Alkaptonuria: an example of a "fundamental disease" – a rare disease with important lessons for more common disorders

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Highlights:

- Fundamental diseases are rare diseases that help elucidate mechanisms of common disorders.
- Alkaptonuria (AKU), an iconic Mendelian disease, is a prototypic fundamental disease.
- Studying AKU has identified new disease mechanisms in osteoarthritis including the exposed collagen hypothesis.
- HDMPs, identified in AKU and then OA, constitute a newly discovered mechanism of joint destruction.

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Abbreviations

advanced glycation end products (AGE**)**

alkaptonuria (AKU)

extracellular matrix (ECM)

high density mineralised protrusions (HDMPs)

homogentisate 1,2 dioxygenase (HGD)

homogentisic acid (HGA)

online Mendelian inheritance in man (OMIM)

osteoarthritis (OA)

proteoglycan (PG)

Abstract

"Fundamental diseases" is a term introduced by the charity Findacure to describe rare genetic disorders that are gateways to understanding common conditions and human physiology. The concept that rare diseases have important lessons for biomedical science has been recognised by some of the great figures in the history of medical research, including Harvey, Bateson and Garrod. Here we describe some of the recently discovered lessons from the study of the iconic genetic disease alkaptonuria (AKU), which have shed new light on understanding the pathogenesis of osteoarthritis. In AKU, ochronotic pigment is deposited in cartilage when collagen fibrils become susceptible to attack by homogentisic acid (HGA). When HGA binds to collagen, cartilage matrix becomes stiffened, resulting in the aberrant transmission of loading to underlying subchondral bone. Aberrant loading leads to the formation of pathophysiological structures including trabecular excrescences and high density mineralised protrusions (HDMPs). These structures initially identified in AKU have subsequently been found in more common osteoarthritis and appear to play a role in joint destruction in both diseases.

1. Introduction: What are Fundamental Diseases?

“Fundamental diseases” is a term introduced by the charity Findacure, which promotes research into development of new treatments for rare diseases (1). They coined the term “fundamental diseases” to capture the concept that rare diseases, especially those that have a genetic cause, are gateways to understanding common conditions and human physiology. Findacure regards the usual terminologies “rare”, “orphan” and “neglected” as contributing to why this group of diseases has been relatively overlooked. However, the concept that rare diseases have important lessons for biomedical science is not new. William Harvey the great English physician of the 17th century wrote in a frequently quoted letter that *“careful investigation of cases of rarer forms of disease”* was the best way *“to advance the proper practice of medicine”* (2). Nearly two and a half centuries later, William Bateson, who was a major advocate of the work of Mendel and first coined the term genetics, repeated Harvey’s advice in his inaugural lecture at the University of Cambridge when he urged young scientists to “*Treasure your exceptions”* (3). Bateson had a major influence on his colleague Archibald Garrod, the father of metabolic disease who introduced the term inborn errors of metabolism. Through discussions with Bateson, Garrod came to recognise that the ultra-rare disease alkaptonuria (AKU) was a recessive disorder and thus became the first human disease shown to follow Mendelian inheritance (4). Garrod also was aware of the wider benefit of the study of rare diseases. In his article “The Lessons of Rare Maladies,” Garrod paraphrased Harvey, *“The study of nature’s experiments is of special value; and many lessons which rare maladies can teach could hardly be learned in other ways”* (4).

In this chapter, we describe some of the recently discovered lessons from the study of AKU (OMIM 203500) which have shed new light on understanding the pathogenesis of osteoarthritis (OA). AKU is one of around 8,000 Mendelian diseases and for every patient in the UK with AKU there are around 100,000 with OA. However by studying rare diseases like AKU which are characterized by severe phenotypes with rapidly developing pathologies, it is easier to identify earlier molecular and microanatomical changes that are also fundamental to the pathogenesis of more common disorders like OA. Pathological changes in OA are less conspicuous because they are not as abundant and progress more slowly. An additional benefit of investigating Mendelian diseases is that it is possible to trace the succession of pathological changes back to the altered function of a single gene.

2. Genetics and Pathophysiology of Alkaptonuria

Half a century after Garrod’s recognition that AKU was a genetic disease with recessive inheritance, La Du and colleagues discovered that the disorder was caused by a deficiency of homogentisate 1,2 dioxygenase (HGD) [E.C.1.13.11.5], an enzyme in the metabolism of tyrosine and phenylalanine (5). The single-copy human HGD gene maps to chromosome 3q21–q23, encompassing 14 exons and encoding a protein of 445 amino acids (6). AKU arises from homozygous or compound heterozygous mutations in the HGD gene, with more than 130 different human mutations now identified (7,8). The prevalence of AKU is calculated to be 1:100,000-250,000 in most ethnic groups. However, in several hotspots including the Dominican Republic and the north western region of Slovakia, the incidence is greater than 1:20,000. Whereas the high incidence in the Dominican Republic appears to be a classical founder effect, the high regional incidence in Slovakia is a baffling result of more than 12 distinct mutations (6). Increased international interest in AKU research over the past few years through the FindAKUre and DevelopAKUre (www.developakure.eu/) (9) consortia has led to the identification of a high incidence of the disease in some regions of Jordan (10) and in specific ethnic groups in India (11). It is likely that there is a vast reservoir of undiagnosed AKU worldwide, particularly in developing countries.

Loss of HGD enzyme activity increases the circulating concentration and urinary excretion of homogentisic acid (HGA), causing urine to darken on exposure to air. Raised HGA levels eventually lead to ochronosis, the deposition of polymers of HGA as pigment in connective tissues including cartilage, heart valves and sclera (12) (see Fig.1). Patients present with disease in early adult life and they are markedly affected in the fourth and fifth decades of life. Over time, patients develop the characteristic external features of ochronosis, blue-black pigmentation of the ear cartilage and sclera

of the eyes. Ear ochronosis can lead to pain in the external ear whilst scleral ochronosis may affect vision. Aortic and mitral valve disease is also common and may require valve replacement (13).

3. Osteoarthropathy in AKU

Joint ochronosis and the subsequent osteoarthritis appear to be an inevitable consequence of AKU causing considerable disability and pain in the peak of adulthood due to premature joint and spine disease. Ochronotic disease of the intervertebral disc develops in the third decade of life causing severe pain, and progressive kyphoscoliosis. Disc degeneration impacts on spinal and thoracic mobility with consequent respiratory problems. Pain due to joint disease is progressive, eventually affecting most synovial joints in the body. Multiple joint replacements are almost inevitable. Other musculoskeletal manifestations of AKU include tendon and ligament ruptures, osteopenia and fractures (13).

4. The Initiation of Ochronotic Pigmentation – the Exposed Collagen Hypothesis

A series of studies on tissue samples from patients with AKU (14,15), an *in vitro* model of ochronosis (16) and a mouse AKU model of the disease (17,18) have revealed that tissues are initially resistant to pigmentation but become susceptible following biomechanical and biochemical influences on the composition or structure of the extracellular matrix. Although these changes have not yet been fully elucidated, early pigmentation has been shown to be associated ultrastructurally with the periodicity of collagen (14), somewhat reminiscent of proteoglycan (PG) binding (19). This pattern strongly indicates that there are specific sites on collagen where HGA can bind but which are protected in native collagen in undamaged extracellular matrix (ECM). PGs and other molecules decorating collagen would repel HGA, an acidic molecule with a pKa of 3.57. However, following structural and compositional changes, including loss of PGs, the potential binding sites become exposed allowing HGA to bind (see Fig. 2). The initial binding appears to be similar to a nucleation event which is followed by rapid deposition of HGA as a pigmented polymer (16). Binding of HGA-derived pigment to the collagen fibres makes them stiffer and susceptible to more mechanical damage. This leads to further ultrastructural changes in collagen, increased exposure of binding sites to HGA and a downward spiral of pigmentation. Transmission electron microscopy (TEM) findings are supported by solid state nuclear magnetic resonance (ssNMR) studies which revealed loss of order at the nanoscale and loss of PGs (20). Ultrastructural and biochemical studies suggest that collagen fibril damage and proteoglycan loss precede pigmentation (14,16 and Taylor et al in preparation). Further support for the exposed collagen hypothesis comes from observations on pigmentation in an *in vitro* model of ochronosis in which bone cells are grown in the presence of HGA at concentrations similar to those observed in AKU. In these cultures pigmentation occurs in days rather than the years it takes to see extensive ochronosis *in vivo* (20). Deposition of ochronotic pigment *in vitro* is much more rapid than *in vivo* because during the synthesis of matrix by cultured cells, the ECM components are not assembled correctly into the highly organised matrix seen *in vivo* in which fibrous proteins, proteoglycans and glycosaminoglycans are arranged. Instead a less well ordered matrix is laid down and thus factors protecting ECM from pigmentation *in situ* are absent and pigmentation is accelerated.

Anatomically, the initial sites of ochronosis are the tissues which have been subjected to the most mechanical loading. In effect HGA behaves like an endogenous marker of repetitive load-induced matrix damage.

In AKU it is possible to follow the process of ECM degeneration which allows pigmentation and eventually leads to joint destruction. However the changes in organisation and composition of the ECM which precede ochronotic pigmentation are not dependent on the presence of HGA. These are independent changes which occur in tissues as a result of ageing, overloading, trauma and degeneration. These changes also occur in non-AKU tissues and just as binding sites become available for HGA to bind, it is likely that other reactive small molecules will form adducts with collagen fibres once the protective molecules have been lost. One example is the formation of advanced glycation end products (AGE) formed by the reaction of ribose and other monosaccharides. As for the reaction with

HGA, AGE modifications affect the biochemistry, the ultrastructure and the mechanical properties of the collagen (21). Thus it is likely that the loss of PGs and other molecules and subsequent exposure of binding sites on collagen underlies degeneration in non-AKU connective tissues.

6. Pathogenesis of Joint Destruction in AKU

Histological studies show that in cartilage, initial pigmentation is focal and located in individual chondrocytes and their territorial matrix in calcified cartilage (15,17,18) but then proliferates throughout the hyaline cartilage in either granular or homogenous conglomerates (15). Once hyaline cartilage is extensively pigmented it becomes stiff and brittle. This is followed by aggressive osteoclastic resorption of the subchondral plate (15). Pigmented unmineralised cartilage becomes impacted on the underlying trabecular bone and embedded in the marrow space. Fragments of pigmented cartilage also become embedded in synovial tissue. Pigmented cartilage is stiffer than normal cartilage, and leads to altered load-distribution within joints including aberrant transmission of loading to the underlying bone. The subchondral plate is subjected to direct damage as a result of the aberrant loading and to load-induced remodelling. Following the resorption of the subchondral bone, the pigmented shell of the remaining articular cartilage eventually fails catastrophically (15).

7. Altered Bone Remodelling – Formation of Trabecular Excrescences

In addition to focal loss and focal sclerosis of the subchondral plate in ochronotic joints, there is also aberrant remodelling of the underlying trabecular bone which leads to further structural changes including the formation of trabecular excrescences (22). These are novel microanatomical structures that were first identified in AKU where they are abundant but have been found subsequently in osteoarthritis but at a lower frequency than in AKU (22). Interestingly adipocytes seem to play a role in the formation of trabecular excrescences either by direct contribution to synthesis or by templating the formation of bone by osteoblasts.

8. Formation of High Density Mineralised Protrusions

Perhaps the most significant lesson from ochronosis to date has been identification of high density mineralised protrusions (HDMPs) (23,24). The first detection of these structures in human joints was in a 50 year old male with AKU who had hip arthroplasty because of lancinating pain in his hip joint. Anatomical examination of his femoral head *ex vivo* revealed no significant loss of cartilage from the articular surface. Subsequent investigation by microCT, MRI and scanning electron microscopy revealed regions of ultra-dense material arising from the mineralising front of calcified cartilage and protruding into the hyaline cartilage. The HDMPs appeared to arise from fluid extruded through microscopic cracks appearing in the subchondral plate which subsequently calcified forming hard, abrasive structures embedded in the hyaline cartilage but not reaching the articular surface (see Fig.3). Initially it was thought that these structures were disease specific for AKU but subsequent studies have revealed that they are present in joints in human OA. They appear to be analogous to mineralised structures previously identified in joints of Thoroughbred racehorses (24) and subsequently in Icelandic horses (25) and Standardbred race horses (26). The protrusions could play a major role in the destruction of cartilage from the subchondral aspect. HDMPs might also be partially responsible for the discordance between pain and cartilage loss in OA. Their formation constitutes a newly recognised mechanism of joint destruction in AKU and in OA and provides potential targets for drug therapy. Furthermore the ability to detect HDMPs in joint tissues *in situ* by MRI holds out the prospect that these recently discovered structures might be a useful imaging biomarker of joint disease progression in AKU and OA.

9. What will be the Future Lessons from AKU and Ochronosis?

One of the the major breakthroughs in AKU research has been the recognition that nitisinone, a drug repurposed from another rare disease, hereditary tyrosinaemia 1 (HT1) (OMIM 276600) might be an

effective treatment. Although there is currently no licensed therapy for AKU there is optimism that Nitisinone is completely effective at lowering HGA and preventing ochronosis in the AKU mouse (18). Nitisinone has also been shown to lower circulating HGA in AKU patients (27). An international clinical trial of the efficacy and safety of nitisinone in AKU is currently underway (www.developakure.eu/). This trial includes a comprehensive metabolomic survey of control and treated patients, which could lead to the discovery of novel biomarkers not just to monitor the progression and response to therapy of AKU, but also has the prospect of identifying markers of cartilage degeneration which might be useful in OA.

Despite its potential beneficial effects, nitisinone causes a large increase in circulating tyrosine. The tyrosinaemia resembles hereditary tyrosinaemia type 3 (OMIM 276710) and can lead to corneal and dermal toxicity (28), and potential neurological damage. Nitisinone will be at best a treatment and the search for a cure should continue.

Mutations causing genetic disease can be repaired or replaced at different levels of biological organisation. Currently there is much excitement about the potential of CRISPR-Cas9-mediated genome editing in Mendelian diseases. If this promise is fulfilled we might be on the brink of a new era in treatment of genetic diseases including AKU.

10. Conclusions

The biomedical literature contains many interesting examples of how lessons from rare diseases have contributed to a better understanding of physiology and pathophysiology. Knowledge gained from rare disease research has also made a significant impact on the discovery of new therapeutic agents. In this article the concept of fundamental disease has been explored with specific focus on how investigating AKU, the first human disorder shown to follow Mendelian inheritance, is contributing to the elucidation of disease mechanisms in the common disorder OA. Joint destruction in OA is one of the major causes of disability worldwide. More focus on the study of the fundamental disease AKU is a good strategy to make progress in OA.

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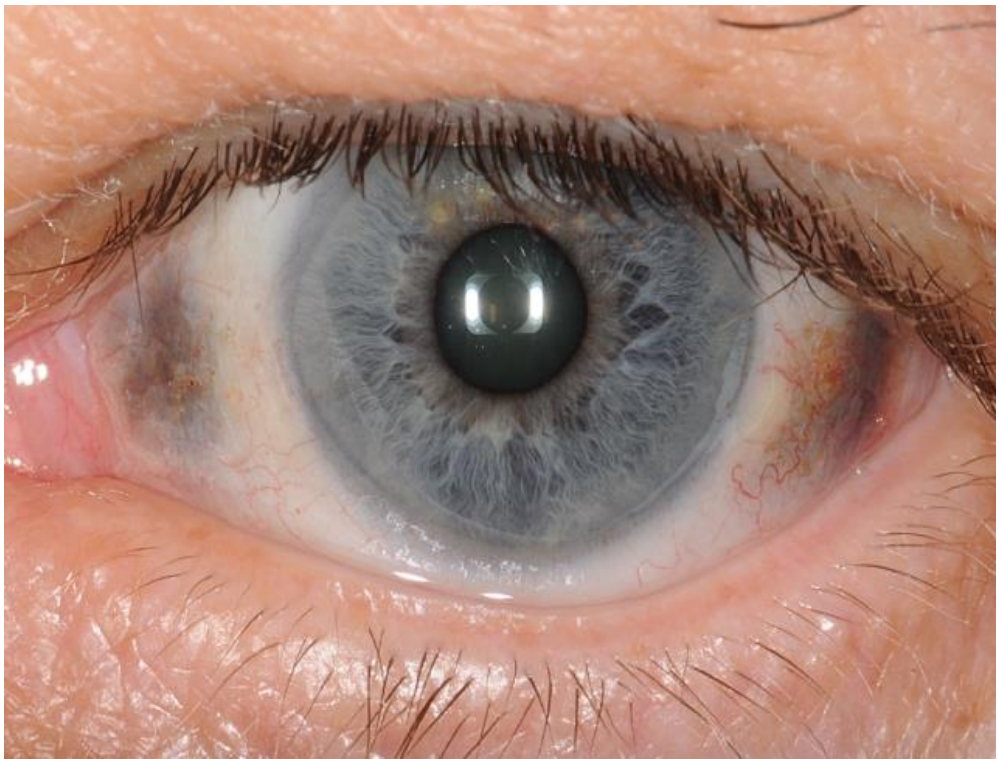
Figures

Fig 1 Ochronosis in tissues of a patient with AKU a). sclera of the eye and b). knee joint. HGA is deposited in collagenous tissues forming pigmented polymers. The pigment cause the extracellular matrix to become stiff leading to severe pathophysiological changes.



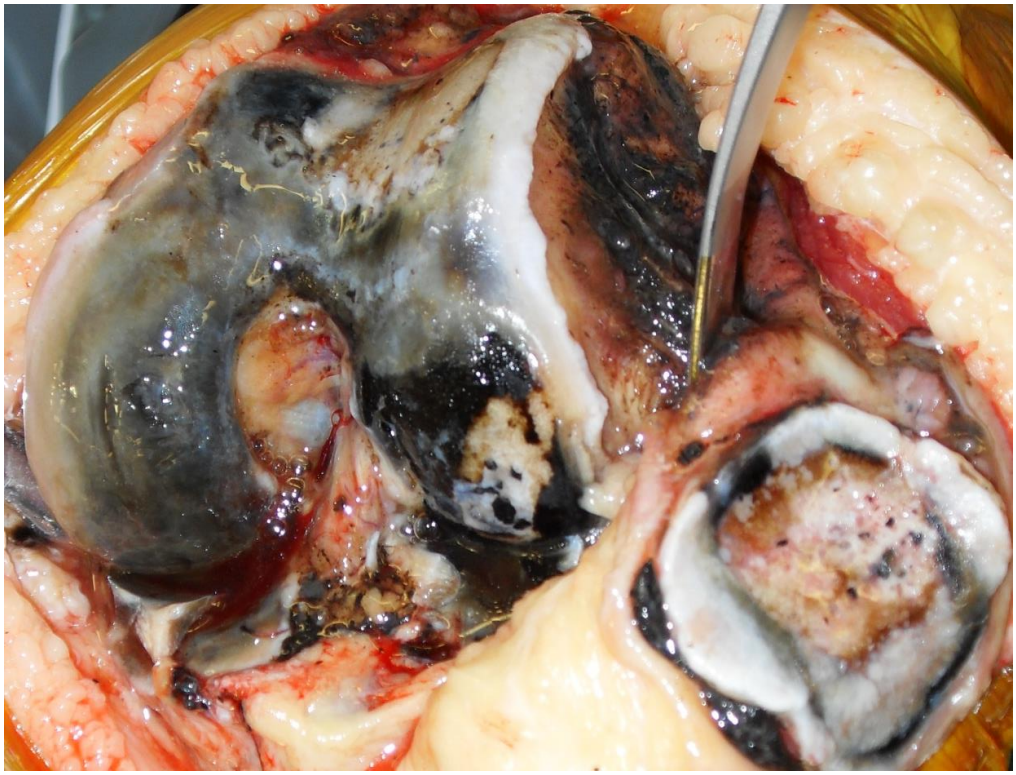


Fig 2. Schematic representation of the exposed collagen hypothesis. Diagrams represent progressive disease progression from upper panel to lower panel. Upper panel. Homogentisic acid (HGA) is present in the extracellular environment but cannot bind to the undamaged collagen fibrils which are decorated with protective molecules including proteoglycans (PGs). Middle panel. Protective molecules including PGs are lost from the collagen fibrils as a result of repetitive mechanical loading, chemical attack or ageing and degeneration. The exposed collagen fibrils are then susceptible to attack from small molecules such as HGA. Lower panel. The initial binding of HGA functions as a nucleation event and is followed by further rapid deposition of HGA as a pigmented polymer. Binding of HGA-derived pigment to the collagen fibres makes them stiffer and susceptible to more mechanical damage. This leads to further ultrastructural changes in collagen, increased exposure to HGA and a downward spiral of intense pigmentation and severe ochronosis.

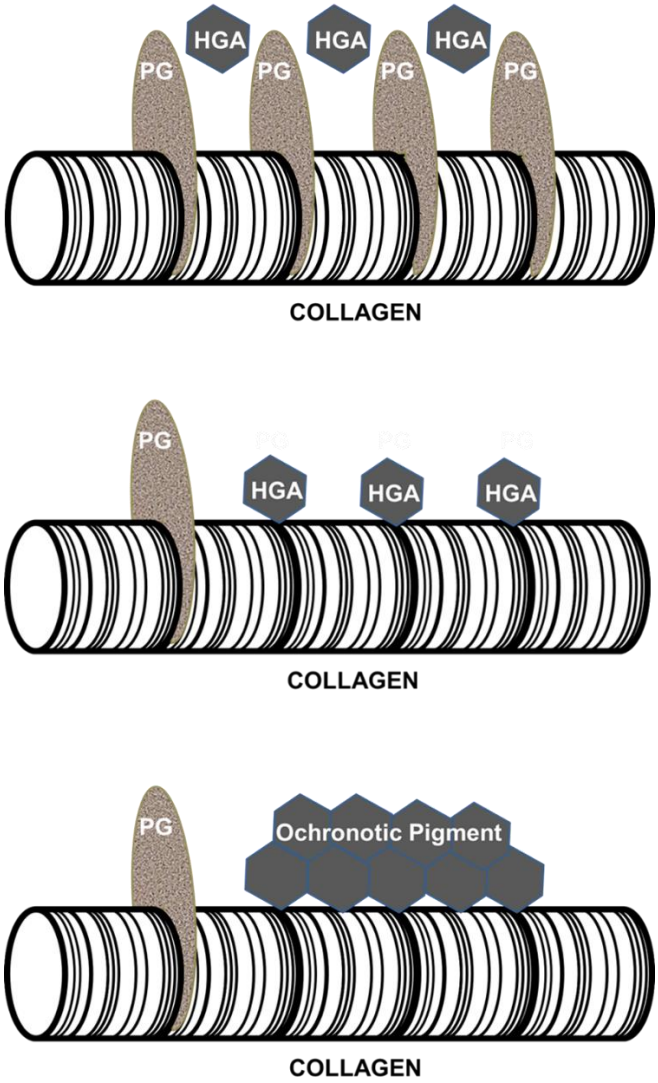


Fig 3. A-D Schematic representation of the stages in formation of high density mineralised protrusions (HDMPs).A. Diagram of hyaline articular cartilage (orange), calcified cartilage (grey) and underlying subchondral and trabecular bone (pink) prior to degeneration. The subchondral plate is intact. B. Collagen in cartilage becomes pigmented (represented by shading) and stiffened leading to aberrant transmission of mechanical loading (large white arrows). Repetitive loading leads to cracking of the subchondral plate (small dark arrows). In addition dysregulated remodeling of underlying bone leads to focal sclerosis and lysis. C. Extrusion of mineralizable matrix through the cracks leads to the formation of HDMPs (stippled grey). D. HDMPs are sharp abrasive and brittle leading to extensive mechanical destruction of the hyaline cartilage (small white arrows).