individual samples were ultimately classified as being deregulated. All statistical tests were performed using GraphPad Prism v 7.02 (GraphPad Software, San Diego, CA).

Bioinformatic analyses

Deregulated miRNAs were used as input in the miRNA Data Integration Portal (mirDIP v 4.1, using default settings integrating 30 different prediction algorithms) (15) to identify their target genes. Corresponding miRNA gene networks were then visualized using NAViGaTOR v 3.0.13 (34). Two lists of target genes were created. The first list included top target genes, i.e., those that were connected to (almost) all *deregulated miRNAs*. The second list included all identified gene targets, i.e. those that were connected to any *deregulated miRNA*. Comprehensive pathway enrichment analysis was performed on both lists with the pathway Data Integration Portal (pathDIP v 4.0.21.2, database v 4.0.7.0, using all pathway sources and only literature curated (core) pathway memberships) (35). All raw data and search results are included as supplementary material (Supplement 2).

Results

In the screening stage, we identified a total of 38 *primary miRNA hits* at one or several time points in one or more condition (Supplement 1). In the validation stage, 20 of these *primary miRNA hits* were confirmed to be deregulated. Table 1 shows the *deregulated miRNAs* for each condition.

[Table 1 near here]

Accidental head impacts in a match

Following non-concussive accidental head impacts, we detected nine *deregulated miRNAs*. Eight of these were changed after 1 h and one after 12 h. Six of the nine miRNAs (miR16-5-p, miR-18a-5p, miR-20a-5p, miR-93-5p, miR-107 and miR-130b-3p) were unaffected by high-intensity exercise (Table 1). Figure 2A shows the miRNAs that were deregulated in response to non-concussive accidental head impacts but were unaffected by high-intensity exercise.

Following concussive accidental head impacts, we detected two *deregulated miRNAs* (miR-204-5p and miR-130b-3p), both unaffected by exercise (Table 1). Both were changed after one hour, while none were changed after 12 h. While miR-130b-3p was also affected by

non-concussive head impacts, increased levels of miR-204-5p were only seen 1 h after concussive head impacts (Figure 2B).

[Figure 2 near here]

Repetitive headers and high-intensity exercise

Following repetitive headers, we detected eight *deregulated miRNAs*. All eight were deregulated after 1 h and one also after 12 h. Six of these eight (miR-24-3p, miR-27a-3p, miR-122-5p, miR-150-5p, miR-499a-5p and miR-885-5p) were unaffected by high-intensity exercise (Table 1). Figure 3A shows the miRNAs that were deregulated in response to repetitive headers but were unaffected by high-intensity exercise. In response to high-intensity exercise, we detected seven *deregulated miRNAs*. Of these, three were deregulated after 1 h and four after 12 h, with no overlap between the two time points (Table 1). Figure 3B shows the change of each of these miRNAs compared to baseline.

[Figure 3 near here]

Identification of miRNA target genes

Figure 4 shows the top-ranked predicted target genes identified in mirDIP (see methods), as determined by the *deregulated miRNAs* in each condition (see Supplement 2 for a comprehensive list). Molecular functions were proportionally similar in all three conditions (Figure 5A). High-intensity exercise involved a higher number of miRNA-targeted genes with catalytic activity, and accidental head impacts were characterized by unique genes belonging to translational regulators and channel regulatory activity. We also identified sets of enriched signaling pathways in all three conditions (Figure 5B). In summary, accidental head impacts was linked to 12 specific signaling pathways, repetitive headers to one pathway, and high-intensity exercise to 31 pathways. Furthermore, accidental head impacts and high-intensity exercise shared 10 pathways, while repetitive headers and high-intensity exercise shared one pathway.

[Figures 4 and 5 near here]

Discussion

This study assessed the short-term effects of accidental head impacts, repetitive headers and high-intensity exercise in soccer on circulating miRNAs. There are three main findings: First, accidental head impacts led to the deregulation of six specific miRNAs, and their target genes were linked to 12 specific signaling pathways, primarily regulating chromatin organization, Hedgehog and Wnt signaling. Second, repetitive headers led to the deregulation of six specific miRNAs, and their target genes were linked to one specific signaling pathway (TGF-β). Third, high-intensity exercise led to the deregulation of seven miRNAs; their target genes were linked to 31 specific signaling pathways. Taken together, these findings indicate that accidental head impacts and repetitive headers in soccer lead to specific alterations in circulating miRNAs that are unaffected by high-intensity exercise. Thus, this exploratory study suggests that miRNAs may serve as biomarkers for brain injury; moreover, miRNAs may have the potential to differentiate injury severity.

Accidental head impacts

Our findings suggest that accidental head impacts may alter the expression of specific miRNAs (Table 1). Interestingly, comparing concussive and non-concussive head impacts, we found that concussions alone caused an increase in miR-204-5p 1 h after injury (Table 1). Conversely, non-concussive events led to the downregulation of several miRNAs

(Figure 2A). The downregulation of miR-130b-3p overlapped between non-concussions and concussions (Table 1); in contrast, we note that Bhomia et al. (17) observed an *increase* in miR-130-3p within 48 h after severe TBI. This provides an opportunity to consider how such differences between studies may potentially arise. While Bhomia et al. (17) described a pronounced upregulation of miR-130-3p (59.04 x), with marked heterogeneity as regards the time from injury (range from 12-48 h), we observed a relatively subtle downregulation (0.66x) *shortly* after concussion. Taken together, differences in head impact severity and specific features of study design are both important to consider. Also, note how Bhomia et al. (17) included healthy controls and orthopedic injuries for comparison. Other studies have also reported increased levels of miR-16-5p (16), miR-20a-5p (17), and miR-93-5p (18) after mTBI. Furthermore, Di Pietro et al. (21) observed an increase in miR-107 in the saliva of concussed rugby players. Note that we observed a *decrease* of these miRNAs, but only after non-concussive impacts. Presumably, these impacts represented less severe impacts than those evaluated in previous studies. Such findings may reflect both adaptive and deleterious changes related to the brain's response to trauma. As part of a broader review on miRNAs in mTBI by Atif and Hicks (10), the authors proposed eight miRNAs as potential biomarkers for mTBI. We included three of these miRNAs in our study. Of note, none of these three were found to be deregulated. However, we identified another potential biomarker for concussion, i.e. miR-204-5p; to our knowledge, this miRNA has not been described in relation to TBI until now. Interestingly, when evaluating tissue expressions patterns, miR-204-5p has been described as being abundant in the brain (32, 33), suggesting a potential pathophysiological relevance of this finding. Also, note that upregulation of this miRNA has been implicated in the pathophysiology of Parkinson disease (36).

Based on the target genes of the different miRNAs, bioinformatic analyses found accidental head impacts to be associated with several biological pathways (Figure 5B). Predominantly, they were linked to pathways regulating chromatin organization, Wnt signaling and Hedgehog signaling. Wnt signaling has been implicated in promoting neuronal regeneration following TBI (37). Hedgehog signaling has been found to inhibit neuronal apoptosis and to reduce damage to tight junctions after TBI in animal studies (38). Although such data are derived from animal models, this may imply the existence of similar mechanisms in response to TBI in humans. Our data thereby indicate an alignment with broader descriptions of disruption of the blood-brain barrier and tight junction complexes as a dominant feature of TBI (39). We also identified pathways associated with signaling by second messengers, regulation of cell adhesion, neuronal apoptosis, circadian rhythm, thermogenesis and repression of pain.

Repetitive headers

Soccer also involves intentionally heading the ball as an inherent part of the game. During matches, male professional players have been reported to head the ball 3 to 4 times per player hour on average (40). However, the potential effects of heading are still largely unknown (1-3, 41). Therefore, to evaluate such exposure, we designed a training session including multiple heading drills with impact characteristics typical in soccer. In our data, we found repetitive headers to alter the levels of six miRNAs (Figure 3A) that remained unaffected by accidental head impacts as well as high-intensity exercise (Table 1). All of these miRNAs were increased acutely (1 h) while returning to baseline after 12 h.

Comparing our findings directly to those of others is challenging due to our unique study design and the specific head impact types we included. However, a recent study by Munoz et al. (42) is of particular interest, describing alterations in extracellular vesicle miRNAs in response to heading in adult soccer players. In line with one of their findings, and despite the methodological differences, both studies observed an increase in miR-24-3p.

Thus, this might be an especially promising target. If reproduced in future studies, such findings could ultimately provide specific biomarkers for subconcussive head impacts in soccer.

We also note that Yang et al. (18) have detected elevated serum levels of miR-499a-5p within 24 h after mild to severe TBI (18). Other studies, however, have found both miR-499-5p and miR-150-5p to be upregulated shortly after aerobic exercise (43, 44), questioning their utility in sport-specific settings. Bhomia et al. (17) found miR-27a-3p to be increased within 12 h after mTBI, and La Rocca et al. (22) have demonstrated upregulation of miR-122-5p in mixed martial arts fighters 1 h and 3 weeks following mTBI. In contrast, we observed an increase in both of these miRNAs immediately after repetitive headers but not after accidental head impacts. Last, to our knowledge, miR-885-5p has not been described in connection to brain injury or exercise to date. Evaluating tissue expression patterns, miR-885-5p has also been described to be abundant in the brain (32, 33). This may suggest a potential release in response to repetitive head impacts.

The absence of overlapping findings between repetitive headers and accidental head impacts in our study is intriguing. Our findings might reflect that different types of head-impact exposures exert differential effects on the brain. Theoretically, variations in impact magnitudes as well as frequencies may lead to different combinations of structural tissue damage and/or adaptive responses. Based on bioinformatic analyses, repetitive headers were associated with TGF-β signaling (Figure 5B). Interestingly, elevated TGF-β levels have been reported in CSF after TBI, suggested to play a role in anti-inflammatory and neuroprotective signaling (45). Of note, previous studies have suggested an association between repetitive head impacts and potential signs of neuroinflammation (46, 47). Ultimately, it is unknown how our findings might translate to specific changes in brain structure, function and metabolism. A possible interpretation could be that repetitive headers have the potential to trigger neuroinflammatory responses in the neuronal tissue. Exploring the association between TFG-β, fluid biomarkers and clinical outcomes could be the subject for future studies.

High-intensity exercise

MiRNA signatures in response to exercise is thought to vary considerably with activity type and intensity (48). Of note, head impacts in soccer often take place in situations also involving strenuous physical activity. High-intensity exercise was therefore included to account for its effects on circulating miRNAs in our specific cohort. Including this as a separate condition ultimately enabled us to isolate miRNAs that may relate to the specific effects of head impacts as compared to the effects of physical activity. As expected, we observed changes in several different miRNAs after both 1 h and 12 h following exercise. The most prominent increase was found for miR-206, which has been shown to be altered in response to physical activity (48) and also to be involved in myogenesis (49). We note that Ko et al. (50) recently suggested miR-206 as part of a panel for detecting TBI. Our results indicate that this miRNA lacks specificity for detecting brain injury in a sport setting. Select findings that support the external validity of our study include increases in miR-1-3p and miR-143-3p, both of which have been previously described to increase after endurance training (51)·(52).

Bioinformatic analyses detected multiple associated pathways. Of these, Toll-like receptor (TLR) signaling, FGF and ErbB signaling were the three most common (Figure 5B). Exercise is well-known to regulate immune processes via several different processes, including TLR signaling (53). Moreover, exercise has been described to activate ErbB signaling, thought to promote cardiac repair (54, 55). Our study also found an association

between exercise and several other biological pathways related to immune processes (e.g. cytokines), as well as neurotrophic and insulin signaling.

Importantly, we accounted for several *shared* pathways among the different conditions. Not surprisingly, high-intensity exercise and accidental head impacts shared the highest number of pathways. These head impacts occurred during matches, which would typically also involve high exercise intensity shortly before the incident. In summary, our findings suggest that the effects of high-intensity exercise on circulating miRNAs are abundant, thus warranting careful consideration.

Strengths and limitations

To our knowledge, this is the first study to explore the effects of different head-impact exposures in soccer on circulating miRNAs. One of the main strengths of our study lies in its design. This allows for the comparison of different exposures in a homogeneous cohort (8), simultaneously accounting for the effects of high-intensity exercise. Despite being a preliminary study with a limited sample size, we applied a two-stage approach – with initial screening for primary miRNA hits and subsequent validation of deregulated miRNAs in individual samples – to reduce the number of false positives.

We also recognize several limitations. First, factors such as sample size, sample timing, biofluid type, demographic characteristics (including previous injuries), injury severity and laboratory methods should be taken into consideration when interpreting findings from any such study. They each potentially explain major discrepancies in findings between studies. The importance of such factors has been previously acknowledged in the literature on miRNAs in TBI (10), and they are all likely to be relevant for some of the differences we observed when comparing our findings to those of others. Second, head-impact exposure was characterized using direct observation and video analysis (8), meaning we were not able to quantify the biomechanical magnitudes of the impacts in the different conditions. Adding to this, the distinction between concussive and non-concussive accidental head impacts was based on a previous consensus definition (28); this definition was slightly modified in 2016 (4). We were also unable to account for any effects from e.g. headers during the match prior to the accidental head impact. Third, even though miRNAs are known to be remarkably stable in peripheral biofluids (14, 56), we were not able to fully account for the effects of long-term storage on the blood samples included in this study. Nevertheless, the substantial overlap of exercise-related miRNAs compared to previous findings suggests adequate sample quality. Fourth, we were unable to account for how the clotting process and potential release of microsomal miRNAs contributed to our findings. Last, as we evaluated a limited number of samples in each group, there is an inherent risk of overfitting the data. For the validation stage, the number of samples was restricted due to a high number of identified primary hits and budgetary constraints. Also, as we considered our study exploratory, we did not correct for multiple comparisons. We therefore emphasize that our observations need confirmation in larger samples and other cohorts.

Conclusion

This exploratory study assessed the short-term effects of accidental head impacts and repetitive headers in soccer on circulating miRNAs, accounting for the effects of high-intensity exercise. We found that different types of head-impact exposure led to specific alterations in miRNA levels, out of which some were associated with signaling pathways suggestive of brain alterations. Thus, miRNAs may have potential as biomarkers for brain injury.

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Table 1. Overview of *deregulated microRNAs* in response to (1) accidental head impacts in a match, (2) repetitive headers during training and (3) high-intensity exercise. MicroRNAs that were unaffected by high-intensity exercise are highlighted with bold font.

Accidental head impacts				Repetitive headers		High-intensity exercise	
Non-concussive		Concussive					
1h	12h	1h	12h[a]	1h	12h	1h	12h
↓ miR-7-5p	↓ **miR-130b-3p** (17)	↑ **miR-204-5p**		↑ **miR-24-3p**	↑ miR-143-3p (57)	↑ miR-1-3p	↓ let-7c-5p
↓ **miR-16-5p** (16)		↓ **miR-130b-3p** (17)		↑ **miR-27a-3p** (17)		↑ miR-143-3p (57)	↓ miR-7-5p
↓ miR-17-5p				↑ **miR-122-5p** (22)		↑ miR-206 (50)	↓ miR-17-5p
↓ **miR-18a-5p** (58)				↑ miR-143-3p (57)			↓ miR-106b-5p (59)
↓ **miR-20a-5p** (17, 22)				↑ **miR-150-5p**			
↓ **miR-93-5p** (18)				↑ miR-206 (50)			
↓ miR-106b-5p (59)				↑ **miR-499a-5p** (18)			
↓ **miR-107** (21)				↑ **miR-885-5p**			

[a]Based on eight samples. Results from all other groups are based on 10 samples. Citations denote studies that have implicated changes in the same microRNA in relation to human TBI.

Figure 1. Flow chart illustrating the sequential stages for identifying *deregulated microRNAs* in response to three conditions: (1) accidental head impacts in a match, (2) repetitive headers during training, and (3) high-intensity exercise. First, screening was performed for 84 microRNAs; for this stage, samples were pooled together. Then, validation was done in individual samples using specific assays for *primary microRNA hits*, allowing for the identification of *deregulated microRNAs*.
Figure 2. *Deregulated microRNAs* in response to (A) non-concussive and (B) concussive accidental head impacts in soccer. Box plots indicate median value and interquartile ranges.
Figure 3. *Deregulated microRNAs* in response to (A) repetitive headers and (B) high-intensity exercise. Box plots indicate median value and interquartile ranges.
Figure 4. Target genes identified in mirDIP for (A) six *deregulated miRNAs* after repetitive headers during training, (B) seven *deregulated miRNAs* after high-intensity exercise, and (C) seven *deregulated miRNAs* after accidental head impacts in a match.
Figure 5. (A) GO Molecular function groups for all target genes identified in each condition. (B) The most important pathways enriched for the top targets. Node size corresponds to number of pathways in each category. Edge color (red, orange, yellow, blue and black) corresponds to low-to-higher p-value.