Identification of microRNAs in equine synovial fluid as potential biomarkers of osteoarthritis

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MicroRNAs (miRs) play an important role in regulating gene expression, but also have a functional role in some diseases for example diabetes, lung and colorectal cancers (1). Altered levels of miRs can be detected and associated with the diagnosis of these diseases. Diagnosis of osteoarthritis (OA) - a complex age-related disease characterised by cartilage degeneration and inflammation (2-4) - by a measurable difference in miR levels may be feasible. Currently there is limited diagnostic ability at early stages due to current clinical imaging methods requiring gross changes to the joint (5), only seen in late stages. Identification of OA-related miRs offers an enhanced ability to diagnose, discover novel treatments and further understand OA development. This project identifies miRs in synovial fluid (SF), the substance that fills and nourishes the joint capsule, in horses for the first time.

Abstract

OA, a degenerative joint disorder, has high prevalence in equines presenting economic and welfare challenges (5,6). With no methods of early OA detection or effective treatment, biomarkers are required. Investigation of differential expression between young and old equine donors was used to determine the suitability of SF miRs in this capacity. Expression of miRs 16, 146a, 140, 132, were detectable, though none demonstrated a statistically significant differential expression between the two age groups (*p*-values= 0.1775, 0.5639, 0.8966, 0.4129 respectively). TRIzol-chloroform RNA extraction in equine SF achieved greater RNA concentrations when compared to the Qiagen miRNeasy serum/plasma kit. Hyaluronidase (HA) treatment improved handling of equine SF, helping maintain RNA quality.

Accounting for 60% of equine lameness (5), and the most common reason for failure to train and race resulting in early equine retirement (6), OA is the greatest single cause of economic loss to the racing industry (7). Annually the horse racing industry is worth over £3.45 billion to the UK economy (8). Most joint injuries in racehorses involve the carpal, metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints which sustain repetitive, focal, high impact loading. In 2010, it was determined one-third of all 2/3-year-old thoroughbred racehorses had partial/full-thickness cartilage lesions and OA of MCP joint (9). Its association with pain and disability levels, has welfare significance applicable to all equines (10), with current treatment options such as non-steroidal anti-inflammatory drugs, selective cyclooxygenase 2 inhibitors, steroids and hyaluronic acid lack efficacy, failing to reverse the loss of articular cartilage (11).

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MiRs are a class of small (20–22 nucleotide) non-coding RNA molecules (12). They regulate genetic expression post-transcriptionally, resulting in either repression of translation or increased mRNA degradation (13). Signalling pathways, critically important in endochondral ossification, articular cartilage homeostasis, and arthritis pathogenesis, are targets for OA associated miRs affecting cartilage biology. Specific miRs have been associated with chondrocyte, cartilage cell, processes of proliferation, differentiation and apoptosis (14), demonstrating they respond to homeostatic changes in the joint as seen in OA, revealing targets for intervention. To date, measurement of miRs in equine SF has not been reported (17, 39, 40). However, there is increasing evidence of miR dysregulation in human OA in cartilage and SF.

SF, a plasma ultra-filtrate found within the joint, nourishes articular cartilage acting as a vector for inflammatory mediators released from the synovium (15). Changes in SF reflect cartilage metabolism and specific miR expression occurs at cartilage level, thus may not be detected systemically in plasma (16). Hence SF could present a sampling alternative. A link between variable expressions of miRs in human knee SF to the staging of OA offers the most significant evidence of miR suitability as an OA biomarker in SF (17).

A single miR regulatory pathway in early tissue healing has highlighted miR replacement as a promising therapy for tendinopathy in rat models (18). In mouse models, the upregulation of miR-483-5p, a miR previously described in oncology (19), initiates and promotes OA development. This could be delayed by intraarticular injection of synthetic antago-miR-483-5p (20).

Ageing has been shown to affect joint pathology, resulting in it being considered among the most consistent risk factors for OA (21). With increasing age in equines, a significant decrease in cartilage thickness, and increase in thickness of calcified cartilage and subchondral bone has been reported (22). Based on published evidence in other species (15, 17), it is hypothesised that miRs can be measured in equine SF and there is an age-related change in targeted miR expression. This study aims to optimise a method of RNA extraction from SF, to allow subsequent RT-qPCR with a select panel of miRs, to determine if differential expression is present between young and old sample groups.

Methods and Materials

Sample Collection

Equine SF was collected from the right MCP joint of ten horses at an abattoir. As an agricultural industry by-product, collection of these samples is not included under regulated procedures in the Animal (Scientific Procedures) Act 1986 (Amendment 2012), Schedule 2 (23). Hence, ethical approval was not required for this project. Horses were non-thoroughbred leisure horses. Disregarding age, no further donor history was available. Age grouping, sample size and mean ± standard deviation (SD) were: young, n=5, age=3.6years ± 0.548 and old, n=5, age=22.4 years ± 2.302. Each joint from which SF was sampled and histologically graded using the Mankins Scoring system (MS).

Hyaluronidase Treatment

HA is used to catalyse the hydrolysis of hyaluronic acid, reducing viscosity of SF, improving ease of sample handling. To determine the effect of HA on RNA yield, 100µl equine SF was HA treated, 100µl of untreated equine SF acted as a control. Samples then underwent RNA extraction using a standard TRIzol (Invitrogen, USA)-chloroform method.

HA treatment was implemented in preparation of the ten equine SF samples (young n=5, old n=5) for RT-qPCR.

RNA extraction

In development of a method that would produce the greatest RNA yield from SF, 200µl HA treated equine SF underwent RNA extraction using the miRNeasy Serum/Plasma kit (Qiagen, UK) per manufacturer's instructions. 100µl HA treated equine SF underwent RNA TRIzol-chloroform extraction, based on previous testing HA treatment was deemed advantageous.

Post extraction, RNA concentration in both experiments was determined using a Nanodrop spectrophotometer (Labtech, UK), with ultraviolet absorbance measurements at 260 nm and 280 nm, allowing RNA purity assessment.

TRIzol-chloroform method was implemented in preparation of the ten equine SF samples (young n=5, old n=5) for RT-qPCR.

Reverse transcription was performed on the extracted RNA to produce polyA-cDNA. The reaction was prepared using the miScriptII-RT Kit (Qiagen, UK) as per manufacturer's instructions, followed by synthesis within a thermal cycler with conditions outlined below.

RT-qPCR

Primer selection was based on current availability (human primers only), validation in existing studies and significance in joint degeneration. MiRBase (24) was used to cross reference the mature sequence of each miR, to ensure primer compatibility between human and equine species. Housekeeping gene U6, commonly described in the literature (25, 26), was used as endogenous control. Qiagen miScript II primer assays (41) included in the study are outlined in table 1.

PolyA-cDNA was diluted with RNase free water. A reaction mix containing miScript universal primer, QuantiTect SYBR green fluorescent dye (both Qiagen, UK) and each of the selected primers was produced. This and the dilute polyA-cDNA was loaded onto a 96-well plate, RT-qPCR was carried out in a thermal cycler.

Statistical analysis

All qPCR reactions were performed in triplicate. Relative miR expression was quantified using the 2^-DCT method (27), normalised to the U6 reference. Data sets were tested for homogeneity using the F max test between equal groups. Normality was tested using the Sharipo-Wilk test for unequal groups, confirming the suitability of parametric testing for this data. Student's t-test was used to calculate a *p*-value. For all data, statistical significance was considered at $p \leq 0.05$. Statistical analysis was undertaken using Microsoft Excel (2013, Microsoft, USA) and Graphpad Prism 7 (2017, Graphpad Software Inc, USA).

Results

Effect of Hyaluronidase Treatment on RNA yield

RNA concentration was markedly increased in non-treated SF when compared to the treated sample (Figure 2). Figure 1 presents RNA quality data showing an increased 260/280 value in HA treated SF due to a small absorbance at A280, indicating fewer contaminants in the 280nm range than the untreated sample. At A260,

the range specific to pure RNA, both samples appeared of poor quality (normal range 1.8-2) although the non-treated exceeded. Low A260/230 values were evident from both samples, more so in the HA treated, due to increased absorbance at 230 nm.

Comparison of RNA extraction methods

Both samples produced a similar total RNA concentration, however 100µl equine SF was used in the TRIzol-chloroform extraction, in comparison to 200µl equine SF by the miRNeasy kit method which produced a slightly reduced yield (Figure 3). The A260/280 value was greater with TRIzol-chloroform due to a lower 280 nm absorbance and a higher absorbance at 260 nm, the normal range for RNA detection.

Table 1. Forward sequences of Qiagen miScript II primers.

Primer ID	Forward sequence
hsa-miR-16-5p	5'-UAGCAGCACGUAAAUAUUGGCG
hsa-miR-140-5p	5'-CAGUGGUUUUACCCUAUGGUAG
hsa-miR-132-3p	5'-UAACAGUCUACAGCCAUGGUCG
hsa-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU
RNU6-1 (human)	5'-CGCAAGGATGACACGCAAATTC

RT-qPCR results

Differences in expression between young and old age groups, in miRs- 16, 146a, 132 were not significant (Figure 4, p=0.1775, 0.5639, 0.4129, respectively). SF miR-140 expression appeared stable between groups (2^-DCT=0.415 and 0.480, young vs old). Large error bars seen for all miRs represent a large SD respective to level of expression, (miR-16 O 25.27±3.501 vs Y 17.79±2.948, miR-132 O 0.9200±0.1315 vs Y 1.788±0.9417, miR-146a O 0.0033±0.0018 vs Y 0.0050±0.0021, miR-140 O 0.4803±0.3491 vs Y 0.4151±0.1804) contributing to non-statistically significant results. An exception to this is miR-132, displaying smaller variation in expression in the old group only, mean ± SD of 0.920 ± 0.227. miR-16 showed the greatest statistical difference between groups (p=0.1775), it showed an increase, mean ± SD, in the old group 25.265 ± 6.063, compared to young 17.785 ± 5.105. For all miRs, data was found to be inconclusive (p > 0.1).

Analysis of MS data revealed that despite the correlation between ageing and OA severity, the samples in this study did not reflect this pattern, (mean ± SD) young 3 ± 1.225 and old 2.8 ± 1.30, all were of normal- low grade joint pathology (MS 1-4). Hence for the miRs investigated, no significant difference could be attributed to age when the MS was similar.

To investigate this further, groups of relative high MS (3/4) (n=7) and low MS (1/2) (n=3) were created to assess any differential miR expression between graded joints, irrespective of age. As this was additional analysis of data collected by the original study design, unequal groups existed.

No significant difference in mean expression between groups for miRs-16, 146a, 140 was seen (Figure 5, p= 0.6703, 0.6923, 0.6138). Large SD was also seen in the above investigated miRs (miR-16 H 23.09±2.344 vs L 26.79±6.072, miR-146a H 0.0041±0.3491 vs L 0.0031±0.0018, miR-140 H 0.4803±0.3491 vs L 0.7511±0.3518) demonstrating inconclusive data. Figure 5 also shows The difference in miR-132 levels between high MS and low MS groups was close to significant (Figure 5, p=0.0792). However, this was not explored further in this study.

Discussion

MiR detection in equine SF

MiR detection from equine SF had not been reported previously, having demonstrated an ability to measure the expression of targeted miRs, this study provides a foundation for future OA biomarker research. This supports the findings of Murata *et al.* (15), who confirmed the presence and stability of miRs in human SF, a promising source for analysing functional miRs in OA. Not only does a potential OA biomarker in the equine patient offer early detection and novel treatment targets, entering the equine joint space is common practice, presenting SF as a convenient and relatively non-invasive diagnostic tool.

Effect of HA treatment on RNA yield

This study has shown HA treatment to be advantageous in preparation for RNA extraction due to improved sample handling and reduced viscosity. Platt *et al.* (28), described a decrease in protein-matrix interaction, resulting from HA breakdown of hyaluronan, chondroitin sulphate and keratin sulphate in SF matrix (29). This can reduce pipetting errors, hence volume variations, maintaining accuracy and precision, important when working with small volumes (30). HA treatment affected RNA values (Figures 1 and 2), despite a decrease in total RNA concentration, treated SF demonstrated fewer contaminants in the A280 range, indicating improved quality. This could be a result of enzymatic digestion of molecules such as proteins, detected at this wavelength, although this was not explored further.

Comparison of RNA extraction methods

This study has optimised a method to process equine SF easily and reliably for RT-qPCR. Improving RNA concentration and quality, will allow a more accurate representation of SF miRs and their differential expression in the future. Commercial kits offer a time efficient RNA extraction method. With no kit currently marketed for SF use

Figure 1: Spectrophotometer results for two 100µl samples of equine SF post TRIzol-chloroform RNA extraction, Non-HA treated vs. HA treated.

	Non HA treated	HA treated
RNA conc (ng/µl)	49.3	32.7
A260	1.23	0.82
A280	1.18	0.57
260/280	1.04	1.43
260/230	0.88	0.34

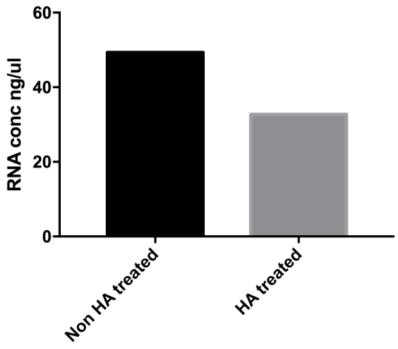

Figure 2: Total RNA concentration (ng/µl), measured by spectrophotometry, from two 100µl equine SF samples post TRIzol-chloroform RNA extraction. Non-HA treated (49.3), HA treated (32.7).

Figure 3: Spectrophotometry results for two equine SF samples, each receiving a different method of RNA extraction. TRIzol-chloroform (100µl) and Qiagen miRNeasy serum/plasma kit (200µl). Note the difference in starting volumes.

	TRIzol-chloroform	Qiagen miRNeasy Kit
Starting volume SF (µl)	100	200
RNA conc (ng/µl)	32.7	31.9
A260	0.817	0.798
A280	0.573	0.605
260/280	1.43	1.32
260/230	0.34	0.73

without protocol modification, it was important to explore their efficacy with this sample medium. Comparison with Qiagen miRNeasy serum/plasma kit, showed the TRIzol-chloroform method to give improved RNA concentration and quality (Figure 3). This is likely due to constituents secreted by synovial tissue, not present in blood plasma, acting as contaminants. The kit contained Guanidinine Thiocyanate whilst TRIzol is phenol based, this could explain quality values seen in Figure 3 as their UV absorption wavelengths differ, 270 nm and 260 nm respectively. More extensive testing using an increased number kits would allow a more thorough conclusion on the best method of RNA extraction from SF.

Use of MS as a histological grading system

MS is a histological grading system developed to evaluate late-stage hip OA (31). Despite its extensive use in degenerative joint disease, in early-stages the full extent of joint surface pathology is not assessed. Healthy, regenerative tissue can also present irregularities that worsen the score (32). Significant observer variability has been described (33), questioning MS reliability and validity. Hence modifications of the traditional MS have been developed, McIlwraith *et al.* (34) described such a system in an equine spontaneous OA model, which was utilised in this study. Each slide was scored twice by two observers. Intra-scorer variability was assessed using Cohen's Kappa (35), this suggested weak agreement. With three observers, a moderate agreement was seen using the Kendall W test however the reliability of this method requires further investigation. The Osteoarthritis Research Society International (OARSI) created a Cartilage Histopathology Assessment System (OOCHAS), intended to be more sensitive to mild OA changes with a more consistent observer application than the MS. Assessment of both severity ("grading") and extent ("staging") of OA lesions allow increased reliability, resulting in its recommendation for use as a premium tool for assessing cartilage, in place of MS (36). In future studies, OOCHAS use would improve the reliability of differential miR expression in association with OA stage. Further analysis could confirm the reliability in the McIlwraith *et al.* method, significant to future studies of this nature due to its specificity in equine OA.

MiR expression in young vs old age groups

None of the miRs studied displayed differential expression between young and old groups. This may be because many factors can predispose an individual to OA, including joint injury, heredity, obesity, ageing, mechanics, and inflammation (37); its complex pathogenesis remains undefined. Alternatively, results in this study may be representative of miR expression with no differential expression of the selected miRs between age groups, miRs in SF could be disease and not age dependent. This would allow further investigations of SF miRs in OA to associate differential expression with disease and not ageing, and in this instance a greater cohort with a variety of ages

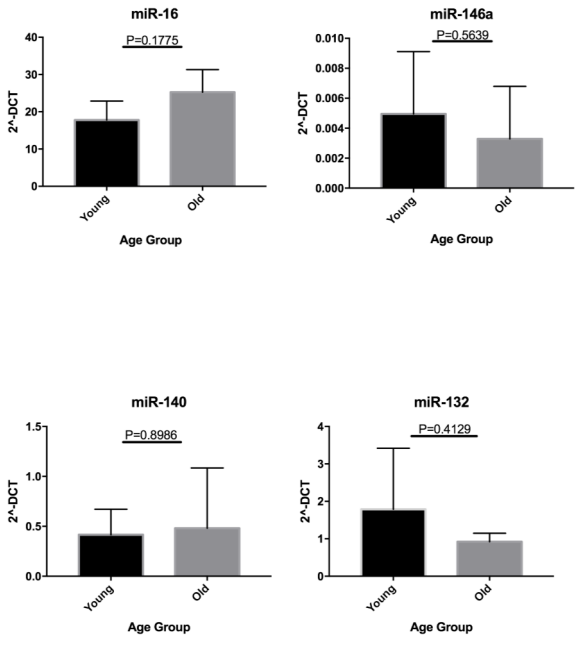

Figure 4: Mean 2^-DCT values in young (n=5) vs old (n=5) equine SF, showing the expression of four miRs (16, 132, 140, 146a), displaying SD and p-value calculated by Student's t-test.

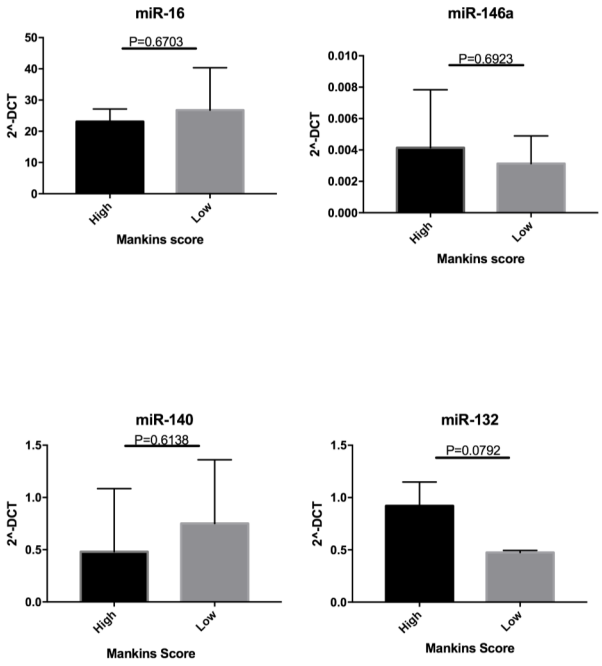

Figure 5: Mean 2^-DCT values of miRs 16, 132, 140, 146a in SF from equine MCP joints of high (n=7) and low (n=3) MS, note the difference in sample size. SD displayed and Student's t-test used to calculate p-value.

could be used, unrestricted by age matching. Further consideration must be that the selected panel of miRs in this study do not alter in ageing, this may not reflect expression of other miRs not included in this study, increasing the panel of miRs investigated would allow this to be determined.

MiR expression in low vs high MS

Murata *et al*. (15) investigated miR-132 expression in SF of OA patients against healthy controls. They found fibroblast-like-synoviocytes and synovial tissues secreted miR-132 with relatively high concentration, hence changes based on damage to these structures could be detectable in SF. Of the selected panel of miRs, differential expression of miR-132 was the most statistically supported (p=0.0792) between high and low MS groups *(Figure 5*). Further investigation could test additional donors for miR -132 expression, to interrogate it as a potential reliable indicator of mild joint pathology. This could help determine patients at risk, improving early management of disease.

Exploration of a suitable housekeeping gene

Although widely used as a housekeeping gene for miR detection in cartilage, research performed in circulating (serum/plasma) miRs, has suggested U6 is not a suitable endogenous control for their quantification (35). In these biofluids, a large fluctuation in U6 expression produced average Ct values below many miRs tested; miR-16 being considered a more stable control. Similar findings existed in this study, where miR-16 was detected earlier with less variation between Ct values (data not shown). Although this finding was neither the purpose of this study nor investigated further, it indicates U6 may be present at different levels endogenously in SF compared to cartilage. Further exploration of miR internal controls in SF is required, as their use in normalisation is critical to determining differential expression.

Limitations

No attempt to match potential confounders, except age, was made due to the lack of animal history. Samples were obtained from an abattoir, with information such as activity status and medical records unknown, however other variables associated with joint change including gender, weight/BCS and breed could be recorded. In future investigations, it will be essential to account biological variability to establish any contributions to differential expression of miRs.

A low sample size of ten equines (old n=5, young n=5) was used, requiring significant expansion to determine the significance and validity of these results. Horses were grouped in wide age ranges, more donors at discrete ages would more reliably allow changes in expression to be tracked in ageing.

Sample collection was not carried out with RNA extraction intended. It is therefore unlikely procedures to prevent RNase degradation were used. This could have created a disparity between samples, where total RNA would not be truly representative.

Understanding of OA pathogenesis is limited. Based on evidence associating miR action to homeostatic processes within cartilage (14, 38), SF miRs could play a direct role in the signalling of changes that occur in the OA affected joint. Mechanisms behind individual miRs are still under investigation. Lack of evidence in this field limits interpretation of this data.

Conclusions

This study has shown miRs are present and detectable in equine SF. HA treatment is beneficial to the quality and handling of SF, whilst RNA values from SF can be achieved using different methods of extraction. Differential expression of SF miRs is not age dependent. A trend in miR-132 expression in equine SF in low grade joint pathologies was seen however significant expansion in sample size is required for further interpretation. This presents a starting point for future studies - revealing SF miRs as potential disease-dependent biomarkers for early diagnosis

Further work

This study used a candidate miR approach to RT-qPCR, universal reverse transcription with specific primer targets, therefore limited to the four miRs selected. Use of another study method could provide a greater understanding of the miRs present in equine SF and their expression profiles. MicroRNA sequencing would allow these advantages, as well as the potential to detect novel miRs in future experiments.

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