Serum snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis

Mandy M. F. Steinbusch1, Yongxiang Fang2, Peter I. Milner3, Peter D. Clegg3, David A. Young4, Tim J. M. Welting1 & Mandy J. Peffers3

The development of effective treatments for the age-related disease osteoarthritis and the ability to predict disease progression has been hampered by the lack of biomarkers able to demonstrate the course of the disease. Profiling the expression patterns of small nucleolar RNAs (snoRNAs) in joint ageing and OA may provide diagnostic biomarkers and therapeutic targets. This study determined expression patterns of snoRNAs in joint ageing and OA and examined them as potential biomarkers. Using SnoRNASeq and real-time quantitative PCR (qRT-PCR) we demonstrate snoRNA expression levels in murine ageing and OA joints and serum for the first time. SnoRNASeq identified differential expression (DE) of 6 snoRNAs in young versus old joints and 5 snoRNAs in old sham versus old experimental osteoarthritic joints. In serum we found differential presence of 27 snoRNAs in young versus old serum and 18 snoRNAs in old sham versus old experimental osteoarthritic serum. Confirmatory qRT-PCR analysis demonstrated good correlation with SnoRNASeq findings. Profiling the expression patterns of snoRNAs is the initial step in determining their functional significance in ageing and osteoarthritis, and provides potential diagnostic biomarkers and therapeutic targets. Our results establish snoRNAs as novel markers of musculoskeletal ageing and osteoarthritis.

Osteoarthritis (OA) is an age-related musculoskeletal disease and a common cause of chronic disability worldwide1. In addition it is a significant contributor to both individual and socioeconomic burden and the number of disability adapted life years globally2. If the deterioration in musculoskeletal health and development of OA can be identified and treated early serious life impairment may be abrogated. Ageing is the time-dependent reduction of functional capacity and stress resistance, associated with an increased risk of morbidity and mortality. The joint and its articular cartilage is particularly affected by ageing3. There is evidence that the rate of ageing, that is the ‘biological age’, differs significantly between individuals’ actual age in years (i.e. the ‘chronological age’). Defining markers of joint ageing may enable a prediction of the risk of onset of OA, enabling early intervention. OA is characterised by a non-symptomatic, pre-radiographical phase that if identified would allow earlier diagnosis. However radiographic changes are only evident later in disease progression. Magnetic resonance imaging techniques have been developed for early-stage evaluation of cartilage damage in OA but are expensive and contraindicated in some individuals.

The development of effective treatments for OA and the ability to predict disease progression has been hampered by the lack of substantive biomarkers, able to demonstrate pathological disturbances preceding identifiable tissue alterations. Others have attempted to identify products of tissue turnover in serum and synovial fluid (reviewed4). This has been challenging due to patient and disease heterogeneity and dilution effects either by tissue fluids or with similar products from other joints or diseases. In addition, the variability of antibody assays has been problematic.

SnoRNAs are a class of evolutionary conserved non-coding small guide RNAs of which the majority direct the chemical modification of other RNA substrates, including ribosomal RNAs and spliceosomal RNAs. In addition, some snoRNAs are involved in the regulation of alternative splicing and post-transcriptional modification of
mRNA, whilst others exhibit miR-like activity. aberrant expression of snoRNAs has been associated with disease development such as lung tumorigenesis.

Emerging evidence shows that there is an increased level of circulating RNAs in the serum of cancer patients. Circulating microRNAs (miRs) have been extensively described as biomarkers for diseases like pancreatic/breast cancer, Alzheimer’s disease and inflammatory diseases like asthma, inflammatory bowel disease and rheumatoid arthritis, but with the recent discovery of stable snoRNAs in serum, interest in their potential as circulating biomarkers of cancers (reviewed) has been stimulated. We have previously identified dysregulation of a defined set of snoRNAs in cartilage and tendon ageing and OA and in man, snoRNA SNORD38 and SNORD48 were identified as potential non-age-dependant serum biomarkers for OA progression following cruciate ligament injury.

Expression profiling of snoRNAs in ageing and OA may help in determining their functional significance in the development and progression of disease and provide much needed diagnostic biomarkers for ageing and OA development. This study compared serum and joint snoRNA expression in ageing and OA from knee joint tissues from young and old adult mice and old mice using a traumatic in vivo model of OA. Because OA involves the whole joint as an organ; we undertook our analysis on whole mouse joints, which included cartilage, meniscus, subchondral bone, and joint capsule with synovium.

Materials and Methods

All reagents were from Thermo-Fisher-Scientific, unless stated.

Animals. C57BL6/J male mice were used for the study. For SnoRNASeq old mice were 18 months old (n = 6), young 8 months old (n = 6) and mice used for destabilisation of the medial meniscus (DMM) 24 months old (sham n = 3; DMM n = 6). Mice were group housed in individually ventilated cages at a 12 hour light/dark cycle, with ad libitum access to food and water. Experimental animal protocols were performed in accordance with the guidelines of the Animals (Scientific Procedures) Act 1986 following ethical review. Animal usage and protocols for this study was approved by the University of Liverpool Animal Welfare Committee.

Surgical induction of OA by DMM in mice. DMM surgery was perform as previously reported. Briefly, under anaesthesia a 3 mm skin incision was made over the medial aspect of the patellar ligament through the joint capsule into the femorotibial joint of the left knee. The medial meniscotibial ligament was transected to destabilise the cranial pole of the medial meniscus from the anterior tibial plateau. In sham operated mice the medial meniscotibial ligament was visualised but not transected. Mice were sacrificed 8 weeks post-surgery.

Joint and serum collection for SnoRNASeq. Following euthanasia, knee joints were collected from young, old, DMM (n = 6 each group) and sham n = 3 for SnoRNASeq. Joints were harvested free of soft tissues at 7 mm from the joint into RNALater. Serum was collected using cardiac puncture. One old serum sample was not processed further due to extensive haemolysis.

OARSI scoring of histological sections of mouse knee joints. For histology, as the total knee joint was used in the study for RNA extraction, joints were collected (into 4% paraformaldehyde) from additional equivalent aged and treated young (n = 8), old (n = 4); sham (n = 5); and DMM (n = 6) mice in order to evaluate the extent of OA. The procedure, surgeon and duration of the studies were identical. Knees were decalcified in 0.5 M ethylenediaminetetraacetic acid (pH 7.4) for 4 weeks at 4 °C and coronally embedded in paraffin. Sectioning, Safranin-O Fast-Green staining and histological scoring (defined as the severity and extent of OA) was undertaken on a scale from 1 to 6 by two blinded independent observers using the OARSI histopathology initiative. All four quadrants of the section (medial tibial plateau, medial femoral condyle, lateral femoral condyle) were scored individually and added for each histological section. For statistical analyses mean summed score values of joints of 3–5 section per knee joint at 4 depths throughout the joint was determined (thus a maximum score of 24 was possible). Inter-observer variability was calculated using Cohen’s Kappa statistics using an online software tool: (http://www.statstodo.com/CohenKappa).

RNA isolation, RNA-Seq analysis, cDNA library preparation and sequencing. Total RNA was isolated from equal weights of joints and 500μl serum using miRNeasy or RNeasy Serum kits respectively with DNase treatment (all Qiagen, Crawley, UK) to remove residual gDNA. Total RNA integrity (RIN) was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Ribosomal RNA was depleted using the Ribo-Zero ™ rRNA Removal Kit (Epicentre, Madison, USA). From 41 samples 100ng of RNA was submitted for library preparation using NEB small RNA library kit (New England Biolabs (NEB), Ipswich, USA). To reduceWorkflow bias we used tobacco acid pyrophosphatase (Epicentre, Madison, USA) to remove potential 3′ caps found on some snoRNAs. Samples were amplified for 15 cycles, mixed into 3 pools, and size selected. The size-selected material was purified with Ampure beads (Agencourt, Beckman-Coulter, High-Wycombe, UK). SnoRNA sequencing was undertaken on the Illumina HiSeq 2000 platform (Illumina, San Diego, USA) using 100 base paired-end reads.

SnoRNASeq data analysis. Sequence data measured from 5 lanes of an Illumina HiSeq2000 were processed through a number of steps to obtain snoRNA expression values. The processes include basecalling and de-multiplexing of indexed reads using CASAVA version 1.8.2; adapter and quality trimming using Cutadapt version 1.2.1 and Sickle version 1.200 to obtain fastq files of trimmed reads; aligning reads to Ensembl GRCm38.77 mouse genome reference sequences which contains 1,555 annotated snoRNA features using Bowtie21 version 2.0.10 with option “--very-sensitive-local”; counting aligned reads against snoRNA features using THSeq-count. The count values were used as snoRNA expression measurements for the DE analysis.
DE analysis was performed in R environment using package edgeR\textsuperscript{22}. 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; 3-D PCA plots were generated using R function in package plot3D) and correlation analysis; handling library size variation respectively for joint samples and serum samples through data normalisation; formulating data variation using negative binomial distributions; modelling data using a generalised linear model; computing log 2 Fold Change (logFC) values for required contrasts based on model fitting results through contrast fitting approach, assigning P-values to logFC values by LR\textsuperscript{23} testing; dealing with the effects of multiple tests using FDR approach to obtain FDR adjusted P-values; and defining significantly DE snoRNAs as those with FDR-adjusted p-value \(< 5\%\). Sequence data have been submitted to National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO); E-MTAB-4878.

RNA isolation, poly(A) cDNA synthesis and snoRNA qRT-PCR. qRT-PCR of snoRNAs was performed\textsuperscript{24}. Total RNA was isolated using the mirVana kit. Isolated RNA samples were polyadenylated at 37 °C for 60 minutes in a 50 μL reaction volume containing 1 μg RNA and 1.5 U poly(A) polymerase (NEB, Ipswich, USA). 500 μL lysis binding buffer was added. Then, an equal volume of acid-phenol:chloroform was added, vortexed and samples were centrifuged for 10 minutes and the aqueous phase removed. The poly(A)-tailed total RNA was extracted using the filter cartridge provided by the mirVana kit. To generate poly(A) cDNA, 500 ng poly(A)-tailed RNA and 250 ng RTQ primer (Eurogentec, Seraing, Belgium) (Table 1) were mixed in a 26 μL reaction volume, incubated at 65 °C for 10 minutes and annealed at 4 °C for 20 minutes. Reverse transcription was performed with 200 U M-MLV reverse transcriptase, 20 U RNasin (both Promega, Southampton, UK), 2 μL dNTP mix (10 mM each; Eurogentec, Seraing, Belgium) and 8 μL 5x M-MLV buffer (Promega, Southampton, USA) in a total reaction volume of 40 μL at 50 °C for 60 minutes. The reverse transcriptase was inactivated at 70 °C for 15 minutes. Finally, 1.5 U of RNase H (NEB, Ipswich, USA) was added to remove small RNAs. A snoRNA-specific forward primer and a universal reverse primer (RTQ-UNIr, matched to the \(T_m\) of each individual snoRNA) were used for the amplification of each snoRNA target (all Eurogentec, Seraing, Belgium) (Table 1). For each cDNA sample a mix was prepared with Mesagreen qPCR Mastermix Plus for SYBR Green (Eurogentec, Seraing, Belgium) and 300 nM forward and reverse oligonucleotides. An ABI-7300 Detection System was used for amplification using the following protocol: denaturation at 95 °C for 5 minutes, followed by 50 cycles of DNA amplification (15 seconds 95 °C and 45 seconds annealing at 62–68 °C). The annealing temperature was optimized for each snoRNAs target. Serially diluted standard curves were utilized to quantify snoRNA expression and data was normalized to a validated housekeeping snoRNA (joint: U2, young-old serum: SNORD85, old sham-old DMM serum and equine serum: U6).

Table 1. Oligonucleotides sequences used in qRT-PCR.

| Name            | Sequence (5′-3′)                           | \(T_m\) (°C) |
|-----------------|--------------------------------------------|--------------|
| RTQ primer poly(A) cDNA synthesis | CGAATTCTAGAGCTCGAGGAGGCAAGC | 75           |
|                 | GACATGGCTGGCTAGTTAAAGCTTG |              |
|                 | GTACCCGAGCTCGGATCCACTAGTCC |              |
|                 | TTTTTTTTTTTTTTTTTTTTTTTTTTTT |              |
| Snora28         | CATGAGACAAAGCGGTATATAGGC | 50           |
| Snora30         | TGTACCGAGCAGCTGGTTACTC | 50           |
| Snora31         | GTTTTGGCCAGTTCAGATTGAATTAG | 50           |
| Snora64         | GTGCCCTCTCTTGGCTAGAG | 65           |
| Snora73         | ACAATGACAGGAGGCAAC | 50           |
| Snord85         | ATATGCAAGACTGTAGGTAG | 50           |
| Snord88         | ACCCTTTGACACCGAGATCCTGA | 50           |
| Snord116        | TGATACCGCCTCTCATCAGG | 65           |
| U2              | TGGATATGCAACAGTCCAGGAACG | 55           |
| U3              | AGTGAAGAGGAAGGACCAGG | 55           |
| U6              | GATGACAGGCAAAATCCTGAGGCTGC | 55           |
| RTQ-UNIr-50     | AATTCTAGAGCAGGACAGG | 50           |
| RTQ-UNIr-55     | CAAATTCATAGCAGCAGGACAGG | 55           |
| RTQ-UNIr-65     | CTAGACGCTCGAGGCAACATCGGGTTCG | 65           |

Validation of SNORD116 as a marker of OA in equine serum. We determined the reproducibility of the expression of SNORD116 in OA serum of another species. There are well-published studies on the application of metacarpophalangeal (MCP) joint OA changes in the horse, a joint with similarities to the human knee joint\textsuperscript{25}. We studied equine serum from normal and MCP OA horses collected from eight normal (mean age ± standard deviation 5.3 ± 2.1 years) and four OA (7.5 ± 1.0 years) castrated male thoroughbred horses at post-mortem. Samples were collected under the regulations of the Hong Kong Jockey Club with owner consent and stored at \(-80 \degree C\). OA diagnosis was based on histological (modified Mankin)\textsuperscript{26} and synovitis scoring\textsuperscript{27} of MCP joint tissues.
Statistical analysis. For statistical evaluation of histological scoring non-parametric Mann-Whitney-U test was used. Inter-observer agreement of histological scoring systems was calculated using Cohen’s kappa coefficient (www.statstodo.com/CohenKappa_Pgm.php). qRT-PCR data was log-transformed prior to statistical evaluation with an independent samples t-test. Statistical evaluation was performed between young and old or old sham versus old DMM using GraphPad Prism 5 (San Diego); p-values are indicated.

Results

OARSI scoring of joints. OARSI scoring of joints (mean ± 95%CI) for young and old were 0.5 ± 0.3 and 2.8 ± 2.7 (p = 0.01), and old sham and old DMM were 1.25 ± 1.1 and 6.5 ± 0.7 (p < 0.001), respectively. Mice exhibited typical histological features of OA in the DMM knees. Cohen’s Kappa statistic was 0.4 indicating a fair agreement. Representative histological images and OARSI scoring are in Fig. 1.

Preliminary analysis SnoRNASeq. To identify DE of snoRNAs in mouse joints and serum in response to age and OA 41 cDNA libraries representing old and young joints (old = HJO; young = HJY) and serum (old = HSO; young = HSY), and old sham and old DMM joints and serum (sham joint = DJC, DMM joint = DJM, sham serum = DSC and DMM serum = DSM) were constructed and subjected to Illumina deep sequencing. Summaries of raw, trimmed reads and sequencing alignment to mouse snoRNAs are in Supplementary files 1 and 2 respectively. Reads mapping percentages for joint libraries were between 12~29%, and for serum libraries 0.03~0.9%. Between 42~53% of the 1555 mouse snoRNA reference sequences were aligned for joints and 16~26% for serum.

Identification of DE snoRNAs using SnoRNASeq. The 3-D PCA plot (Fig. 2A) shows that the joint samples and serum samples are clearly separated by the 1st component, which explains 93.02% of the data variation. For serum samples, the group sham and the group DMM scatter separately on the 2nd component, furthermore, based on the 3rd component a clear separation between serum samples of young-healthy and samples of old-healthy exists. For joint samples, there is also a separation between DMM and sham samples, though the separation is not as clear as shown for serum samples. In addition, 5 DMM joint samples scatter far away from other joint samples on the 3rd component. Therefore, it can be expected that disease response small RNA can be detected from this data set. The heat map of hierarchical clusters of correlations among samples (Fig. 2B) depicts that the joint and serum groups of samples are very different in snoRNA expression. In addition, joint samples correlated to each other much more closely than serum samples confirming the phenomena revealed by the PCA analysis shown in Fig. 2A. This indicates that major disease responses in the data were contributed from serum samples. A read length distribution graph was generated to highlight the constitutional difference of non-coding RNAs in joint and serum samples at 100 bp or below (Fig. 2C). For this one serum and one joint sample with approximately the same library size were chosen. The plots of the read length distribution for joint and serum
samples reveal that a peak value of frequency for read length in joint samples is 22 bp (vertical, dotted black line). Therefore the corresponding reads are generally well annotated miRNAs. Another peak is noted at around 100 bp, which is consistent with non-coding RNAs whose length is a hundred bp and over. In contrast, a peak is presented at 30 bp long for serum samples (vertical, dotted red line). More than 50% of the reads are 30 bp long in the serum library (data not shown). In contrast, the joint library has just 1.14% of reads that are 30 bp long (data not shown). Such reads may come from piwi-interacting RNAs (piRNAs). There were 498–646 snoRNAs expressed in serum samples and 1068–1286 in joint samples. The DE snoRNAs between contrasts are in Table 2. These included 6 snoRNAs in young versus old joints, 5 snoRNAs in old sham versus old DMM joints. In serum we identified DE of 27 snoRNAs in young versus old serum and 18 snoRNAs in old sham versus old DMM serum.

Validation of SnoRNASeq data using qRT-PCR. Very little is known about the roles of snoRNAs as biomarker for disease or about the functional roles of snoRNAs in mammalian cell biology. We thus could not define functional criteria to select snoRNAs for validation based on prior knowledge. To stay unbiased we thus selected snoRNAs from the RNAseq data that displayed a moderate fold significant expression difference, higher fold significant expression difference, non-significant expression difference and selected box H/ACA (SNORAs) as well as box C/D (SNORDs) snoRNAs for validation. Levels of candidate snoRNAs for further qRT-qPCR analysis were determined using the original RNA from all donors used to perform the SnoRNASeq experiment. There was good concordance between SnoRNASeq and qRT-PCR platforms (Table 2 and Fig. 3). SNORD88
| Contrast                  | Gene ID                | Name                  | Log2 FC | FDR   | Host Gene                                      |
|---------------------------|------------------------|-----------------------|---------|-------|-----------------------------------------------|
| Young vs. Old joint       | ENSMUSG00000098372     | SNORD113              | −0.92   | 0.05  | Predicted gene                                |
|                           | ENSMUSG00000065016     | SNORA3                | −0.80   | 0.01  | Ribosomal protein L27A                        |
|                           | ENSMUSG00000080352     | SNORD88               | −0.55   | 0.00  | RIKEN cDNA 2410002F23 gene                    |
|                           | ENSMUSG00000064380     | SNORA73               | 0.56    | 0.03  | Ribosomal protein S4                          |
|                           | ENSMUSG00000087883     | SNORA17               | 1.21    | 0.05  | Contactin associated protein-like S8          |
|                           | ENSMUSG00000064536     | SNORD38               | 1.49    | 0.05  | PDZ domain containing 4                       |
|                           | ENSMUSG00000089255     | SNORA78               | −2.29   | 0.01  | Ribosomal protein S2                          |
|                           | ENSMUSG00000080396      | SNORD111              | −1.38   | 0.01  | Splicing factor 3b, subunit 3                 |
|                           | ENSMUSG00000065883     | U3                    | −2.53   | 0.00  | Predicted gene                                |
|                           | ENSMUSG00000064500     | SNORD95               | 1.16    | 0.01  | Guanine nucleotide binding protein, beta polypeptide 2 like 1 |
|                           | ENSMUSG00000095118     | SNORD14D              | 1.23    | 0.00  | Heat shock protein 8                          |
|                           | ENSMUSG00000065219     | SNORD32A              | 1.55    | 0.04  | Ribosomal protein L13A                        |
|                           | ENSMUSG00000088678     | SNORA17               | 1.90    | 0.03  | Predicted gene                                |
|                           | ENSMUSG00000064938     | miR3068/SNORA58       | 1.96    | 0.03  | AlkB, alkylation repair homolog 1              |
|                           | ENSMUSG00000065282     | SNORA18               | 1.99    | 0.00  | Predicted gene                                |
|                           | ENSMUSG00000064387     | SNORA73A              | 2.02    | 0.01  | Regulator of chromosome condensation 1        |
|                           | ENSMUSG00000064602     | SNORA41               | 2.12    | 0.00  | Eukaryotic translation elongation factor 1     |
|                           | ENSMUSG00000088670     | SNORA31               | 2.14    | 0.03  | Regulatory associated protein of MTOR, complex 1 |
|                           | ENSMUSG00000064918     | SNORD18               | 2.18    | 0.02  | Ribosomal protein L4                          |
|                           | ENSMUSG00000065158     | SNORA73               | 2.26    | 0.02  | ERO1-like beta                                |
|                           | ENSMUSG00000064493     | SNORA28               | 2.39    | 0.03  | Eukaryotic translation initiation factor 5     |
|                           | ENSMUSG00000077167     | SNORA53               | 2.43    | 0.00  | Solute carrier family 25                      |
|                           | ENSMUSG00000064495     | SNORA5                | 2.49    | 0.00  | Transforming growth factor beta regulated gene 4 |
|                           | ENSMUSG00000064569     | SNORD71               | 2.56    | 0.00  | Adaptor-related protein complex 1, gamma 1     |
|                           | ENSMUSG00000064791     | SNORD14E              | 2.63    | 0.00  | Heat shock protein 8                          |
|                           | ENSMUSG00000077714     | SNORD17               | 2.76    | 0.00  | Sorting nexin 5                               |
|                           | ENSMUSG00000065353     | SNORD73B              | 2.79    | 0.00  | Regulator of chromosome condensation 1 and an lnc |
|                           | ENSMUSG00000064696     | SNORD20               | 3.09    | 0.00  | Nucleolin                                    |
|                           | ENSMUSG00000065778     | SNORA55               | 3.10    | 0.02  | Poly(A) binding protein, cytoplasmic 4         |
|                           | ENSMUSG00000080478     | SNORD23               | 4.42    | 0.00  | Glioma tumor suppressor candidate region gene 2 |
|                           | ENSMUSG00000095176     | SNORA44               | 5.65    | 0.00  | Hypothetical protein PNAS-123                  |
|                           | ENSMUSG000000888040     | SNORA17              | 12.14   | 0.00  | Hypothetical protein MGC16037, homology human collagen alpha 2 |
|                           | ENSMUSG00000065852     | SNORA2                | 13.40   | 0.00  | Hypothetical protein, 152aa                     |
| Sham vs. DMM joint        | ENSMUSG00000096017     | SNORD116              | 10.56   | 0.01  | Predicted gene                                |
|                           | ENSMUSG00000064938     | miR3068               | 3.31    | 0.03  | AlkB, alkylation repair homolog 1              |
|                           | ENSMUSG00000089014     | SNORA36               | 3.05    | 0.01  | Dyskerin                                     |
|                           | ENSMUSG00000077722     | SNORA66               | 2.78    | 0.05  | Ribosomal protein L5                          |
|                           | ENSMUSG00000064918     | SNORD18               | 2.21    | 0.01  | Ribosomal protein L4                          |
|                           | ENSMUSG00000064400     | U3                   | 1.92    | 0.01  | Predicted gene                                |
|                           | ENSMUSG00000089417     | U90                  | 1.90    | 0.01  | Importin                                     |
|                           | ENSMUSG00000077709     | SNORA64               | 1.85    | 0.04  | Small nuclear ribonucleoprotein N              |
|                           | ENSMUSG00000065734     | SNORD49A              | −1.03   | 0.05  | RIKEN cDNA 2410006H16 gene                    |
|                           | ENSMUSG00000064450     | SNORD68               | −1.06   | 0.04  | Ribosomal protein L13                         |
|                           | ENSMUSG00000064444     | SNORD58               | −1.18   | 0.01  | Ribosomal protein L17                         |
|                           | ENSMUSG00000064453     | SNORD21               | −1.22   | 0.01  | Ribosomal protein L5                          |
|                           | ENSMUSG00000065281     | SNORD27               | −1.31   | 0.01  | Small nuclear RNA host gene 1                  |
|                           | ENSMUSG00000064871     | SNORD58B              | −1.40   | 0.05  | Ribosomal protein L17                         |
|                           | ENSMUSG00000087819     | SNORA48               | −1.41   | 0.04  | Nucleosome assembly protein 1-like 1           |
|                           | ENSMUSG00000064751     | SNORD46               | −1.46   | 0.01  | Ribosomal protein S8                          |
|                           | ENSMUSG00000077756     | SNORD90               | −1.48   | 0.01  | Ring finger and CCCH-type zinc finger domains 2 |
|                           | ENSMUSG00000089093     | SNORD11               | −1.86   | 0.03  | NOP58 ribonucleoprotein                       |

Table 2. Differentially expressed snoRNAs between contrasts including host gene identification.
Explanation: positive Log2 FC = increased in old or DMM. Negative Log2 FC = is decreased in old or DMM.
was significantly decreased in young versus old joint (Fig. 3A), while SNORA73 was validated to be increased in young versus old joint (Fig. 3A). In agreement with its absence in the DE group (Table 2), SNORA30 was not differentially expressed in young versus old joint (Fig. 3A) and SNORD88 was not differentially expressed in sham versus DMM joint (Fig. 3A). SNORA31, SNORA28, SNORD225 and SNORA73 were confirmed to be significantly increased in young versus old serum (Fig. 3B) and SNORD116, SNORA64 and U3 were significantly increased in sham versus DMM serum (Fig. 3C). SNORD46 was confirmed to be significantly decreased in DMM serum as compared to sham serum (Fig. 3C).

**SNORD116 in equine serum in OA.** To confirm increased SNORD116 in OA serum levels in a different species using qRT-PCR, we measured SNORD116 in equine serum samples. Normal equine donors had a Mankin’s score 1.25 ± 0.9 (mean ± 95% CI) and OA donors 7.75 ± 7.6. Synovial membrane from normal donors had a synovitis score 7 of 3.25 ± 2.3 and OA donors 3.25 ± 3.2. There was a significant increase in serum expression of SNORD116 in OA compared to normal donors (p = 0.0010) (Fig. 4).

**Discussion**

SnoRNAs are emerging as important regulators of cell functions, such as alternative splicing, metabolic stress and development of disease; in cancer, Prader-Willi Syndrome and autism. Through expression profiling of snoRNAs using deep-sequencing we reveal novel molecular features relating to joint ageing and OA.

Whilst gene expression has been evaluated in animal models of OA, including the rat anterior cruciate transection and meniscal tear models, and mouse DMM model these were primarily interrogating protein-coding genes. Furthermore, apart from the final study these experiments evaluated a single tissue, primarily articular cartilage. In musculoskeletal ageing single tissues have been investigated. OA is a process that involves the whole joint as an organ; therefore we undertook our analysis on whole mouse joints, which included cartilage, meniscus, subchondral bone, and joint capsule with synovium.

Mice are considered skeletally mature at around 3-months-old (approximate equivalent of a teenage human) while a 12-month-old mouse would signify a 40 to 50 year human. Thus, to investigate the effects of joint age we used 8-month-old equivalent to 25 to 28 year human (referred to as young) and 24-month-old mice (referred to as old). To study the development of post-traumatic OA we measured OA severity histologically and analysed snoRNAs expression in joints and serum from 24-month-old mice following DMM. Limitations of this study were that we did not additionally undertake the DMM model in young mice. Additionally the ‘old’ age in young versus old contrast was not the same age as ‘old’ age in sham versus DMM. OARSI scores that were observed in the sham group (DMM surgery cohort; 24 months old) were lower than the old group (young versus old cohort; 18 months old). At this moment we cannot explain this difference, but different housing and environments of the two cohorts may have influenced this. The DMM model is a post-injury model in which the histological lesions within the affected joint are similar to those observed in human OA. Mild OA-like pathology was present in the old and sham mice implying that mice at this age in the early stages of acquiring naturally occurring OA comparable to studies of human knees at the equivalent age of approximately 40 years-old. However the effect of the DMM model exacerbated these changes.

Detection of snoRNAs in serum has previously been demonstrated. SnoRNAs are serum stable, and although normally resident in the nucleolus it is thought in serum they are present as unidentified protein complexes. It is however not clear whether disease-associated RNAs detected in the circulation result from local tissue disturbances and cell death, or whether they are actively locally secreted via exosomes or microvesicles or are a systemic response upon local tissue damage. This may even depend on the specific pathology or specific RNA species. Determining the average read-length distribution of representative joint and serum samples revealed an unexpected finding. When looking at the read-length distribution the serum reads specifically contain a large peak of snoRNAs with a length of approximately 30 nt. The combined realisation that quite a large portion of the reads could not be mapped back to the used genome database, that a large portion of the piRNA class of non-coding RNAs lacks annotation in the used genome database and that piRNAs are typically 30 nt long, makes it is reasonable to think that this explains the presence of the large 30 nt peak specifically in serum. Only recently the presence of piRNAs in human blood has been described and this significant difference in the constitution of small RNAs for joint and serum samples is a phenomenon calling for further investigation.

In the young versus old serum (27 snoRNAs) more snoRNAs were significantly up-regulated compared to the old sham versus old DMM serum (18 snoRNAs) indicating ageing per se had a greater effect on differential snoRNA presence in serum than OA. This could be due to different ‘old’ ages in the comparisons, but also maybe expected as in ageing serum snoRNAs will be from many tissues whereas in the DMM model we are most likely highlighting primarily OA joint-related snoRNAs. As we were studying the whole joint this may be due to varying expression of snoRNAs in joint tissues, as it is known that there is tissue-specific snoRNA expression. We are unable to determine the amount each tissue type contributed to overall expression of snoRNAs. Each tissue will vary in its cellularity and hence RNA and snoRNA content. Thus whilst a novel aspect of this study was that snoRNAs were extracted from the multiple tissues that form the joint, our approach may be less sensitive in detecting snoRNAs that change in a single tissue. However it has the advantage of determining snoRNAs that could be more globally implicated in OA. Despite the potential limitations, we identified a number of potentially interesting snoRNAs for future studies.

SNORA73 was increased in old joint and serum (Table 2, Fig. 3) and represents a potential joint ‘biological ageing’ marker. A reliable measurement of the state of ageing and a prediction of the risk of the onset of morbidity for chronic age-related diseases such as OA would be beneficial. Such a strategy could serve as a measure of ‘biological age’ and predict an age-related biological response more accurately than chronological age. SNORA64 was increased and SNORD46 was decreased in DMM serum, but both were not DE in young versus old serum,
indicating these snoRNAs as possible OA markers. SNORD18 was increased in serum both in ageing and following DMM (Table 2) signifying that they are affected in ageing and also OA. It is difficult to speculate how much this snoRNA changes in age-related OA versus age from this study. Histology identified mild increase in OARSI score with age and the level of OA changes in the DMM model was mild. It would be beneficial to investigate this snoRNAs further in tissues and serum in more severe OA.

An interesting finding was an increase in SNORD38 in ageing joint. Zhang et al.\textsuperscript{12} demonstrated a strong association between serum levels of SNORD38 and severe cartilage damage, in anterior cruciate ligament (ACL) injury, enabling distinction between ACL injury patients from normal donors. They were unable to determine...
The expression of snoRNAs in musculoskeletal tissues potentially modulates the ageing process as previously described. While determining the transcriptomic signature of ageing equine cartilage, we found that in musculoskeletal tissues snoRNAs potentially modulate the ageing process as previously described. Interestingly, snoRNA host-genes were identified in this study including transforming growth factor 

1 (with roles in joint homeostasis). Therefore, an alteration of snoRNA expression may result from changes in transcriptional activity of the host-gene related to joint homeostasis or disease.

Our results implicate specific changes in snoRNA abundance in joint ageing (SNORD88 and SNORD38 were respectively decreased and increased) and OA suggesting the potential use of snoRNAs such as SNORA73 and SNORD23 as a novel biomarker for joint ageing. SNORA64, SNORD46 and SNORD116 for OA, SNORD18 for ageing and OA.

References

1. Woolf, A. D. & Pfleger, B. Burden of major musculoskeletal conditions. *Bull World Health Organ* **81**, 646–656 (2003).

2. Hunter, D. J., Schofield, D. & Callander, E. The individual and socioeconomic impact of osteoarthritis. *Nat Rev Rheumatol* **10**, 437–441, doi: 10.1038/nrrheum.2014.44 (2014).

3. Li, Y., Wei, X., Zhou, J. & Wei, L. The age-related changes in cartilage and osteoarthritis. *Biomed Res Int* 2013, 916530, doi: 10.1155/2013/916530 (2013).

4. Iwashima, M., Kaneko, H. & Kaneko, K. The evolving role of biomarkers for osteoarthritis. *Ther Adv Musculoskelet Dis* **6**, 144–153, doi: 10.1177/1759720X14541175 (2014).

5. Stepanov, G. A. et al. Regulatory role of small nucleolar RNAs in human diseases. *Biomed Res Int* **2015**, 206849, doi: 10.1155/2015/206849 (2015).

6. Williams, G. T. & Farzaneh, F. Are snoRNAs and snoRNA host genes new players in cancer? *Nat Rev Cancer* **12**, 84–88, doi: 10.1038/nrc3195 (2012).

7. Tada, M. et al. [IPMN and pancreatic cyst as high risk of pancreatic cancer]. *Nihon Shokakibyo Gakkai Zasshi* **112**, 1474–1478, doi: 10.11405/nisshoshi.112.1474 (2015).

8. Kishikawa, T. et al. Circulating RNAs as new biomarkers for detecting pancreatic cancer. *World J Gastroenterol* **21**, 8527–8540, doi: 10.3748/wjg.v21.i28.8527 (2015).

9. Cortez, M. A., Walsh, J. W. & Calin, G. A. Circulating microRNAs as noninvasive biomarkers in breast cancer. *Recent Results Cancer Res* **195**, 151–161, doi: 10.1007/978-3-642-28160-0_13 (2012).

10. Galimberti, D. et al. Circulating miRNAs as potential biomarkers in Alzheimer’s disease. *J Alzheimers Dis* **20**, 1261–1267, doi: 10.3233/JAD-140756 (2014).

11. Mi, S., Zhang, J., Zhang, W. & Huang, R. S. Circulating microRNAs as biomarkers for inflammatory diseases. *MicroRNA* **2**, 63–71, doi: 10.2174/2211536613021010007 (2013).

12. Zhang, L. et al. Serum non-coding RNAs as biomarkers for osteoarthritis progression after ACL injury. *Osteoarthritis Cartilage* **20**, 1631–1637, doi: 10.1016/j.joca.2012.08.016 (2012).

13. Peffers, M., Liu, X. & Clegg, P. Transcriptomic signatures in cartilage ageing. *Arthritis Res Ther* **15**, R98, doi: 10.1186/ar4278 (2013).

14. Peffers, M. J. et al. Transcriptome analysis of ageing in uninjured human Achilles tendon. *Arthritis Res Ther* **17**, 33, doi: 10.1186/s13075-015-0544-2 (2015).
Acknowledgements

We thank George Bou-Gharios, Alan Carter, James Anderson and Yalda Ashraf Kharaz for assistance with histological scoring and Andy Cremer for technical assistance with the snoRNA qRT-PCR. The authors wish to thank the Wellcome Trust for MJP’s Wellcome Trust Clinical Intermediate Fellowship (grant 107471/Z/15/Z) and the Medical Research Council (MRC) and Arthritis Research UK as part of the MRC-Arthritis Research UK Centre for Integrated research into Musculoskeletal Ageing (CIMA) (grant MR/K006312/1) (MJP/PC), NWO-DFG (grant #DN82-304; NL; TJMW) and the Dutch Arthritis Foundation (grant #LLP14; NL; TJMW).
Author Contributions
M.J.P., M.M.F.S.; conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript. Y.F.; assembly of data, data analysis, manuscript writing, final approval of the manuscript. P.M.; provision of study material, assembly of data, manuscript writing and final approval of the manuscript. P.D.C., T.J.M.W., D.Y.; conception and design, manuscript writing, final approval of the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Steinbusch, M. M. F. et al. Serum snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis. Sci. Rep. 7, 43558; doi: 10.1038/srep43558 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017