1 **Title:**

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

3

4 **Authors:**

5 Catarina Castanheira^{1*}, C.Castanheira@liverpool.ac.uk

6 Panagiotis Balaskas¹, panbalas@liverpool.ac.uk

7 Charlotte Falls¹, hlcfalls@liverpool.ac.uk

8 Yalda Ashraf-Kharaz¹, yalda@liverpool.ac.uk

9 Peter Clegg¹, pclegg@liverpool.ac.uk

10 Kim Burke², kim.burke@vetsouth.co.nz

11 Yongxiang Fang³, fangy@liverpool.ac.uk

12 Philip Dyer⁴, pddyer@liv.ac.uk

13 Tim JM Welting⁵, t.welting@maastrichtuniversity.nl

14 Mandy J Peffers¹, peffs@liverpool.ac.uk

15

16 **Author affiliations:**

17 ¹Department of Musculoskeletal and Ageing Science, Institute of Life Course and Medical

18 Sciences, William Henry Duncan Building, 6 West Derby Street, Liverpool, L7 8TX, UK

19 ²Institute of Veterinary Science, University of Liverpool, Chester High Road, Neston, CH64

20 7TE

21 ³Centre for Genomic Research, Institute of Integrative Biology, Biosciences Building, Crown

22 Street, University of Liverpool, Liverpool, L69 7ZB, UK

23 ⁴Institute of Infection and Global Health, University of Liverpool, 8 West Derby Street,

24 Liverpool, L7 3EA, UK

25 ⁵Department of Orthopaedic Surgery, Maastricht University Medical Centre, 6202 AZ

26 Maastricht, The Netherlands

27

28 ***Corresponding author:**

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

30

31 **ABSTRACT**

32 **Background:** Osteoarthritis remains one of the greatest causes of morbidity and mortality in

33 the equine population. The inability to detect pre-clinical changes in osteoarthritis has been

34 a significant impediment to the development of effective therapies against this disease.

35 Synovial fluid represents a potential source of disease-specific small non-coding RNAs

36 (sncRNAs) that could aid in the understanding of the pathogenesis of osteoarthritis. We

37 hypothesised that early stages of osteoarthritis would alter the expression of sncRNAs,

38 facilitating the understanding of the underlying pathogenesis and potentially provide early

39 biomarkers.

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

47 **Results:** Results revealed 22 differentially expressed sncRNAs including 13 microRNAs; miR-
 48 10a, miR-223, let7a, miR-99a, miR-23b, miR-378, miR-143 (and six novel microRNAs), four
 49 small nuclear RNAs; U2, U5, U11, U12, three small nucleolar RNAs; U13, snoR38, snord96,
 50 and one small cajal body-specific RNA; scarna3. Five sncRNAs were validated; miR-223 was
 51 significantly reduced in early OA and miR-23b, let-7a-2, snord96A and snord13 were
 52 significantly upregulated. Significant cellular functions deduced by the differentially
 53 expressed microRNAs included apoptosis ($P < 0.0003$), necrosis ($P < 0.0009$), autophagy ($P <$
 54 0.0007) and inflammation ($P < 0.00001$). A conservatively filtered list of 57 messenger RNA
 55 targets was obtained; the top biological processes associated were regulation of cell
 56 population proliferation ($P < 0.000001$), cellular response to chemical stimulus ($P <$
 57 0.000001) and cell surface receptor signalling pathway ($P < 0.000001$).

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

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

64

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

66

67 **BACKGROUND**

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

81

82 In the search for molecular biomarkers that could reveal pre-clinical phases of the disease,
83 scientists have focused much of their attention on microRNAs (miRNAs), the best

characterised family of small non-coding RNAs. Evolutionarily conserved, these 17-22 nucleotide long molecules regulate gene expression at post-transcriptional level generally by repressing translation or increasing degradation of messenger RNAs (mRNAs). They are involved in different cellular pathways and intercellular communication thus influencing tissue homeostasis [8]. As such, miRNA profiles can be altered as a result of cellular damage and/or tissue injury and altered expression of certain miRNAs is implicated in several diseases, including OA [9–11]. Specific miRNAs have been found to modulate osteoblastogenesis and osteoclastogenesis, chondrogenesis and cartilage degradation, synovial inflammation and neurogenesis, thus contributing to the development and progression of OA; comprehensive reviews of which miRNAs are involved in each of these processes can be found elsewhere [12–14]. miRNAs can be found intracellularly or extracellularly, circulating in virtually any biological fluid in a remarkably stable manner [15–17]. Because biological fluids are generally obtainable through minimally invasive techniques, circulating miRNAs are attractive candidates for disease diagnosis, monitoring and prognostication [18, 19]. Interest in other classes of small non-coding RNAs such as small nucleolar RNAs (snoRNAs) has recently 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 [20]. 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 [21].

Equine miRNAs have been identified in numerous healthy tissues [22, 23] and their potential role in different diseases such as osteochondrosis, rhabdomyolysis and insulin resistance has also been investigated [24–26]. 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 [27]. In 2010, Murata et al. investigated the presence and stability of miRNAs in synovial fluid for the first time, and found five differentially expressed miRNAs in human 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 [28]. Intra-articular treatment with hyaluronic acid was shown to modified miRNA expression in OA patients [29]. 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 [30].

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. 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) and early OA (27±7.5) groups were not significantly different. Because OA is an age-related disease, horses included in the control group presented minor macroscopic or histological changes. For samples used for small RNA sequencing there was a significant increase in the macroscopic score between control 1.0±0.5, early OA; 5.4±1.9 (P=0.04) donors. There was a significant increase in histological score between control; 2.1±0.7, 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) donors and in 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).

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 using an independent cohort of synovial fluid samples from control (n=6, histological score 1.5 ± 1.3) and early OA (n=6, histological score 5.8 ± 2.5) synovial fluid. 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.

Identification of potential target mRNA genes of the differentially expressed miRNAs

To explore potential biological associations of 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 functions 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), 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 [31]. 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.

196

197 Due to the considerable interest in miRNA-mediated gene regulation in recent years, the list
198 of miRNAs possibly implicated in OA and other joint related pathologies has grown [13].
199 miRNAs that are differentially expressed in joint tissues of patients with OA are likely to
200 contribute to OA pathophysiology and may be utilised as diagnostic factors [32]. One
201 example is miR-140, which is significantly downregulated in human OA cartilage [10]; miR-
202 140 knockdown in mice promoted proteoglycan loss by disrupting A Disintegrin-like and
203 Metalloproteinase with Thrombospondin Type 1 Motif 5 (ADAMTS-5) expression [33]; intra-
204 articular injection of miR-140 in rats attenuated OA progression by modulating extracellular
205 matrix (ECM) homeostasis [34]; pretransfection with miR-140 partially retarded chondrocyte
206 senescence in an interleukin(IL)-1 β induced *in vitro* model of OA [35]; dysregulation of miR-
207 140-3p and-5p in synovial fluid has been correlated with OA severity [36]; and a miRNA
208 assay on human serum has identified miR-140-3p as a potential biomarker for OA [37].

209

210 In our study, we compared two groups of horses (control vs early OA) based on macroscopic
211 and histological scoring of joints in relation to arthritic changes. Because OA is an age-
212 related disease, both groups were selected from an elderly population and age-matched to
213 account for age-related changes; hence classifying the normal group as “control” as
214 opposed to “healthy”.

215

216 Among the differentially expressed miRNAs found in our study, miR-23b was significantly
217 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 [38]. 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) [39, 40].

Likewise, we found let-7a-2 to be upregulated in early OA. In an experiment comparing miRNA expression in synovial fluid from OA patients undergoing hyaluronic acid treatment, let-7a was significantly upregulated in synovial fluid of OA samples compared to healthy controls; levels of let-7a in affected patients returned to normal after hyaluronan injection [29]. Let-7a is thought to regulate IL-6 receptor (IL6R), and its inhibition can enhance cell proliferation, reduce apoptosis and inhibit inflammatory response in ATDC5 cells in a lipopolysaccharide-induced *in vitro* model of OA [41]. Members of the let-7 family have often been described in studies involving OA; a large population-based study identified serum let-7e as a promising candidate to predict OA risk, independent of age, sex and body mass index [42]. A recent investigation supported this claim, providing further evidence of decreased expression of let-7e in serum of patients affected with knee OA [43]. The exact roles of miRNAs of the let-7 family remain unclear, but the evidence for their use as biomarkers for OA is growing.

miR-223 was also significantly upregulated in synovial fluid of OA patients prior to intra-articular injection of hyaluronan [29]. miR-223 participates in cartilage homeostasis and structure by targeting growth differentiation factor 5 (GDF5) [38]. Early-stage OA patients showed upregulation of miR-223 in peripheral blood mononuclear cells, with its expression

decreasing as OA progressed [44]. In our study, we found miR-223 to be downregulated in the synovial fluid of the early OA cohort, which supports the involvement of this miRNA in the early osteoarthritic process. miR-223 is also enriched in the synovial fluid of patients with rheumatoid arthritis when compared to OA, and can differentiate between patient cohorts [11].

We have previously shown the involvement of snoRNAs in cartilage ageing and OA and their potential use as biomarkers for OA [21]. 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, 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 [45]. In another microarray study of young compared to old OA cartilage we identified SNORD13 was increased in OA cartilage [46]. 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 [47] and our results support our earlier work for the potential use of snoRNAs as novel biomarkers in OA [21].

Profiling circulating, cell-free small non-coding RNAs is generally a challenging task due to the limited amount of RNA present in biofluids, as well as presence of inhibitory compounds which potentially hinder downstream enzymatic processes. However, liquid biopsies have

gained prominence due to their ease of collection and potential use as diagnostic tools. The horse is a well-established model for spontaneous OA and the horse joint is often used to investigate OA pathogenesis and therapeutics [7]. The metacarpophalangeal joint's relatively simple structure is ideal for the investigation of early joint modifications because we are unable to determine which tissues are contributing to the overall expression of small non-coding RNAs. This allowed us to more easily determine the origin of small non-coding RNAs that are specifically implicated in OA to cartilage, subchondral bone and synovium.

Future studies would benefit from analysing larger cohorts of patients; our study was limited by the availability of joints with early OA, resulting in a small sample size. Nevertheless, it enabled us to identify small non-coding RNA changes in the initial and an additional cohort and revealed, for the first time, the potential use of small non-coding RNAs as biomarkers for early OA.

Predicted targets of the miRNAs of interest appear to be involved in processes of cellular destruction and inflammation; comparable processes have been previously shown to contribute to the pathogenesis of OA such as degeneration of ECM promoted through pro-inflammatory cytokines [48], synovial inflammation [49] and chondrocyte apoptosis [50], among others. Experimental validation of the predicted target genes can clarify biological mechanisms behind these small non-coding RNAs and elucidate their role in the pathogenesis of OA, which is critical for the success of future interventions, as these molecules can be targeted in a specific manner [51, 52].

286

287 These results support the use of synovial fluid small non-coding RNAs as molecular
288 biomarkers for early disease in OA joints. Our future research is currently ascertaining the
289 applicability of these findings in a clinical setting.

290

291 **CONCLUSIONS**

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

300

301 **METHODS**

302 All reagents were from ThermoFisher Scientific, unless stated.

303

304 **Sample collection and preparation**

305 Samples were collected from the metacarpophalangeal joints of horses from an abattoir as
306 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.

Synovial fluid was collected from the metacarpophalangeal joints of control (non-OA), n= 3 (age mean± standard deviation; 22±2 years) and early OA, n=3 (22±7.5 years) non-Thoroughbred horses. At collection synovial fluid was immediately placed on ice and then centrifuged for 10 min at 3000 g and 4°C. The supernatant was removed and stored at -80°C. Donors were assigned to groups based on scoring of the metacarpophalangeal joint using a macroscopic grading system as previously described [53] and histological scoring system [54].

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 [21] 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 [55] 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 [56] with option “-g 1”; counting aligned reads using HTSeq-count [57] against the features defined in horse genome GTF file (release 90).

Differential expression analysis was performed in R using package DESeq2 [58]. 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 [59] 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.

qRT-PCR validation

Validation of the selected small RNA sequencing results in an independent cohort of equine metacarpophalangeal synovial fluid was undertaken using qRT-PCR. Six control (non-OA), mean±standard deviation (20.2±2.4 years) and six early OA (20.8±4.1) with macroscopically and histologically graded sample scores similar to those used for sequencing were used.

Total RNA was extracted as above. Small non-coding RNAs were chosen based on our current work, level of differential expression and following a literature review of differentially expressed genes. These were miR-143, miR-223, miR-99a, miR-23b, let-7a-2, snord96A and snord13. Primer sequences/assays used can be found in Additional File 5.

PolyA cDNA was synthesized using 200ng RNA and the miScript II RT Kit. A mastermix was prepared using the miScript SYBR Green PCR Kit (Qiagen, Crawley, UK) and the appropriate bespoke designed miScript Primer Assays (Qiagen, Crawley, UK). Real-time PCR was undertaken using a LightCycler® 96 system (Roche). Steady-state transcript abundance of potential endogenous control genes was measured in the small RNA sequencing data.

Assays for four genes – miR-181a, miR-100, miR-191a and U6 were selected as potential reference genes because their expression was unaltered in this study. Stability of this panel of genes was assessed by applying a gene stability tool RefFinder [60]. The geometric mean of miR-100 and miR-191a was selected as the stable endogenous control. miR-100 has been previously used as a normaliser in a similar study as it was identified by NormFinder as the

most stable [28]. Relative expression levels were normalised to the geometric mean of miR-100 and miR-191 and calculated using the $2^{-\Delta\Delta CT}$ method [61].

miRNA target prediction and pathway analysis

Potential biological associations of the differentially expressed miRNAs in early OA synovial fluid were identified using Ingenuity Pathway Analysis (IPA) (IPA, Qiagen Redwood City, CA, USA) 'Core Analysis'. Canonical pathways, 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) [62] 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 [63] with allowed similarity of 0.4 and visualised using Cytoscape [64].

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.30 [65]. 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

ADAMTS5 = A Disintegrin-like and Metalloproteinase with Thrombospondin Type 1 Motif 5, CRTAP = cartilage associated protein, DNA = deoxyribonucleic acid, ECM = extracellular matrix, FDR = false discovery rate, GDF5 = growth differentiation factor 5, GO = gene ontology, IPA = Ingenuity Pathway Analysis, IL = interleukin, IL6R = interleukin 6 receptor, logFC = log2 fold change, miRNAs = micro RNAs, OA = osteoarthritis, PKA = protein kinase A, qRT-PCR = quantitative reverse transcription polymerase chain reaction, RNA = ribonucleic acid, 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.

Funding

Catarina Castanheira is funded through a Horse Trust PhD studentship (G5018) and Mandy Peffers funded through a Wellcome Trust Intermediate Clinical Fellowship (107471/Z/15/Z). This work was also supported by the MRC and Versus Arthritis as part of the Medical Research Council Versus Arthritis Centre for Integrated Research into Musculoskeletal Ageing (CIMA) [MR/R502182/1]. The MRC Versus Arthritis Centre for Integrated Research

into Musculoskeletal Ageing is a collaboration between the Universities of Liverpool, Sheffield and Newcastle.

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. PD, CAF, 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.

Acknowledgments

The authors would like to thank staff at both the F Drury and Sons abattoir, Swindon for assistance in sample collection and processing, Valerie Tilston for preparation of histology slides, and Aibek Smagul for bioinformatics support.

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 $-\log_{10}$ 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 $2^{-\Delta\Delta CT}$ 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. $P < 0.05$; *

Figure 3. (A) Ingenuity Pathway Analysis (IPA) derived functions of differentially expressed miRNAs in early OA synovial fluid. IPA identified that cellular functions 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 functions 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.

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

489

490 **ADDITIONAL FILES**

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

495

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

499

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

504

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

508

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

513

514 REFERENCES

- 515 1. Ireland JL, Clegg PD, McGowan CM, Mckane SA, Chandler KJ, Pinchbeck GL. Disease
516 prevalence in geriatric horses in the United Kingdom: Veterinary clinical assessment of 200
517 cases. *Equine Vet J.* 2012;44:101–6.
- 518 2. Ireland JL, Clegg PD, McGowan CM, Platt L, Pinchbeck GL. Factors associated with
519 mortality of geriatric horses in the United Kingdom. *Prev Vet Med.* 2011;101:204–18.
- 520 3. Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. *Bull World Health*
521 *Organ.* 2003;81:646–56.
- 522 4. Mobasheri A, Batt M. An update on the pathophysiology of osteoarthritis. *Ann Phys*
523 *Rehabil Med.* 2016;59:333–9.
- 524 5. Ashkavand Z, Malekinejad H, Vishwanath BS. The pathophysiology of osteoarthritis. *J*
525 *Pharm Res.* 2013;7:132–8.
- 526 6. Goodrich LR, Nixon AJ. Medical treatment of osteoarthritis in the horse - A review. *Vet J.*
527 *2006;171:51–69.*
- 528 7. McIlwraith CW, Frisbie DD, Kawcak CE. The horse as a model of naturally occurring

529 osteoarthritis. *Bone Joint Res.* 2012;1:297–309.

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

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

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

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

541 12. Yu X-M, Meng H-Y, Yuan X-L, Wang Y, Guo Q-Y, Peng J, et al. MicroRNAs' Involvement in
542 Osteoarthritis and the Prospects for Treatments. *Evid Based Complement Alternat Med.*
543 2015;2015:236179.

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

546 14. Peffers MJ, Balaskas P, Smagul A. Osteoarthritis year in review 2017: genetics and
547 epigenetics. *Osteoarthr Cartil.* 2018;26:304–11.

548 15. Ge Q, Zhou Y, Lu J, Bai Y, Xie X, Lu Z. miRNA in Plasma Exosome is Stable under Different
549 Storage Conditions. *Molecules.* 2014;19:1568–75.

550 16. Wang K. The Ubiquitous Existence of MicroRNA in Body Fluids. Clin Chem. 2017;63:784–
551 5.

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

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

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

559 20. Stepanov GA, Filippova JA, Komissarov AB, Kuligina E V, Richter VA, Semenov D V.
560 Regulatory Role of Small Nucleolar RNAs in Human Diseases. Biomed Res Int.
561 2015;2015:206849. doi:10.1155/2015/206849.

562 21. Steinbusch MMF, Fang Y, Milner PI, Clegg PD, Young DA, Welting TJM, et al. Serum
563 snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis. Sci Rep. 2017;7:1–
564 11.

565 22. Kim M-C, Lee S-W, Ryu D-Y, Cui F-J, Bhak J, Kim Y. Identification and Characterization of
566 MicroRNAs in Normal Equine Tissues by Next Generation Sequencing. PLoS One.
567 2014;9:e93662. doi:10.1371/journal.pone.0093662.

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

570 24. Barrey E, Bonnamy B, Barrey EJ, Mata X, Chaffaux S, Guerin G. Muscular microRNA
571 expressions in healthy and myopathic horses suffering from polysaccharide storage

572 myopathy or recurrent exertional rhabdomyolysis. *Equine Vet J.* 2010;42 SUPPL. 38:303–10.

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

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

579 27. McIlwraith CW. Use of synovial fluid and serum biomarkers in equine bone and joint
580 disease: a review. *Equine Vet J.* 2010;37:473–82.

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

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

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

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

592 32. Zhang M, Lygrissea K, Wang J. Role of MicroRNA in Osteoarthritis. *J Arthritis.* 2017;06.
593 doi:10.4172/2167-7921.1000239.

594 33. Miyaki S, Sato T, Inoue A, Otsuki S, Ito Y, Yokoyama S, et al. MicroRNA-140 plays dual
595 roles in both cartilage development and homeostasis. *Genes Dev.* 2010;24:1173–85.

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

599 35. Si H bo, Yang T min, Li L, Tian M, Zhou L, Li D ping, et al. miR-140 Attenuates the
600 Progression of Early-Stage Osteoarthritis by Retarding Chondrocyte Senescence. *Mol Ther -*
601 *Nucleic Acids.* 2020;19:15–30.

602 36. Yin C-M, Suen W-C-W, Lin S, Wu X-M, Li G, Pan X-H. Dysregulation of both miR-140-3p
603 and miR-140-5p in synovial fluid correlate with osteoarthritis severity. *Bone Joint Res.*
604 2017;6:612–8.

605 37. Ntoumou E, Tzetis M, Braoudaki M, Lambrou G, Poulou M, Malizos K, et al. Serum
606 microRNA array analysis identifies miR-140-3p, miR-33b-3p and miR-671-3p as potential
607 osteoarthritis biomarkers involved in metabolic processes. *Clin Epigenetics.* 2017;9:127.

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

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

614 40. Karlsen TA, Jakobsen RB, Mikkelsen TS, Brinchmann JE. MicroRNA-140 targets RALA and
615 regulates chondrogenic differentiation of human mesenchymal stem cells by translational

- 616 enhancement of SOX9 and ACAN. *Stem Cells Dev.* 2014;23:290–304.
- 617 41. Sui G, Zhang L, Hu Y. MicroRNA-let-7a inhibition inhibits LPS-induced inflammatory injury
618 of chondrocytes by targeting IL6R. *Mol Med Rep.* 2019;20:2633–40.
- 619 42. Beyer C, Zampetaki A, Lin NY, Kleyer A, Perricone C, Iagnocco A, et al. Signature of
620 circulating microRNAs in osteoarthritis. *Ann Rheum Dis.* 2015;74:e18–e18.
- 621 43. Feng L, Feng C, Wang CX, Xu DY, Chen JJ, Huang JF, et al. Circulating microRNA let-7e is
622 decreased in knee osteoarthritis, accompanied by elevated apoptosis and reduced
623 autophagy. *Int J Mol Med.* 2020;45:1464–76.
- 624 44. Okuhara A, Nakasa T, Shibuya H, Niimoto T, Adachi N, Deie M, et al. Changes in
625 microRNA expression in peripheral mononuclear cells according to the progression of
626 osteoarthritis. *Mod Rheumatol.* 2012;22:446–57.
- 627 45. Peffers MJ, Chabronova A, Balaskas P, Fang Y, Dyer P, Cremers A, et al. SnoRNA
628 signatures in cartilage ageing and osteoarthritis. Preprint at
629 <https://www.biorxiv.org/content/10.1101/2020.04.01.019505v1> (2020).
- 630 46. Peffers MJ, Ripmeester E, Caron M, Steinbusch M, Balaskas P, Cremers A, et al. A role for
631 the snoRNA U3 in the altered translational capacity of ageing and osteoarthritic
632 chondrocytes. *Osteoarthr Cartil.* 2018;26:S45–6.
- 633 47. McMahon M, Contreras A, Ruggero D. Small RNAs with big implications: New insights
634 into H/ACA snoRNA function and their role in human disease. *Wiley Interdiscip Rev RNA.*
635 2015;6:173–89.
- 636 48. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in
637 osteoarthritis. In: *Clinical Orthopaedics and Related Research*. Lippincott Williams and

638 Wilkins; 2004. p. S27–36.

639 49. Wang X, Hunter DJ, Jin X, Ding C. The importance of synovial inflammation in
640 osteoarthritis: current evidence from imaging assessments and clinical trials. *Osteoarthr*
641 *Cartil.* 2018;26:165–74.

642 50. Hwang HS, Kim HA. Chondrocyte apoptosis in the pathogenesis of osteoarthritis. *Int J*
643 *Mol Sci.* 2015;16:26035–54.

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

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

650 53. Kawcak CE, Frisbie DD, Werpy NM, Park RD, McIlwraith CW. Effects of exercise vs
651 experimental osteoarthritis on imaging outcomes. *Osteoarthr Cartil.* 2008;16:1519–25.

652 54. McIlwraith CW, Frisbie DD, Kawcak CE, Fuller CJ, Hurtig M, Cruz A. The OARSI
653 histopathology initiative e recommendations for histological assessments of osteoarthritis in
654 the horse. *Osteoarthr Cartil.* 2010;18:S93–105.

655 55. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing
656 reads. *EMBnet.journal.* 2011;17:10. doi:10.14806/ej.17.1.200.

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

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

662 58. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome*
663 *Biol*. 2010;11. doi:10.1186/gb-2010-11-10-r106.

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

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

668 61. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
669 quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods*. 2001;25:402–8.

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

673 63. Supek F, Bošnjak M, Škunca N, Šmuc T. REVIGO Summarizes and Visualizes Long Lists of
674 Gene Ontology Terms. *PLoS One*. 2011;6:e21800.
675 <https://doi.org/10.1371/journal.pone.0021800>.

676 64. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A
677 software Environment for integrated models of biomolecular interaction networks. *Genome*
678 *Res*. 2003;13:2498–504.

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

681

682