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Ochronosis in a murine model of alkaptonuria is synonymous to that in the human condition

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SUMMARY

Objective: Alkaptonuria (AKU) is a rare genetic disease which results in severe early onset osteo-arthropathy. It has recently been shown that the subchondral interface is of key significance in disease pathogenesis. Human surgical tissues are often beyond this initial stage and there is no published murine model of pathogenesis, to study the natural history of the disease. The murine genotype exists but it has been reported not to demonstrate ochronotic osteoarthropathy consistent with the human disease. Recent anecdotal evidence of macroscopic renal ochronosis in a mouse model of tyrosinaemia led us to perform histological analysis of tissues of these mice that are known to be affected in human AKU.
Design: The homogentisate 1,2-dioxygenase $Hgd^{+/-}Fah^{-/-}$ mouse can model either hereditary tyrosi-naemia type I (HT1) or AKU depending on selection conditions. Mice having undergone Hgd reversion were sacrificed at various time points, and their tissues taken for histological analysis. Sections were stained with haematoxylin eosin (H&E) and Schmorl's reagent.
Results: Early time point observations at 8 months showed no sign of macroscopic ochronosis of tissues. Macroscopic examination at 13 months revealed ochronosis of the kidneys. Microscopic analysis of the kidneys revealed large pigmented nodules displaying distinct ochre colouration. Close microscopic examination of the distal femur and proximal fibula at the subchondral junctions revealed the presence of numerous pigmented chondrocytes.
Conclusions: Here we present the first data showing ochronosis of tissues in a murine model of AKU. These preliminary histological observations provide a stimulus for further studies into the natural history of the disease to provide a greater understanding of this class of arthropathy.

Introduction

Alkaptonuria (AKU) is a rare autosomal recessive condition that is caused by a single enzyme deficiency in the tyrosine metabolic pathway[1]. The absent enzyme is homogentisate 1,2-dioxygenase (HGD), which is responsible for the conversion of homogentisic acid (HGA) to 4-maleylacetoacetic acid (MAA)[2]. Enzyme loss results in accumulation of HGA in both tissues and circulating blood[1]. HGA shows a particularly high affinity for collagenous tissues; initial deposits are associated with the periodicity of collagen fibres[3]. Between the third and fourth decade of life, accumulation of HGA polymers begins to manifest in joint disorders, culminating in devastating arthropathy. Furthermore, HGA-associated pigment known as ochronosis has detrimental effects on other collagenous matrices in the body[4,5]. Ochronosis of cartilage was recently shown to be initiated in individual chondrocytes and their territorial matrix, before progressing to a more widespread pigmentation of the cartilage matrix[6]. Elucidation of the mechanism by which ochronosis begins and progresses will provide new targets for intervention. It has been suggested that 2-[2-nitro-4-(tri-fluoromethyl)benzoyl]cyclohexane-1,3-dione (NTBC), the thera-peutic agent used in treating a related tyrosine catabolic disorder hereditary tyrosinaemia type I (HT1), may prove beneficial in treating AKU by blocking the production of HGA in the breakdown of tyrosine[7] (Fig. 1). The efficacy and safety associated with the use of this drug to treat AKU has yet to be determined, but testing would be facilitated by access to a mouse model of joint pathology.

A murine model of AKU was generated in 1994 by ENU-induced mutagenesis[8]. The AKU mutation was then backcrossed onto both

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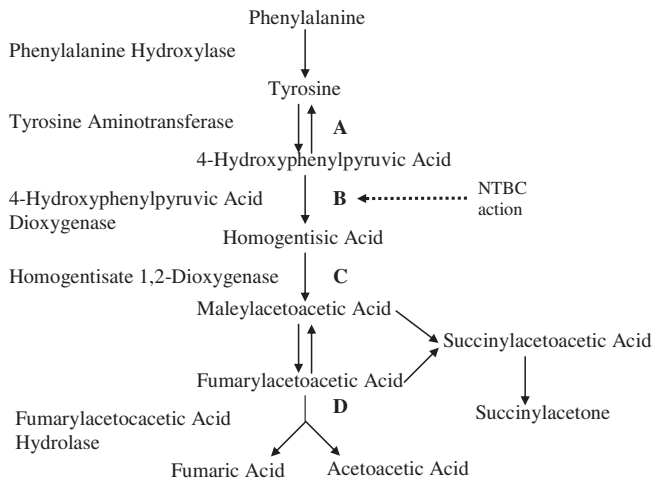
Fig. 1. Diagram of the phenylalanine and tyrosine metabolic pathway highlighting enzymes and associated defects. A = Tyrosinemia type II, B = Tyrosinemia type III, C = AKU, D = Tyrosinemia type I. NTBC is shown at its site of action.

the BALB/cByJ and the C57/BL/6J backgrounds. These animals were previously thought not to exhibit ochronosis, despite excreting sufficient HGA to cause darkening of urine[9]. The mice have truncated HGD protein resulting from a splice mutation[8].

The related tyrosine disorder HT1 is caused by a deficiency of (Fah) — the terminal enzyme in the pathway — that causes progressive liver disease and renal tubular dysfunction[10]. In both HT1 patients and Fah$^{-/-}$ mice, toxic metabolites like fumarylacetoacetate (FAA) accumulate causing death in a cell-autonomous manner. Treatment with NTBC, an inhibitor that blocks upstream of FAH, prevents metabolite accumulation and rescues the phenotype[11]. Hgd$^{-/-}$ and FAA hydrolase (Fah) deficient mice have been crossed for several generations to produce Hgd$^{-/-}$Fah$^{-/-}$ mice[12]. Interestingly, some mice with the intermediate genotype Hgd$^{+/-}$Fah$^{-/-}$, were resistant to liver failure characteristic of HT1 when withdrawn from NTBC. Histological examination showed healthy liver nodules in these mice, suggesting reversion had occurred at the Hgd locus. The reversion is thought to arise as a response to the production of reactive compounds in the liver and kidneys, namely FAA, and other subsequent spontaneously formed derivatives. Individual hepatocytes that revert to the double knockout genotype have a selective advantage within the liver as they do not produce the toxic derivatives[12]. The accumulation of such hepatotoxic compounds can be prevented by NTBC administration. Hgd$^{+/-}$Fah$^{-/-}$ neonates must nurse from a mother on NTBC as neonates are acutely sensitive to HT1-related hepatotoxin accumulation during this developmental window and will die from acute liver failure within 24 h without NTBC from the breast milk. However, when Hgd$^{+/-}$Fah$^{-/-}$ adult mice are withdrawn from NTBC, a small percentage will undergo a loss of heterozygosity (LOH) event at the Hgd locus generating an Hgd$^{-/-}$Fah$^{-/-}$ hepatocyte that is resistant to effects of the FAH deficiency. These reverted hepatocytes now have a selective advantage and can clonally expand to repopulate the liver. As a consequence, the block to HGD leads to an accumulation of HGA in the liver and blood stream.

Other models of ochronosis have been reported, but are generally not useful or viable for experimental studies[13,14]. Following anecdotal descriptions of ochronosis in Hgd$^{+/-}$Fah$^{-/-}$ mice withdrawn from NTBC, we undertook both a macro- and microscopic study of their tissues. Here we describe the first detection of ochronosis in a murine model of AKU and similarities between the initial stages of pigmentation in the murine model and those of the human condition. We expect that this murine model will prove beneficial for screening therapeutic agents for treating the condition.

Materials and methods

Mouse strains and animal husbandry

Hgd$^{-/-}$ and Fah$^{-/-}$ mice were bred to generate Hgd$^{+/-}$Fah$^{-/-}$ mice. The Hgd mutation was generated by an ENU-mutagenesis screen[8], while the Fah mutation was generated by neo-cassette insertion into exon 5[15]. The resultant Hgd$^{+/-}$Fah$^{-/-}$ can model either HT1 or AKU depending on selection conditions from the time spent off NTBC. Hgd$^{+/-}$Fah$^{-/-}$ neonates will die from acute HT1−related liver failure if NTBC is not continually administered. NTBC treatment at 4-mg/L in the drinking water prevents hepatorenal injury and rescues the phenotype. Short-term NTBC withdrawal allows modelling of HT1 as mice rapidly develop hepatorenal disease over the course of several weeks and die of end-stage liver disease in 6−8 weeks[10]. Long-term NTBC withdrawal can stimulate LOH at the Hgd locus resulting in mice with an Hgd$^{-/-}$Fah$^{-/-}$ genotype. These mice model AKU and are protected from HT1-related damage from the upstream Hgd mutation[9]. Depending on the selection conditions desired, mice were maintained on either 4-mg/L NTBC in the drinking water or water with no added drug, and fed irradiated high-fat low-protein mouse chow (Lab Diet Cat#Picolab 5LJ5) ad libitum to decrease flux through the tyrosine pathway. The Institutional Animal Care and Use Committee of Oregon Health & Science University approved all procedures and mouse experiments.

Histology

Hgd$^{+/-}$ Fah$^{-/-}$ mice having undergone Hgd reversion were sacrificed at various time points; one mouse (male) at 8 months and six mice (four males and two females) at 13 months, and their tissues taken for analysis. Kidneys were sectioned through the midline, dividing into an anterior and posterior half prior to processing. Tissue samples were washed in phosphate buffer saline (PBS) and then fixed in 10% phosphate buffered formol saline (PBFS). Mineralized tissues were decalcified with 12% ethylenediaminetetraacetic acid (EDTA) in 10% formalin. Following fixation and/or decalcification, tissues were routinely processed for histology and paraffin embedded. Five-micrometre sections were cut and mounted on glass slides, knees were sectioned transversely from lateral to medial. Sections were stained with either haematoxylin eosin (H&E) (every third section) or Schmorl's reagent (every fourth section) which we have previously shown to be a sensitive stain for tissue ochronosis[16]. Stained sections were dehydrated and mounted in Dibutyl phthalate in Xylene (DPX) (Sigma, UK).

Results

Macroscopic findings

Early time point observations of survivors at 8 months off NTBC showed no sign of macroscopically visible ochronosis to the skin, eyes, ears, femur, knee joint, vertebrae or various cartilages. An examination of the liver showed a mosaic pattern of nodules of relatively normal tissue among areas of damage. Histological analysis of early time point animals was not undertaken.

Interestingly, macroscopic examination of the liver at 13 months off NTBC revealed yellow-brown discolouration with signs of
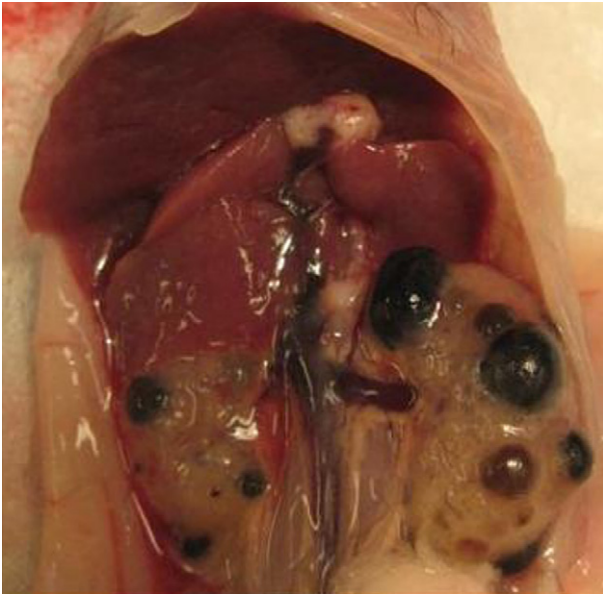
Fig. 2. Macroscopic image of large ochronotic renal nodules from an $Hgd^{-/-}Fah^{-/-}$ (reverted from Hgd$^{+/-}$ Fah$^{-/-}$) mouse harvested after 13 months off NTBC. Dark deposits can be seen on both kidneys. Note the pale discolouration of the surrounding non-ochronotic renal tissues.

necrosis and inflammation. Deeper dissection and examination of the retroperitoneal structures revealed large nodules on both kidneys (Fig. 2). These nodules were black, indicative of ochronosis. In the regions of the kidneys not affected by ochronosis, necrosis and inflammation were observed. Following kidney dissection, macroscopic observations showed pigmented nodules in the renal columns, cortices and medulla. There were no grossly visible signs of ochronosis in the skin, ears, adipose tissue in the abdominal cavity, digestive tract, vertebral column, weight-bearing joints or

other large cartilages at 13 months. Additionally, tendons inserting into the large joints, as well as heart and aortic vasculature revealed no signs macroscopically of brown colouring that would imply ochronotic pigmentation.

Microscopic findings

Renal system

Microscopic analysis of the kidneys revealed a large inflammatory reaction across the organ. Large pigmented nodules within the kidneys displayed distinct ochre colouration (Fig. 3A). Within and surrounding these nodules were abundant mononuclear cells, all of which showed dense intracellular pigmentation in their cytoplasm. Within these cells was uniform ochronotic pigmentation along with some easily identifiable dense crystals. Schmorl's staining of near serial sections confirmed the presence of ochronotic pigmentation within the cells (Fig. 3B).

A cross section of the renal tubules showed the presence of both pigmented urine and crystals within collecting ducts, distal, proximal and thin tubules by H&E (Fig. 4A, C). Glomeruli lacked signs of ochronosis. Schmorl's staining of near serial sections of these areas confirmed the presence of ochronosis within these structures (Fig. 4B). Interestingly Schmorl's staining also identified early pigmentation in some of the epithelial cells in distal tubules, not identified by H&E. Within these tubules, mononuclear cells with pigmented cytoplasm could also be seen.

Examination of the renal extracellular matrix showed no evidence of ochronosis by H&E. However, when near serial sections were stained with Schmorl's reagent, low levels of early pigmentation were detected in and peripheral to the regions where the densely pigmented nodules were found (Fig. 4D).

Musculoskeletal system

Low power microscopic examination of the knee joints revealed normal bone and cartilage architecture with no evidence of dense ochronotic pigmentation of the articular cartilage matrix at any of
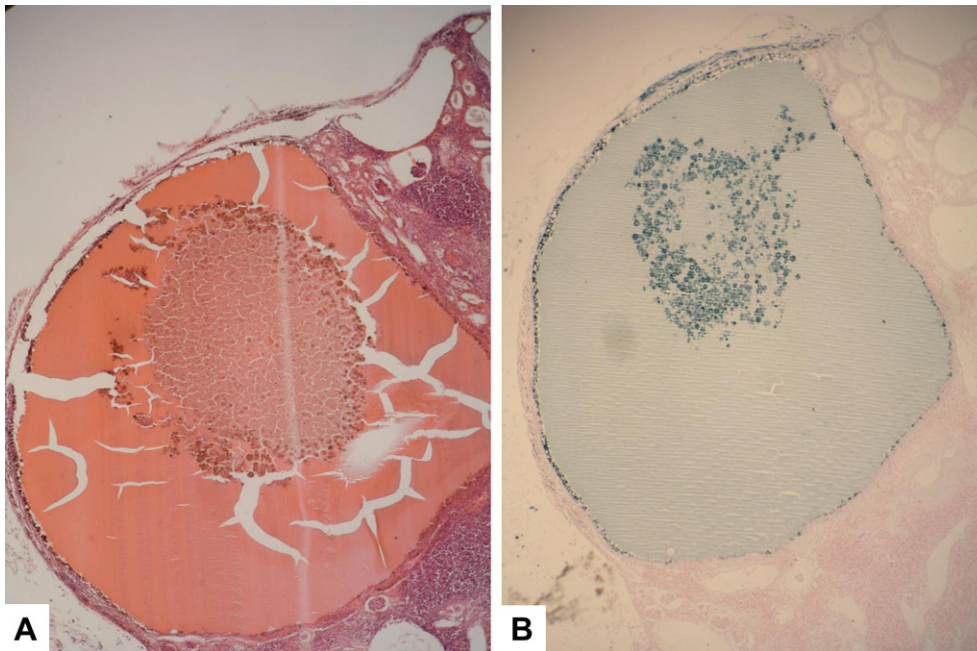

Fig. 3. Near serial kidney sections from an $Hgd^{-/-}Fah^{-/-}$ (reverted from Hgd$^{+/-}$ Fah$^{-/-}$) mouse harvested after 13 months off NTBC. (A) Dense ochronosis can be seen in the nodules formed in the distended tubules by H&E. Pigmentation is seen both within the nodule itself and lining the periphery. (B) Confirmation of HGA presence and its polymeric ochronotic derivative in these structures and the cells within is visible by Schmorl's stain. Field widths 0.84 mm.
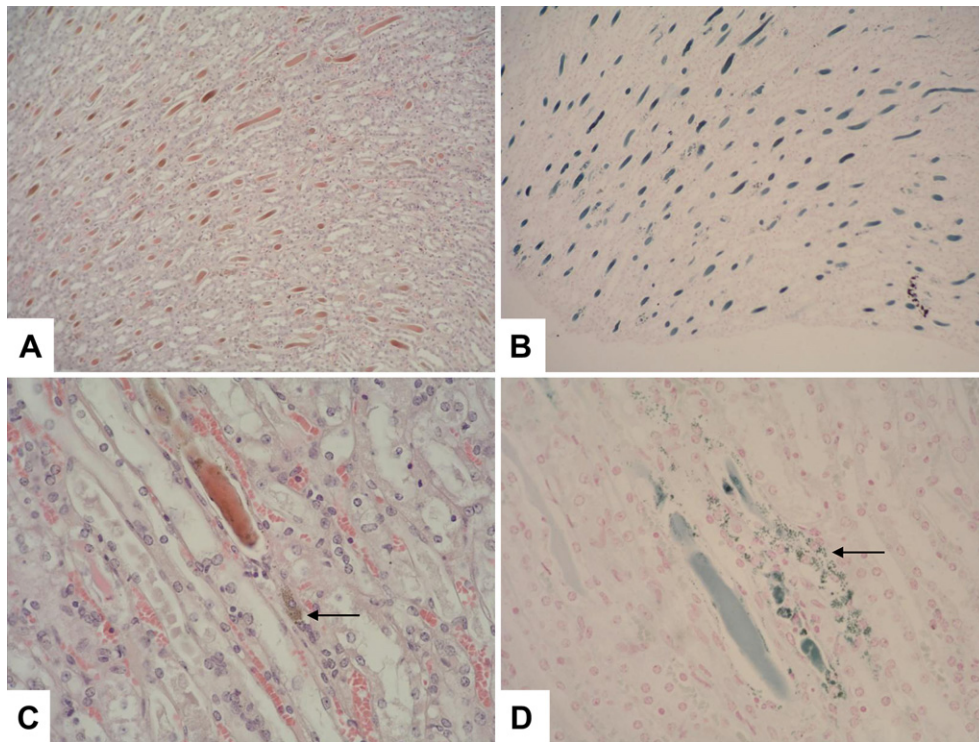
Fig. 4. Near serial sections showing the presence of polymerized HGA urine in the tubules of the kidneys from $Hgd^{-/-}Fah^{-/-}$ (reverted from $Hgd^{+/-} Fah^{-/-}$) mice. (A) Presence of numerous ochronotic deposits across the kidney by H&E. (B) Schmorl's staining confirming the presence of ochronotic deposits within the tubules. (C) Intra-tubular and intracellular deposition (arrow) within the cells by H&E. (D) Schmorl's staining confirming the presence of ochronotic pigmentation within the tubules and intracellular deposits. Many smaller deposits associated with the surrounding cells and possibly the collagenous matrices are present (arrow). (Field widths: A & B 1.2 mm, C & D 0.3 mm).

the joint surfaces. However, closer examination of the distal femur at the subchondral junction revealed the presence of numerous pigmented chondrocytes (Fig. 5). In many cases, these cells were necrotic or dead; they occupied a smaller fraction of their lacunae than their non-ochronotic neighbouring cells. It was also difficult to distinguish nucleus from cytoplasm in the ochronotic cells. They displayed dense granular pigmentation intracellularly and more uniform pigmentation of the territorial matrix. Numerous groups of
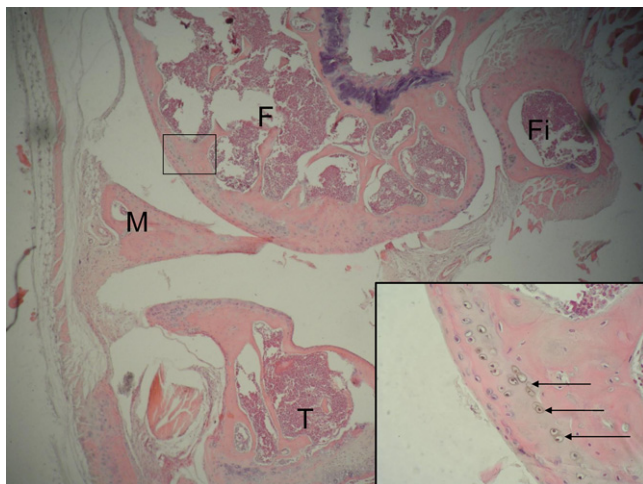
Fig. 5. Histological section showing the overview of a knee joint from an $Hgd^{-/-}Fah^{-/-}$ (reverted from $Hgd^{+/-} Fah^{-/-}$) mouse. No obvious signs of ochronosis are present in the cartilages of the knee joint. F = Femur, M = Meniscus, T = Tibia, Fi = Fibula. **Inset**: initial ochronosis associated with the pericellular matrix of the deep chondrocytes (arrows). Intracellular deposits can also be seen. Note the absence of pigmentation from the interterritorial matrix and bone matrix (Field width 3 mm).

chondrocytes with pericellular pigmentation were observed in close proximity. In the interterritorial matrix between these pigmented groups, there was no extracellular pigmentation associated with the cartilage matrix. There were also numerous chondrocytes in these regions that showed intracellular pigmentation without the pericellular pigmentation (Fig. 5 inset). The majority of the latter chondrocytes were still alive, with a clearly identifiable nucleus. Superficial to these were chondrocytes that displayed intracellular and faint pericellular pigmentation. At the articular surface, no sign of pigmentation was visible, either intra- or extra-cellularly. Deep to these chondrocytes, the bone was examined for the presence of ochronotic pigmentation, however no comparable pigmentation was seen within the calcified matrix, nor in the osteocytes, osteoblasts or osteoclasts examined.

Similar histological examination was undertaken in the proximal tibia and fibula, alongside the distal femur. Ochronotic pigment was seen both intracellularly and pericellularly (Fig. 6A). The osteocytes located in the bone beneath these pigmented cells were free of pigmentation (Fig. 6B). In articular regions, there was identical presentation of ochronotic pigmentation in the deep chondrocytes, as well as intra- and pericellular pigmentation of the more superficial chondrocytes. There was no form of pigmentation at the articular surface. The entheses inserting into the bones of the knee joint were also examined, but displayed no sign of ochronosis. These same regions were then examined using Schmorl's reagent. Schmorl's stain highlighted the presence of early pigment associated with the chondrocytes in the fibrocartilaginous insertion of the entheses (Fig. 6C), which was not detected by H&E. Dense pigmentation was associated with the deep chondrocytes and their pericellular matrix. It also showed the presence of pigment in and around the more superficial chondrocytes (Fig. 6D). Interestingly, this staining also confirmed the absence of pigmentation from the
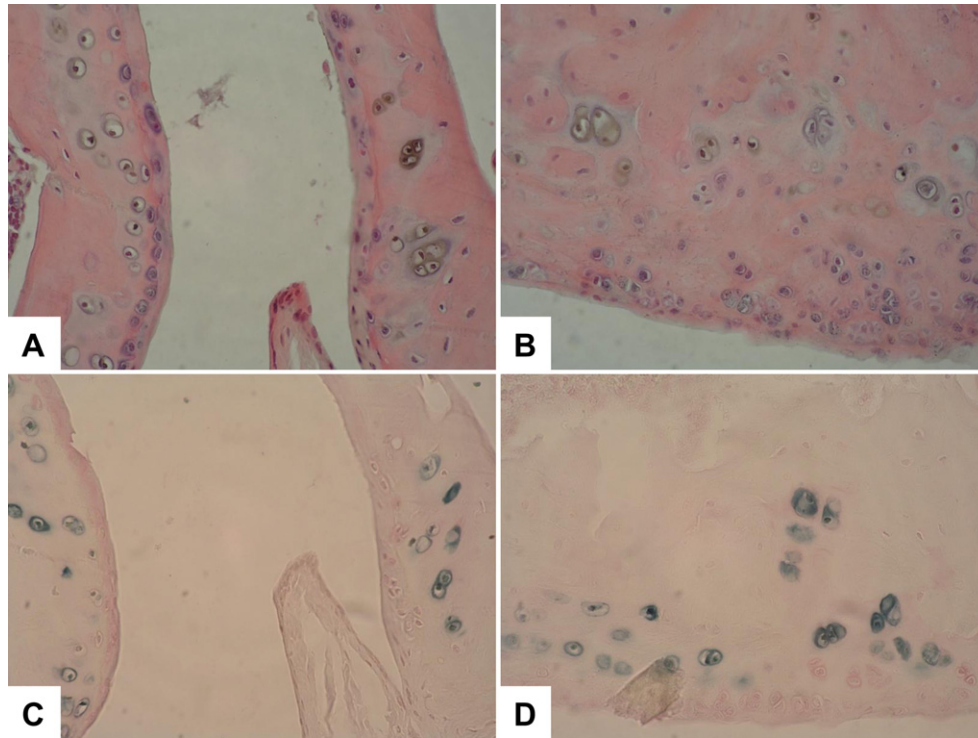
Fig. 6. Near serial sections showing ochronosis in the distal femur and fibula of $Hgd^{-/-}Fah^{-/-}$ (reverted from $Hgd^{+/-}Fah^{-/-}$) mice by H&E and Schmorl's. (A) Ochronosis can be seen in the pericellular and territorial matrices of individual chondrocytes in the deep layer of both the femur and fibula (H&E). (B) Ochronosis in the pericellular and territorial matrices of individual chondrocytes in the deep layer of the distal femur (H&E). Note the absence of pigmentation at the superficial and articular surfaces. (C) Near serial section of (A) showing the distal femur and fibular head stained with Schmorl's to identify ochronosis around and within chondrocytes, including those in the superficial layer of the distal femur. (D) Near serial section of (B) showing the distal femur stained with Schmorl's reagent. Pigmentation is identified intracellularly and within the pericellular and territorial matrices of chondrocytes of the deep layer, but also seen more readily in the superficial zone with both intracellular and pericellular deposits identifiable (Field width 0.3 mm).

articular surface and chondrocytes. No evidence for pigment in any of the mineralized bone matrices or any of the bone cells was seen.

Examination of the shoulder by H&E at low power demonstrated an interesting presentation of ochronosis in a small number of chondrocytes at varied locations. Even greater numbers of cells demonstrating early pigmentation could be seen when near serial sections were stained with Schmorl's than by H&E. These stained chondrocytes were most notable in the columns of chondrocytes located within the entheses. There were fewer stained chondrocytes peripheral to these columns, those that were stained in these peripheral regions were stained to a lesser intensity. The staining was most prominent in intracellular and pericellular regions, while little staining could be seen in the interterritorial matrices. Through utilization of both H&E and Schmorl's stain, it was concluded that no pigmentation was present in the chondrocytes of the articular surface across the shoulder joint. The epiphyseal plate showed dense proteoglycan staining, but all cells and extracellular matrix were devoid of pigmentation when examined using both stains.

Analysis of the thoracic and lumbar vertebrae revealed an absence of pigmentation both intra- and extra-cellularly when observed by H&E. Analysis of the vertebral articulations with Schmorl's reagent revealed a small number of chondrocytes with intracellular and pericellular pigmentation when viewed in sections. The spinal cord also displayed no ochronosis when analyzed using either stain.

Cardiovascular and respiratory system

Microscopic examination of the laryngeal cartilages revealed dense proteoglycan staining in the thyroid cartilage matrix and numerous chondrocytes appeared necrotic/apoptotic, but they demonstrated no detectable sign of ochronosis. The muscular insertions onto the laryngeal cartilages displayed no sign of ochronosis. Examination of the tracheal ring cartilages, tracheal epithelia, and fibrous tissues showed no ochronotic pigmentation.

Microscopic examination of the heart revealed dense muscular tissue with no sign of ochronosis. Examination of the heart valves and their attachment to the muscular tissue displayed normal connective tissues and no sign of intracellular or extracellular ochronosis. Analysis of the ascending and descending aorta revealed no signs of ochronosis associated with any layer of the vessel architecture. There was no sign of cellular ochronosis in the tissues.

Discussion

Here we present the first data showing ochronosis of tissues in a murine model of AKU. It has previously been published that mice with AKU exist, however these animals have not previously been reported to display the usual phenotypic ochronosis seen in the adult human, even though they have elevated levels of urinary HGA[8,17]. Several hypothesis have been suggested to explain the discrepancy including that the endogenous production of ascorbic acid is a protective mechanism against the gradual ochronosis of tissues in these animals[18], that these mouse models do not live long enough to allow deposition to occur in their tissues, or that urinary excretion is so complete that tissues do not experience high enough concentrations of HGA. Here we show that tissues, including those of the joints, show ochronosis in mice carrying the mutation for the disease, when combined with the effects of a second mutation in *Fah*.

It has been proposed that endogenously produced ascorbic acid may be protective for these mice. Our results show that mere presence of ascorbic acid is not sufficient to completely prevent pigmentation in all mice. While mice that have been alkaptonuric from birth have not yet been shown to display pigmentation, longitudinal studies covering the whole of their lifespan do not exist, so it is difficult to completely rule out pigmentation in aged specimens. It has also been suggested that HGA is not the sole determining factor in pigmentation[3]. The non-uniform distribution of pigmentation in $Hgd^{-/-}Fah^{-/-}$ (reverted from $Hgd^{+/-}$ $Fah^{-/-}$) mice supports this idea as neither these mice nor regions of these mice show pigmentation in the first 8 months following the initial reversion to the AKU phenotype many months before sampling.

Detection of pigmentation within the kidneys in these animals is not surprising from a functional point of view as this is the site of blood filtration and high metabolic activity. It is well documented that the darkening of urine is a sign of ochronosis and that renal manifestations in humans are not uncommon. For example, documented cases of passing pigmented kidney stones, pigmentation within human kidneys, and histologically-associated manifestations of renal deposition have been described[4,19].

The kidneys in these animals are of interest as the effects of NTBC withdrawal and FAA accumulation may seriously affect their ability to function. The loss of function may also be similar to the renal decline experienced by human AKU patients with ageing, limiting the ability of the kidneys to excrete HGA[20]. However, there is currently no longitudinal data detailing the urinary and plasma levels of HGA in AKU patients prior to known ochronosis, or the change over time once pigmentation starts. Observations made in adolescence that can then be compared following removal of a known ochronotic joint or repeated measurement of ochronosis in the sclera or pinna are needed.

It is widely accepted that there is HGA present in the urine of the $Hgd^{-/-}$ and $Hgd^{-/-}Fah^{-/-}$ mice. Whilst data exists detailing urinary HGA concentrations in these mice[17], there is currently no published data describing the concentration of the HGA in the plasma of these animals. Despite this, we show that regardless of the concentration of HGA in these animals, levels are sufficient to initiate polymerization and deposition of ochronotic pigment in multiple tissues. Absence of deposition in the cardiac connective tissues is not surprising, given that not all AKU patients present with cardiac involvement, suggesting that lifestyle factors may play a role in the deposition process. Whatever these factors may be, they are yet to be elucidated.

It has recently been shown that deposition in the human condition starts focally, associated with single chondrocytes in the calcified cartilage, progressing towards the articular surface with intracellular deposition appearing to precede the extracellular deposition[6]. It appears that the initiation in these animals is the same, demonstrating that these animals may be valuable for screening therapeutic strategies for inhibiting or reversing pigment deposition in AKU. Whilst bone changes are seen in the human disorder[6], they were not observed in these mice: bone matrix and bone cells were devoid of pigmentation. This also adds weight to the hypothesis that mineralization is protective in the disorder. We hold that bone changes are secondary to the cartilage pathology in the progression of AKU, so while bone changes were not observed in these animals, they may occur at a later stage, once the cartilaginous changes become more widespread.

The observations within the chondrocytes of two distinctly different types of pigmentation (granular and uniform) are interesting, as this suggests that similar to the human condition, pigmentation may actually arise under two different mechanisms within these tissues[5]. This adds further weight to our claim of synonymy with the human condition. Murine models have proved useful for investigation into disorders on the tyrosine metabolic pathway, particularly with the AKU mouse model which was influential in decoding the location of the then unknown human mutation[8,21,22]. The proposed treatment of AKU using NTBC came about from investigations into mice deficient in Fah. These animals are exposed to the build up of toxic tyrosine metabolites neonatally, once FAH production begins around day 16 of foetal development. Untreated $Fah^{-/-}$ mice do not survive beyond 24 h of birth; however administration of NTBC to mothers whilst pregnant results in the neonatal effect of FAH deficiency being abolished[11]. NTBC for treatment of HT1 is now widely used, although it is not without some risks. Treated patients are still vulnerable to other dermatologic, ophthalmologic and neurodevelopmental complications associated with elevated blood tyrosine levels[9]. In addition, paediatric patients who go undiagnosed and are without NTBC treatment for greater than 6 months are at high risk of developing hepatocarcinoma[12]. $Fah^{-/-}$ and $Hgd^{+/-}Fah^{-/-}$ mice show general health deterioration following withdrawal from NTBC. In contrast, $Hgd^{-/-}Fah^{-/-}$ mice are healthy at 4, 7, 9 and 11 weeks off NTBC[12]. Localised reversion from $Hgd^{+/-}$ to $Hgd^{-/-}$ in liver cells can take place spontaneously during the crisis invoked by withdrawal of $Hgd^{+/-}Fah^{-/-}$ mice from NTBC. A similar phenotype and spontaneous mutation has also been described in $Aspergillus$[23]. The reversion to the AKU phenotype in this model system showed the distinct darkening of culture media[23]. Furthermore, it has been shown that kidneys from $Fah^{-/-}$ mice off NTBC are pale and enlarged, confirming that they are abnormal[10]. This may result from an increase in the stresses to the kidneys, which in turn promotes ochronosis in the surrounding renal regions.

It is encouraging that the earliest signs of pigmentation in these animals have been observed and that they are synonymous with those in the human condition. It is within this critical time that ochronosis would need to be treated in human AKU, to prevent full ochronotic osteoarthropathy from developing. Based on our published evidence and that described in the literature, it is unlikely that deposition of pigment can be reversed[6]. This time point in the murine model will act as a useful tool for screening potential therapeutic agents. Detection of ochronosis in murine tissues represents a significant advance in the knowledge and understanding of alkaptonuric osteoarthropathy and will allow us to gain a better understanding into the molecular pathogenesis of the disorder.

Author contributions

Design of the study: AMT, NKP, MG, JAG, JCJ.
Data acquisition: AMT, AP, NKP, HS, CMK, PJMW, BW.
Data analysis: AMT, NKP, JAG, JCJ.
Interpretation of results: AMT, NKP, MG, LRR, JAG, JCJ.
Drafting and revising of article: all authors.
Final approval: all authors.
Author responsible for manuscript: JCJ.

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Competing interests

Animal study approval

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