# Subclinical Ochronosis Features In Alkaptonuria: A Cross-Sectional Study

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**Abstract:**

**BACKGROUND:** Alkaptonuria (AKU) is present from birth, yet clinical effects are considered to appear later in life. Morbidity of AKU, considered irreversible, is secondary to ochronosis. Age of ochronosis onset is not clearly known. Nitisinone profoundly lowers homogentisic acid (HGA), the metabolic defect in AKU. Nitisinone also arrests ochronosis and slows progression of AKU. However, tyrosinaemia post-nitisinone has been associated with corneal keratopathy, rash and cognitive impairment in HT1. The optimal time to start nitisinone in AKU is unknown.

**METHODS:** In an open, cross-sectional, single site study, 32 AKU patients were to be recruited. The primary outcome was presence of ochronosis in an ear biopsy. Secondary outcomes included analysis of photographs of eyes/ears, serum/urine HGA, markers of tissue damage/inflammation/oxidation, MRI imaging, gait, quality of life and Alkaptonuria Severity Score Index (qAKUSSI).

**RESULTS:** Thirty patients, with mean age (SD) 38 (14) years were recruited. Percentage pigmentation within ear biopsies increased with age. Ear pigmentation was detected in a 20 year old female implying ochronosis can start in patients before the age of 20. Gait and qAKUSSI were outside the normal range in all the AKU patients.

**CONCLUSIONS:** Ochronosis can be present before age 20 years.

What does this study add: It shows for the first time that features of AKU and subclinical ochronosis can be present from age 16 years.

Impact on clinical practice: This study supports nitisinone therapy from age 16 years in AKU. An AKU paediatric study is needed in those younger than 16 years.

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Subclinical Ochronosis Features In Alkaptonuria: A Cross-Sectional Study

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Key words: Alkaptonuria, AKUSSI, ochronosis, natural history, ear cartilage biopsy

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JAG and LFT: Quantitative analysis of eye ochronosis
EW: ear biopsies
AMM and ATH: assay of HGA,
AM: scoring of MRI
GJB, HS and MR: Gait analysis
FG: Bone and cartilage biomarkers
DB, DG, RR, AS: inflammatory markers
MK: Quality of life assessments
ST: Analysis of Questionnaires
ATH: HGA assays
TFC and EE: Statistical analysis
TFC: First drafting and revision of the manuscript

All authors contributed to analysis of the data, edited the manuscript and approved the final version.

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Abstract

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RESULTS: Thirty patients, with mean age (SD) 38 (14) years were recruited. Percentage pigmentation within ear biopsies increased with age. Ear pigmentation was detected in a 20 year old female implying ochronosis can start in patients before the age of 20. Gait and qAKUSSI were outside the normal range in all the AKU patients.

CONCLUSIONS: Ochronosis can be present before age 20 years.

What does this study add: It shows for the first time that features of AKU and subclinical ochronosis can be present from age 16 years.

Impact on clinical practice: This study supports nitisinone therapy from age 16 years in AKU. An AKU paediatric study is needed in those younger than 16 years.
Key messages

- Nitisinone arrests ochronosis and decreases progression of alkaptonuria
- Nitisinone increases circulating tyrosine and consequences such as corneal keratopathy, rashes and cognitive impairment
- Presence of irreversible ochronosis can justify nitisinone therapy in younger patients
- AKU features established in patients as young as 16 – 20 years, suggesting that nitisinone therapy can be justified from age 16 years
- Further studies needed to determine whether nitisinone should be administered to AKU patients younger than 16 years
Background

Alkaptonuria (AKU) (OMIM#203500) is a rare genetic deficiency of homogentisate dioxygenase (HGD) (EC:1.13.11.5), characterised by high circulating homogentisic acid (HGA).[1] The frequency of AKU is around 1 in 250,000 to 1 in a 1,000,000 in most populations worldwide. Deposition of HGA in connective tissue as pigment in AKU is termed ochronosis.[2] Debilitating manifestations of AKU are due to ochronosis including premature arthritis, cardiac valve disease, fractures, muscle and tendon ruptures.[3] Despite the presence of the defect from birth, apart from dark urine, and sometimes renal stones, there are few osteoarticular symptoms until around age 25 years.[4]

A recent study has shown that nitisinone, an inhibitor of p-hydroxyphenylpyruvate dioxygenase (EC:1.13.11.27) is effective in AKU.[5, 6, 7, 8] Nitisinone decreases circulating HGA, inhibits ochronosis in AKU mice and humans, slowing progression of human AKU, but is expensive.[9, 10] It is an imperfect treatment producing a different metabolic block, characterised by tyrosinaemia; toxic consequences, such as corneal keratopathy, eczema like skin rash and cognitive impairment can ensue.[11]

AKU is not fully reversible and would benefit from early treatment. Mouse studies show that nitisinone can prevent onset of ochronosis when started soon after birth, and arrest ochronosis when started later.[9, 10] Mouse studies revealed ochronotic pigment laid down in knee joints as early as 15 weeks.[9] These findings highlight the concern that subclinical ochronosis could be damaging connective tissues early in life. Starting nitisinone later in AKU is expected to result in residual disease as AKU is irreversible.[4] Identifying when ochronosis takes hold in AKU is important, as this information would allow optimal use of nitisinone, in terms of when to begin treatment.

The SOFIA (Subclinical Ochronosis Features In Alkaptonuria) study was designed to identify the earliest age when ochronosis, microscopic and macroscopic, can be detected in patients and at what age it might be appropriate to begin nitisinone treatment.
Methods

PATIENTS

AKU patients, verified by elevated HGA levels, and at least 16 years old were eligible for inclusion. A non-AKU control group was also selected. The United Kingdom NRES granted ethics approval (REC NO:15/NW/0749; IRAS:180968).

STUDY DESIGN

SOFIA was an open, cross-sectional, single-site (Royal Liverpool University Hospital) study, 32 AKU patients were to be recruited (2 males, 2 females in each age interval: 16-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, over-50) covering the age spread of non-ochronotic and ochronotic groups. The primary outcome was the amount of ochronosis measured in ear biopsies. The secondary outcomes were: visible ochronosis quantification in eyes and ears; MRI examination; deviation in gait; modified qAKUSSI (questionnaire Alkaptonuria Severity Score Index); circulating and urine HGA; inflammation, cartilage damage, bone and other tissue biomarkers, and quality of life.

ASSESSMENTS

Ear cartilage: a 4mm diameter, 1-2mm thick biopsy was taken from the conchal bowl of the ear and stored using a standardised protocol. Ochronosis was measured by % light absorbance in microscope photographs (non-ochronotic tissue=0, completely ochronotic tissue=100) (see Supplementary Material).

Standardised photographs of eyes and ears were scored for ochronosis: for eyes using qualitative and quantitative systems, for ears, a qualitative system (Supplementary Figure S1, Tables S1, S2).

Modified Pfirrmann Grading System and Spondyloarthritis Research Consortium of Canada (SPARCC) scores of the spine and whole-organ MRI scores (WORMS) of the knee were calculated (Supplementary Figures S2-S5, Table S3).[12, 13, 14] Institutional MRI protocols were followed.
3D gait analysis was performed for 36 AKU patients (29 from SOFIA, 7 from the UK National Alkaptonuria Centre) and for a control group of 10 volunteers free from gait problems.[15, 16] Mean deviation of AKU gait from normality (MDP mean) was calculated using the MDP program (Supplementary Material, Figure S6).[17, 18]

Disease questionnaires were used to calculate the modified questionnaire-based qAKUSSI (Supplementary Table S4).[19]

Fasting blood samples and 24-hour urine samples were collected to measure metabolites and biomarkers of inflammation, cartilage, bone and tissue. Serum and urine HGA were quantitated by liquid chromatography tandem mass spectrometry (LC-MS/MS) methods.[20, 21] Serum amyloid A (SAA) was analysed using a commercial assay. Quantitative determination of serum protein thiols and S-thiolated proteins was carried out.[22] Serum protein thiols were measured by colorimetric reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).[23] S-thiolated proteins were measured.[24] Protein Thiolation Index (PTI) was calculated as the molar ratio between total S-thiolated proteins [RSSP, where RS is cysteine (CySSP), cysteinylglycine (CyGlySSP), homocysteine (HcySSP), γ-glutamylcysteine (γGluCySSP) and glutathione (GSSP)] and the concentration of protein thiols.[25]

The rate of connective tissue remodelling was analysed by the measurement in serum and urine of biomarkers of collagen and other extracellular matrix protein formation and degradation using competitive and sandwich ELISAs developed at Nordic Bioscience (Supplementary Table S5). The assays were run according to the standard procedure detailed in the references in Supplementary Table S5.

Quality of Life was assessed using HAQ, SF-36 and KOOS questionnaires.[26, 27, 28]

STATISTICAL ANALYSIS

Multiple linear and LOESS regression were the main analyses used. Outcome variables were plotted against age and regression analyses carried out. Comparisons with controls were made as appropriate. All analyses were conducted using SAS® version 9.3 and R version 3.3.2.
Results

Thirty AKU patients (15 male, 15 female) were recruited. Mean age: 39 years for males, 37 years for females, mean BMI: 25 kg/m$^2$ for males and 23 kg/m$^2$ for females (Supplementary Tables S6-S8).

**EAR CARTILAGE BIOPSY, EYE AND EAR PHOTOGRAPHS**

Ear biopsies from 28 samples were processed. Figure 1 shows ear and eye ochronosis measured by biopsy and photographs plotted against age, together with regression lines. Figure 1(a) shows percentage of pigmentation of whole ear biopsy. Fitted regression lines were:

% ear pigmentation = -23.5 + 1.43 × age for males (age: p=0.001, gender: p=0.742, R$^2=0.39$)

% ear pigmentation = -26.9 + 1.43 × age for females.

**FIGURE 1 ABOUT HERE**

Age was statistically significant (p=0.001), but not gender. The fit of this model, and some of the following models, was not particularly good, but it does give a strong indication of the increase in pigmentation with age.

Assessment of pigment intensity by measuring light absorption of whole cartilage biopsy revealed pigmentation in a patient aged 20.

Figure 1(b) shows ear ochronosis measured from photographs. Ochronosis was not detected until the middle of the third decade; a LOESS (Local Regression) reflects this. Figure 1(c) shows qualitative eye ochronosis scores. Regression lines were:

Eye pigmentation = -7.3 + 0.36 × age for males (age: p<0.001, gender: p=0.448, R$^2=0.50$)

Eye pigmentation = -8.7 + 0.36 × age for females.

Age was statistically significant, but gender not. Figure 1(d) shows the correlation of the qualitative and quantitative eye ochronosis scores (R$^2=0.83$).
MRI SCANS AND GAIT ANALYSES

MRI spine and left knee imaging were performed on 27 and 26 AKU patients respectively. Disc degeneration, measured using the modified Pfirrmann score, is shown in Figure 2(a) and marrow oedema results, indicated by the modified SPARCC score, are shown in Figure 2(b). Knee scores are shown in Figure 2(c). Increases of the MRI scores with age are clearly not linear; LOESS regressions are shown for the two spine scores. Both show significant increases starting in the third decade of life. The WORMS scores generally stay low over the age range with a few exceptions.

FIGURE 2 ABOUT HERE

Figure 2(d) shows the mean movement deviation profile (MDP<sub>mean</sub>) plotted against age for AKU patients and controls together with a linear regression line for controls and a LOESS regression for the AKU patients. Deviation from normal gait was found even in the younger patients, and a steep incline after 50 years.

MDP<sub>mean</sub> = 2.44 − 0.01×age for Controls.

Supplementary Figures S7 and S8 show a linear clustering and a 2D visualisation of the AKU patients and controls.

MODIFIED qAKUSSI

Figure 3(a) shows total modified qAKUSSI plotted against age; Figures 3(b)-(d) show its components parts of clinical features, spine rheumatology and non-spine rheumatology. The corresponding regressions are:

qAKUSSI = -8.53 + 1.15×age for males  (age: p<0.001, gender: p=0.780, R<sup>2</sup>=0.47)
qAKUSSI = -10.41 + 1.15×age for females
qAKUSSI<sub>clin</sub> = -7.31 + 0.67×age for males  (age: p=0.001, gender: p=0.316, R<sup>2</sup>=0.36)
qAKUSSI<sub>clin</sub> = -12.35 + 0.67×age for females.
There were no significant differences for gender; all regressions showed a significant increase with age.

**FIGURE 3 ABOUT HERE**

**HGA, BIOMARKERS AND METABOLITE ANALYSES**

Figure 4 shows some results obtained for HGA measurements, the inflammation biomarker, SAA, and connective tissue damage markers. Figures 4(a), (b) show serum HGA and HGA clearance against age. The regression equations are:

- **Serum_HGA**  =17.4+0.28×age for males  
  (age: p=0.006, gender: p=0.465, R^2=0.26)

- **Serum_HGA**  =19.2+0.28×age for females

- **HGA_clearance**  =1322-11.8×age for males  
  (age: p=0.007, gender: p=0.934, R^2=0.26)

- **HGA_clearance**  =1313-11.8×age for females

Gender was not significant, but serum HGA significantly increased and HGA clearance significantly decreased with age. Supplementary Figure S9 shows 24-hour urine HGA plotted against age.

Figures 4(c-e) show SAA, CyGlySSP and PTI plotted against age and fitted regression lines. SAA and PTI significantly increased with age, but with no significant difference between groups. The regression lines for CyGlySSP showed significantly different slopes and intercepts for groups.[19-22]

- **SAA**  = 8.5+0.43×age for AKU  
  (age: p=0.047, group: p=0.667, R^2=0.07)*

- **SAA**  =10.8+0.43×age for Controls

- **CyGlySSP**  =22.1-0.20×age for AKU  
  (age: p<0.001, group: p=0.005,

- **CyGlySSP**  =25.4+0.04×age for Controls  
  (age*group interaction: p=0.002, R^2=0.25)
PTI = 0.31 + 0.002 × age for AKU (age: p = 0.015, group: p = 0.090, \( R^2 = 0.14 \))

PTI = 0.28 + 0.002 × age for Controls

* Two outliers removed.

Supplementary Figure S10 shows extra inflammatory and oxidative markers.

**FIGURE 4 ABOUT HERE**

Figures 4(f)-(h) show uC1M_cr, TIM and P1NP plotted against age together with regression lines. The regression lines for the two groups for uC1M_cr have marginally significantly different slopes and significantly different intercepts. TIM has significantly different slopes and intercepts for the two groups. P1NP significantly decreases with age for AKU patients and controls at the same rate, but with mean P1NP higher for AKU patients.

- uC1M_cr = 3.59 + 0.05 × age for AKU (age: p = 0.004, group: p = 0.004, \( R^2 = 0.30 \))
- uC1M_cr = 1.65 + 0.03 × age for Controls (age: p = 0.047, group: p = 0.326, \( R^2 = 0.30 \))
- TIM = 157 + 4.09 × age for AKU (age: p = 0.047, group: p = 0.039, \( R^2 = 0.22 \))
- TIM = 223 + 0.52 × age for Controls (age: p = 0.022, group: p = 0.002, \( R^2 = 0.22 \))
- P1NP = 172 - 1.43 × age for AKU (age: p = 0.022, group: p = 0.002, \( R^2 = 0.22 \))
- P1NP = 121 - 1.43 × age for Controls

Supplementary Figure S11 shows the biomarkers not covered in the main text, plotted against age for AKU patients and controls.

**QUALITY OF LIFE**

Figure 5 shows 6 of the 33 quality of life measurements: HAQ Disability Index, HAQ Pain score, SF36 Physical Functioning score, SF36 General Health score, KOOS Sport and Recreation score and KOOS Quality of Life score. The regression equations were:

- HAQ_DI = -0.73 + 0.03 × age for males (age: p < 0.001, gender: p = 0.676, \( R^2 = 0.61 \))
- HAQ_DI = -0.68 + 0.03 × age for females
- HAQ_Pain = -0.81 + 0.05 × age for males (age: p < 0.001, gender: p = 0.533, \( R^2 = 0.71 \))
- HAQ_Pain = -0.65 + 0.05 × age for females.
SF36_PF   =  109.8 -1.17×age for males   (age: p<0.001, gender: p=0.390, R^2=0.36)
SF36_PF   =  117.3 -1.17×age for females.
SF36_GH   =  83.3 -1.03×age for males   (age: p=0.001, gender: p=0.775, R^2=0.35)
SF36_GH   =  85.5 -1.03×age for females.
KOOS_Sport_Rec = 144.6 -2.18×age for males   (age: p<0.001, gender: p=0.938, R^2=0.60)
KOOS_Sport_Rec = 145.3 -2.18×age for females.
KOOS_QoL   = 115.1 -1.37×age for males   (age: p<0.001, gender: p=0.815, R^2=0.45)
KOOS_QoL   = 117.0 -1.37×age for females.

FIGURE 5 ABOUT HERE

All these measures show significant worsening of health with age but no difference between genders. The other QoL measures are reported in the Supplementary Material (Figures S12-S14).

Discussion

The SOFIA study objective was to identify the earliest age when ochronosis, microscopic and macroscopic, can be detected in AKU patients. Firstly, we review the published information in childhood AKU.

A natural history study in 2002 reported on 64 patients (ages 4 to 80 years), but described no clinical features in childhood.[29] A Slovak study, described childhood AKU 35 years ago, in 39 patients.[30] Dark urine was present in all. Pigmentary changes in axillary regions appeared at 8-10 years. Dark brown to black staining of ear cerumen was present even in childhood. Ear cartilage pigmentation in a 12 year old patient, and scleral pigmentation in a 13 year old patient, was reported, although no photographs were taken. The reported youngest age of arthropathy was 24 years. One 6-month old presented with a kidney stone.[31]

We are the first to report on direct tissue studies in AKU, reasoning that it might be possible to identify pigmentation in tissue that could not be identified through overlying skin. Percentage pigmentation within ear biopsies increases with age, females lagging behind males. Difference between genders was not statistically different, probably due to the small sample size. Pigmentation
was detected in a 20 year old female, so ochronosis can start in AKU patients before the age of 20. Eye ochronosis increased with age and was first detected at age 22 years. Externally, visible ear ochronosis was only detected after age 34 years.

AKU patients had 24-hour urine HGA and serum HGA values much higher than those of controls, showing no correlation with age, or gender. Serum HGA increased with age, but with no difference between genders, co-existing with worsening AKU. Conversely, HGA clearance decreased with age. The inflammation biomarkers, SAA and serum protein thiols, increased with age with no significant difference between AKU patients and controls.

uC1M, a marker of collagen type I degradation measured in urine, decreased with age in AKU patients and P1NP, a marker of collagen type I formation measured in serum, decreased with age in both AKU and control groups. Bones are the main source of collagen type I in the human body, therefore the results suggest the rate of bone remodelling decreases with age. C1M measured in urine could reflect the remodelling of the renal tissue, suggesting the degradation of collagen type I in the kidney is decreased compatibly with an increase in fibrogenesis in the kidneys.[32] This hypothesis needs further investigation. TIM, a marker of MMP-mediated titin degradation describing cardiac remodelling, increased with age in AKU patients but not in controls, indicating increased remodelling of heart muscle with disease progression. At present, it is not possible to establish a precise age at which the biomarkers change dramatically. Further studies are needed with more patients at younger age with matched controls.

The modified Pfirrmann spine scores on MRI analysis show AKU patients are unlikely to demonstrate degenerative lesions before age 30 years, however above 35 years they frequently show degenerative disc changes and overall worse Pfirrmann scores.[12] Marrow oedema lesion results (modified SPARCC scores) also appear to significantly rise from age of 30 years.[13] Degeneration of the knee (WORMS score) did not appear to be associated with age up to the middle of the fourth decade.[14] Despite the lack of change in spine and joints at ages younger than 30 years, the gait analysis was abnormal early on. The mean of Mean Deviation Profiles (MDP) for gait was significantly higher for
AKU patients than for controls.[17, 18] The younger AKU patients all had high MDP values indicating that gait is affected at this early age.

qAKUSSI scores increase with age, the mean increasing at a rate of 1.14 units per year. All qAKUSSI components (clinical, spine and non-spine) increase with age. Even at the ages between 16 and 20, scores well above zero have been observed. Clinically, this means that as AKU patients get older, they score higher on q-AKUSSI regardless of their gender, reflecting a worsening disease burden.

Three QOL questionnaires showed pain increases and general quality of life decreases with age for AKU patients. For the HAQ questionnaire, deterioration starts around age 30 years, but this can be at the start of the second decade for some domains for some patients. For the SF36 questionnaire, physical functions steadily decrease with age. For the KOOS questionnaire, the functions decrease with age. Overall, the quality of life appears to seriously deteriorate from the third decade.

The overall conclusion is that this study has shown that ochronosis starts at an early age, before adulthood, but was unable to assess the earliest age that it may start. Further data is needed from a paediatric study if this start point is to be established.

The difficulties of carrying out a human natural history study of AKU can be overcome to some extent by studying mouse AKU.[9, 10] With biochemistry similar to human, mice with AKU also develop ochronosis, results suggesting that ochronosis begins a short time after weaning and progresses linearly over time. The use of nitisinone from early on in the life of an AKU mouse completely prevented the appearance of ochronosis. When nitisinone was administered after ochronosis was established, it arrested further progression.

The reason for carrying out this AKU study is because of the availability of a HGA-lowering and a disease modifying therapy in the form of an enzyme inhibitor nitisinone. To consider using nitisinone early in life requires justification in the form of early ochronosis or early morbidity. The present study suggests the presence of apparently irreversible ochronosis and morbidity early in life.
REFERENCES


32. Papasotiriou M, Genovese F, Klinkhammer BM, Kunter U, Nielsen SH, Karsdal MA, Floege
J, Boor P. Serum and urine markers of collagen degradation reflect renal fibrosis in
LEGENDS TO FIGURES

Figure 1. (a) Ochronosis score from ear biopsy plotted against age with regression lines, (b) ear ochronosis measured from photographs plotted against age with LOESS regression line, (c) eye ochronosis measured from photographs plotted against age with regression lines, (d) total quantitative eye pigmentation expressed as pixel value score plotted against total qualitative eye pigmentation score.

Figure 2. (a) MRI Pfirrmann scores plotted against age, (b) SPARCC scores plotted against age, (c) WORMS scores plotted against age, (d) MDP\textsubscript{mean} plotted against age.

Figure 3. Modified qualitative alkaptonuria Severity Score Index (modified qAKUSSI): (a) total modified qAKUSSI, (b) qAKUSSI, clinical features, (c) aAKUSSI, spine rheumatology, (d) qAKUSSI, non-spine rheumatology.

Figure 4. (a) Serum HGA (µmol/L), (b) HGA clearance (ml/min), (c) SAA (ng/ml), (d) CyGlySSP (µM), (e) PTI, (f) Urine C1M\_cr (ng/(µmol), (g) Serum TIM (ng/ml), (h) Serum P1NP (ng/ml)

Figure 5. Quality of life scores: (a) HAQ Disability Index, (b) HAQ Pain Score, (c) SF36 Physical Functioning score, (d) SF36 General Health score, (e) KOOS Sport and Recreation score, (d) KOOS Quality of Life score.
Supplementary Material: Subclinical Ochronosis Features In Alkaptonuria (SOFIA): An Open Cross-sectional study to determine age of onset of ochronosis and morbidity

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**ASSESSMENTS**

**Ear Biopsy**

Each biopsy contained a disc of cartilage 4mm in diameter and 1-2 mm thick. The disc was bisected along the diameter and a thin slice of 0.8mm was taken from the cut face. This sample was examined using an Olympus SZH binocular microscope in darkfield mode at 7.5 X magnification. The biopsy section was photographed using a 9M pixels DCM 900 camera and images stored as TIFFs. TIFFs were opened in Image J as 8-bit RGB images. An oval region of interest 3 mm long by 1 mm wide was selected and the mean colour intensity in the blue channel was quantified on 255 scale, transformed so that white=0 and black=255. Following subtraction of the absorbance of non-ochronotic tissue the % absorbance was calculated (non-ochronotic tissue=0 and completely ochronotic tissue =100). Presence of ochronotic pigmentation was confirmed by histology on serial sections followed by Schmorl staining and microscopy (data not shown).

**Eye and Ear quantitative and qualitative assessments**

For quantitative analysis. JPEG image files were converted into 8-bit grey scale images using Image J. The images were calibrated for both size and colour for standardisation. Using the zoom function the temporal and nasal parts of the right and left eyes were analysed for conjunctival scleral pigmentation. Upon identification of pigmentation the free hand tool was used to draw around the area if a definitive boundary could be defined. The area, perimeter, standard deviation, mean, minimum and maximum pixel values were recorded for each region of pigmentation. If no definitive boundary was evident a sample within the area of pigmentation was taken for pixel value comparisons. The total quantitative score is the sum of the scleral and conjunctival pixel values in the temporal and nasal parts of the right and left eyes. The maximum pixel value for both the scleral and conjunctival pigmentation is 100 representing the darkest intensity, therefore the maximum score for each eye is 400.

**Figure S1. An example of eye ochronosis**
Table S1 Qualitative scoring system for eye photographs. The score for a patient is the sum of scores over the four regions.

Table S2 Qualitative scoring system for ear photographs.
**Assessment of MRI scans**

Figure S2. Sagittal STIR image of an example of high T2 signal within the disc with disc height loss.

![Sagittal STIR image](image1)

Figure S3. Sagittal T1 image of an example of high T1 signal (arrows) crossing the L3/4 and L4/5 intervertebral discs representing interbody fusion.

![Sagittal T1 image](image2)
Figure S4. Sagittal 3D volume spoiled echo sequence showing low signal foci in the hyaline cartilage consistent with chondrocalcinosis.

Figure S5. Sagittal STIR sequence of the whole spine in three study participants. 5a is a 26 year old female, 5b is a 43 year old male and 5c is a 67 year old female.

5a: the youngest, shows no focus of marrow oedema and normal thoracic and lumbar intervertebral discs. 5b: 43 year old shows relatively advance disc degenerative changes with foci of bone marrow oedema at multiple vertebrae. 5c: with advanced endstage degeneration, there is interbody fusion at multiple levels but little oedema like signal return, mainly confined to the mobile non-fused levels (T2/3, T7/8 & T8/9). Note also the kyphotic deformity is much wore in 5c associated with the advanced disc degeneration.
Table S3 Scoring system for MRI scan

### Analysis of the thoracic and lumbar spine:

**Modified Pfirrmann Grading System for thoracolumbar intervertebral disc degeneration**

- Each intervertebral disc space (12 thoracic, 5 lumbar) will be graded for degenerative changes using a modified Pfirrmann grading system which is an 8 point score on T2 weighted images. A total score for the T/L spine will be recorded.
  - Max score for a thoracolumbar spine = 136
- Additional observation per disc score 1
  - Presence of increase T2 signal within nucleus with disc height loss
  - Presence of increase T1 signal crossing disc (fusion)
  - Presence of insufficiency fracture
    - Max score = 51

**SPARCC (Spondyloarthritis Research Consortium of Canada) grading of increase T2 signal abnormality on STIR for 6 of the most affected discovertebral units (DVU)**

- Maximum score per unit = 18
- Total maximum score for 6 DVUs = 108

### Analysis of knee:

**WORMS score – semiquantitative multifeature scoring system**

- Cartilage signal & morphology – 8 point scale (0, 1, 2, 2.5, 3, 4, 5, 6), score 0-6
  - Max scores:
    - MFTJ = 30, LFTJ = 30, PFJ = 24
- Subarticular BM abnormality – score 0-3
  - Max scores
    - MFTJ = 15, LFTJ = 15, PFJ = 12, Region S = 3
- Subarticular cysts – score 0-3
  - Max scores
    - MFTJ = 15, LFTJ = 15, PFJ = 12, Region S = 3
- Bone attrition – score 0-3
  - Max scores
    - MFTJ = 15, LFTJ = 15, PFJ = 12
- Osteophytes – score 0-7
  - Max scores
    - MFTJ = 35, LFTJ = 35, PFJ = 28
- Ligament scores
  - Cruciates (anterior and posterior) 0/1 – intact/torn
  - Collaterals (medial and lateral) 0/1 – intact/torn
    - Combined ligament score = Cruciates + ½ * collaterals
- Menisci – score 0-6 (depending on scores for tears present in regions)
- Synovial thickening and effusion – Score 0-3 for entire knee
- Loose bodies – Score 0-3 for entire knee

Maximum WORMS score for knee = 335

### Additional factors

- Presence of low intrachondral signal
  - Max scores
    - MFTJ = 5, LFTJ = 5, PFJ = 4
**Gait Analysis**

Data were collected using Vicon MX motion capture with nine optoelectronic cameras (T160 and T10) sampling at a frame rate of 100 Hz (Vicon, Oxford, UK). Fifteen reflective markers were placed on the lower limbs in accordance with the Helen Hayes model\(^\text{14}\) and patients were asked to walk barefoot at a self-selected speed along a 10m walkway (Supplementary Figure S6). Pre-processed data from the 45 (X, Y, Z) marker coordinates were analysed using a self-organising map (SOM Toolbox 2.0\(^\text{15}\)) in MATLAB (R2017a, The MathWorks Inc., Massachusetts, US) to calculate the multi-dimensional deviation of AKU gait from normality. The marker coordinate data of patients and controls were expressed relative to a straight line fitted onto the progression of the centre of their pelvis followed by calculation of z-scores for each waveform to compensate for differences in marker offsets and amplitudes. The self-organising neural network was then used to calculate the mean deviation of AKU gait from normality (MDP\(_{\text{mean}}\))\(^\text{16,17}\) during three gait cycles of each patient, using the MDP freeware program\(^\text{16}\). Gait deviation for each patient was then calculated as the average of three walks, plotted against age.

**Figure S6. Marker trajectories during gait and a stick figure of the pelvis and legs**

![Marker trajectories during gait and a stick figure of the pelvis and legs](https://mc.manuscriptcentral.com/bmjinnov)
Modified qAKUSSI assessment

Table S4: Modified qAKUSSI scoring system: the overall scores are the totals over individual feature scores

<table>
<thead>
<tr>
<th>Non-spine Non-Rheumatology Clinical features</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye Pigments (conjunctival, scleral, temporal and nasal)</td>
<td>0,1,2,3,4,6,8</td>
</tr>
<tr>
<td>Ear Pigments</td>
<td>0,1,2,3</td>
</tr>
<tr>
<td>Prostate and kidney Stones</td>
<td>4 (per episode)</td>
</tr>
<tr>
<td>Heart valve disease</td>
<td>8</td>
</tr>
<tr>
<td>Hearing impairment</td>
<td>4</td>
</tr>
<tr>
<td>Fracture</td>
<td>8 per fracture</td>
</tr>
<tr>
<td>Muscle/ tendon/ ligament rupture</td>
<td>8 per rupture</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-spine Rheumatology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical joint pain score</td>
</tr>
<tr>
<td>Number of joint replacements</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spine Rheumatology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Spinal pain score</td>
</tr>
</tbody>
</table>

Table S4 summarises the clinical aspects that were assessed in the modified q-AKUSSI in SOFIA. Eye and ear pigmentationst were scored from digital images taken by the clinical photography department at the Royal Liverpool University Hospital. They were independently scored by one assessor who was blinded to patient demographics and clinical details. For the other elements of the modified q-AKUSSI, case notes were reviewed.
Biomarkers

Serum and urine HGA were quantitated by liquid chromatography tandem mass spectrometry (LC-MS/MS) methods using an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream electrospray ionisation coupled with an Agilent 1290 Infinity II UHPLC pump and autosampler. Ten microliters of sample were diluted either with 0.4μmol/L \(^{13}\)C\(_6\)-HGA in 0.1% formic acid (v/v) in deionised water (acidified urine samples) or 0.2μmol/L \(^{13}\)C\(_6\)-HGA in 0.1% formic acid (v/v) in deionised water (deproteinised serum samples). Separation was achieved on an Atlantis dC18 column (100 x 3.0mm, 3μm, Waters) maintained at 35°C. Quantitation was achieved using a matrix-matched seven point calibration curve and two product ion transitions for both HGA and its internal standard \(^{13}\)C\(_6\)-HGA.

Serum amyloid A (SAA) was analysed using a commercial 96-well plate solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) (Human SAA, KHA0011, Invitrogen Life Technologies) according to manufacturer’s instruction. Plates were read on a VersaMax microplate reader using Ascent software (Thermo Scientific). SAA quantification was obtained against a second order polynomial standard curve generated with SAA standards. All the blanks, standards and samples were tested in duplicate. Quantitative determination of serum protein thiols and S-thiolated proteins was carried out. Serum protein thiols were measured by colorimetric reaction with 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB). S-thiolated proteins were measured. Protein Thiolation Index (PTI) was calculated as the molar ratio between total S-thiolated proteins [RSSP, where RS is cysteine (CySSP), cysteinylglycine (CyGlySSP), homocysteine (HcySSP), \(\gamma\)-glutamylcysteine (\(\gamma\)GluCySSP) and glutathione (GSSP)] and the concentration of protein thiols.
Table S5. Markers of connective tissue damage measured in SOFIA

<table>
<thead>
<tr>
<th>Assay</th>
<th>Specifications</th>
<th>Measuring</th>
<th>Measured in</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1M</td>
<td>MMP-generated fragment of type I collagen</td>
<td>Inflammation related to synovial membrane remodeling</td>
<td>Serum</td>
<td>(1)</td>
</tr>
<tr>
<td>CTX-I</td>
<td>Cathepsin-generated and cross-linked fragment of type I collagen</td>
<td>Bone resorption</td>
<td>Serum</td>
<td>(2)</td>
</tr>
<tr>
<td>P1NP</td>
<td>N-terminal pro-peptide of type I collagen</td>
<td>Bone formation</td>
<td>Serum</td>
<td>(3)</td>
</tr>
<tr>
<td>C2M</td>
<td>MMP-generated fragment of type II collagen</td>
<td>Cartilage remodeling</td>
<td>Serum</td>
<td>(4)</td>
</tr>
<tr>
<td>C3M</td>
<td>MMP-generated fragment of type III collagen</td>
<td>Inflammation related to synovial membrane remodeling</td>
<td>Serum</td>
<td>(5)</td>
</tr>
<tr>
<td>CRPM</td>
<td>MMP-generated fragment of C-reactive protein</td>
<td>Local inflammation</td>
<td>Serum</td>
<td>(6)</td>
</tr>
<tr>
<td>C6M</td>
<td>MMP-generated fragment of type VI collagen</td>
<td>General fibrosis</td>
<td>Serum</td>
<td>(7)</td>
</tr>
<tr>
<td>VCANM</td>
<td>MMP-generated fragment of versican</td>
<td>Cardiovascular remodeling</td>
<td>Serum</td>
<td>(8)</td>
</tr>
<tr>
<td>MIM</td>
<td>MMP-generated fragment of mimecan</td>
<td>Cardiovascular remodeling</td>
<td>Serum</td>
<td>(9)</td>
</tr>
<tr>
<td>TIM</td>
<td>MMP-generated fragment of cardiac-specific titin</td>
<td>Cardiovascular remodeling</td>
<td>Serum</td>
<td>(10)</td>
</tr>
<tr>
<td>U-CTX-II</td>
<td>C-telopeptide of type II collagen</td>
<td>Cartilage remodeling</td>
<td>Urine</td>
<td>(11)</td>
</tr>
<tr>
<td>U-C3M</td>
<td>MMP-generated fragment of type III collagen</td>
<td>Renal fibrosis</td>
<td>Urine</td>
<td>(12)</td>
</tr>
<tr>
<td>U-C1M</td>
<td>MMP-generated fragment of type I collagen</td>
<td>Renal fibrosis</td>
<td>Urine</td>
<td>n/a</td>
</tr>
</tbody>
</table>
RESULTS

Demographics

A total of 30 AKU patients were recruited. Half of them were males. The mean age for all patients was 38.1 years ranging from 16 years to 67 years. Tables S6, S7 and S8 summarise the demographics of this cohort.

Table S6. Demographics of AKU cohort in SOFIA-age

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>N</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKU</td>
<td>M</td>
<td>15</td>
<td>36.9</td>
<td>13.2</td>
<td>20.0</td>
<td>35.0</td>
<td>67.0</td>
</tr>
<tr>
<td>AKU</td>
<td>F</td>
<td>15</td>
<td>39.4</td>
<td>14.7</td>
<td>16.0</td>
<td>43.0</td>
<td>62.0</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>30</td>
<td>38.1</td>
<td>13.8</td>
<td>16.0</td>
<td>36.5</td>
<td>67.0</td>
</tr>
</tbody>
</table>

Table S7. Demographics of AKU cohort in SOFIA-weight

<table>
<thead>
<tr>
<th>Gender</th>
<th>N</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>15</td>
<td>60.0</td>
<td>7.6</td>
<td>50.2</td>
<td>59.6</td>
<td>79.4</td>
</tr>
<tr>
<td>M</td>
<td>15</td>
<td>74.2</td>
<td>7.2</td>
<td>64.2</td>
<td>74.0</td>
<td>86.4</td>
</tr>
<tr>
<td>All</td>
<td>30</td>
<td>67.1</td>
<td>10.2</td>
<td>50.2</td>
<td>66.2</td>
<td>86.4</td>
</tr>
</tbody>
</table>

Table S8. Demographics of AKU cohort in SOFIA-BMI

<table>
<thead>
<tr>
<th>Gender</th>
<th>N</th>
<th>Mean</th>
<th>Std</th>
<th>Min</th>
<th>Median</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>15</td>
<td>23.0</td>
<td>2.8</td>
<td>18.9</td>
<td>22.8</td>
<td>29.3</td>
</tr>
<tr>
<td>M</td>
<td>15</td>
<td>25.3</td>
<td>3.3</td>
<td>18.8</td>
<td>25.3</td>
<td>30.8</td>
</tr>
<tr>
<td>All</td>
<td>30</td>
<td>24.1</td>
<td>3.2</td>
<td>18.8</td>
<td>24.0</td>
<td>30.8</td>
</tr>
</tbody>
</table>

Gait Analyses

Figure S7:A 1D self-organising neural network forced a linear clustering of AKU patients (red) and controls (blue). Their age is indicated by the size of circles.
Figure S8: A 2D scatter plot visualising the topological relationships between AKU patients (red) and controls (blue) as a function of their age (circle size).

Figure S9. 24-hour urine HGA plotted against age
Figure S10. Inflammatory and oxidative markers

- **PSH**: $497.1 - 1.54 \times \text{age for AKU}$ (age: $p=0.008$, group: $p=0.103$, $R^2 = 0.16$)
- **PSH**: $520.7 - 1.54 \times \text{age for Controls}$
- **CySSP**: $133.5 + 0.22 \times \text{age for AKU}$ (age: $p=0.383$, group: $p=0.209$, $R^2 = 0.04$)
- **CySSP**: $125.5 + 0.22 \times \text{age for Controls}$
- **HcySSP**: $3.76 + 0.11 \times \text{age for AKU}$ (age: $p=0.121$, group: $p=0.792$, $R^2 = 0.04$)
- **HcySSP**: $4.24 + 0.11 \times \text{age for Controls}$
- **γGluCySSP**: $0.81 - 0.01 \times \text{age for AKU}$ (age: $p=0.001$, group: $p=0.0827$)
- **γGluCySSP**: $0.54 - 0.00 \times \text{age for Controls}$ (age*group interaction: $p=0.008$, $R^2 = 0.19$)
- **GSSP**: $1.60 + 0.00 \times \text{age for AKU}$ (age: $p=0.907$, group: $p=0.395$, $R^2 = 0.01$)
- **GSSP**: $1.51 + 0.00 \times \text{age for Controls}$
- **RSSP**: $157.8 - 0.22 \times \text{age for AKU}$ (age: $p=0.446$, group: $p=0.348$, $R^2 = 0.03$)
- **RSSP**: $150.8 - 0.22 \times \text{age for Controls}$
Connective tissue biomarkers

Figure S11: Levels of the biomarkers plotted against age in AKU and control samples
C1M = 22.01 + 0.11 × age for AKU  (age:p=0.269, group:p=0.933, R²=0.02)

C1M = 22.23 + 0.11 × age for Controls

C2M = 0.43 + 0.00 × age for AKU  (age:p=0.335, group:p=0.458, R²=0.03)

C2M = 0.39 + 0.00 × age for Controls

C3M = 10.61 + 0.01 × age for AKU  (age:p=0.869, group:p=0.043, R²=0.07)

C3M = 12.33 + 0.01 × age for Control

C6M = 15.74 + 0.01 × age for AKU  (age:p=0.893, group:p=0.080, R²=0.05)

C6M = 18.42 + 0.01 × age for Controls

CRPM = 9.85 + 0.01 × age for AKU  (age:p=0.684, group:p=0.008, R²=0.12)

CRPM = 11.70 + 0.01 × age for Controls

CTX_I = 1.22 - 0.02 × age for AKU  (age:p<0.001, group:p=0.527, R²=0.22)

CTX_I = 1.15 - 0.02 × age for Controls

MIM = 26.57 + 0.11 × age for AKU  (age:p=0.543, group:p=0.634, R²=0.01)

MIM = 28.84 + 0.11 × age for Controls

VCANM = 1.40 + 0.00 × age for AKU  (age:p=0.152, group:p=0.343, R²=0.05)

VCANM = 1.47 + 0.00 × age for Controls

uC1M = 15.57 - 0.09 × age for AKU  (age:p=0.195, group:p=0.003, R²=0.16)

uC1M = 10.29 - 0.09 × age for Controls

uC3M = 606 + 0.16 × age for AKU  (age:p=0.953, group:p=0.001, R²=0.48)

uC3M = 120 + 0.16 × age for Controls

uCTX_II = -4.19 + 0.27 × age for AKU  (age:p<0.001, group:p=0.202, age*group interaction:p=0.034, R²=0.45)**

uCTX_II = 1.27 + 0.06 × age for Controls

uC3M_cr = 107 - 0.79 × age for AKU  (age:p=0.121, group:p<0.001, R²=0.35)

uC3M_cr = 40 - 0.79 × age for Controls

uCTX_II_cr = 2.19 - 0.02 × age for AKU  (age:p=0.091, group:p=0.011, R²=0.14)

uCTX_II_cr = 1.30 - 0.02 × age for Controls

** For patients with age > 25
Quality of Life

**HAQ**

A total of thirty patients completed the HAQ questionnaire. This index considers how arthritis has an impact on everyday life. Total score is between 0–3.0, in 0.125 increments. Increasing scores indicate worse functioning (score 0=no functional impairment; score 3=complete impairment). These scores were correlated with age. The sub-figures in Figure S11 show a clear pattern: as AKU patients become older, they struggle with their Activities of Daily Living (ADLs); with the exception of “eating” all other domains show significant correlation with age. Sub-Figure, HAQ-Pain, shows that as patients get older, the burden of AKU arthritis pain increases and the debilitating effects of the ochronosis become overt (Sub-Figure, HAQ-Disability Index).

**SF36**

A total of 29 patients completed this questionnaire. Correlations with age are shown in the sub-figures of Figure S12. All questions are scored on a scale from 0 to 100 (highest level of functioning possible=100, complete loss of function=0). It is clear that as AKU patients get older, their energy levels and physical functions decrease. In addition, their general health deteriorates and they experience more pain. Nonetheless, emotionally, they remain resilient (Sub-Figure, SF36-Limitations due to emotional problems).

**KOOS**

Twenty seven patients completed this questionnaire. Correlations with age are shown in the sub-figures of Figure S13. As seen with the previous two tools, there is a clear deterioration in QoL, across all domains, with age. This can be detected as early as 35 year of age.
Figure S12: HAQ
Figure S13: SF_36

[Graphs showing SF36 outcomes (Emotional well-being, Emotional functioning, Energy/Fatigue, Social functioning, Pain, General Health) vs Age with gender differentiation.]
Figure S14: KOOS
REFERENCES


5th October 2018

The Editor
BMJ Innovations

Dear Sir/madam

I submit this manuscript entitled ‘Subclinical Ochronosis Features In Alkaptonuria: A Cross-Sectional Study’ on behalf of my colleagues (and my co-authors) for consideration of publication in the British Medical Journal Innovations. All of my co-authors have contributed to the manuscript. Since a disease modifying therapy is now available for alkaptonuria, we believe that this paper is timely in addressing the age to begin this disease modifying therapy.

Yours sincerely

Dr Lakshminarayan Ranganath
Chief Investigator in SOFIA
Fig 1

169x169mm (72 x 72 DPI)
Fig 2

169x169mm (72 x 72 DPI)
Fig 3

169x169mm (72 x 72 DPI)
Fig 4

169x169mm (72 x 72 DPI)
Fig 5

169x169mm (72 x 72 DPI)
SFig 1 - Supplementary Fig 1

75x49mm (72 x 72 DPI)
SFig 3 - Supplementary Fig 3

111x111mm (72 x 72 DPI)
SFig 4 - Supplementary Fig 4

168x95mm (72 x 72 DPI)
SFig 5 a, b, c - Supplementary Fig 5 a, b, c

115x94mm (72 x 72 DPI)
Sfig 6 - Supplementary Fig 6

104x131mm (72 x 72 DPI)
SFig 7 - Supplementary Fig 7

155x20mm (72 x 72 DPI)
SFig 8 - Supplementary Fig 8

106x84mm (72 x 72 DPI)
24 Hour Urine HGA vs Age

SFig 9 - Supplementary Fig 9

169x169mm (72 x 72 DPI)
SFig 10 - Supplementary Fig 10

169x169mm (72 x 72 DPI)
SFig 11 - Supplementary Fig 11

169x169mm (72 x 72 DPI)
SFig 11.1 - Supplementary Fig 11.1

169x169mm (72 x 72 DPI)
SFig 11.2 - Supplementary Fig 11.2

169x169mm (72 x 72 DPI)
SFig 12 - Supplementary Fig 12

169x229mm (72 x 72 DPI)
SFig 13 - Supplementary Fig 13

169x176mm (72 x 72 DPI)
SFig 14 - Supplementary Fig 14

169x176mm (72 x 72 DPI)