ORIGINAL RESEARCH

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 patients with AKU 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 woman implying ochronosis can ensue.11

Conclusions Ochronosis can be present before age 20 years.

BACKGROUND

Alkaptonuria (AKU) (OMIM #203500) is a rare genetic deficiency of homogentisate dioxygenase (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 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, and 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.5–8 Nitisinone decreases circulating HGA, and 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.12 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
Diagnostics

in residual disease as AKU is irreversible. 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

Patients with AKU, verified by elevated HGA levels, and at least 16 years old were eligible for inclusion. A non-AKU control group was also selected. None of the patients in this study received nitisinone at the time of participation.

Study design

SOFIA was an open, cross-sectional, single-site (Royal Liverpool University Hospital) study; 32 patients with AKU were to be recruited (two males, two 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 (QoL).

Assessments

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

Standardised photographs of eyes and ears were scored for ochronosis: for eyes using qualitative and quantitative systems; for ears, a qualitative system (online 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 (online supplementary figures S2–S5, table S3). Institutional MRI protocols were followed.

3D gait analysis was performed for 36 patients with AKU (29 from SOFIA, 7 from the UK National Alkaptonuria Centre) and for a control group of 10 volunteers free from gait problems. Mean deviation of AKU gait from normality (MDP mean) was calculated using the MDP program (online supplementary material, online supplementary figure S6).

Disease questionnaires were used to calculate the modified questionnaire-based qAKUSSI (online supplementary table S4).

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 methods. Serum amyloid A (SAA) was analysed using a commercial assay. Quantitative determination of serum protein thiol and S-thiolated proteins was carried out. Serum protein thiol concentrations were measured by colorimetric reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). 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), cysteynglycine (CyGlySSP), homocysteine (HcySSP), γ-glutamylcysteine (γGluCySSP) and glutathione (GSSP)) and the concentration of protein thiols.

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 (online supplementary table S5). The assays were run according to the standard procedure detailed in the references in online supplementary table S5.

QoL was assessed using HAQ, SF-36 and Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaires.

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 V.9.3 and R V.3.3.2.

Results

Thirty patients with AKU (15 male, 15 female) were recruited. Mean age was 39 years for males and 37 years for females; mean body mass index (BMI) was 25 kg/m² for males and 23 kg/m² for females (online 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 1A 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, R²=0.39).

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

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

- Eye pigmentation = $-7.3 + 0.36 \times \text{age}$ for males (age: $p<0.001$, gender: $p=0.448$, $R^2=0.50$).
- Eye pigmentation = $-8.7 + 0.36 \times \text{age}$ for females.

Age was statistically significant, but gender was not. Figure 1D 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 patients with AKU, respectively. Disc degeneration, measured using the modified Pfirrmann score, is shown in figure 2A and marrow oedema results, indicated by the modified SPARCC score, are shown in figure 2B. Knee scores are shown in figure 2C. 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 generally stay low over the age range with a few exceptions.

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

- $\text{MDP mean} = 2.44 - 0.01 \times \text{age}$ for controls.

Online supplementary figures S7 and S8 show a linear clustering and a 2D visualisation of the patients with AKU and controls.

Modified qAKUSSI

Figure 3A shows total modified qAKUSSI plotted against age; figure 3B–D shows its component parts of clinical features, spine rheumatology and non-spine rheumatology. The corresponding regressions are:
Figure 2  (A) MRI Pfirrmann scores plotted against age, (B) Spondyloarthritis Research Consortium of Canada (SPARCC) scores plotted against age, (C) whole-organ MRI score (WORMS) plotted against age, (D) mean movement deviation profile (MDP\textsubscript{mean}) plotted against age. AKU, alkaptonuria.

\begin{align*}
q_{\text{AKU_SI}} &= -8.53 + 1.15 \times \text{age} \text{ for males} \\
& (\text{age: } p<0.001, \text{ gender: } p=0.780, \ R^2=0.47); \\
q_{\text{AKU_SI}} &= -10.41 + 1.15 \times \text{age} \text{ for females}.
\end{align*}

\begin{align*}
q_{\text{AKU_SI\_clin}} &= -7.31 + 0.67 \times \text{age} \text{ for males} \\
& (\text{age: } p=0.001, \text{ gender: } p=0.316, \ R^2=0.36); \\
q_{\text{AKU_SI\_clin}} &= -12.35 + 0.67 \times \text{age} \text{ for females}.
\end{align*}

\begin{align*}
q_{\text{AKU_SI\_spine}} &= -1.32 + 0.22 \times \text{age} \text{ for males} \\
& (\text{age: } p=0.002, \text{ gender: } p=0.076, \ R^2=0.35); \\
q_{\text{AKU_SI\_spine}} &= 1.91 + 0.22 \times \text{age} \text{ for females}.
\end{align*}

\begin{align*}
q_{\text{AKU_SI\_nonspine}} &= 0.05 + 0.26 \times \text{age} \text{ for males} \\
& (\text{age: } p=0.017, \text{ gender: } p=0.995, \ R^2=0.20); \\
q_{\text{AKU_SI\_nonspine}} &= 0.03 + 0.26 \times \text{age} \text{ for females}.
\end{align*}

There were no significant differences for gender; all regressions showed a significant increase with age.

**HGA, biomarkers and metabolite analyses**

Figure 4 shows some results obtained for HGA measurements, the inflammation biomarker, SAA and connective tissue damage markers. Figure 4A,B shows serum HGA and HGA clearance against age. The regression equations are:

\begin{align*}
\text{Serum\_HGA} &= 17.4 + 0.28 \times \text{age} \text{ for males} \\
& (\text{age: } p=0.006, \text{ gender: } p=0.465, \ R^2=0.26).
\end{align*}

\begin{align*}
\text{Serum\_HGA} &= 19.2 + 0.28 \times \text{age} \text{ for females}.
\end{align*}

\begin{align*}
\text{HGA\_clearance} &= 1322 - 11.8 \times \text{age} \text{ for males} \\
& (\text{age: } p=0.007, \text{ gender: } p=0.934, \ R^2=0.26).
\end{align*}

\begin{align*}
\text{HGA\_clearance} &= 1313 - 11.8 \times \text{age} \text{ for females}.
\end{align*}

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

Figure 4C–E shows 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. \(19-22\)

\begin{align*}
\text{SAA} &= 8.5 + 0.43 \times \text{age} \text{ for AKU} \ (\text{age: } p=0.047, \text{ group: } p=0.667, \ R^2=0.07) *
\end{align*}

\begin{align*}
\text{SAA} &= 10.8 + 0.43 \times \text{age} \text{ for controls}
\end{align*}

\begin{align*}
\text{CyGlySSP} &= 22.1 - 0.20 \times \text{age} \text{ for AKU} \ (\text{age: } p<0.001, \text{ group: } p=0.005) \\
\text{CyGlySSP} &= 25.4 + 0.04 \times \text{age} \text{ for controls} \\
& (\text{age\times} \text{group interaction: } p=0.002, \ R^2=0.25).
\end{align*}

\begin{align*}
\text{PTI} &= 0.31 + 0.002 \times \text{age} \text{ for AKU} \ (\text{age: } p=0.015, \text{ group: } p=0.090, \ R^2=0.14).
\end{align*}

\begin{align*}
\text{PTI} &= 0.28 + 0.002 \times \text{age} \text{ for controls}.
\end{align*}
Figure 3  Modified qualitative Alkaptonuria Severity Score Index (qAKUSSI): (A) Total modified qAKUSSI; (B) qAKUSSI, clinical features; (C) aAKUSSI, spine rheumatology; (D) qAKUSSI, non-spine rheumatology.

*Two outliers removed.

Online supplementary figure S10 shows extra inflammatory and oxidative markers.

Figure 4F–H shows 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 patients with AKU and controls at the same rate, but with mean P1NP higher for patients with AKU. uC1M_cr=3.59–0.05×age for AKU (age: p=0.004, group: p=0.004, uC1M_cr=1.65–0.03×age for controls (age×group interaction: p=0.056, R²=0.30).

TIM=157+4.09×age for AKU (age: p=0.047, group: p=0.326).

TIM=223+0.52×age for controls (age×group interaction: p=0.039, R²=0.30).

P1NP=172–1.43×age for AKU (age: p=0.022, group: p=0.002, R²=0.22).

P1NP=121–1.43×age for controls.

Online supplementary figure S11 shows the biomarkers not covered in the main text, plotted against age for patients with AKU and controls.

Quality of life

Figure 5 shows 6 of the 33 QoL measurements: Health assessment questionnaire (HAQ) Disability Index, HAQ Pain score, Short-Form Health Survey (SF)-36 Physical Functioning score, SF-36 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, R²=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, R²=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, R²=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, R²=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, R²=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, R²=0.45)

KOOS_QoL=117.0–1.37×age for females.
All these measures show significant worsening of health with age but no difference between genders. The other QoL measures are reported in online supplementary figures S12–S14.

DISCUSSION

The SOFIA study objective was to identify the earliest age when ochronosis, microscopic and macroscopic, can be detected in patients with AKU. First, we review the published information in childhood AKU. It is important to clarify that none of the patients in this study received nitisinone at the time of participation.

A natural history study in 2002 reported on 64 patients (ages 4 to 80 years), but described no clinical features in childhood. A Slovak study described childhood AKU 35 years ago in 39 patients. 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 patient presented with a kidney stone.

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 patient.

Figure 4  (A) Serum homogentisic acid (HGA) (μmol/L), (B) HGA clearance (mL/min), (C) serum amyloid A (SAA) (ng/mL), (D) CyGlySSP (μM), (E) Protein Thiolation Index (PTI), (F) urine C1M_cr (ng/μmol), (G) serum TIM (ng/mL), (H) serum P1NP (ng/mL). AKU, alkaptonuria.
female, so ochronosis can start in patients with AKU 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.

Patients with AKU 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, coexisting 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 patients with AKU and controls. Both circulating SAA (inflammation marker) and PTI (oxidative stress marker) increased with age with no significant difference between patients with AKU and controls, confirming previous findings in AKU. However, the highest SAA concentrations were found in younger subjects (CTR 29 years, SAA 132 mg/L; AKU 20 years, SAA 121 mg/L). Additionally, in both groups, SAA was above the reference threshold of 10 mg/L in 21/30 subjects. Since no additional data apart from gender and age were made available on control subjects, we cannot rule out the hypothesis of underlying inflammatory conditions, however unlikely, raising SAA levels also in controls, for example, BMI; this, however, again excludes a major role for SAA in AKU.

uC1M, a marker of collagen type I degradation measured in urine, decreased with age in patients with

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**Figure 5** Quality of life (QoL) scores: (A) HAQ Disability Index, (B) HAQ Pain score, (C) SF-36 Physical Functioning score, (D) SF-36 General Health score, (E) Knee injury and Osteoarthritis Outcome Score (KOOS) Sport and Recreation score, (F) KOOS Quality of Life score.
AKU, 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. This hypothesis needs further investigation. TIM, a marker of MMP-mediated titin degradation describing cardiac remodelling, increased with age in patients with AKU 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.

Identifying ochronosis externally by examining the eyes and ears does not tell us about what is happening inside the body. It is possible that highly stressed tissues of the musculoskeletal system could be affected by ochronosis earlier but not easily accessible to assessment. Conventional imaging is not sensitive enough to identify internal ochronosis early as the changes are late; newer approaches such as Raman spectroscopy which are non-invasive may provide solutions. We were hoping to find biomarkers that could inform on potential changes in connective tissue in this study. The usefulness of such markers is limited in advanced AKU where there are other revealing features. In early AKU, that is, in young patients, biomarkers are potentially more likely to prove valuable to monitor the disease, as well as inform when the disease has taken hold. Unfortunately, in the young, the musculoskeletal system is changing enormously due to physiological growth, as well as the gender differences in growth, maturation and development. In the present study, the low numbers of young patients with AKU did not allow a clear distinction between AKU and non-AKU controls, even though there was an apparent difference. The formation and deposition of the ochronotic pigment in the cartilaginous tissue renders the cartilage stiffer and would cause an increased remodelling of proteins from the connective tissue of cartilage, bone and possibly other soft tissues. The examined markers of connective tissue remodelling, however, fail to show a direct correlation with initiation of ochronosis. TIM, a marker of titin degradation reflecting a remodelling of the cardiac sarcomere, could be an indirect marker of ochronosis of the cardiac valve, causing impairment in the cardiac function. AKU is an ultrarare disease with a frequency of 1 in 250 000. This is the first study to examine a wide variety of AKU features in a cohort of sufficient size to provide reliable data. Bone, cartilage and tissue markers are likely to be beneficial if they can be validated in a larger young cohort of patients with AKU matched to a normal cohort for age and gender. Plans for this further study are advanced and likely to begin shortly in the SOFIA-Paediatric study.

The modified Pfirrmann spine scores on MRI analysis show patients with AKU 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. Marrow oedema lesion results (modified SPARCC scores) also appear to significantly rise from age of 30 years. Degeneration of the knee (WORMS) did not appear to be associated with age up to the middle of the fourth decade. Despite the lack of change in spine and joints at ages younger than 30 years, the gait analysis was abnormal early on. The mean deviation profiles (MDPs) for gait was significantly higher for patients with AKU than for controls. The younger patients with AKU 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 patients with AKU get older, they score higher on qAKUSSI regardless of their gender, reflecting a worsening disease burden.

Three QOL questionnaires showed pain increases and general QoL decreases with age for patients with AKU. 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 SF-36 questionnaire, physical functions steadily decrease with age. For the KOOS questionnaire, the functions decrease with age. Overall, the QoL 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 are 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. With biochemistry similar to humans, 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.
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