**Title:**

Equine synovial fluid small non-coding RNA signatures in early osteoarthritis

**Authors:**

Catarina Castanheira[1]*, C.Castanheira@liverpool.ac.uk

Panagiotis Balaskas[1], panbalas@liverpool.ac.uk

Charlotte Falls[1], hlcfalls@liverpool.ac.uk

Yalda Ashraf-Kharaz[1], yalda@liverpool.ac.uk

Peter Clegg[1], pclegg@liverpool.ac.uk

Kim Burke[2], kim.burke@vetsouth.co.nz

Yongxiang Fang[3], fangy@liverpool.ac.uk

Philip Dyer[4], pddyer@liv.ac.uk

Tim JM Welting[5], t.welting@maastrichtuniversity.nl

Mandy J Peffers[1], peffs@liverpool.ac.uk

**Author affiliations:**

[1]Department of Musculoskeletal and Ageing Science, Institute of Life Course and Medical Sciences, William Henry Duncan Building, 6 West Derby Street, Liverpool, L7 8TX, UK

[2]Institute of Veterinary Science, University of Liverpool, Chester High Road, Neston, CH64 7TE

[3]Centre for Genomic Research, Institute of Integrative Biology, Biosciences Building, Crown Street, University of Liverpool, Liverpool, L69 7ZB, UK

[4]Institute of Infection and Global Health, University of Liverpool, 8 West Derby Street, Liverpool, L7 3EA, UK

[5]Department of Orthopaedic Surgery, Maastricht University Medical Centre, 6202 AZ Maastricht, The Netherlands

***Corresponding author:**

Catarina Castanheira (C.Castanheira@liverpool.ac.uk), Tel: 07470515799

## ABSTRACT

**Background:** Osteoarthritis remains one of the greatest causes of morbidity and mortality in the equine population. The inability to detect pre-clinical changes in osteoarthritis has been a significant impediment to the development of effective therapies against this disease. Synovial fluid represents a potential source of disease-specific small non-coding RNAs (sncRNAs) that could aid in the understanding of the pathogenesis of osteoarthritis. We hypothesised that early stages of osteoarthritis would alter the expression of sncRNAs, facilitating the understanding of the underlying pathogenesis and potentially provide early biomarkers.

**Methods:** Small RNA sequencing was performed using synovial fluid from the metacarpophalangeal joints of both control and early osteoarthritic horses. A group of differentially expressed sncRNAs was selected for further validation through qRT-PCR using an independent cohort of synovial fluid samples from control and early osteoarthritic horses. Bioinformatic analysis was performed in order to identify putative targets of the differentially expressed microRNAs and to explore potential associations with specific biological processes.

**Results:** Results revealed 22 differentially expressed sncRNAs including 13 microRNAs; miR-10a, miR-223, let7a, miR-99a, miR-23b, miR-378, miR-143 (and six novel microRNAs), four small nuclear RNAs; U2, U5, U11, U12, three small nucleolar RNAs; U13, snoR38, snord96, and one small cajal body-specific RNA; scarna3. Five sncRNAs were validated; miR-223 was significantly reduced in early osteoarthritis and miR-23b, let-7a-2, snord96A and snord13 were significantly upregulated. Significant cellular actions deduced by the differentially expressed microRNAs included apoptosis ($P < 0.0003$), necrosis ($P < 0.0009$), autophagy ($P < 0.0007$) and inflammation ($P < 0.00001$). A conservatively filtered list of 57 messenger RNA targets was obtained; the top biological processes associated were regulation of cell population proliferation ($P < 0.000001$), cellular response to chemical stimulus ($P < 0.000001$) and cell surface receptor signalling pathway ($P < 0.000001$).

**Conclusions:** Synovial fluid sncRNAs may be used as molecular biomarkers for early disease in equine osteoarthritic joints. The biological processes they regulate may play an important role in understanding early osteoarthritis pathogenesis. Characterising these dynamic molecular changes could provide novel insights on the process and mechanism of early

osteoarthritis development and is critical for the development of new therapeutic approaches.

**KEYWORDS:** equine, synovial fluid, osteoarthritis, small non-coding RNAs

## BACKGROUND

Osteoarthritis (OA) remains one of the greatest causes of morbidity and mortality for horses in the UK [1, 2]. Additionally, it is the most common disease affecting the joints in humans, and a significant cause of pain and disability worldwide [3]. This degenerative, age-related joint disease is characterised by a progressive degradation of articular cartilage and concomitant structural and functional change of all joint constituents, including the synovial membrane, the subchondral bone and periarticular tissues [4]. Of multifactorial origin, OA is a product of genetic, mechanical and environmental factors such as age, trauma and occupation [4, 5]. Despite its high prevalence and significant welfare and economic impact, its pathophysiology remains poorly understood and currently available diagnostic tools can only identify the disease when cartilage has already exceeded its capacity for intrinsic repair, and changes can no longer be reversed [6, 7]. As a result, the development of effective treatments is also compromised, and currently recommended therapies are mainly symptomatic.

In the search for molecular biomarkers that could reveal pre-clinical phases of the disease, scientists have focused much of their attention on microRNAs (miRNAs), the best characterised family of small non-coding RNAs. Evolutionarily conserved, these 17-22

84 nucleotide long molecules regulate gene expression at post-transcriptional level generally
85 by repressing translation or increasing degradation of messenger RNAs (mRNAs). They are
86 involved in different cellular pathways and intercellular communication thus influencing
87 tissue homeostasis [8]. As such, miRNA profiles can be altered as a result of cellular damage
88 and/or tissue injury and altered expression of certain miRNAs is implicated in several
89 diseases, including OA [9–11]. miRNAs can promote cell differentiation by modulating
90 expression of catabolic genes; for instance, miR-139 which is increased in OA cartilage
91 inhibits cell proliferation by suppressing expression of insulin-like growth factor 1 receptor
92 (IGF1R) and eukaryotic translation initiation factor 4 gamma 2 (EIF4G2) [12]. Furthermore,
93 miRNA expression can also regulate proinflammatory cytokines, again contributing to
94 altered expression of OA-inducing genes; for example, in lipopolysaccharide (LPS)-treated
95 mouse chondrocytes, inhibition of miR-203 increases apoptosis and further stimulates the
96 production of inflammatory cytokines [13]. Additionally, miRNAs can play cartilage-
97 protective roles; miR-193b-3p inhibits extracellular matrix (ECM) degradation through
98 inhibition of inducible nitric oxide (NO) synthesis [14]; dysregulation of miR-193b-3p can
99 therefore promote cartilage degeneration. Comprehensive reviews on miRNAs involved in
100 osteoblastogenesis and osteoclastogenesis, chondrogenesis and cartilage degradation,
101 synovial inflammation and neurogenesis can be found elsewhere [15–17].

102 miRNAs can be found intracellularly or extracellularly, circulating in virtually any biological
103 fluid in a remarkably stable manner [18–20]. Because biological fluids are generally
104 obtainable through minimally invasive techniques, circulating miRNAs are attractive
105 candidates for disease diagnosis, monitoring and prognostication [21, 22]. Interest in other
106 classes of small non-coding RNAs such as small nucleolar RNAs (snoRNAs) has recently
107 emerged. Mostly known for their housekeeping functions, snoRNAs have canonical roles in

the chemical modification of RNA substrates such as ribosomal RNAs, but can also exhibit miRNA-like activity [23]. Aberrant expression of snoRNAs has also been associated with the development of different diseases and a recent study found alterations in the snoRNA profile of OA joints in mice when compared to healthy controls, highlighting the potential of snoRNAs to be used as novel markers for this disease [24].

Equine miRNAs have been identified in numerous healthy tissues [25, 26] and their potential role in different diseases such as osteochondrosis, rhabdomyolysis and insulin resistance has also been investigated [27–29]. However, information on miRNA influence on the pathogenesis of equine OA is still lacking. Synovial fluid represents a reliable source of chemical information that can accurately reflect pathological conditions affecting the joint due to its functional proximity within joint tissues [30]. In 2010, Murata et al. investigated the presence and stability of miRNAs in human synovial fluid for the first time, and found five differentially expressed miRNAs in OA patients compared to healthy controls, supporting the potential use of synovial fluid miRNAs as diagnostic biomarkers [11]. More recently, a screening of 752 miRNAs in synovial fluid from human patients with early- and late-stage OA demonstrated seven upregulated miRNAs in late-stage OA, irrespective of age, gender and body mass index [31]. Intra-articular treatment with hyaluronic acid was shown to modify miRNA expression in OA patients [32]. Although miRNA expression has not yet been investigated in equine OA, a preliminary study has recently described a reproducible method for miRNA isolation from equine synovial fluid and blood plasma [33].

With growing evidence of alterations in small non-coding RNA patterns in the synovial fluid of OA joints, we theorised that early stages of OA would affect these molecules and potentially provide early biomarkers for OA in equine patients. Examining expression of

small non-coding RNAs in synovial fluid in early OA may also provide further insights on the pathological changes that occur. Therefore, we investigated the profile of small non-coding RNAs of early equine OA synovial fluid using next generation sequencing.

## RESULTS

### Macroscopic and histological assessment

The donors used for small RNA sequencing were selected from an elderly population of horses to account for any age-related changes. The ages of the control (mean±standard deviation; 22±2 years) and early OA (27±7.5 years) groups were not significantly different. Horses included in the control group presented minor macroscopic or histological changes, which are to be expected in healthy older animals. Horses included in the early OA group presented intermediate OA scores and were not obviously lame prior slaughter, suggestive of primary OA.

For samples used for small RNA sequencing there was a significant increase in the macroscopic score between control (1.0±0.5) and early OA (5.4±1.9, P=0.04). Likewise, there was a significant increase in the histological score between control (2.1±0.7) and early OA (6.1±1.5, P=0.01) (Additional File 1).

For the independent cohort the ages of the control and early OA groups were not significantly different (Additional File 1). There was a significant increase in the macroscopic score between control (1.75±1.5) and early OA (3.6±0.9, P=0.04) samples. Similarly, there was a significantly increase in the histological score between control (1.5 ±1.3) and early OA (5.8±2.5, P=0.02) (Additional File 1).

## Analysis of small RNA sequencing data

Summaries of raw, trimmed reads and mapped reads to the *Equus caballus* database are in Additional File 2. There were 323 small non-coding RNAs identified. The categories of RNA identified are in Figure 1A and included small non-coding RNAs; miRNAs, snoRNAs and small nuclear RNAs (snRNAs).

In total, the expression of 22 small noncoding RNAs; snoRNAs, snRNAs and miRNAs were significantly different in early OA synovial fluid (±1.3 log2 fold change (logFC), and $P < 0.05$) (Figure 1B; Table 1). We further generated a heatmap of the differentially expressed small non-coding RNAs (Figure 1C).

**Table 1. Differentially expressed small non-coding RNAs in early OA synovial fluid.**

| Ensembl Gene Identification | Gene Name | Gene Biotype | logFC early versus control | P value early versus control |
|---|---|---|---|---|
| ENSECAG00000025823 | eca-let-7a-2 | miRNA | 1.39 | 0.02 |
| ENSECAG00000026330 | eca-mir-10a | miRNA | -2.49 | 0.00 |
| ENSECAG00000026319 | eca-mir-125a | miRNA | -1.43 | 0.05 |
| ENSECAG00000026274 | eca-mir-143 | miRNA | -1.87 | 0.04 |
| ENSECAG00000026469 | eca-mir-223 | miRNA | -2.00 | 0.01 |
| ENSECAG00000025270 | eca-mir-23b | miRNA | 1.77 | 0.03 |
| ENSECAG00000025913 | eca-mir-378 | miRNA | -1.34 | 0.04 |
| ENSECAG00000025243 | eca-mir-99a-2 | miRNA | -1.29 | 0.02 |
| ENSECAG00000025456 | ENSECAG00000025456 | miRNA | -1.74 | 0.02 |
| ENSECAG00000025697 | ENSECAG00000025697 | miRNA | 1.31 | 0.03 |
| ENSECAG00000025869 | ENSECAG00000025869 | miRNA | 1.44 | 0.02 |
| ENSECAG00000026713 | ENSECAG00000026713 | miRNA | -7.27 | 0.03 |
| ENSECAG00000027105 | ENSECAG00000027105 | miRNA | -1.34 | 0.04 |
| ENSECAG00000027634 | ENSECAG00000027634 | miRNA | -1.77 | 0.04 |
| ENSECAG00000027641 | SCARNA3 | snoRNA | -7.28 | 0.03 |
| ENSECAG00000026609 | snoR38 | snoRNA | 8.01 | 0.01 |
| ENSECAG00000025929 | SNORD96 | snoRNA | 7.61 | 0.01 |
| ENSECAG00000027243 | snoU13 | snoRNA | 2.02 | 0.04 |
| ENSECAG00000025371 | U11 | snRNA | 1.45 | 0.03 |

| | | | | |
|---|---|---|---|---|
| ENSECAG00000025759 | U12 | snRNA | 2.70 | 0.00 |
| ENSECAG00000025571 | U2 | snRNA | 2.57 | 0.00 |
| ENSECAG00000025679 | U2 | snRNA | 2.49 | 0.00 |
| ENSECAG00000026075 | U2 | snRNA | 2.52 | 0.00 |
| ENSECAG00000026524 | U2 | snRNA | 2.43 | 0.00 |
| ENSECAG00000026243 | U2 | snRNA | 2.51 | 0.00 |
| ENSECAG00000025523 | U2 | snRNA | 1.48 | 0.03 |
| ENSECAG00000025597 | U2 | snRNA | 1.57 | 0.04 |
| ENSECAG00000025663 | U5 | snRNA | 3.11 | 0.00 |
| ENSECAG00000026081 | U5 | snRNA | 1.92 | 0.01 |

**Confirmation of differential gene expression using qRT-PCR**

Seven small non-coding RNAs (miR-143, miR-223, miR-99a, miR-23b, let-7a-2, snord96A, snord13) were selected for further validation based on our current work, level of differential expression ($P < 0.05$ and $logFC > 1.2$) and following a literature review of differentially expressed genes. An independent cohort of synovial fluid samples was used, comprising of control (n=6, histological score 1.5 ±1.3) and early OA (n=6, histological score 5.8±2.5) synovial fluid samples. In agreement with the sequencing data miR-223 was significantly reduced in early OA and miR-23b, let-7a-2, snord96A and snord13 were significantly increased in early OA (Figure 2). For two miRNAs, miR-143 and miR-99a-2 quantitative reverse transcription-polymerase chain reaction (qRT-PCR) findings did not validate sequencing findings; despite being decreased in the OA group in our sequencing data, qRT-PCR showed increased expression of both miRNAs in the independent OA group compared to controls, although this was not statistically significant (Figure 2).

**Identification of potential target mRNA genes of the differentially expressed miRNAs**

With the goal of exploring potential biological associations with the differentially expressed miRNAs in early OA synovial fluid we undertook an Ingenuity Pathway Analysis (IPA) 'Core Analysis' on these. Interesting features were determined from the gene networks inferred. Significant cellular actions deduced by the differentially expressed miRNAs included apoptosis ($P<0.0003$), necrosis ($P<0.0009$), autophagy ($P<0.0007$) and inflammation ($P<0.00001$) (Figure 3A).

Next, we undertook analysis to determine the mRNA targets of the differentially expressed miRNAs. Eight miRNAs were differentially expressed in early OA compared to non-OA controls. Once a conservative filter was applied (only miRNAs with experimentally confirmed or highly conserved predicted targets), miR-let7a-2 and miR-378 were excluded. Six miRNAs remained which collectively putatively target 993 mRNAs. We then additionally added the filters chondrocytes, fibroblast and osteoblasts, removed duplicates and obtained a list of 57 mRNA targets (Additional File 3).

The presumed target mRNAs were input into the gene ontology (GO) tool PANTHER and the biological processes were summarised in REViGO and visualised using Cytoscape (Figure 3B). The top biological processes were regulation of cell population proliferation (false discovery rate (FDR)-adjusted $P= 6.24E^{-13}$), cellular response to chemical stimulus ($FDR = 4.54E^{-12}$) and cell surface receptor signalling pathway ($FDR= 6.39E^{-12}$) (Additional File 4).

## DISCUSSION

The inability to detect pre-clinical changes in OA has been one of the main impediments to the development of effective therapies against this disease [34]. From a biomarker perspective, profiling synovial fluid circulating locally within the affected joint cavity at an early stage may provide new insights into pathological changes occurring during OA initiation and progression, and ultimately allow for the implementation of new therapeutic approaches. Our study is, to the best of our knowledge, the first to characterise the small non-coding RNA profile of synovial fluid in early OA in horses, providing evidence of a pattern of differential expressed synovial fluid miRNAs and other small non-coding RNAs in early OA synovial fluid when compared to our control samples.

Osteoarthritis is a highly heterogeneous disease and can be broadly divided into primary, naturally occurring OA, which is chronic and associated with age; and post-traumatic OA, usually related to athletic use [35]. Whilst post-traumatic OA is highly prevalent and has a significant economic impact particularly for younger and athletic horses [36], animals over 15 years comprise up to one third of the equine population and represent a growing proportion of referral hospital admissions [37]. Musculoskeletal conditions are a major reason for euthanasia in older horses, suggesting that the social economic burden of age-related osteoarthritis is rising [2]. While trauma might be one of the causative factors of OA in older horses, it is difficult to ascertain whether the body's response to external sources of stress is being affected by ageing or whether age-related changes are actually a predisposing factor for traumatic injuries by increasing their likelihood, for example through altered biomechanics. For this reason, in our study we sought to exclude the cofounding effect of a traumatic injury by selecting older donors originating from the abattoir; although

this does not guarantee absence of a traumatic injury, horses can only enter the food chain if they are not obviously lame prior to slaughter, making it more likely that these OA cases arise from age-related molecular mechanisms. Selected donors from both control and early OA groups were age-matched to account for any age-related changes. Donors from the control group presented minor changes in their joints which are to be expected in older horses, hence classifying this group as “control” as opposed to “healthy”.

Due to the considerable interest in miRNA-mediated gene regulation in recent years, the list of miRNAs possibly implicated in OA and other joint related pathologies has grown [16]. miRNAs that are differentially expressed in joint tissues of patients with OA are likely to contribute to OA pathophysiology and may be utilised as diagnostic factors [38]. One example is miR-140, which is significantly downregulated in human OA cartilage [10] and is thought to attenuate OA progression by modulating ECM homeostasis [39]; also, dysregulation of miR-140-3p and-5p in synovial fluid has been correlated with OA severity [40].

Among the differentially expressed miRNAs found in our study, miR-23b was significantly increased in the early OA cohort. miR-23b is thought to be involved in OA progression by targeting cartilage-associated protein (CRTAP) and thus influencing cartilage homeostasis [41]. This miRNA has also been shown to positively regulate the chondrogenic differentiation of mesenchymal stem cells by regulating the expression of sex-determining region Y-Box 9 (SOX9) and protein kinase A (PKA) [42, 43].

Likewise, we found let-7a-2 to be upregulated in early OA. In an experiment comparing miRNA expression in synovial fluid from human OA patients undergoing hyaluronic acid treatment, let-7a was significantly upregulated in synovial fluid of OA samples compared to

249 healthy controls; levels of let-7a in affected patients returned to normal after hyaluronan
250 injection [32]. Let-7a is thought to regulate IL-6 receptor (IL6R), and its inhibition can
251 enhance cell proliferation, reduce apoptosis and inhibit inflammatory response in ATDC5
252 cells in a LPS-induced *in vitro* model of OA [44]. Members of the let-7 family have often been
253 described in studies involving OA; a large population-based study identified serum let-7e as
254 a promising candidate to predict OA risk, independent of age, sex and body mass index [45].
255 A recent investigation supported this claim, providing further evidence of decreased
256 expression of let-7e in serum of patients affected with knee OA [46]. The exact roles of
257 miRNAs of the let-7 family remain unclear, but the evidence for their use as biomarkers for
258 OA is growing.

259 In the above mentioned paper by Xu et al. (2015) [32], miR-223 was also significantly
260 upregulated in synovial fluid of OA patients prior to intra-articular injection of hyaluronan.
261 miR-223 participates in cartilage homeostasis and structure by targeting growth
262 differentiation factor 5 (GDF5) [41]. Early-stage OA patients showed upregulation of miR-
263 223 in peripheral blood mononuclear cells, with its expression decreasing as OA progressed
264 [47]. In our study, we found miR-223 to be downregulated in the synovial fluid of the early
265 OA cohort, which supports the involvement of this miRNA in the early osteoarthritic
266 process. miRNA regulation is complex and differences between our results and previously
267 published literature in human patients may be due to different stages in osteoarthritic
268 process; species variation may also partially justify these disparities. Additionally, an
269 increasing body of evidence demonstrates that long non-coding RNAs (lncRNAs) can act as
270 sponges for microRNAs [48]; a previous study found that the expression of miR-223 was
271 restrained by lncRNA activated by transforming growth factor beta (lncRNA-ATB) [49] which
272 might contribute to variations in miRNA expression.

We have previously shown the involvement of snoRNAs in cartilage ageing and OA and their potential use as biomarkers for OA [24]. In this study we identified for the first time snord13 and snord96a as highly expressed small non-coding RNAs in early OA. Our previous work in human OA cartilage identified a dysregulation in SNORD96A expression in ageing and OA. In addition, we demonstrated changes in chondrogenic, hypertrophic, ribosomal RNA (rRNA) and OA related gene expression following overexpression and knockdown of SNORD96A in human chondrocytes. Interestingly we also identified an increase in SNORD96A in chondrocytes treated with OA synovial fluid [50]. In another microarray study of young compared to old OA cartilage we identified SNORD13 was increased in OA cartilage [51]. Together these findings indicate that changes in synovial fluid snoRNAs could in part be due to a dysregulation in their expression in cartilage in OA. snoRNAs are emerging with unappreciated functional roles in cell physiology [52] and our results support our earlier work for the potential use of snoRNAs as novel biomarkers in OA [24].

Predicted targets of the miRNAs of interest appear to be involved in processes of inflammation and cellular destruction including necrosis, apoptosis and autophagy, which have been previously shown to contribute to the pathogenesis of OA in human patients through pro-inflammatory cytokines production [53], synovial inflammation [54] and chondrocyte apoptosis [55]; subchondral bone changes [56] and chondrocyte apoptosis [57] have also been implicated in the pathogenesis of OA in horses.

For example, a disintegrin and metalloproteinase with thrombospondin motif 1 (ADAMTS1) is known to cleave aggrecan, a critical component for cartilage structure [58]; bone morphogenetic protein (BMP) receptor type 1B (BMPRB) is a receptor for BMP, and BMP signalling is essential for chondrocyte proliferation, survival and differentiation [59]; and IL-

296 6R interacts with IL-6, one of the pro-inflammatory cytokines increased in osteoarthritis
297 [60]. Experimental validation of these and other predicted target genes can clarify biological
298 mechanisms behind small non-coding RNAs of interest and elucidate their role in the
299 pathogenesis of OA; this is critical for the success of future interventions, as these molecules
300 can be targeted in a specific manner [61, 62].

301 Profiling circulating, cell-free small non-coding RNAs is generally a challenging task due to
302 the limited amount of RNA present in biofluids, as well as presence of inhibitory compounds
303 which potentially hinder downstream enzymatic processes. However, liquid biopsies for the
304 investigation of non-coding RNA profiles have gained prominence due to their ease of
305 collection and potential use as diagnostic tools. Future studies in this field would benefit
306 from analysing larger cohorts of patients; our study was limited by the availability of joints
307 with early OA, resulting in a small sample size. Notwithstanding, a previous study on the
308 subject of RNA-Seq analysis performance [63] has shown the number of genes called
309 significant increases as the sample number increases; this suggests that for pipelines such as
310 the one used in this study, having a slightly underpowered approach means we are more
311 likely to underestimate rather than overestimate the number of differentially expressed
312 miRNAs. That fact that we were able to validate our findings through qRT-PCR in an
313 independent cohort solidifies our findings, despite the small sample size. Further work is
314 unquestionably needed, yet this experiment enabled us to identify small non-coding RNA
315 changes in the initial and an additional cohort and revealed, for the first time, the potential
316 use of small non-coding RNAs as biomarkers for early OA. These results support the use of
317 synovial fluid small non-coding RNAs as molecular biomarkers for early disease in OA joints.
318 Our future research is currently ascertaining the applicability of these findings in a clinical
319 setting.

## CONCLUSIONS

This study demonstrates that equine synovial fluid displays a pattern of small non-coding RNA differential expression in early OA when compared to controls, as defined by gross and histological scoring and many of these small non-coding RNAs have previously been demonstrated to have a role in OA. The affected biological cellular processes in response to changing miRNAs and their target genes might play an important role in early OA pathogenesis. This opens the possibility of a relatively non-invasive method for early detection of OA. Furthermore, characterisation of these dynamic molecular changes could provide novel insights on the process and mechanism of early OA development.

## METHODS

All reagents were from ThermoFisher Scientific, unless stated.

### Sample collection and preparation

Samples were collected from the metacarpophalangeal joints of horses from an abattoir as a by-product of the agricultural industry. Specifically, the Animal (Scientific procedures) Act 1986, Schedule 2, does not define collection from these sources as scientific procedures. Ethical approval was therefore not required.

The joints were aseptically dissected to allow visual inspection of the metacarpus, the proximal phalange and the sesamoids. All joints were photographed and macroscopic changes were scored based on a scoring system as previously described [64]. Synovial fluid was aseptically collected directly from the open joint with a 5 ml sterile syringe, immediately

placed on sterile microcentrifuge tubes on ice and centrifuged for 10 min at 3000 g and 4°C to remove cells and debris. The supernatant was collected and stored at -80°C. A cartilage and subchondral bone fragment was collected from the palmar aspect of one of the metacarpal condyles, fixed on paraformaldehyde and sent for histology; histological scoring was performed using the previously described scoring system [65].

Donors were assigned to groups based on the macroscopic and histologic scoring. The control (non-OA) group was comprised of 3 donors with age mean± standard deviation 22±2 years; while the early OA group was comprised of 3 donors with 22±7.5 years.

**RNA isolation, cDNA library preparation and small RNA sequencing**

Synovial fluid was treated to reduce viscosity with 1µg/ml of hyaluronidase at 37˚C for 1 hr, centrifuged at 1000 g for 5 min, and supernatant used for total RNA extraction using miRNeasy serum kits (Qiagen, Crawley, UK). The integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer system using an RNA Pico chip. 100ng samples were submitted for library preparation using NEBNext® Small RNA Library Prep Set for Illumina (New England Biosciences (NEB), Ipswich, USA) but with the addition of a Cap-Clip™ Acid Pyrophosphatase (Cell script, Madison, USA) step to remove any 5' cap structures [24] and size selected using a range 120-300bp. This enabled both miRNAs and snoRNAs to be identified in a non-biased approach. The pooled libraries were sequenced on an Illumina HiSeq4000 platform with version 1 chemistry to generate 2 x 150 bp paired-end reads. Data has been submitted to National Centre for Biotechnology Information; accession E-MTAB-8409.

## Small RNA sequencing data analysis

Sequence data were processed through a number of steps to obtain non-coding RNA expression values including; basecalling and de-multiplexing of indexed reads using CASAVA version 1.8.2; adapter and quality trimming using Cutadapt version 1.2.1 [66] and Sickle version 1.200 to obtain fastq files of trimmed reads; aligning reads to horse genome reference sequences (release 90) from Ensembl using Tophat version 2.0.10 [67] with option "–g 1"; counting aligned reads using HTSeq-count [68] against the features defined in horse genome GTF file (release 90).

Differential expression analysis was performed in R using package DESeq2 [69]. The processes and technical details of the analysis include; assessing data variation and detecting outlier samples through comparing variations of within and between sample groups using principle component analysis (PCA) and correlation analysis; handling library size variation using DESeq2 default method; formulating data variation using negative binomial distributions; modelling data using a generalised linear model; computing logFC values for control versus early OA based on model fitting results through contrast fitting approach, evaluating the significance of estimated logFC values by Wald test; adjusting the effects of multiple tests using FDR approach [70] to obtain FDR adjusted P-values.

The Ensembl horse genome GTF file release 90 does not have mature miRNA features. We linked the defined miRNA primary transcripts to miRBase horse miRNA GFF3 file by feature's genome coordinates so as to obtain the corresponding mature miRNA.

386 **qRT-PCR validation**

387 Validation of the selected small RNA sequencing results in an independent cohort of equine
388 metacarpophalangeal synovial fluid was undertaken using qRT-PCR. Six control (non-OA),
389 mean±standard deviation (20.2±2.4 years) and six early OA (20.8±4.1) with macroscopically
390 and histologically graded sample scores similar to those used for sequencing were used.
391 Total RNA was extracted as above. Small non-coding RNAs were chosen based on our
392 current work, level of differential expression ($P < 0.05$ and $logFC > 1.2$) and following a
393 literature review of differentially expressed genes. These were miR-143, miR-223, miR-99a,
394 miR-23b, let-7a-2, snord96A and snord13. Primer sequences/assays used can be found in
395 Additional File 5. PolyA cDNA was synthesized using 200ng RNA and the miScript II RT Kit. A
396 mastermix was prepared using the miScript SYBR Green PCR Kit (Qiagen, Crawley, UK) and
397 the appropriate bespoke designed miScript Primer Assays (Qiagen, Crawley, UK). Real-time
398 PCR was undertaken using a LightCycler® 96 system (Roche). Steady-state transcript
399 abundance of potential endogenous control genes was measured in the small RNA
400 sequencing data. Assays for four genes – miR-181a, miR-100, miR-191a and U6 were
401 selected as potential reference genes because their expression was unaltered in this study.
402 Stability of this panel of genes was assessed by applying a gene stability tool RefFinder [71].
403 The geometric mean of miR-100 and miR-191a was selected as the stable endogenous
404 control. miR-100 has been previously used as a normaliser in a similar study as it was
405 identified by NormFinder as the most stable [31]. Relative expression levels were
406 normalised to the geometric mean of miR-100 and miR-191 and calculated using the 2^-DCT
407 method [72].

408

## miRNA target prediction and pathway analysis

Potential biological associations of the differentially expressed miRNAs in early OA synovial fluid were identified using IPA (IPA, Qiagen Redwood City, CA, USA) 'Core Analysis'. Canonical pathways, novel networks, and common upstream regulators were then queried. Additionally in order to identify putative miRNA targets, bioinformatic analysis was performed by uploading differentially expressed miRNA data into the MicroRNA Target Filter module within IPA software. This identifies experimentally validated miRNA-mRNA interactions from TarBase, miRecords, and the peer-reviewed biomedical literature, as well as predicted miRNA-mRNA interactions from TargetScan. We used a conservative filter at this point, using only experimentally validated and highly conserved predicted mRNA targets for each miRNA. Targets were then also filtered on the cells chondrocyte, osteoblasts and fibroblasts (the latter two settings were the nearest to bone and synovial cells available for selection), to represent joint cells in contact with synovial fluid.

PANTHER (GO Ontology database 2020-02-21) [73] was used for overrepresentation analysis of the mRNA targets using Fisher's Exact test with FDR correction. This tests whether the input mRNAs associate significantly with specific pathways and generates a list of biological process GO terms. Terms with FDR adjusted $P < 0.05$ were summarised using REViGO [74] with allowed similarity of 0.4 and visualised using Cytoscape [75].

## Statistical analysis

The heatmap and volcano plots were made using MetaboAnalyst 3.5 (http://www.metaboanalyst.ca) which uses the R package of statistical computing software

[76]. For statistical evaluation of gene expression data, following normality testing, Mann-Whitney tests were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com); P values are indicated.

## LIST OF ABBREVIATIONS

ADAMTS1 = a disintegrin and metalloproteinase with thrombospondin motif 1, BMP = bone morphogenic protein, BMPRB = BMP receptor type 1B, CRTAP = cartilage associated protein, DNA = deoxyribonucleic acid, ECM = extracellular matrix, EIF4G2 = eukaryotic translation initiation factor 4 gamma 2, FDR = false discovery rate, GDF5 = growth differentiation factor 5, GO = gene ontology, IGF1R = insulin-like growth factor 1 receptor; IPA = Ingenuity Pathway Analysis, IL = interleukin, IL6R = interleukin 6 receptor, LPS = lipopolysaccharide, logFC = log2 fold change, lncRNA = long non-coding RNA, lncRNA-ATB = lncRNA activated by transforming growth factor beta, miRNAs = micro RNAs, NO = nitric oxide, OA = osteoarthritis, PCA = principal component analysis, PKA = protein kinase A, qRT-PCR = quantitative reverse transcription polymerase chain reaction, RNA = ribonucleic acid, Rrna = ribosomal RNA, sncRNAs = small non-coding RNAs, snoRNAs = small nucleolar RNAs, snRNAs = small nuclear RNAs, SOX9 = sex-determining region Y – box 9.

## DECLARATIONS

### Ethics approval and consent to participate

Synovial fluid was collected as a by-product of the agricultural industry. Specifically, the Animal (Scientific procedures) Act 1986, Schedule 2, does not define collection from these sources as scientific procedures. Ethical approval was therefore not required.

## Consent for publication

Not applicable.

## Availability of data and materials

Data has been submitted to National Centre for Biotechnology Information; accession E-MTAB-8409.The datasets supporting the conclusions of this article are included within the article and its additional files.

## Competing interests

The authors declare no competing interests.


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into Musculoskeletal Ageing is a collaboration between the Universities of Liverpool, Sheffield and Newcastle. The funding bodies approved the study design and collection of data and were not involved in either the analysis and interpretation of data nor the writing of the manuscript.

## Authors' contributions

MP, PC and TW designed and coordinated the study. MP, KB, PD collected the samples. PB, PD and KB processed the samples for small RNA sequencing. YF assembled the sequencing data and performed sequencing data analysis. PD, CF, YA and CC processed the samples for validation and performed qRT-PCR. MP and CC conducted the statistical analysis and drafted the manuscript. All authors revised the draft critically and read and approved the final submitted manuscript.

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## REFERENCES

1. Ireland JL, Clegg PD, Mcgowan CM, Mckane SA, Chandler KJ, Pinchbeck GL. Disease prevalence in geriatric horses in the United Kingdom: Veterinary clinical assessment of 200

493 cases. Equine Vet J. 2012;44:101–6.

494 2. Ireland JL, Clegg PD, McGowan CM, Platt L, Pinchbeck GL. Factors associated with
495 mortality of geriatric horses in the United Kingdom. Prev Vet Med. 2011;101:204–18.

496 3. Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. Bull World Health
497 Organ. 2003;81:646–56.

498 4. Mobasheri A, Batt M. An update on the pathophysiology of osteoarthritis. Ann Phys
499 Rehabil Med. 2016;59:333–9.

500 5. Ashkavand Z, Malekinejad H, Vishwanath BS. The pathophysiology of osteoarthritis. J
501 Pharm Res. 2013;7:132–8.

502 6. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - A review. Vet J.
503 2006;171:51–69.

504 7. McIlwraith CW, Frisbie DD, Kawcak CE. The horse as a model of naturally occurring
505 osteoarthritis. Bone Joint Res. 2012;1:297–309.

506 8. Adams BD, Parsons C, Walker L, Zhang WC, Slack FJ. Targeting noncoding RNAs in disease.
507 J Clin Invest. 2017;127:761–71.

508 9. Díaz-Prado S, Cicione C, Muiños-López E, Hermida-Gómez T, Oreiro N, Fernández-López C,
509 et al. Characterization of microRNA expression profiles in normal and osteoarthritic human
510 chondrocytes. BMC Musculoskelet Disord. 2012;13:144.

511 10. Miyaki S, Nakasa T, Otsuki S, Grogan SP, Higashiyama R, Inoue A, et al. MicroRNA-140 is
512 expressed in differentiated human articular chondrocytes and modulates interleukin-1
513 responses. Arthritis Rheum. 2009;60:2723–30.

514 11. Murata K, Yoshitomi H, Tanida S, Ishikawa M, Nishitani K, Ito H, et al. Plasma and
515 synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis.
516 Arthritis Res Ther. 2010;12:R86.

517 12. Hu W, Zhang W, Li F, Guo F, Chen A. miR-139 is up-regulated in osteoarthritis and
518 inhibits chondrocyte proliferation and migration possibly via suppressing EIF4G2 and IGF1R.
519 Biochem Biophys Res Commun. 2016;474:296–302.
520 doi:https://doi.org/10.1016/j.bbrc.2016.03.164.

521 13. Wang Z, Chi X, Liu L, Wang Y, Mei X, Yang Y, et al. Long noncoding RNA maternally
522 expressed gene 3 knockdown alleviates lipopolysaccharide-induced inflammatory injury by
523 up-regulation of miR-203 in ATDC5 cells. Biomed Pharmacother. 2018;100:240–9.
524 doi:https://doi.org/10.1016/j.biopha.2018.02.018.

525 14. Chang Z, Meng F, Zhang Z, Mao G, Huang Z, Liao W, et al. MicroRNA-193b-3p regulates
526 matrix metalloproteinase 19 expression in interleukin-1β-induced human chondrocytes. J
527 Cell Biochem. 2018;119:4775–82. doi:10.1002/jcb.26669.

528 15. Yu X-M, Meng H-Y, Yuan X-L, Wang Y, Guo Q-Y, Peng J, et al. MicroRNAs' Involvement in
529 Osteoarthritis and the Prospects for Treatments. Evid Based Complement Alternat Med.
530 2015;2015:236179.

531 16. Endisha H, Rockel J, Jurisica I, Kapoor M. The complex landscape of microRNAs in
532 articular cartilage: biology, pathology, and therapeutic targets. JCI insight. 2018;3.

533 17. Peffers MJ, Balaskas P, Smagul A. Osteoarthritis year in review 2017: genetics and
534 epigenetics. Osteoarthr Cartil. 2018;26:304–11.

535 18. Ge Q, Zhou Y, Lu J, Bai Y, Xie X, Lu Z. miRNA in Plasma Exosome is Stable under Different

536 Storage Conditions. Molecules. 2014;19:1568–75.

537 19. Wang K. The Ubiquitous Existence of MicroRNA in Body Fluids. Clin Chem. 2017;63:784–
538 5.

539 20. Zhang Z, Qin YW, Brewer G, Jing Q. MicroRNA degradation and turnover: Regulating the
540 regulators. Wiley Interdiscip Rev RNA. 2012;3:593–600.

541 21. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological
542 challenges in utilizing miRNAs as circulating biomarkers. J Cell Mol Med. 2014;18:371–90.

543 22. Buschmann D, Haberberger A, Kirchner B, Spornraft M, Riedmaier I, Schelling G, et al.
544 Toward reliable biomarker signatures in the age of liquid biopsies - How to standardize the
545 small RNA-Seq workflow. Nucleic Acids Res. 2016;44:5995–6018.

546 23. Stepanov GA, Filippova JA, Komissarov AB, Kuligina E V, Richter VA, Semenov D V.
547 Regulatory Role of Small Nucleolar RNAs in Human Diseases. Biomed Res Int.
548 2015;2015:206849. doi:10.1155/2015/206849.

549 24. Steinbusch MMF, Fang Y, Milner PI, Clegg PD, Young DA, Welting TJM, et al. Serum
550 snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis. Sci Rep. 2017;7:1–
551 11.

552 25. Kim M-C, Lee S-W, Ryu D-Y, Cui F-J, Bhak J, Kim Y. Identification and Characterization of
553 MicroRNAs in Normal Equine Tissues by Next Generation Sequencing. PLoS One.
554 2014;9:e93662. doi:10.1371/journal.pone.0093662.

555 26. Pacholewska A, Mach N, Mata X, Vaiman A, Schibler L, Barrey E, et al. Novel equine
556 tissue miRNAs and breed-related miRNA expressed in serum. BMC Genomics. 2016;17:1–15.

557 27. Barrey E, Bonnamy B, Barrey EJ, Mata X, Chaffaux S, Guerin G. Muscular microRNA
558 expressions in healthy and myopathic horses suffering from polysaccharide storage
559 myopathy or recurrent exertional rhabdomyolysis. Equine Vet J. 2010;42 SUPPL. 38:303–10.

560 28. Desjardin C, Vaiman A, Mata X, Legendre R, Laubier J, Kennedy SP, et al. Next-generation
561 sequencing identifies equine cartilage and subchondral bone miRNAs and suggests their
562 involvement in osteochondrosis physiopathology. BMC Genomics. 2014;15:798.
563 doi:10.1186/1471-2164-15-798.

564 29. da Costa Santos H, Hess T, Bruemmer J, Splan R. Possible Role of MicroRNA in Equine
565 Insulin Resistance: A Pilot Study. J Equine Vet Sci. 2018;63:74–9.

566 30. McIlwraith CW. Use of synovial fluid and serum biomarkers in equine bone and joint
567 disease: a review. Equine Vet J. 2010;37:473–82.

568 31. Li Y-H, Tavallaee G, Tokar T, Nakamura A, Sundararajan K, Weston A, et al. Identification
569 of synovial fluid microRNA signature in knee osteoarthritis: differentiating early- and late-
570 stage knee osteoarthritis. Osteoarthr Cartil. 2016;24:1577–86.

571 32. Xu JF, Zhang SJ, Zhao C, Qiu BS, Gu HF, Hong JF, et al. Altered microRNA Expression
572 Profile in Synovial Fluid from Patients with Knee Osteoarthritis with Treatment of Hyaluronic
573 Acid. Mol Diagnosis Ther. 2015;19:299–308.

574 33. Antunes J, Koch TG, Koenig J, Cote N, Dubois M-S. On the road to biomarkers:
575 developing a robust system for miRNA evaluation in equine blood and synovial fluid.
576 Osteoarthr Cartil. 2019;27:S110–1.

577 34. Chu CR, Williams AA, Coyle CH, Bowers ME. Early diagnosis to enable early treatment of
578 pre-osteoarthritis. Arthritis Res Ther. 2012;14:212. doi:10.1186/ar3845.

579 35. McCoy AM. Animal Models of Osteoarthritis: Comparisons and Key Considerations. Vet
580 Pathol. 2015;52:803–18. doi:10.1177/0300985815588611.

581 36. Neundorf RH, Lowerison MB, Cruz AM, Thomason JJ, McEwen BJ, Hurtig MB.
582 Determination of the prevalence and severity of metacarpophalangeal joint osteoarthritis in
583 Thoroughbred racehorses via quantitative macroscopic evaluation. Am J Vet Res. 2010;71.

584 37. Ireland JL. Demographics, Management, Preventive Health Care and Disease in Aged
585 Horses. Vet Clin North Am Equine Pract. 2016;32:195–214.
586 doi:https://doi.org/10.1016/j.cveq.2016.04.001.

587 38. Zhang M, Lygrissea K, Wanga J. Role of MicroRNA in Osteoarthritis. J Arthritis. 2017;06.
588 doi:10.4172/2167-7921.1000239.

589 39. Si HB, Zeng Y, Liu SY, Zhou ZK, Chen YN, Cheng JQ, et al. Intra-articular injection of
590 microRNA-140 (miRNA-140) alleviates osteoarthritis (OA) progression by modulating
591 extracellular matrix (ECM) homeostasis in rats. Osteoarthr Cartil. 2017;25:1698–707.

592 40. Yin C-M, Suen W-C-W, Lin S, Wu X-M, Li G, Pan X-H. Dysregulation of both miR-140-3p
593 and miR-140-5p in synovial fluid correlate with osteoarthritis severity. Bone Joint Res.
594 2017;6:612–8.

595 41. Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative MicroRNA and Proteomic
596 Approaches Identify Novel Osteoarthritis Genes and Their Collaborative Metabolic and
597 Inflammatory Networks. PLoS One. 2008;3:e3740. doi:10.1371/journal.pone.0003740.

598 42. Ham O, Song BW, Lee SY, Choi E, Cha MJ, Lee CY, et al. The role of microRNA-23b in the
599 differentiation of MSC into chondrocyte by targeting protein kinase A signaling.
600 Biomaterials. 2012;33:4500–7.

601 43. Karlsen TA, Jakobsen RB, Mikkelsen TS, Brinchmann JE. MicroRNA-140 targets RALA and
602 regulates chondrogenic differentiation of human mesenchymal stem cells by translational
603 enhancement of SOX9 and ACAN. Stem Cells Dev. 2014;23:290–304.

604 44. Sui G, Zhang L, Hu Y. MicroRNA-let-7a inhibition inhibits LPS-induced inflammatory injury
605 of chondrocytes by targeting IL6R. Mol Med Rep. 2019;20:2633–40.

606 45. Beyer C, Zampetaki A, Lin NY, Kleyer A, Perricone C, Iagnocco A, et al. Signature of
607 circulating microRNAs in osteoarthritis. Ann Rheum Dis. 2015;74:e18–e18.

608 46. Feng L, Feng C, Wang CX, Xu DY, Chen JJ, Huang JF, et al. Circulating microRNA let–7e is
609 decreased in knee osteoarthritis, accompanied by elevated apoptosis and reduced
610 autophagy. Int J Mol Med. 2020;45:1464–76.

611 47. Okuhara A, Nakasa T, Shibuya H, Niimoto T, Adachi N, Deie M, et al. Changes in
612 microRNA expression in peripheral mononuclear cells according to the progression of
613 osteoarthritis. Mod Rheumatol. 2012;22:446–57.

614 48. Tu J, Huang W, Zhang W, Mei J, Zhu C. The emerging role of lncRNAs in chondrocytes
615 from osteoarthritis patients. Biomed Pharmacother. 2020;131:110642.
616 doi:https://doi.org/10.1016/j.biopha.2020.110642.

617 49. Ying H, Wang Y, Gao Z, Zhang Q. Long non-coding RNA activated by transforming growth
618 factor beta alleviates lipopolysaccharide-induced inflammatory injury via regulating
619 microRNA-223 in ATDC5 cells. Int Immunopharmacol. 2019;69:313–20.
620 doi:https://doi.org/10.1016/j.intimp.2019.01.056.

621 50. Peffers MJ, Chabronova A, Balaskas P, Fang Y, Dyer P, Cremers A, et al. SnoRNA
622 signatures in cartilage ageing and osteoarthritis. Sci Rep. 2020;10:10641.

623 doi:10.1038/s41598-020-67446-z.

624 51. Peffers MJ, Ripmeester E, Caron M, Steinbusch M, Balaskas P, Cremers A, et al. A role for
625 the snoRNA U3 in the altered translational capacity of ageing and osteoarthritic
626 chondrocytes. Osteoarthr Cartil. 2018;26:S45–6.

627 52. Mcmahon M, Contreras A, Ruggero D. Small RNAs with big implications: New insights
628 into H/ACA snoRNA function and their role in human disease. Wiley Interdiscip Rev RNA.
629 2015;6:173–89.

630 53. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in
631 osteoarthritis. In: Clinical Orthopaedics and Related Research. Lippincott Williams and
632 Wilkins; 2004. p. S27–36.

633 54. Wang X, Hunter DJ, Jin X, Ding C. The importance of synovial inflammation in
634 osteoarthritis: current evidence from imaging assessments and clinical trials. Osteoarthr
635 Cartil. 2018;26:165–74.

636 55. Hwang HS, Kim HA. Chondrocyte apoptosis in the pathogenesis of osteoarthritis. Int J
637 Mol Sci. 2015;16:26035–54.

638 56. Stewart HL, Kawcak CE. The Importance of Subchondral Bone in the Pathophysiology of
639 Osteoarthritis . Frontiers in Veterinary Science . 2018;5:178.
640 https://www.frontiersin.org/article/10.3389/fvets.2018.00178.

641 57. Thomas CM, Fuller CJ, Whittles CE, Sharif M. Chondrocyte death by apoptosis is
642 associated with cartilage matrix degradation. Osteoarthr Cartil. 2007;15:27–34.
643 doi:https://doi.org/10.1016/j.joca.2006.06.012.

644 58. Lin EA, Liu C-J. The role of ADAMTSs in arthritis. Protein Cell. 2010;1:33–47.

645 doi:10.1007/s13238-010-0002-5.

646 59. Yoon BS, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, Lyons KM. Bmpr1a and
647 Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. Proc Natl
648 Acad Sci U S A. 2005;102:5062–7. doi:10.1073/pnas.0500031102.

649 60. Akeson G, Malemud CJ. A Role for Soluble IL-6 Receptor in Osteoarthritis. J Funct
650 Morphol Kinesiol. 2017;2:27. doi:10.3390/jfmk2030027.

651 61. Nakamura A, Rampersaud YR, Nakamura S, Sharma A, Zeng F, Rossomacha E, et al.
652 MicroRNA-181a-5p antisense oligonucleotides attenuate osteoarthritis in facet and knee
653 joints. Ann Rheum Dis. 2019;78:111–21.

654 62. Baek D, Lee KM, Park KW, Suh JW, Choi SM, Park KH, et al. Inhibition of miR-449a
655 Promotes Cartilage Regeneration and Prevents Progression of Osteoarthritis in In Vivo Rat
656 Models. Mol Ther - Nucleic Acids. 2018;13:322–33.

657 63. Baccarella A, Williams CR, Parrish JZ, Kim CC. Empirical assessment of the impact of
658 sample number and read depth on RNA-Seq analysis workflow performance. BMC
659 Bioinformatics. 2018;19:423. doi:10.1186/s12859-018-2445-2.

660 64. Kawcak CE, Frisbie DD, Werpy NM, Park RD, Mcilwraith CW. Effects of exercise vs
661 experimental osteoarthritis on imaging outcomes. Osteoarthr Cartil. 2008;16:1519–25.

662 65. McIlwraith CW, Frisbie DD, Kawcak CE, Fuller CJ, Hurtig M, Cruz A. The OARSI
663 histopathology initiative - recommendations for histological assessments of osteoarthritis in
664 the horse. Osteoarthr Cartil. 2010;18:S93–105.

665 66. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing
666 reads. EMBnet.journal. 2011;17:10. doi:10.14806/ej.17.1.200.

667 67. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate
668 alignment of transcriptomes in the presence of insertions, deletions and gene fusions.
669 Genome Biol. 2013;14. doi:10.1186/gb-2013-14-4-r36.

670 68. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput
671 sequencing data. Bioinformatics. 2015;31:166–9.

672 69. Anders S, Huber W. Differential expression analysis for sequence count data. Genome
673 Biol. 2010;11. doi:10.1186/gb-2010-11-10-r106.

674 70. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful
675 Approach to Multiple Testing. J R Stat Soc Ser B. 1995;57:289–300.

676 71. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: A miRNA analysis tool for deep
677 sequencing of plant small RNAs. Plant Mol Biol. 2012;80:75–84.

678 72. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
679 quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25:402–8.

680 73. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more
681 genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic
682 Acids Res. 2018;47:D419–26.

683 74. Supek F, Bošnjak M, Škunca N, Šmuc T. REVIGO Summarizes and Visualizes Long Lists of
684 Gene Ontology Terms. PLoS One. 2011;6:e21800.
685 https://doi.org/10.1371/journal.pone.0021800.

686 75. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A
687 software Environment for integrated models of biomolecular interaction networks. Genome
688 Res. 2003;13:2498–504.

76. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 2009;37. doi:10.1093/nar/gkp356.

## FIGURE LEGENDS

**Figure 1.** Overview of HiSeq data from equine synovial fluid in control and early OA. (A) Categories of RNAs identified in normal and early OA synovial fluid. (B) Volcano plot of small non-coding RNAs identified represents logFC and –log10 P value. Pink dots represent differentially expressed small non-coding RNAs. (C) A heatmap representation of the differentially expressed small non-coding RNA reads from control (non-OA) and early OA equine synovial fluid. Two-dimensional grid matrix displaying columns referring to the control (non-OA) and early OA samples and rows of small non-coding RNAs identified by their Ensembl identification. The heatmap was generated using log-transformed normalised read counts, normalisation was performed by EdgeR's trimmed mean of M values. The colour of each entry is determined by the number of reads, ranging from red (negative values) to yellow (positive values).

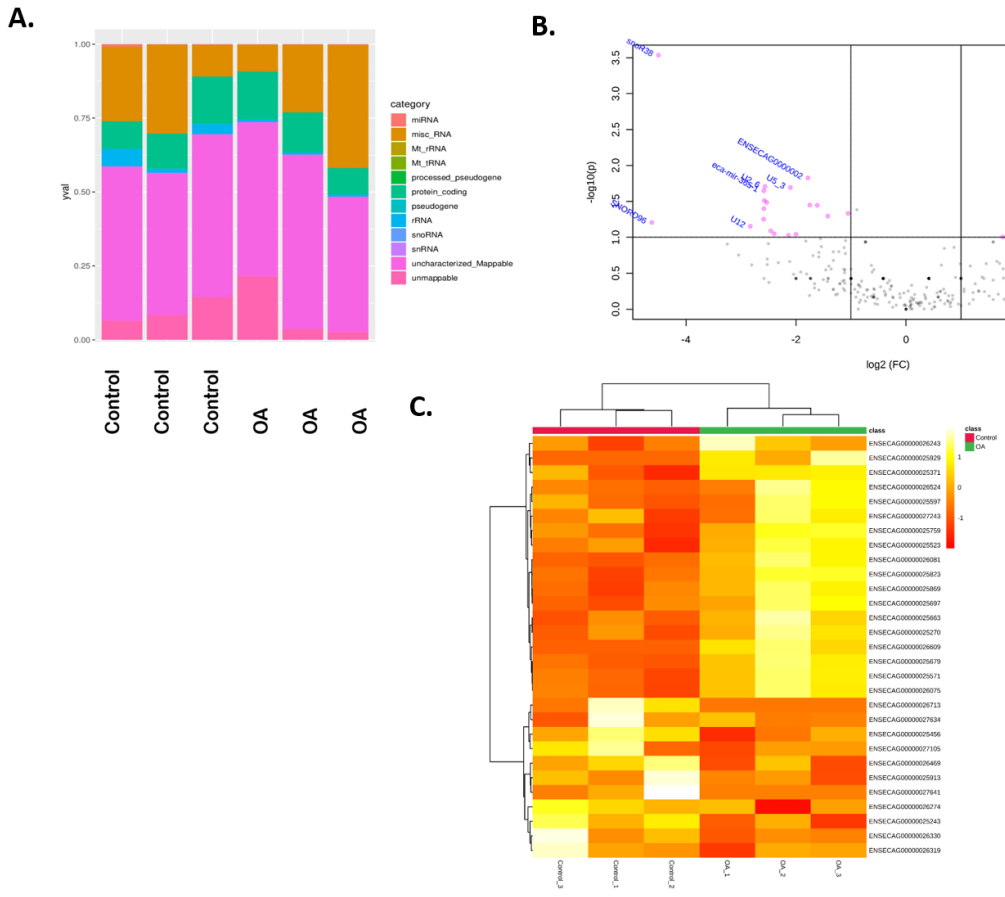
**Figure 2.** Validation of small non-coding RNAs differentially expressed following small RNA sequencing in an independent cohort using qRT-PCR. RNA extracted from the synovial fluid of six healthy control donors and six early OA donors. Histograms of the relative expression calculated using 2^-DCT method using the geometric mean of miR-100 and miR191 as an endogenous control. All qRT-PCR reactions were performed in triplicate. Statistical

significance was tested in Graphpad Prism using a Mann Whitney test. Bars represent means with standard error of the mean. P<0.05; *

**Figure 3.** (A) Ingenuity Pathway Analysis (IPA) derived actions of differentially expressed miRNAs in early OA synovial fluid. IPA identified that cellular actions apoptosis, necrosis, autophagy and inflammation were associated with the differentially expressed miRNAs. Figures are graphical representations of molecules identified in our data in their respective networks. Red nodes; upregulated in early OA, and green nodes; downregulated gene expression in early OA synovial fluid. Intensity of colour is related to higher fold-change. Legends to the main features in the networks are shown. The actions colour is dependent on whether it is predicted to be activated or inhibited. (B) The position of differentially expressed miRNAs in the chondrocyte/fibroblast/osteoblast expression network. PANTHER was used to identify gene ontology (GO) biological processes associated with predicted mRNA targets and perform overrepresentation analysis to highlight the GO terms most significantly affected by dysregulated miRNA-mRNA interactions in early OA synovial fluid. GO terms (FDR< 0.05) were summarised and visualised using REViGO and Cytoscape. Allowed similarity setting in REViGO was tiny (0.4). The line width specified the amount of similarity.

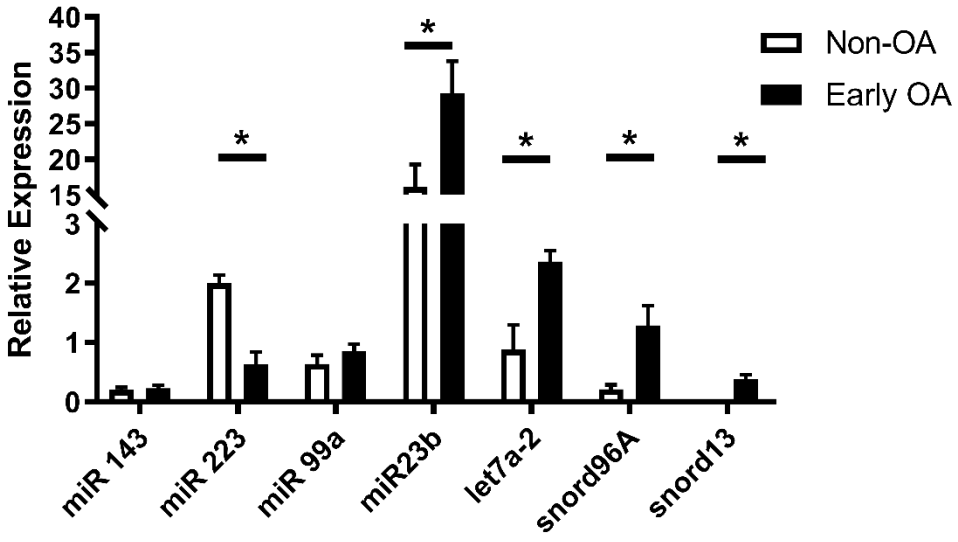
**FIGURES**

**Figure 1**




730

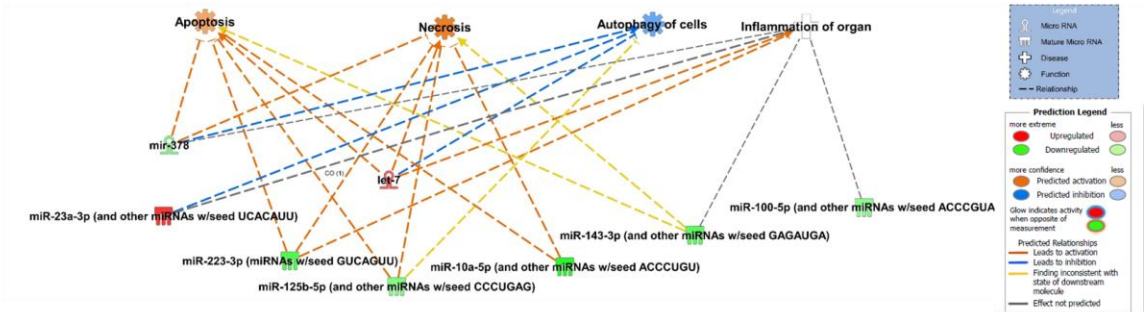
731 **Figure 2**



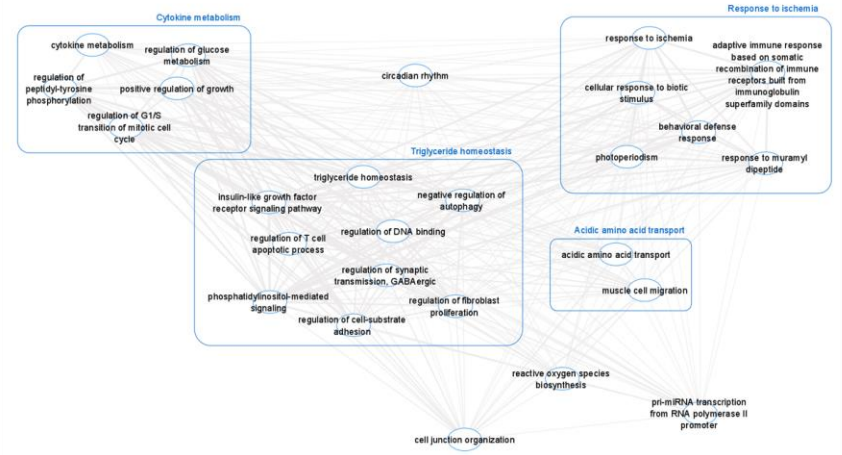

732

733 **Figure 3**

A.




B.

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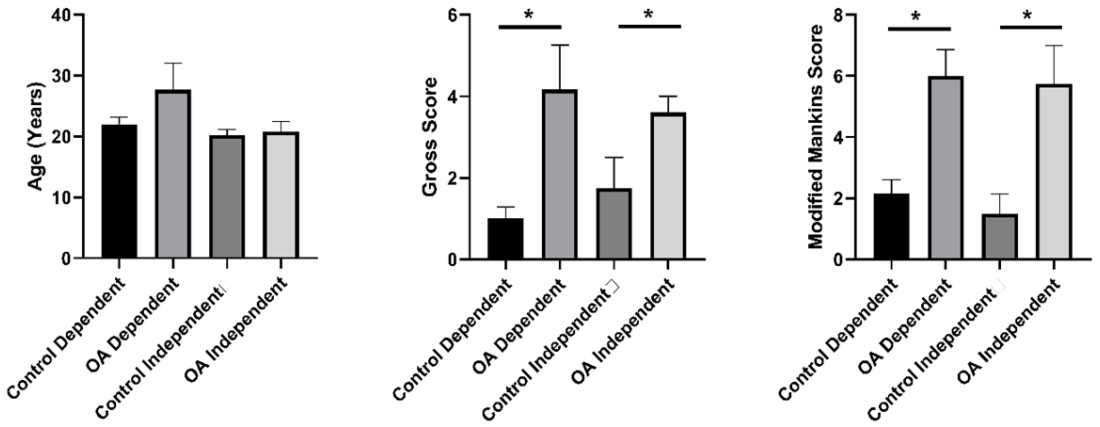
736 **Supp 1 figure**

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738 **ADDITIONAL FILES LEGENDS**

**Additional file 1.** Histograms of age, gross score and Modified Mankin's Score for dependent and independent equine donor cohorts (.tiff). Expressions are means and error bars ± standard error means. Statistical analysis undertaken in GraphPad Prism 8.0 using a Mann Whitney Test. P values *; P <0.05.

**Additional file 2.** Summary of raw, trimmed reads and mapped reads (.xlsx). Summary of raw, trimmed reads and mapped reads to *Equus caballus* database, from analysis of small RNA sequencing data.

**Additional file 3.** mRNA targets predicted by IPA (.xlsx). List of mRNA targets predicted by bioinformatic analysis with IPA software, using a conservative filter of only experimentally validated and highly conserved predicted mRNA targets for each miRNA. Targets were then also filtered on the cells chondrocyte, osteoblasts and fibroblasts

**Additional file 4.** PANTHER GO terms FDR-adjusted P < 0.05 (.xlsx). List of GO terms with FDR-adjusted P < 0.05, obtained with PANTHER overrepresentation analysis of the mRNA targets using Fisher's Exact test.

**Additional file 5.** Primer sequences/assays used for detection of small non-coding RNAs through qRT-PCR analysis (.xlsx). For miRNAs and snoRNAs with sequences homologous to human, Qiagen primer assays were used. Remaining miRNA primers were customised using Eurogentec primer design.