1 **Cartilage Biomarkers in the Osteoarthropathy of Alkaptonuria Reveal Low**

2 **Turnover and Accelerated Ageing**

3

4 Adam M Taylor[1,2], Ming-Feng Hsueh[3], Lakshminarayan R Ranganath[4], James A

5 Gallagher[4], Jane P Dillon[4], Janet L Huebner[2], Jon B Catterall[2] & Virginia B Kraus[2,3,5].

6

7 [1]Lancaster Medical School, Faculty of Health & Medicine, Lancaster University,

8 Lancaster, UK; [2]Duke Molecular Physiology Institute, Department of Medicine,

9 [3]Department of Pathology, and [5]Department of Orthopaedic Surgery, Duke University

10 School of Medicine, Durham, North Carolina, USA; [4]Musculoskeletal Biology,

11 University of Liverpool, Liverpool, UK.

12

13 **Short title**: Biomarkers of cartilage ageing in alkaptonuria

14 **Corresponding Author**

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

16 Dr Adam Taylor

17 Clinical Anatomy Learning Centre

18 Lancaster Medical School

19 Furness College

20 Lancaster University

21 Bailrigg, LA1 4YB

22 England, UK **Keywords**: Alkaptonuria, Osteoarthritis, Biomarkers, Ageing,

23 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-arthritic (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

1   demonstrates a very low turnover state and has low amounts of extractable matrix

2   proteins.

3

4

1   **<u>Introduction</u>**

2   Alkaptonuria (AKU), a rare autosomal recessive condition, is classified as a rare form of

3   osteoarthritis (OA) [1]. AKU results from ~~a~~ deficiency of a single enzyme, homogentisate

4   1,2-dioxygenase (HGD), responsible for opening the benzene ring of homogentisic acid

5   (HGA), an intermediate in tyrosine metabolism. The absence or lack of HGD function

6   results in HGA accumulation in tissues. HGA is excreted in the urine, but renal excretion

7   is insufficient to completely clear the burden of HGA in AKU [2]. Elevated HGA

8   concentrations in urine, plasma and body tissues result in a classic triad of clinical

9   presentations. These include the earliest presentation, from birth, of urine darkening upon

10   standing or exposure to alkaline conditions [3, 4]. Over many years of HGA exposure,

11   the second feature occurs–termed ochronosis or darkening of collagenous tissues–often

12   seen in the ear pinna, eye sclerae and other less uniformly and readily examined areas [5,

13   6]. The process of ochronosis is not completely understood, however it's suggested that

14   HGA undergoes polymerisation to a benzoquinone intermediate before assuming its final

15   polymeric form in collagenous tissues [7]. The final presenting feature of the disease,

16   namely early age onset of severe and rapidly progressing OA, results from ochronotic

17   pigment deposition in articular cartilages whose presence alters cartilage biomechanical

18   and biochemical properties [8]. The presence of this pigment appears to induce large-

19   scale aggressive resorption of the subchondral bone plate and articular calcified cartilage

20   due to stress-shielding in AKU joints [8].

21

22   A novel ultrastructural bone turnover mechanism has been identified in both AKU and

23   OA joint tissues, providing further evidence to support the similarities between the

4

1 diseases [1,2&8]. In both cases, novel structures result from un-coupled bone turnover,

2 deviating from the widely accepted Frostian bone turnover model. These structures also

3 appear attached to the existing trabecular stock, without prior preparation of the

4 trabecular surface by osteoclasts [8, 9]. The presence of ochronotic pigment in hyaline

5 cartilage makes it impervious to enzymatic degradation by Tergazyme (a bacterial

6 alkaline pronase); this may prevent the type of catabolism normally observed in OA

7 cartilage [8]. Shown by ultrastructural analyses, pigment associates closely with collagen

8 fibres in joint tissues, specifically in ligamentous capsule [10] and articular cartilage [AM

9 Taylor, PhD thesis, 2011]. Along with ultrastructural evidence of a disorder of collagen

10 fibres, solid state NMR demonstrates that there is disorder of extracellular proteins in

11 ochronotic cartilage when compared to control [11]. However, little is known about the

12 effects of ochronotic pigment on other matrix components, the biological ageing process

13 and joint tissue turnover of these patients [8].

14

15 AKU is iconic in medicine as the first disease, demonstrated by Archibald Garrod in

16 1902, to conform to Mendelian inheritance [3]. The symptoms [1, 2], gene responsible

17 [12, 13] and enzyme [14] are all known and well documented; even with this knowledge,

18 a viable treatment is yet to be available to patients. Trials on a potential therapy,

19 Nitisinone have been undertaken, but the outcome was deemed not beneficial [15, 16].

20 Nitisinone can inhibit ochronosis in a mouse model [17, 18]. However, Nitisinone is not

21 without its side-effects, including elevated plasma tyrosine [19]. Moreover, Nitisinone is

22 not likely to benefit individuals with established pigmentation and tissue impregnation

23 with a large burden of cross-linked HGA. A clearer understanding of the molecular

1 changes occurring in cartilage could provide more options for treating this disease and

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

3

4 Ageing leads to non-enzymatic post-translational modification of proteins including

5 racemization and isomerization [20, 21, 22]. Racemization refers to the time-dependent

6 conversion of amino acids in proteins from the L form to D form (only L amino acids are

7 incorporated into mammalian proteins). Cartilage, a tissue with low turnover due to its

8 aneural and avascular nature, undergoes quantifiable racemization of some amino acids

9 during the lifespan [23, 24]. Recent advances in the field of biomarkers of ageing and

10 turnover have demonstrated that cartilage oligomeric matrix protein (COMP) is a useful

11 determinant of differential turnover between hip and knee, with knee cartilage showing a

12 greater turnover response than hip cartilage [20]. In AKU, where cartilage and its

13 constituent matrix components become bound by HGA polymeric pigment, we

14 considered it likely that these biomarkers of ageing and turnover would provide novel

15 insights into the pathophysiology of this iconic disease. Given that pigmented cartilage

16 appears to be impervious to enzymatic degradation, we hypothesized that cartilage

17 turnover of alkaptonuria specimens will be reduced compared to osteoarthritic and non-

18 osteoarthritic comparators.

19

20 The aim of this study was to investigate the impact of ochronotic pigment on cartilage

21 matrix turnover in joint samples from patients with AKU. Through investigation of

22 aspartate racemization, glycosaminoglycan (GAG) and COMP, we characterized the

1 turnover of tissue relative to amount of pigmentation and compared results to non-

2 pigmented tissue from Non-OA and OA samples.

3

4 **<u>Materials & Methods</u>**

5 ***Cartilage sample collection.*** Alkaptonuric knees (n=2, mean age 58.5 years, range 58-59

6 years) and hips (n=4, mean age 61.0 years, range 54-70 years) were obtained at the time

7 of surgery from individuals undergoing total joint arthroplasty to alleviate symptoms

8 associated with ochronotic osteoarthropathy. Alkaptonuric cartilage samples were

9 acquired from macroscopically ochronotic areas of tissue. All alkaptonuric joint

10 specimens showed extensive pigmentation of all zones of cartilage. One specimen

11 showed complete pigmentation of all the cartilage and absence of subchondral bone in

12 many areas. Osteophytes were present in one of the samples where cartilage absence had

13 exposed underlying osseous tissues. Pigmentation of connective tissues including

14 ligaments was seen in all samples. All AKU samples were obtained with informed patient

15 consent according to the Declaration of Helsinki and under ethical approval from the

16 Liverpool Research Ethics Committee. Waste articular cartilage specimens were obtained

17 from randomly selected total knee (n=5, mean age 68.6 years, range 54-88 years) and

18 total hip (n=7, mean age 81.9 years, range 72-90 years) arthroplasties performed at Duke

19 University Medical Center to alleviate symptoms of OA. From each arthritic joint,

20 cartilage was harvested from around the lesion (lesion cartilage). Non-arthritic control

21 cartilage samples from knee (n=1, mean age 36 years) and hip (n=5, mean age 63.4 years,

22 range 23-82 years) joints were obtained at the time of reconstructive surgery for trauma

23 from patients without evidence of OA as determined by macroscopic inspection of the

1   specimens by the surgeon. All samples from Duke University were collected under Duke

2   Institutional Review Board approval as waste surgical specimens and according to the

3   Declaration of Helsinki. Due to the early onset of the arthropathy in AKU we were

4   unable to directly age match samples. Instead, we utilized a matching strategy to ensure

5   that OA and non-OA samples encompassed the AKU sample ages. All samples in each

6   group were from different donors.

7

8   *Sample preparation.* Soluble and insoluble cartilage proteins were prepared as previously

9   described with minor modifications [20]. Cartilage was pulverized under liquid nitrogen

10 and extracted for 24 h at $4^{o}C$ with gentle mixing in 4M Guanidine-HCl (Gu-HCl) in

11 sodium acetate buffer pH 4.0 with protease inhibitor cocktail (Sigma-Aldrich, St Louis,

12 MO) at a ratio of 2 ml of Gu-HCL extraction buffer per 1g of wet weight cartilage.

13 Following centrifugation and collection of the supernatant, the remaining cartilage was

14 extracted a second time with Gu-HCl extraction buffer for a further 24 h. Both first and

15 second Gu-HCl extractions were combined and stored at $-80^{o}C$ until needed. A 200 µl

16 aliquot of extract was buffer exchanged on an AKTA purifier 10 (GE Healthcare,

17 Pittsburgh, PA) using a HiTrap Desalting column (GE, Pittsburgh, PA) into PBS and

18 stored at $-80^{o}C$ constituting the soluble protein fraction. The residual Gu-HCl insoluble

19 protein fraction was washed a further 5 times in nanopure water over a 24 hr period at

20 $4^{o}C$ with rotation mixing to remove any residual Gu-HCl and extracted proteins. Excess

21 water was removed and the samples stored at $-80°C$ until required for hydrolysis and

22 HPLC analysis.

23

1 *Determination of protein content.* Total protein was determined using the commercially

2 available BCA^{TM} Protein Assay Kit performed according to the manufacturer's

3 instructions (Pierce, Rockford, IL). The presence of HGA was assessed by addition of

4 10M NaOH to sample extract. No color change indicative of the presence of HGA was

5 seen in any samples, assuring the lack of interference by HGA with the BCA assay for

6 protein determination.

7

8 *Determination of GAG content.* GAG content of cartilage extracts was quantified by dye

9 binding assay with dimethylmethylene blue (Sigma, St. Louis, MO) as previously

10 described [25]. Our analyses demonstrated no confounding of the DMMB reaction by

11 HGA (unpublished results).

12

13 *Quantification of racemized amino acids.* Racemized Asx (Aspartate and Asparagine)

14 concentrations in cartilage extracts were quantified using a previously described high

15 performance liquid chromatography (HPLC) approach developed and validated in our

16 laboratory [23]. Briefly, the insoluble and soluble cartilage proteins were acid hydrolyzed

17 in 6M HCl at 105$^{°}$C for 6h. The resulting free D- and L-amino acids were derivatized to

18 fluorescent compounds by addition of o-phthaldialdehyde and N-tert-butyloxycarbonyl-

19 L-cysteine. The subsequent derivatives were separated by reversed-phase HPLC using a

20 C18 column and mobile phases of 0.2M acetic acid adjusted to a pH of 6.0 and an

21 acetonitrile gradient. The resulting peaks were quantified fluorometrically (ex340nm,

22 em440nm) by comparison to commercially available pure D- and L-amino acids (Fluka

23 Biochemicals).

1

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

1 mixing. Excess avidin-HRP was discarded, the plate washed and signal detected using

2 tetramethyl benzidine (TMB, Sigma-Aldrich, St Louis, MO) reagent after stopping with

3 2M HCl and detection at 450nm.

4

5 *Statistical analysis* was performed using GraphPad Prism Version 5 software (La Jolla,

6 CA). All results were analysed as unpaired data using the nonparametric Mann-Whitney

7 U test.

8

9 *Histological analysis of alkaptonuric samples* was undertaken by fixing joint tissues at

10 surgery in 10% PBFS for H&E staining or by mounting with tissue-tek and freezing in

11 liquid N_2 for von Kossa; 5μm and 10μm sections were cut respectively. Non-decalcified

12 cryostat sections of AKU tissue were stained in aqueous 5% silver nitrate for 30mins

13 under a bench lamp, then washed with dH$_2$O and a 5% solution of sodium thiosuphate

14 was applied for 1 min to terminate the reaction. Sections were dehydrated through graded

15 alcohols and mounted in DPX (Sigma Aldrich, UK)

16

17 **Results**

18

19 Our results show significantly more total protein was extractable from AKU cartilage

20 than either Non-OA and OA samples, p=0.0022 and 0.035 respectively (Figure 1). There

21 was no significant difference between the mg of soluble protein/ml between Non-OA and

22 OA (p=0.4245).

23

1 However, the mean amount of extractable GAG (as a proportion of total protein) was

2 significantly less from AKU cartilage samples compared to either Non-OA or OA

3 samples, p=0.0022 and p=0.0012 respectively (Figure 2A). Similar significant disease-

4 related differences were also reflected in the smaller hip only set; AKU vs Non-OA and

5 OA, p=0.0159 and p=0.0121 respectively (Figure 2B).

6

7 A greater amount of aged COMP protein, expressed as a proportion of deamidated to

8 total COMP (D/T ratio), was extractable from AKU cartilage compared to OA samples

9 (p=0.0441) (Figure 3A). There was no significant difference between Non-OA and AKU

10 cartilage (p=0.1797). Analysis of the hip only set showed no significant differences

11 between AKU and OA (p=0.5556) or Non-OA cartilages (p=0.2303) (Figure 3B).

12

13 Significantly less total COMP (as a proportion of total protein) was extractable from

14 AKU cartilage than either Non-OA or OA samples, p=0.0022 and p=0.0009 respectively

15 (Figure 4A). Similar significant disease-related differences were reflected in the smaller

16 hip only set; AKU vs Non-OA and OA, p=0.0159 and p=0.0061 respectively (Figure 4B).

17

18 Significantly less D-COMP (as a proportion of total protein) was extractable from AKU

19 cartilage than either Non-OA or OA samples, p=0.043 and p=0.0009 respectively (Figure

20 4C). Similar significant disease-related differences were also reflected in the smaller hip

21 only set; AKU vs Non-OA and OA, p=0.0317 and p=0.0061 respectively (Figure 4D).

22

23 A general measure of aged protein, (Asx D/D+L), representing a ratio of racemized Asx

24 (D form) to non-racemized Asx (L form) was used to further assess the biological age of

1 two cartilage fractions: the Gu-HCl extractable protein from cartilage (Figure 5 A-B),

2 primarily representing proteoglycan; and the Gu-HCl insoluble protein from cartilage

3 (Figure 5 C-D), representing collagen and collagen associated proteins. Compared to OA,

4 the soluble protein fractions from AKU cartilage were significantly enriched in aged

5 (racemized) protein represented by the mean Asx D/D+L ratio, p=0.0076. Compared to

6 Non-OA, the mean Asx ratio from AKU cartilage was higher but this was not statistically

7 significant (p=0.1797) (Figure 5A). Similar disease-related differences were not reflected

8 in the smaller OA vs AKU hip only set (p=0.1091) or between Non-OA and AKU

9 (p=0.0931) (Figure 5B). The mean Asx ratio in the insoluble cartilage fraction was

10 significantly higher for AKU vs OA for all samples (p=0.0277), and also trended higher

11 for AKU vs Non-OA for all samples (p=0.0931) (Figure 5C). The hip only set also

12 showed similar trends with higher Asx ratios in AKU vs Non-OA and OA (p=0.0635 and

13 0.0727, respectively) (Figure 5D).

14 Histological examination of alkaptonuric cartilages revealed full depth pigmentation of

15 numerous areas across all samples, with many empty chondrocyte lacunae; regions with

16 retained chondrocytes displayed intracellular pigmentation (Figure 6A). Some areas of

17 calcified cartilage matrix displayed a granular like pigment, often in proximity to empty

18 chondrocyte lacunae. The bone domain showed mature bone with characteristic lamellar

19 structure and multiple osteocytes without pigmentation. In a number of areas there were

20 regions where pigmented cartilage was observed without articular calcified cartilage or

21 subchondral bone beneath it. In areas where articular cartilage was absent from the bone

22 surface, pieces of impacted cartilage were observed within the marrow space, surrounded

23 by a variety of mono- and multinucleated cells, many of which displayed intracellular

1 pigmentation (Figure 6B). Histological examination by von Kossa staining and alizarin

2 red staining (data not shown) revealed that there was no deposition of calcium salts such

3 as hydroxyapatite associated with pigment. This contrasted with the mineralised bone

4 seen beneath the cartilage (Figure 6C).

5

6 **<u>Discussion</u>**

7 Although few in absolute numbers, the AKU samples in this study represent 0.6% of the

8 currently known global population with AKU and 9.375% of the currently known UK

9 AKU population, highlighting one of the difficulties in studying rare diseases [2,4]. Here

10 we present the first data analyzing biomarkers of ageing and turnover comparing

11 alkaptonuric to OA and non-OA cartilage samples. AKU samples demonstrated an older

12 profile of biomarkers of ageing across a number of analyzed parameters, both by ratio of

13 aged (deamidated) to total COMP and by ratio of aged (racemized) aspartate to total

14 aspartate in protein. These results were consistent with lower anabolism of AKU

15 cartilage, i.e. low turnover resulting in enrichment of age-related post-translation

16 modification of cartilage protein including the higher ratios of deamidated to total COMP

17 and higher ratios of racemized aspartate to total aspartate. This was of interest

18 considering that the mean age and range of age was higher for OA than AKU, which

19 would be expected to yield more age-related changes in OA.

20

21 Previously it has been suggested that turnover in articular cartilage is greatest at the

22 superficial layer and the turnover rate decreases as depth from the articular surface

23 increases [27]. This may initially be true in AKU, but once pigmentation commences, the

1 highest rate of turnover, in the form of catabolism, appears in the deepest cartilage, with

2 resorption of hyaline articular calcified cartilage, following resorption of the subchondral

3 bone [8]. With the biochemical tools of deamidated COMP and racemized aspartate, it

4 would be possible in future to evaluate turnover by depth of cartilage to determine if

5 protein turnover reflects apparent turnover based on calcified cartilage and subchondral

6 bone pathology.

7

8 Interestingly, despite the presence of pigment and potential for matrix cross-linking via

9 pigment adducts, the amount of protein extractable from AKU cartilage was higher than

10 for OA and non-OA. Higher levels of molecular degeneration could lead to more readily

11 extractable protein. In the case of AKU, a large proportion of both non-collagen and

12 collagen bound proteins might be affected resulting in a higher amount of extractable

13 protein relative to OA and non-OA. This explanation is plausible given the trend for OA

14 cartilage to yield more extractable protein than non-OA and for AKU to yield still higher

15 amounts of extractable protein.

16

17 Until recently, little was known about the molecular events occurring in AKU cartilage

18 [8]. Most information available is representative of end stage of the disease when the

19 molecular mechanisms are almost/already complete [8]. Our observation of lower levels

20 of extractable GAG in AKU are consistent with an OA trajectory [28, 29], however, the

21 extractable amounts we observed in our AKU patient samples were significantly less than

22 for OA. Similarly, total COMP was significantly less extractable from AKU cartilage.

1   The significantly reduced amounts of extractable GAG and COMP in AKU cartilage

2   could be imagined to arise through two scenarios:

3   1) They are lost very early or steadily, due to accelerated molecular degeneration,

4   ageing, trauma, biochemical alteration of the extracellular milieu, or some

5   combination of these factors, and not replaced due to chondrocyte pathology induced

6   by the pigment. One of the symptoms of AKU is an early onset, rapidly progressing

7   osteoarthropathy. If proteoglycan in AKU is lost very early and much more rapidly

8   than general OA, this may be a factor in the accelerated disease progression observed

9   in AKU.

10   2) They are bound to the cartilage extracellular matrix by the presence of ochronotic

11   polymer and cannot be extracted. If proteoglycans are bound within the ochronotic

12   matrix, this is likely a result of the polymerization process occurring in water spaces

13   of the cartilage. HGA, which will polymerize to form ochronotic pigment, is a small

14   very highly water soluble molecule and likely binds matrix proteins that are anchored

15   in and around collagen fibres [10]. However, this latter possibility seems unlikely

16   given the ability to extract more total protein from AKU cartilage than from OA or

17   Non-OA.

18

19   Regardless of scenario, both possibilities lead to a cartilage matrix in AKU that is far

20   from normal. It has already been shown that pigmentation commences pericellularly [8].

21   Given the intricate relationship that exists between chondrocytes and their pericellular

22   matrices [30, 31], this likely exacerbates processes that drive alteration in tissue

23   composition. Alterations in the pericellular matrix have been linked to OA [32].

1   Secondly, pigmented cartilage in AKU is significantly stiffer than that seen in OA and

2   normal cartilage [8]. It is likely that the observed changes in cartilage matrix composition

3   described here precede microscopic changes in the articular cartilage and subchondral

4   bone plate and may contribute to their progression by allowing HGA to polymerize in

5   spaces once occupied by GAG, or utilize GAG as its nucleation site. There is a possibility

6   that the greater loss of GAG in the AKU allows for greater extracellular protein

7   penetration and this, at least in part, might account for higher total protein content in

8   AKU samples. This, potentially combined with an increased crosslinking in the matrix

9   binding larger amounts of protein in. One could also posit increased protein production

10   due to a cellular attempt to mount a repair response, producing more proteins

11   incorporated into the matrix. The latter explanation seems unlikely given the apparent

12   low turnover of AKU cartilage specimens based on COMP D/T and racemized Asp

13   results.

14   The histological images presented demonstrate typical changes observed previously in

15   alkaptonuric joints [8]. Cartilage is full of ochronotic pigment; existing in association

16   with collagen [10] or with other matrix proteins. The bone changes indicate altered

17   mechanical loading, with resorption of subchondral bone and calcified cartilage [9,10].

18   We did not see levels of calcium deposition consistent with mineralised tissue, compared

19   to bone within our sections. Lack of calcium associated with pigmentation is consistent

20   with our previous work showing calcium is not present in association with ochronotic

21   pigment by BSE-SEM or IKI enhanced BSE-SEM [8, 32]. These morphological changes

22   are likely contributed to by the matrix changes described here.

23

1   Whilst proteoglycan loss in these patients has physiological significance, there are

2   clinical implications. Radiographic presentation of AKU patients is almost always

3   representative of some degenerative changes that mimic OA; joint space narrowing is

4   often described, particularly in the intervertebral disc spaces [8]. Cartilage thinning is not

5   well documented, the majority of specimens obtained at surgery are mostly normal, apart

6   from their ochronosis [8]. Those that do possess what would be described as lesions,

7   appear ochronotic and are typified by mechanical damage from contact between brittle

8   cartilage on opposing surfaces, rather than enzymatic degradation and lesions typically

9   observed with OA. Because the presence of pigment initially protects cartilage from overt

10 breakdown, early on radiographs appear normal whilst cartilage is

11 pigmenting/pigmented. Only when mechanical trauma dislodges cartilage may

12 radiological changes be observed; therefore, other diagnostic measures for assessing the

13 state of joints of individuals with AKU, particularly during early joint pathological

14 stages, may be more appropriate. Along with HGA [8], the damaged cartilage fragments

15 are often seen embedded in the synovium and synovial fluid aspirate [34, 35].

16

17 Our data demonstrate the alterations that occur in the cartilage of alkaptonuric patients.

18 The loss of numerous matrix proteins raises potential issues for treating AKU. Nitisinone

19 has been suggested as a potential therapy [15, 16, 18, 36] however, it should be borne in

20 mind that this would most benefit individuals without established pigmentation. These

21 data represent an advance in understanding AKU cartilage turnover that could stimulate

22 new therapeutic options. For instance, our results suggest individuals may also benefit

23 from growth factor and other anabolic treatments as there appears to be a lack of an

1   anabolic response to the pigmentation process, particularly in those who have some

2   established pigmentation.

3   **Key messages**

4   1) AKU cartilage demonstrates an older profile of ageing biomarkers compared to OA

5   and non-OA comparators.

6   2) AKU cartilage shows a low turnover profile.

7   3) Anabolic therapy may be beneficial to patients with AKU.

23   **Author Contributions**

1   AT, JAG, LRR and VBK conceived the study.

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

3   All authors contributed to interpreting data, drafting, reading and approving the final

4   version of the manuscript.

5   **References**

6   *1. Kraus, VB. 2014. Rare Osteoarthritis: Ochronosis and Kashin-Beck Disease. In:*

7   *Rheumatology, 6th edition, Ed. MC Hochberg, AJ Silman, JS Smolen, ME*

8   *Weinblatt, MH Weisman (Mosby Elsevier, Philadelphia) Chapter 185:1536-1540.*

9   *2. Mistry JB, Bukhari M, Taylor AM. Alkaptonuria. Rare Dis. 2013 Dec*

10   *18;1:e27475. doi: 10.4161/rdis.27475.*

11   *3. Garrod, Archibald E. The Incidence of Alkaptonuria: A Study in Chemical*

12   *Individuality. Lancet, 1902. vol. ii, pp. 1616-1620.*

13   *4. Ranganath L, Taylor AM, Shenkin A, , et al. Identification of alkaptonuria in the*

14   *general population: a United Kingdom experience describing the challenges,*

15   *possible solutions and persistent barriers. J Inherit Metab Dis. 2011*

16   *Jun;34(3):723-30.*

17   *5. Taylor AM, Wilson PJ, Ingrams DR, Helliwell TR, Gallagher JA, Ranganath LR.*

18   *Calculi and intracellular ochronosis in the submandibular tissues from a patient*

19   *with alkaptonuria. J Clin Pathol. 2010 Feb;63(2):186-8.*

20   *6. Taylor AM, Batchelor TJ, Adams VL, Helliwell TR, Gallagher JA, Ranganath LR.*

21   *Ochronosis and calcification in the mediastinal mass of a patient with*

22   *alkaptonuria. J Clin Pathol. 2011 Oct;64(10):935-6.*

1 *7. La Du BN, Zanonni VG, Laster L, Seegmiller JE. The nature of the defect in*

2   *tyrosine metabolism in alcaptonuria. J Biol Chem. 1958 Jan;230(1):251-60.*

3 *8. Taylor AM, Boyde A, Wilson PJ, et al. The role of calcified cartilage and*

4   *subchondral bone in the initiation and progression of ochronotic arthropathy in*

5   *alkaptonuria. Arthritis Rheum. 2011 Dec;63(12):3887-96.*

6 *9. Taylor AM, Boyde A, Davidson JS, Jarvis JC, Ranganath LR, Gallagher JA.*

7   *Identification of trabecular excrescences, novel microanatomical structures,*

8   *present in bone in osteoarthropathies. Eur Cell Mater. 2012 Apr 21;23:300-9.*

9 *10. Taylor AM, Wlodarski B, Prior IA, et al. Ultrastructural examination of tissue in*

10   *a patient with alkaptonuric arthropathy reveals a distinct pattern of binding of*

11   *ochronotic pigment. Rheumatology (Oxford). 2010 Jul;49(7):1412-4.*

12 *11. Chow WY, Taylor AM, Reid DG, Gallagher JA, Duer MJ. Collagen atomic scale*

13   *molecular disorder in ochronotic cartilage from an alkaptonuria patient,*

14   *observed by solid state NMR. J Inherit Metab Dis. 2011 Dec;34(6):1137-40.*

15 *12. Pollak MR, Chou YH, Cerda JJ, et al. Homozygosity mapping of the gene for*

16   *alkaptonuria to chromosome 3q2. Nat Genet. 1993 Oct;5(2):201-4.*

17 *13. Janocha S, Wolz W, Srsen S, et al. The human gene for alkaptonuria (AKU) maps*

18   *to chromosome 3q. Genomics. 1994 Jan 1;19(1):5-8.*

19 *14. Fernández-Cañón JM, Granadino B, Beltrán-Valero de Bernabé D, et al. The*

20   *molecular basis of alkaptonuria Nat Genet. 1996 Sep;14(1):19-24.*

21 *15. Suwannarat P, O'Brien K, Perry MB, et al. Use of nitisinone in patients with*

22   *alkaptonuria. Metabolism. 2005 Jun;54(6):719-28.*

1   16. *Introne WJ, Perry MB, Troendle J, et al. A 3-year randomized therapeutic trial of*

2   *nitisinone in alkaptonuria. Mol Genet Metab. 2011 Aug;103(4):307-14.*

3   17. *Taylor AM, Preston AJ, Paulk NK, et al. Ochronosis in a murine model of*

4   *alkaptonuria is synonymous to that in the human condition. Osteoarthritis*

5   *Cartilage. 2012 Aug;20(8):880-6.*

6   18. *Preston AJ, Keenan CM, Sutherland H, et al. Ochronotic osteoarthropathy in a*

7   *mouse model of alkaptonuria, and its inhibition by nitisinone. Ann Rheum Dis.*

8   *2014 Jan;73(1):284-9.*

9   19. *Hughes AT, Milan AM, Christensen P, et al. Urine homogentisic acid and*

10   *tyrosine: simultaneous analysis by liquid chromatography tandem mass*

11   *spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2014 Jul*

12   *15;963:106-12.*

13   20. *Catterall JB, Hsueh MF, Stabler TV, et al. Protein modification by deamidation*

14   *indicates variations in joint extracellular matrix turnover. J Biol Chem. 2012 Feb*

15   *10;287(7):4640-51*

16   21. *Catterall JB, Barr D, Bolognesi M, Zura RD, Kraus VB. Post-translational aging*

17   *of proteins in osteoarthritic cartilage and synovial fluid as measured by*

18   *isomerized aspartate. Arthritis Res Ther. 2009;11(2):R55.*

19   22. *McCudden C and VB Kraus. Biochemistry of amino acid racemization and*

20   *clinical application to musculoskeletal disease. Clinical Biochemistry 2006;*

21   *39(12):1112-30.*

1   23. *Stabler TV, Byers SS, Zura RD, Kraus VB. Amino acid racemization reveals*

2   *differential protein turnover in osteoarthritic articular and meniscal cartilages.*

3   *Arthritis Res Ther. 2009;11(2):R34.*

4   24. *Catterall, JB, Zura RD, Bolognesi MP, Kraus VB. Amino acid racemization*

5   *reveals a high state of repair in knee compared with hip osteoarthritic cartilage.*

6   *Osteoarthritis Cartilage. 2015 Sep 28. doi: 10.1016/j.joca.2015.09.003.*

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

8   *proteoglycans and glycosaminoglycans in the presence of guanidine*

9   *hydrochloride. Anal Biochem, 1987. 161(1): p. 103-8.*

10   26. *Vilím V, Olejárová M, Machácek S, Gatterová J, Kraus VB, Pavelka K. Serum*

11   *levels of cartilage oligomeric matrix protein (COMP) correlate with radiographic*

12   *progression of knee osteoarthritis. Osteoarthritis Cartilage. 2002 Sep; 10(9):707-*

13   *13.*

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

15   *Soc Lond B Biol Sci. 1975 Jul 17; 271(912):293-313.*

16   28. *Malemud CJ. Changes in proteoglycans in osteoarthritis: biochemistry,*

17   *ultrastructure and biosynthetic processing. J Rheumatol Suppl. 1991 Feb;27:60-*

18   *2.*

19   29. *Ling W, Regatte RR, Navon G, Jerschow A. Assessment of glycosaminoglycan*

20   *concentration in vivo by chemical exchange-dependent saturation transfer*

21   *(gagCEST). Proc Natl Acad Sci U S A. 2008 Feb 19;105(7):2266-70.*

22   30. *Vincent TL, McLean CJ, Full LE, Peston D, Saklatvala J. FGF-2 is bound to*

23   *perlecan in the pericellular matrix of articular cartilage, where it acts as a*

chondrocyte mechanotransducer. Osteoarthritis Cartilage. 2007 Jul;15(7):752-63.

31. *Alexopoulos LG, Haider MA, Vail TP, Guilak F. Alterations in the mechanical properties of the human chondrocyte pericellular matrix with osteoarthritis. J Biomech Eng. 2003 Jun;125(3):323-33.*

32. *Guilak F, Alexopoulos LG, Upton ML, et al. The pericellular matrix as a transducer of biomechanical and biochemical signals in articular cartilage. Ann N Y Acad Sci. 2006 Apr; 1068:498-512*

33. *Gallagher JA, Ranganath LR, Boyde A. Lessons from rare diseases of cartilage and bone. Curr Opin Pharmacol. 2015 Jun;22:107-14.*

34. *Maathuis PG, Driessen AP. Painted black. Ann Rheum Dis. 2002 Feb;61(2):100-1.*

35. *Damian LO, Felea I, Boloşiu C, Botar-Jid C, Fodor D, Rednic S. A case of alkaptonuria - ultrasonographic findings. Med Ultrason. 2013 Dec;15(4):321-5.*

36. *Mistry JB, Jackson DJ, Bukhari M, Taylor AM. A role for interleukins in ochronosis in a chondrocyte in vitro model of alkaptonuria. Clin Rheumatol. 2015 Oct 16.*

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 $\pm$95% CI.*

24

1 Figure 2: Lower GAG in AKU cartilage compared to OA and Non-OA samples. *Graph*

2 *showing extractable glycosaminoglycan (GAG) (ug GAG/mg protein) from AKU (n=6),*

3 *OA (n=12) and Non-OA (n=6) for independent cartilage specimens. **A**; all samples; both*

4 *hips and knees. **B**; hip only samples. Error bars represent the mean of the n in each*

5 *group ±95% CI.*

6 Figure 3: Higher D/T COMP ratio in AKU cartilage compared to OA. *Graph showing*

7 *extractable D-COMP as a proportion of total COMP (D/T) from AKU (n=6), OA (n=12)*

8 *and Non-OA (n=6) for independent cartilage specimens. **A**; all samples; both hips and*

9 *knees. **B**; hip only samples. Error bars represent the mean of the n in each group ±95%*

10 *CI.*

11 Figure 4: Lower total COMP and lower deamidated COMP in AKU cartilage compared

12 to OA and Non-OA. *Graph showing extractable total COMP (T-COMP) (T-COMP/mg*

13 *protein) from AKU (n=6), OA (n=12) and Non-OA (n=6) cartilage specimens. **A**; all*

14 *samples; both hips and knees. **B**; hip only samples. Graph showing extractable*

15 *deamidated-COMP (D-COMP) (D-COMP/mg protein) from AKU (n=6), OA (n=12) and*

16 *Non-OA (n=6) cartilage specimens. **C**; all samples; both hips and knees. **D**; hip only*

17 *samples. Error bars represent the mean of the n in each group ±95% CI.*

18 Figure 5: Higher racemized Asx content in PBS and insoluble extract fractions of AKU

19 cartilage compared to OA and Non-OA samples. *Graphs showing the amount of*

20 *racemized Asx (D form) as a proportion of non-racemized Asp (L form) represented by*

21 *the D/D+L ratio in AKU, OA and Non-OA cartilage specimens. **A**; Asx Ratio (D/D+L) in*

22 *PBS extract of all samples; both hips and knees from AKU (n=6), OA (n=12) and Non-*

23 *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 $\pm$ 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 ochronontic colouration but absence of similar black staining indicative of calcium deposition.*