Manuscript ID ijms-2189846 – Authors' response to reviewer 2

The authors would like to thank the reviewer for their time in assessing the manuscript and for the useful and insightful comments. We have addressed each of the comments below with responses highlighted in red.

Analysis of RNA polyadenylation in healthy and osteoarthritic human articular cartilage described by Phaedra Winstanley-Zarach et al. is a very interesting paper. However, it is insufficient for publication in several respects.

First, numerous papers have been reported on the regulation of polyadenylation and its involvement in various life events and diseases such as cancer and development. However, in the Introduction section, the authors present few previous reports.

The reviewer is correct that this is an area that has been well studied. We feel that we have provided appropriate level citations to direct readers to articles and reviews which are representative of this field, and which allow further reading for those interested in learning more about it (References 14-17).

Also, there is a lack of explanation as to why they decided to analyze APA for cartilage in OA.

We state in the introduction "…there has been no study into how chondrocytes utilise this method of transcript mRNA regulation or whether it is affected by degenerative diseases such as osteoarthritis". In the revised manuscript we have added some additional text in the final paragraph to re-enforce the potential applications of the study.

In the pathogenesis of OA, it is more likely that long-term physical loading and aging are responsible for the onset of OA, rather than gene expression changes in cartilage. Therefore, the authors should clearly state that this study tested whether mRNA modifications are altered in the pathogenesis.

Whilst the reviewer is correct that abnormal physical loading and ageing can contribute to osteoarthritis, the disease is more multi factorial and can also be influenced by factors such as diet, obesity, gender and genetics. There are detailed high powered GWAS studies that have identified genetic linkages with osteoarthritis showing that gene expression or function can be causative of the disease. All the influencing factors result in widespread gene expression changes in diseased tissue and there are numerous transcriptomic studies (including ours) that show this. We have explained in the final paragraph of the introduction that we are able to use Quantseq-Rev data to not only look at transcript levels but to also determine polyadenylation site usage. We have further emphasised this by introducing the word "Furthermore" into line 84.

Regarding the fact that there were only 20 genes with changes in polyadenylation shown in Table 2, it should be made clear in the database-derived experimental data that this is not due to mere individual differences.

We utilised statistical analysis with corrected p values to ensure that regulated polyadenylation sites were identified consistently across samples in each group.

The lack of description of cartilage samples does not guarantee the reproducibility of this study. For example, the patient's race, height, weight, BMI, disease grade, presence of synovial fluid retention, and course of treatment up to sampling should be specifically described. OA is a disease that is diagnosed and treated according to the patient's complaints. Therefore, even if radiographs show obvious abnormalities in the knee cartilage, there may be cases in which the patient does not necessarily experience severe pain. For this reason, the absence of abnormalities in the knee cartilage of a healthy person should be described based on objective clinical data, not the complaint.

All healthy tissue was macroscopically intact and was obtained post-mortem from joints with no gross signs of cartilage damage, ulceration or inflammation. Control samples were provided with age, sex, significant co-morbidities and cause of death. All osteoarthritic tissue was from patients undergoing

knee replacement operations for clinically diagnosed osteoarthritis. We have added this information to the methodology at line 497. Our ethical approval for tissue collection from joint replacement operations only allows us to obtain the age and sex of the patient. Due to the limited sample number, we are not able to stratify the data based upon these factors. We only studied cartilage from male patients to reduce sample variation, a limitation that we discuss in line 467. Measurement of transcript levels has shown that these two groups have distinct changes in their transcript levels, consistent with other studies examining the effects of osteoarthritis. We have also commented on the limitation of the study indicating that the effects represent the results of comparing clinically osteoarthritic with clinically healthy joints but do not allow us to consider factors such as co-morbidities because of limited sample size.

Regarding changes in polyadenylation, for representative genes (OSMR, KMT2A, etc.), it will help the reader's understanding if the gene structures is illustrated.

We have included a gene structure schematic for OSMR, KMT2A and ANOS1 in figure 3A of the revised manuscript.

For genes with low expression, the possibility of missing them due to low read counts should be explained.

This has been commented on at line 462.

The significance of the study is unclear because the potential for better treatment and diagnosis is not well-described.

We have already described in the discussion the limited changes in alternative polyadenylation that occur as a result of osteoarthritis, whilst also identifying the potential disease associated mechanisms that might result for some of the genes where regulation has been shown to occur (e.g., KMT2A and OSMR). We have added some additional text to the final paragraph of the discussion to re-enforce this (line 463). We also do state in line 478 that, because of our study, widespread regulation of polyadenylation processes per se would not appear to be something that would be useful to target in osteoarthritis. Furthermore, we have added a limitations section to the discussion which mentions this (line 463)

It should describe whether the various mRNA variants detected are in the cell nucleus or in the cytoplasm, taking into account mRNA degradation pathways due to nonsense mutations, etc.

Our data does not allow us to determine the subcellular location of the mRNAs we have identified. However, the reviewer raises a very good point here, as increasingly, alternative polyadenylation is being shown to impact strongly on mRNA localisation within cells. This is something that we are very much aware of and is part of our plans for future study of these genes. We have added a limitations paragraph to the discussion which mentions this (line 471)