Cartilage Biomarkers in the Osteoarthropathy of Alkaptonuria Reveal Low Turnover and Accelerated Ageing

Adam M Taylor¹,², Ming-Feng Hsu³, Lakshminarayan R Ranganath⁴, James A Gallagher⁴, Jane P Dillon⁴, Janet L Huebner², Jon B Catterall² & Virginia B Kraus²,³,⁵.

¹Lancaster Medical School, Faculty of Health & Medicine, Lancaster University, Lancaster, UK; ²Duke Molecular Physiology Institute, Department of Medicine, ³Department of Pathology, and ⁵Department of Orthopaedic Surgery, Duke University School of Medicine, Durham, North Carolina, USA; ⁴Musculoskeletal Biology, University of Liverpool, Liverpool, UK.

Short title: Biomarkers of cartilage ageing in alkaptonuria

Corresponding Author
Correspondence to Dr Adam Taylor: a.m.taylor@lancs.ac.uk

Dr Adam Taylor
Clinical Anatomy Learning Centre
Lancaster Medical School
Furness College
Lancaster University
Bailrigg, LA1 4YB

England, UK Keywords: Alkaptonuria, Osteoarthritis, Biomarkers, Ageing, Glycosaminoglycan, Cartilage oligomeric matrix protein, Racemization, Ochronosis.
Abstract

Objective: Alkaptonuria (AKU) is a rare autosomal recessive disease resulting from a single enzyme deficiency in tyrosine metabolism. As a result, homogentisic acid (HGA) cannot be metabolized, causing systemic increases. Over time, HGA polymerizes and deposits in collagenous tissues leading to ochronosis. Typically, this occurs in joint cartilages leading to an early onset, rapidly progressing osteoarthropathy. We examined tissue turnover in cartilage affected by ochronosis and its role in disease initiation and progression.

Methods: Hip and knee cartilages were obtained with informed patient consent at surgery for arthropathy due to alkaptonuria (AKU n=6; 2 knees/4 hips) and osteoarthritis (OA n=12; 5 knees/7 hips); healthy non-arthritis (Non-OA n=6; 1 knee/5 hips) cartilages were obtained as waste from trauma surgery. We measured cartilage concentrations (normalized to dry weight) of racemized aspartate, glycosaminoglycan (GAG), cartilage oligomeric matrix protein (COMP) and deamidated COMP (D-COMP). Unpaired alkaptonuria, OA and Non-OA samples were compared by nonparametric Mann-Whitney U test.

Results: Despite more extractable total protein from AKU cartilage than from OA or Non-OA, there was significantly less extractable GAG, COMP and D-COMP in AKU samples compared to OA and Non-OA comparators. Racemized Asx (Aspartate and Asparagine) was significantly enriched in AKU cartilage compared with OA.

Conclusions: These novel data represent the first examination of cartilage matrix components in a sample of patients with AKU, representing almost 10% of the known UK alkaptonuric population. Compared with OA and Non-OA, AKU cartilage
demonstrates a very low turnover state and has low amounts of extractable matrix proteins.
**Introduction**

Alkaptonuria (AKU), a rare autosomal recessive condition, is classified as a rare form of osteoarthritis (OA) [1]. AKU results from a deficiency of a single enzyme, homogentisate 1,2-dioxygenase (HGD), responsible for opening the benzene ring of homogentisic acid (HGA), an intermediate in tyrosine metabolism. The absence or lack of HGD function results in HGA accumulation in tissues. HGA is excreted in the urine, but renal excretion is insufficient to completely clear the burden of HGA in AKU [2]. Elevated HGA concentrations in urine, plasma and body tissues result in a classic triad of clinical presentations. These include the earliest presentation, from birth, of urine darkening upon standing or exposure to alkaline conditions [3, 4]. Over many years of HGA exposure, the second feature occurs–termed ochronosis or darkening of collagenous tissues–often seen in the ear pinna, eye sclerae and other less uniformly and readily examined areas [5, 6]. The process of ochronosis is not completely understood, however it’s suggested that HGA undergoes polymerisation to a benzoquinone intermediate before assuming its final polymeric form in collagenous tissues [7]. The final presenting feature of the disease, namely early age onset of severe and rapidly progressing OA, results from ochronotic pigment deposition in articular cartilages whose presence alters cartilage biomechanical and biochemical properties [8]. The presence of this pigment appears to induce large-scale aggressive resorption of the subchondral bone plate and articular calcified cartilage due to stress-shielding in AKU joints [8].

A novel ultrastructural bone turnover mechanism has been identified in both AKU and OA joint tissues, providing further evidence to support the similarities between the
diseases [1, 2, 8]. In both cases, novel structures result from un-coupled bone turnover, deviating from the widely accepted Frostian bone turnover model. These structures also appear attached to the existing trabecular stock, without prior preparation of the trabecular surface by osteoclasts [8, 9]. The presence of ochronotic pigment in hyaline cartilage makes it impervious to enzymatic degradation by Tergzyme (a bacterial alkaline pronase); this may prevent the type of catabolism normally observed in OA cartilage [8]. Shown by ultrastructural analyses, pigment associates closely with collagen fibres in joint tissues, specifically in ligamentous capsule [10] and articular cartilage [AM Taylor, PhD thesis, 2011]. Along with ultrastructural evidence of a disorder of collagen fibres, solid state NMR demonstrates that there is disorder of extracellular proteins in ochronotic cartilage when compared to control [11]. However, little is known about the effects of ochronotic pigment on other matrix components, the biological ageing process and joint tissue turnover of these patients [8].

AKU is iconic in medicine as the first disease, demonstrated by Archibald Garrod in 1902, to conform to Mendelian inheritance [3]. The symptoms [1, 2], gene responsible [12, 13] and enzyme [14] are all known and well documented; even with this knowledge, a viable treatment is yet to be available to patients. Trials on a potential therapy, Nitisinone have been undertaken, but the outcome was deemed not beneficial [15, 16]. Nitisinone can inhibit ochronosis in a mouse model [17, 18]. However, Nitisinone is not without its side-effects, including elevated plasma tyrosine [19]. Moreover, Nitisinone is not likely to benefit individuals with established pigmentation and tissue impregnation with a large burden of cross-linked HGA. A clearer understanding of the molecular
changes occurring in cartilage could provide more options for treating this disease and

shed new light on our understanding of the disease pathogenesis and progression.

Ageing leads to non-enzymatic post-translational modification of proteins including
racemization and isomerization [20, 21, 22]. Racemization refers to the time-dependent
conversion of amino acids in proteins from the L form to D form (only L amino acids are
incorporated into mammalian proteins). Cartilage, a tissue with low turnover due to its
aneural and avascular nature, undergoes quantifiable racemization of some amino acids
during the lifespan [23, 24]. Recent advances in the field of biomarkers of ageing and
turnover have demonstrated that cartilage oligomeric matrix protein (COMP) is a useful
determinant of differential turnover between hip and knee, with knee cartilage showing a
greater turnover response than hip cartilage [20]. In AKU, where cartilage and its
constituent matrix components become bound by HGA polymeric pigment, we
considered it likely that these biomarkers of ageing and turnover would provide novel
insights into the pathophysiology of this iconic disease. Given that pigmented cartilage
appears to be impervious to enzymatic degradation, we hypothesized that cartilage
turnover of alkaptonuria specimens will be reduced compared to osteoarthritic and non-
osteoarthritic comparators.

The aim of this study was to investigate the impact of ochronotic pigment on cartilage
matrix turnover in joint samples from patients with AKU. Through investigation of
aspartate racemization, glycosaminoglycan (GAG) and COMP, we characterized the
turnover of tissue relative to amount of pigmentation and compared results to non-pigmented tissue from Non-OA and OA samples.

**Materials & Methods**

*Cartilage sample collection.* Alkaptonuric knees (n=2, mean age 58.5 years, range 58-59 years) and hips (n=4, mean age 61.0 years, range 54-70 years) were obtained at the time of surgery from individuals undergoing total joint arthroplasty to alleviate symptoms associated with ochronotic osteoarthropathy. Alkaptonuric cartilage samples were acquired from macroscopically ochronotic areas of tissue. All alkaptonuric joint specimens showed extensive pigmentation of all zones of cartilage. One specimen showed complete pigmentation of all the cartilage and absence of subchondral bone in many areas. Osteophytes were present in one of the samples where cartilage absence had exposed underlying osseous tissues. Pigmentation of connective tissues including ligaments was seen in all samples. All AKU samples were obtained with informed patient consent according to the Declaration of Helsinki and under ethical approval from the Liverpool Research Ethics Committee. Waste articular cartilage specimens were obtained from randomly selected total knee (n=5, mean age 68.6 years, range 54-88 years) and total hip (n=7, mean age 81.9 years, range 72-90 years) arthroplasties performed at Duke University Medical Center to alleviate symptoms of OA. From each arthritic joint, cartilage was harvested from around the lesion (lesion cartilage). Non-arthritic control cartilage samples from knee (n=1, mean age 36 years) and hip (n=5, mean age 63.4 years, range 23-82 years) joints were obtained at the time of reconstructive surgery for trauma from patients without evidence of OA as determined by macroscopic inspection of the
specimens by the surgeon. All samples from Duke University were collected under Duke
Institutional Review Board approval as waste surgical specimens and according to the
Declaration of Helsinki. Due to the early onset of the arthropathy in AKU we were
unable to directly age match samples. Instead, we utilized a matching strategy to ensure
that OA and non-OA samples encompassed the AKU sample ages. All samples in each
group were from different donors.

Sample preparation. Soluble and insoluble cartilage proteins were prepared as previously
described with minor modifications [20]. Cartilage was pulverized under liquid nitrogen
and extracted for 24 h at 4°C with gentle mixing in 4M Guanidine-HCl (Gu-HCl) in
sodium acetate buffer pH 4.0 with protease inhibitor cocktail (Sigma-Aldrich, St Louis,
MO) at a ratio of 2 ml of Gu-HCL extraction buffer per 1g of wet weight cartilage.
Following centrifugation and collection of the supernatant, the remaining cartilage was
extracted a second time with Gu-HCl extraction buffer for a further 24 h. Both first and
second Gu-HCl extractions were combined and stored at -80°C until needed. A 200 µl
aliquot of extract was buffer exchanged on an AKTA purifier 10 (GE Healthcare,
Pittsburgh, PA) using a HiTrap Desalting column (GE, Pittsburgh, PA) into PBS and
stored at -80°C constituting the soluble protein fraction. The residual Gu-HCl insoluble
protein fraction was washed a further 5 times in nanopure water over a 24 hr period at
4°C with rotation mixing to remove any residual Gu-HCl and extracted proteins. Excess
water was removed and the samples stored at -80°C until required for hydrolysis and
HPLC analysis.
Determination of protein content. Total protein was determined using the commercially available BCA™ Protein Assay Kit performed according to the manufacturer's instructions (Pierce, Rockford, IL). The presence of HGA was assessed by addition of 10M NaOH to sample extract. No color change indicative of the presence of HGA was seen in any samples, assuring the lack of interference by HGA with the BCA assay for protein determination.

Determination of GAG content. GAG content of cartilage extracts was quantified by dye binding assay with dimethylmethylene blue (Sigma, St. Louis, MO) as previously described [25]. Our analyses demonstrated no confounding of the DMMB reaction by HGA (unpublished results).

Quantification of racemized amino acids. Racemized Asx (Aspartate and Asparagine) concentrations in cartilage extracts were quantified using a previously described high performance liquid chromatography (HPLC) approach developed and validated in our laboratory [23]. Briefly, the insoluble and soluble cartilage proteins were acid hydrolyzed in 6M HCl at 105°C for 6h. The resulting free D- and L-amino acids were derivatized to fluorescent compounds by addition of o-phthaldialdehyde and N-tert-butyloxycarbonyl-L-cysteine. The subsequent derivatives were separated by reversed-phase HPLC using a C18 column and mobile phases of 0.2M acetic acid adjusted to a pH of 6.0 and an acetonitrile gradient. The resulting peaks were quantified fluorometrically (ex340nm, em440nm) by comparison to commercially available pure D- and L-amino acids (Fluka Biochemicals).
COMP ELISA. Total COMP (T-COMP) concentrations of the Gu-HCl soluble protein fraction of cartilage were quantified by ELISA using the mAb 16F12 for capture and biotinylated mAb 17C10 for detection as previously described [26] with the following minor modifications. Plates were blocked with 3% BSA/PBS/0.02% sodium azide for 2 hours. Streptavidin-alkaline phosphatase conjugate (ExtrAvadin, Sigma, St. Louis, MO) was diluted 30,000x in 0.01% BSA/PBS/0.02% sodium azide and the phosphatase substrate (4-nitrophenyl phosphate disodium salt hexahydrate, Sigma, St. Louis, MO) was used as the detection agent. Plates were read at wavelength 405 nm after 20 mins of incubation.

D-COMP sandwich ELISA. D-COMP concentrations of the Gu-HCl soluble protein fraction of cartilage were determined using the D-COMP sandwich ELISA as previously described [20]. Briefly, 96 well plates were coated with the deamidated COMP specific 6-1A12 D-COMP mAb in 0.02M sodium carbonate coating buffer, pH 9.6 overnight at 4°C [20]. Plates were blocked for at least 2 h with 5% w/v BSA in PBS, pH 7.4 at 37°C, prior to use. Samples or standard were diluted in 0.1% w/v BSA in PBS as required prior to incubation of 50 µl of sample or standard overnight at 4°C. Unbound sample was discarded and the plate washed with PBS-tween wash buffer before incubation with the biotinylated 17-C10 COMP detection mAb (a gift from Dr. V. Vilim). Unbound 17-C10 was discarded and the plate washed. Due to the lower levels of D-COMP, the D-COMP ELISA sensitivity was increased using avidin poly-horseradish peroxidase (poly-HRP, Thermo Scientific, Rockford, IL), added for 30 min at room temperature with gentle
mixing. Excess avidin-HRP was discarded, the plate washed and signal detected using tetramethyl benzidine (TMB, Sigma-Aldrich, St Louis, MO) reagent after stopping with 2M HCl and detection at 450nm.

Statistical analysis was performed using GraphPad Prism Version 5 software (La Jolla, CA). All results were analysed as unpaired data using the nonparametric Mann-Whitney U test.

Histological analysis of alkaptonuric samples was undertaken by fixing joint tissues at surgery in 10% PBFS for H&E staining or by mounting with tissue-tek and freezing in liquid N₂ for von Kossa; 5µm and 10µm sections were cut respectively. Non-decalcified cryostat sections of AKU tissue were stained in aqueous 5% silver nitrate for 30mins under a bench lamp, then washed with dH₂O and a 5% solution of sodium thiosuphate was applied for 1 min to terminate the reaction. Sections were dehydrated through graded alcohols and mounted in DPX (Sigma Aldrich, UK)

Results

Our results show significantly more total protein was extractable from AKU cartilage than either Non-OA and OA samples, p=0.0022 and 0.035 respectively (Figure 1). There was no significant difference between the mg of soluble protein/ml between Non-OA and OA (p=0.4245).
However, the mean amount of extractable GAG (as a proportion of total protein) was significantly less from AKU cartilage samples compared to either Non-OA or OA samples, \( p = 0.0022 \) and \( p = 0.0012 \) respectively (Figure 2A). Similar significant disease-related differences were also reflected in the smaller hip only set; AKU vs Non-OA and OA, \( p = 0.0159 \) and \( p = 0.0121 \) respectively (Figure 2B).

A greater amount of aged COMP protein, expressed as a proportion of deamidated to total COMP (D/T ratio), was extractable from AKU cartilage compared to OA samples (\( p = 0.0441 \)) (Figure 3A). There was no significant difference between Non-OA and AKU cartilage (\( p = 0.1797 \)). Analysis of the hip only set showed no significant differences between AKU and OA (\( p = 0.5556 \)) or Non-OA cartilages (\( p = 0.2303 \)) (Figure 3B).

Significantly less total COMP (as a proportion of total protein) was extractable from AKU cartilage than either Non-OA or OA samples, \( p = 0.0022 \) and \( p = 0.0009 \) respectively (Figure 4A). Similar significant disease-related differences were reflected in the smaller hip only set; AKU vs Non-OA and OA, \( p = 0.0159 \) and \( p = 0.0061 \) respectively (Figure 4B).

Significantly less D-COMP (as a proportion of total protein) was extractable from AKU cartilage than either Non-OA or OA samples, \( p = 0.043 \) and \( p = 0.0009 \) respectively (Figure 4C). Similar significant disease-related differences were also reflected in the smaller hip only set; AKU vs Non-OA and OA, \( p = 0.0317 \) and \( p = 0.0061 \) respectively (Figure 4D).

A general measure of aged protein, \((\text{Asx D/D+L})\), representing a ratio of racemized Asx (D form) to non-racemized Asx (L form) was used to further assess the biological age of
two cartilage fractions: the Gu-HCl extractable protein from cartilage (Figure 5 A-B), primarily representing proteoglycan; and the Gu-HCl insoluble protein from cartilage (Figure 5 C-D), representing collagen and collagen associated proteins. Compared to OA, the soluble protein fractions from AKU cartilage were significantly enriched in aged (racemized) protein represented by the mean Asx D/D+L ratio, p=0.0076. Compared to Non-OA, the mean Asx ratio from AKU cartilage was higher but this was not statistically significant (p=0.1797) (Figure 5A). Similar disease-related differences were not reflected in the smaller OA vs AKU hip only set (p=0.1091) or between Non-OA and AKU (p=0.0931) (Figure 5B). The mean Asx ratio in the insoluble cartilage fraction was significantly higher for AKU vs OA for all samples (p=0.0277), and also trended higher for AKU vs Non-OA for all samples (p=0.0931) (Figure 5C). The hip only set also showed similar trends with higher Asx ratios in AKU vs Non-OA and OA (p=0.0635 and 0.0727, respectively) (Figure 5D).

Histological examination of alkaptonuric cartilages revealed full depth pigmentation of numerous areas across all samples, with many empty chondrocyte lacunae; regions with retained chondrocytes displayed intracellular pigmentation (Figure 6A). Some areas of calcified cartilage matrix displayed a granular like pigment, often in proximity to empty chondrocyte lacunae. The bone domain showed mature bone with characteristic lamellar structure and multiple osteocytes without pigmentation. In a number of areas there were regions where pigmented cartilage was observed without articular calcified cartilage or subchondral bone beneath it. In areas where articular cartilage was absent from the bone surface, pieces of impacted cartilage were observed within the marrow space, surrounded by a variety of mono- and multinucleated cells, many of which displayed intracellular
pigmentation (Figure 6B). Histological examination by von Kossa staining and alizarin red staining (data not shown) revealed that there was no deposition of calcium salts such as hydroxyapatite associated with pigment. This contrasted with the mineralised bone seen beneath the cartilage (Figure 6C).

Discussion

Although few in absolute numbers, the AKU samples in this study represent 0.6% of the currently known global population with AKU and 9.375% of the currently known UK AKU population, highlighting one of the difficulties in studying rare diseases [2,4]. Here we present the first data analyzing biomarkers of ageing and turnover comparing alkaptonuric to OA and non-OA cartilage samples. AKU samples demonstrated an older profile of biomarkers of ageing across a number of analyzed parameters, both by ratio of aged (deamidated) to total COMP and by ratio of aged (racemized) aspartate to total aspartate in protein. These results were consistent with lower anabolism of AKU cartilage, i.e. low turnover resulting in enrichment of age-related post-translation modification of cartilage protein including the higher ratios of deamidated to total COMP and higher ratios of racemized aspartate to total aspartate. This was of interest considering that the mean age and range of age was higher for OA than AKU, which would be expected to yield more age-related changes in OA.

Previously it has been suggested that turnover in articular cartilage is greatest at the superficial layer and the turnover rate decreases as depth from the articular surface increases [27]. This may initially be true in AKU, but once pigmentation commences, the
highest rate of turnover, in the form of catabolism, appears in the deepest cartilage, with
resorption of hyaline articular calcified cartilage, following resorption of the subchondral
bone [8]. With the biochemical tools of deamidated COMP and racemized aspartate, it
would be possible in future to evaluate turnover by depth of cartilage to determine if
protein turnover reflects apparent turnover based on calcified cartilage and subchondral
bone pathology.

Interestingly, despite the presence of pigment and potential for matrix cross-linking via
pigment adducts, the amount of protein extractable from AKU cartilage was higher than
for OA and non-OA. Higher levels of molecular degeneration could lead to more readily
extractable protein. In the case of AKU, a large proportion of both non-collagen and
collagen bound proteins might be affected resulting in a higher amount of extractable
protein relative to OA and non-OA. This explanation is plausible given the trend for OA
cartilage to yield more extractable protein than non-OA and for AKU to yield still higher
amounts of extractable protein.

Until recently, little was known about the molecular events occurring in AKU cartilage
[8]. Most information available is representative of end stage of the disease when the
molecular mechanisms are almost/already complete [8]. Our observation of lower levels
of extractable GAG in AKU are consistent with an OA trajectory [28, 29], however, the
extractable amounts we observed in our AKU patient samples were significantly less than
for OA. Similarly, total COMP was significantly less extractable from AKU cartilage.
The significantly reduced amounts of extractable GAG and COMP in AKU cartilage could be imagined to arise through two scenarios:

1) They are lost very early or steadily, due to accelerated molecular degeneration, ageing, trauma, biochemical alteration of the extracellular milieu, or some combination of these factors, and not replaced due to chondrocyte pathology induced by the pigment. One of the symptoms of AKU is an early onset, rapidly progressing osteoarthropathy. If proteoglycan in AKU is lost very early and much more rapidly than general OA, this may be a factor in the accelerated disease progression observed in AKU.

2) They are bound to the cartilage extracellular matrix by the presence of ochronotic polymer and cannot be extracted. If proteoglycans are bound within the ochronotic matrix, this is likely a result of the polymerization process occurring in water spaces of the cartilage. HGA, which will polymerize to form ochronotic pigment, is a small very highly water soluble molecule and likely binds matrix proteins that are anchored in and around collagen fibres [10]. However, this latter possibility seems unlikely given the ability to extract more total protein from AKU cartilage than from OA or Non-OA.

Regardless of scenario, both possibilities lead to a cartilage matrix in AKU that is far from normal. It has already been shown that pigmentation commences pericellularly [8]. Given the intricate relationship that exists between chondrocytes and their pericellular matrices [30, 31], this likely exacerbates processes that drive alteration in tissue composition. Alterations in the pericellular matrix have been linked to OA [32].
Secondly, pigmented cartilage in AKU is significantly stiffer than that seen in OA and normal cartilage [8]. It is likely that the observed changes in cartilage matrix composition described here precede microscopic changes in the articular cartilage and subchondral bone plate and may contribute to their progression by allowing HGA to polymerize in spaces once occupied by GAG, or utilize GAG as its nucleation site. There is a possibility that the greater loss of GAG in the AKU allows for greater extracellular protein penetration and this, at least in part, might account for higher total protein content in AKU samples. This, potentially combined with an increased crosslinking in the matrix binding larger amounts of protein in. One could also posit increased protein production due to a cellular attempt to mount a repair response, producing more proteins incorporated into the matrix. The latter explanation seems unlikely given the apparent low turnover of AKU cartilage specimens based on COMP D/T and racemized Asp results.

The histological images presented demonstrate typical changes observed previously in alkaptonuric joints [8]. Cartilage is full of ochronotic pigment; existing in association with collagen [10] or with other matrix proteins. The bone changes indicate altered mechanical loading, with resorption of subchondral bone and calcified cartilage [9,10]. We did not see levels of calcium deposition consistent with mineralised tissue, compared to bone within our sections. Lack of calcium associated with pigmentation is consistent with our previous work showing calcium is not present in association with ochronotic pigment by BSE-SEM or IKI enhanced BSE-SEM [8, 32]. These morphological changes are likely contributed to by the matrix changes described here.
Whilst proteoglycan loss in these patients has physiological significance, there are clinical implications. Radiographic presentation of AKU patients is almost always representative of some degenerative changes that mimic OA; joint space narrowing is often described, particularly in the intervertebral disc spaces [8]. Cartilage thinning is not well documented, the majority of specimens obtained at surgery are mostly normal, apart from their ochronosis [8]. Those that do possess what would be described as lesions, appear ochronotic and are typified by mechanical damage from contact between brittle cartilage on opposing surfaces, rather than enzymatic degradation and lesions typically observed with OA. Because the presence of pigment initially protects cartilage from overt breakdown, early on radiographs appear normal whilst cartilage is pigmenting/pigmented. Only when mechanical trauma dislodges cartilage may radiological changes be observed; therefore, other diagnostic measures for assessing the state of joints of individuals with AKU, particularly during early joint pathological stages, may be more appropriate. Along with HGA [8], the damaged cartilage fragments are often seen embedded in the synovium and synovial fluid aspirate [34, 35].

Our data demonstrate the alterations that occur in the cartilage of alkaptonuric patients. The loss of numerous matrix proteins raises potential issues for treating AKU. Nitisinone has been suggested as a potential therapy [15, 16, 18, 36] however, it should be borne in mind that this would most benefit individuals without established pigmentation. These data represent an advance in understanding AKU cartilage turnover that could stimulate new therapeutic options. For instance, our results suggest individuals may also benefit from growth factor and other anabolic treatments as there appears to be a lack of an
anabolic response to the pigmentation process, particularly in those who have some established pigmentation.

**Key messages**

1) AKU cartilage demonstrates an older profile of ageing biomarkers compared to OA and non-OA comparators.
2) AKU cartilage shows a low turnover profile.
3) Anabolic therapy may be beneficial to patients with AKU.

**Acknowledgements**

We are extremely grateful to all patients who donated their tissues as part of this study. The authors would like to thank Dr Hazel Sutherland for her technical assistance with histology.

**Disclosure statement**

The authors declare no conflicts of interest.

**Funding**

This work was supported by an OARSI scholarship award [to AT] and the AKU Society & Rosetrees Trust. This work was also supported by National Institute of Health /National Institute of Arthritis Musculoskeletal and Skin Diseases [AR050245] [to VBK] and the National Institute of Health /National Institute on Aging [AG028716] [to VBK].

**Role of the funding source**

Funders had no input into study design, collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

**Author Contributions**
AT, JAG, LRR and VBK conceived the study.

AT, JBC, JPD, MFH & JLH undertook experimental procedures.

All authors contributed to interpreting data, drafting, reading and approving the final version of the manuscript.

References


23. Stabler TV, Byers SS, Zura RD, Kraus VB. Amino acid racemization reveals
differential protein turnover in osteoarthritic articular and meniscal cartilages.

24. Catterall, JB, Zura RD, Bolognesi MP, Kraus VB. Amino acid racemization
reveals a high state of repair in knee compared with hip osteoarthritic cartilage.

25. Chandrasekhar, S., M.A. Esterman, and H.A. Hoffman, Microdetermination of

levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic
progression of knee osteoarthritis. Osteoarthritis Cartilage. 2002 Sep; 10(9):707-
13.

27. Maroudas A. Glycosaminoglycan turn-over in articular cartilage. Philos Trans R

28. Malemud CJ. Changes in proteoglycans in osteoarthritis: biochemistry,
ultrastructure and biosynthetic processing. J Rheumatol Suppl. 1991 Feb;27:60-
2.

29. Ling W, Regatte RR, Navon G, Jerschow A. Assessment of glycosaminoglycan
concentration in vivo by chemical exchange-dependent saturation transfer

30. Vincent TL, McLean CJ, Full LE, Peston D, Saklatvala J. FGF-2 is bound to
perlecan in the pericellular matrix of articular cartilage, where it acts as a


**Figure Legends**

Figure 1: Higher total protein in AKU and OA cartilage compared to Non-OA samples.

Graph showing extractable protein (mg soluble protein/ml) from AKU (n=6), OA (n=12) and Non-OA (n=6) for independent cartilage specimens. Error bars represent the mean of the n in each group ±95% CI.
Figure 2: Lower GAG in AKU cartilage compared to OA and Non-OA samples. *Graph showing extractable glycosaminoglycan (GAG) (ug GAG/mg protein) from AKU (n=6), OA (n=12) and Non-OA (n=6) for independent cartilage specimens. A; all samples; both hips and knees. B; hip only samples. Error bars represent the mean of the n in each group ±95% CI.*

Figure 3: Higher D/T COMP ratio in AKU cartilage compared to OA. *Graph showing extractable D-COMP as a proportion of total COMP (D/T) from AKU (n=6), OA (n=12) and Non-OA (n=6) for independent cartilage specimens. A; all samples; both hips and knees. B; hip only samples. Error bars represent the mean of the n in each group ±95% CI.*

Figure 4: Lower total COMP and lower deamidated COMP in AKU cartilage compared to OA and Non-OA. *Graph showing extractable total COMP (T-COMP) (T-COMP/mg protein) from AKU (n=6), OA (n=12) and Non-OA (n=6) cartilage specimens. A; all samples; both hips and knees. B; hip only samples. Graph showing extractable deamidated-COMP (D-COMP) (D-COMP/mg protein) from AKU (n=6), OA (n=12) and Non-OA (n=6) cartilage specimens. C; all samples; both hips and knees. D; hip only samples. Error bars represent the mean of the n in each group ±95% CI.*

Figure 5: Higher racemized Asx content in PBS and insoluble extract fractions of AKU cartilage compared to OA and Non-OA samples. *Graphs showing the amount of racemized Asx (D form) as a proportion of non-racemized Asp (L form) represented by the D/D+L ratio in AKU, OA and Non-OA cartilage specimens. A; Asx Ratio (D/D+L) in PBS extract of all samples; both hips and knees from AKU (n=6), OA (n=12) and Non-OA (n=6) for independent cartilage specimens B; Asx Ratio (D/D+L) in PBS extract*
from hip only samples from AKU (n=4), OA (n=7) and Non-OA (n=5) for independent cartilage specimens C; Asx Ratio (D/D+L) in Insoluble extract all samples; both hips and knees from AKU (n=6), OA (n=12) and Non-OA (n=6) for independent cartilage specimens D; Asx Ratio (D/D+L) in Insoluble extract from hip only samples from AKU (n=4), OA (n=7) and Non-OA (n=5) for independent cartilage specimens Error bars represent the mean of the n in each group ± 95% CI.

Figure 6: H&E stained section of alkaptonuric femoral head showing pigmented articular cartilage and pigmented articular cartilage impacted in the marrow. A; Histological section showing full depth articular cartilage pigmentation with numerous empty chondrocyte lacunae (arrows). There is pericellular pigmentation around chondrocyte lacunae in the calcified cartilage. There are also areas where the calcified cartilage is not continuous with either the subchondral bone below it or hyaline articular cartilage above it. Bar = 50 µm. B; Shards of pigmented cartilage can be seen impacted within the marrow space of the bone marrow cavity (C). The cartilage is surrounded by inflammatory cells, some of which show intracellular pigmentation (arrow). Fibrous tissue response of the marrow cavity can be seen by numerous strands of matrix peripherally around the cartilage (asterisk). Bar = 10 µm. C; 10 micron non-decalcified cryostat section of AKU tissue stained with von Kossa. Densely mineralised bone, black, can be seen beneath isogenous zones of chondrocytes located in the hyaline articular cartilage which shows ochronotic colouration but absence of similar black staining indicative of calcium deposition.