Ochronotic pigmentation is caused by homogentisic acid and is the key event in Alkaptonuria leading to the destructive consequences of the disease – a review

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Abstract

Ochronosis is the process in Alkaptonuria (AKU) that causes all the debilitating morbidity. The process involves selective deposition of homogentisic acid-derived pigment in tissues altering the properties of these tissues, leading to their failure. Some tissues like cartilage are more easily affected by ochronosis while others such as the liver and brain are unaffected for reasons that are still not understood. *In vitro* and mouse models of ochronosis have confirmed the dose relationships between homogentisic acid and ochronosis and also their modulation by HPPD inhibition. Ochronosis cannot be fully reversed and is a key factor in influencing treatment decisions. Earlier detection of ochronosis preferably by non-invasive means is desirable. A cause-effect relationship between HGA and ochronosis is discussed. The similarity in AKU and familial hypercholesterolaemia is explored, and lessons learnt. More research is needed to more fully understand the crucial nature of ochronosis.

Introduction: Archibald Garrod is the father of inborn errors of metabolism and studied four disorders, namely alkaptonuria, pentosuria, cystinuria and albinism. Pentosuria (OMIM#260800) is a defect in l-xylulose reductase, necessary for xylitol metabolism, leading to overproduction of pentose sugars and pentosuria, but is otherwise harmless as it does not accumulate in the body or produce a disease process of its own (Knox 1958). This is unlike alkaptonuria (AKU) (Figure 1) (OMIM#203500) where ochronosis is the key pathophysiological event (Garrod 1902; Galdston et al. 1952; O'Brien et al. 1963). Ochronosis-like processes have also been described in non-AKU states and termed pseudo-ochronosis or exogenous ochronosis (as opposed to endogenous ochronosis in AKU). Exogenous ochronosis has been the subject of previous reviews and will not be discussed further here (Levin and Maibach 2001; Bhattar et al. 2015).

Ochronosis is the term used to describe a process by which a yellowish (ochre) discoloration develops (Figure 2A), due to the deposition of pigment. First described by Rudolf Virchow in 1866, ochronosis was observed in miscroscopic examination of connective tissues (Virchow 1866). The tissues affected by ochronosis macroscopically, however, appear to be blue grey or black when large amounts of pigment are present. Virchow noticed that the pigment also specially accumulated in damaged or inflamed sites, such as irritated joint synovia and arteriosclerotic plaques. He hypothesised that non-crystalline colouring matter, whose nature was then unknown, slowly saturates the cartilage, a special target in AKU.

It is now known that AKU is a condition characterised by the absence of homogentisate dioxygenase enzyme (HGD) (EC:1.13.11.5) (Figure 1) leading to an inability to metabolise homogentisic acid (HGA) (La Du et al. 1958). HGA, an intermediary in the phenylalanine/tyrosine pathway, is normally completely and rapidly metabolised to yield fumarate and acetoacetate (Phornphutkul et al. 2002). The degradation of phenylalanine (an essential amino acid) and tyrosine (a non-essential amino acid) proceeds to fumarate and acetoacetate through homogentisic acid so rapidly that in normal subjects there is no increase in circulating HGA and very little in urine (Davison et al. 2015). The HGD deficiency in AKU causes excessive HGA production, causing homogentisicaciduria, while also increasing HGA concentrations within body tissues. Alkaptonuria is an autosomal recessive disorder with a delayed slowly-progressive multisystemic damage for which there is still no approved disease modifying therapy, even though a drug called nitisinone decreases HGA (Introne et al. 2011). Although HGA by itself causes morbidity such as renal, prostate, gall bladder and salivary

stones, the main disease process leading to tissue destruction and debilitating clinical sequelae is ochronosis, the focus of this review.

Formation of ochronotic pigment (OP): *Overproduction of HGA:* With an inability to metabolise HGA, there is an increase in whole body HGA despite massively increased renal excretion of HGA (Figure 1). The most important pathophysiological consequence in tyrosine pathway in AKU is conversion of accumulating HGA to OP. HGA is a reducing agent and especially under alkaline conditions it is itself rapidly oxidised via benzoquinone acetic acid turning solutions and tissues black (Zannoni et al. 1969). Slow spontaneous blackening of urine is usual in AKU, but adding alkali instantly turns urine black; acidifying the black urine does not return the black colour to normal, suggesting a potential irreversible change.

Apparently slow formation of pigment: Despite the metabolic defect existing from birth, pigmentation of eyes and ears is slow to develop externally, taking up to two or three decades to appear, presumably a reflection of the gradual accretion of the pigment. Similarly, back and knee pain, due to development of critical ochronosis in these sites, are also apparent in the second and third decades (Cox and Ranganath 2011). Formation of OP is markedly increased in renal failure, consistent with increased retention of HGA, and accelerates severe morbidity and debility (Introne et al. 2002).

Factors supporting molecular interaction of HGA with cartilage matrix (Figure 2B, C): The molecular mechanism through which HGA interacts with collagen matrix is unknown. Ultrastructural examination of pigmented cartilage showed that initial pigmentation is closely associated with the periodicity of collagen fibres. A periodic banding pattern of OP was observed on individual collagen fibres, with very early pigmentation appearing as small granules on the surface of fibres (Taylor et al. 2010b). These findings suggest that a nucleation-like event underlies ochronosis, where initial granular deposits on individual collagen fibres is followed by further rapid pigment deposition (Gallagher et al. 2016). The data also suggest that collagen fibrils provide specific binding sites for pigment.

Close observation of tissues obtained from AKU patients (Taylor et al. 2011b, 2012) and mice (Taylor et al. 2012; Preston et al. 2014) and *in vitro* (Tinti et al. 2011b) models of AKU show that cartilage is initially resistant to pigmentation. It is proposed that biomechanical and biochemical changes, such as those that occur in cartilage as part of the natural ageing process, render tissues susceptible to ochronosis. The ‘exposed collagen hypothesis’ theorises that binding sites become available for HGA following the loss of protective molecules such as

proteoglycans and glycosaminoglycans (Figure 2C) (GAGs) (Gallagher et al. 2016). In support of this hypothesis, AKU cartilage is shown to have lower levels of extractable GAGs and oligomeric matrix protein than osteoarthritic and non-osteoarthritic cartilage (Taylor et al. 2017). There is also evidence that the structure and maturity of the collagen matrix can influence pigment deposition. Newly-synthesised aberrant matrix proteins in scar tissue also appear to pigment rapidly, as reported in the case of a mediastinal mass from an AKU patient (Taylor et al. 2011a). Solid-state NMR analysis of AKU articular joint cartilage also observed spectra indicative of marked collagen disorder at the atomic level (Chow et al. 2011), further supporting the idea that disruption to the collagen matrix supports OP deposition.

Further work is required to elucidate a specific binding site for OP within the collagen matrix. It is also not known whether the initial binding occurs as HGA, the oxidised intermediate benzoquinoneacetic acid (BQA) or OP.

Reversibility of pigment: It is currently believed that the OP process is not fully reversible, although there are indications of some reversal of pigment; the mechanism of such reversal is currently unknown (Ranganath et al. 2018). It is not known if OP process is dynamic i.e. formation and removal co-existing, although macrophages with pigment has been described in case reports (Gaines et al. 1987; Fisher and Davis 2004; Damian et al. 2013).

Diet and OP: Tyrosine and phenylalanine are not limiting amino acids in the diet and are consumed in vast excess of requirement. The attempts to retard progression of AKU by reducing the amounts phenylalanine and tyrosine flux is in keeping with the belief that amounts of protein intake could influence AKU morbidity.

Generation of OP in vitro and *ex vivo (Figure 2D):* Model systems have been developed for investigating various effects of HGA exposure under controlled conditions *in vitro* and *ex vivo*. *In vitro* models include human serum (Braconi et al. 2010a, 2011) and osteoblastic (cell line) and chondrocytic (cell line and isolated from human cartilage) cell cultures (Braconi et al. 2010b; Tinti et al. 2010, 2011b; Braconi et al. 2012; Millucci et al. 2012; Spreafico et al. 2013; Millucci et al. 2015b; Mistry et al. 2016). Incubation of cell cultures with HGA leads to OP formation by four weeks. The amount of OP formed is proportionate to the concentrations of HGA in the medium (Tinti et al. 2011b). *Ex vivo* 'organotypic' approaches have modelled AKU by studying the effect of HGA on human cartilage explants (Tinti et al. 2011a; Millucci et al. 2012; Bernardini et al. 2019). In these studies, pericellular pigmentation of cartilage explants is visible after two months of incubation with HGA (Tinti et al. 2011a).

Development of these model systems has not only been useful in studying the development of OP under controlled conditions, but also a number of other pathophysiological events closely associated with ochronosis including secondary amyloidosis (Millucci et al. 2012; Spreafico et al. 2013; Millucci et al. 2015b), perturbed redox homeostasis (Braconi et al. 2010a, b, 2011, 2012, 2015; Tinti et al. 2010; Spreafico et al. 2013) and disorder of the cartilage extracellular matrix, including proteoglycan loss and collagen fibril re-arrangement (Tinti et al. 2010; Millucci et al. 2015b; Bernardini et al. 2019). Oxidative stress is a frequent and major consequence of HGA exposure in these studies, as indicated by direct oxidative modification to proteins and depletion of endogenous antioxidants such as glutathione (Braconi et al. 2010a, b, 2011, 2012, 2015; Spreafico et al. 2013). *In vitro* studies have also been important in firmly establishing the pro-oxidant role of ascorbic acid in the presence of HGA. These studies indicate that ascorbic acid is not efficacious as a mediator of HGA-induced oxidative stress and secondary amyloidosis, unless combined with other reducing agents (Tinti et al. 2010).

Animal model of OP formation: In addition, mouse models of AKU, previously only considered to be models of AKU biochemistry, have been shown to develop OP (Taylor et al. 2012; Preston et al. 2014). Despite a marked elevation in plasma and urinary HGA, these mice do not develop the striking macroscopic ochronosis observed in adult humans, except in the kidneys after 13 months (Taylor et al. 2012). Several potential explanations for the lack of widespread macroscopic pigmentation in AKU mice have been postulated. We suggest that the most likely explanations are a) reduced joint-loading of quadrupedal mice, b) faster cellular turnover for removal of pigment and c) that the shorter lifespan of mice compared with humans might be insufficient for gross pigment deposition (Preston et al. 2014). However, pigmentation is observed in AKU mice microscopically; chondrocytes within the articular cartilage begin to show pigmentation at 15 weeks (Preston et al. 2014). This pigmentation progressively increases over the lifespan of the AKU mouse. Pigmentation of individual chondrocytes in AKU mice mirrors the focal initiation of ochronosis observed in human AKU, in which pigment deposition begins within single chondrocytes of the calcified cartilage and eventually results in widespread extracellular pigmentation (Taylor et al. 2011b). An exact serum HGA concentration threshold at which OP develops is not known, but a threshold range of 40-60 µM/L has been reported in mice (Lewis 2018).

In AKU mice administration of p-hydroxyphenylpyruvate dioxygenase (EC:1.13.11.27) (HPPD) inhibitors, such as nitisinone, soon after birth resulted in failure to pigment, while a similar administration of HPPD inhibitors during the middle of the life span of the mouse, when

pigmentation has already developed, prevented progression, but not reversal, of pigmentation (Keenan et al. 2015). This suggests that HPPD inhibitors, by decreasing HGA, can modify the OP process in AKU mice.

Structure of OP: *The classic view of OP formation:* A fundamental question in understanding the process of ochronosis and the development of therapeutic interventions aimed at its prevention or potential reversal concerns the structure of OP itself. The prevailing view on the mechanism by which HGA produces OP in AKU largely comes from work carried out by Zannoni and colleagues in the 1960's. These authors stated that guinea pig cartilage and skin contain enzymes, namely HGA polyphenol oxidases, which were shown to catalyse the *in vitro* oxidation of HGA into a dark, ochronotic-like pigment (Zannoni et al. 1969). BQA was demonstrated as an intermediate in the *in vitro* enzymatic oxidation of HGA, and it was proposed that BQA may polymerize to form OP in AKU (Zannoni et al. 1962, 1969). However, it is important to note the lack of evidence that polyphenol oxidase enzymes are expressed in human or other mammals (Taylor et al. 2016). Enzymes that can oxidise HGA, 'HGA-oxidases', are observed in various species of bacteria known to produce 'pyomelanin' pigment derived from HGA (Hunter and Newman 2010; Roberts et al. 2015). In these species, HGA-derived pyomelanin is thought to serve various adaptive functions including resistance to environmental stress such as UV light and oxidising agents. Subsequent research showed that HGA oxidation can occur non-enzymatically between pH 6.8 and 9.5 in the presence of oxygen (Martin and Batkoff 1987); it is still widely-stated in the literature that oxidation of HGA to benzoquinone intermediates occurs spontaneously in AKU and results in a polymeric pigment structure.

An alternative view of OP chemical structure: Given the general assumption that OP is a product of HGA oxidative polymerisation, it is important to define the term 'polymer'. The accepted definition of a polymer is a large molecule, or 'macromolecule', composed of multiple repeating subunits of a relatively lower molecular weight monomer (McNaught and Wilkinson 2014). The classic concept of a polymer is that the monomer subunits are covalently bound (Allcock et al. 2003).

Roberts et al. (2015) question the widely-held assumption that OP is polymeric. These authors cite the lack of conclusive evidence for this in the literature, and make the point that a polymeric structure is not necessarily required to produce the visual properties of a dark pigment; there are numerous examples of low molecular weight biological pigments. More recent analysis of

synthetically-derived OP solutions using size exclusion chromatography suggest that OP has greater molecular weight than HGA, as indicated by a peak at shorter retention time. However, visually pigmented solutions could be formed from HGA (over a shorter period of time; 10 days as opposed to 2 years) without evidence of the peak corresponding to OP and no observed decrease in the HGA peak (Taylor and Vercruysse 2017). This suggests that the visual darkening of the solution due to increased pH can be due to presence of the low molecular weight oxidised form of HGA over a relatively shorter period.

OP has been referred to as 'melanin-like' in the literature (Roberts et al. 2015); largely because melanin is another dark biological pigment derived from tyrosine and classically considered to be formed by polymerisation. However there is a growing body of evidence that melanin and OP are not covalently bound structures, and that their macromolecules do not comprise regularly repeating monomers. A recent study reports data from physicochemical analyses on the 'pyomelanin' pigment produced in the bacteria *rubrivivax benzoatilyticus* (strain JA2) (Mekala et al. 2019). This bacteria mirrors the conditions of OP pigment production in AKU; absence of the *HGD* gene causes accumulation of HGA, resulting in a brown pigment under aerobic conditions and in the presence of phenylalanine (Mekala et al. 2018). Fourier-transform infrared spectroscopy (FTIR) showed a range of band stretching vibrations indicating various chemical groups (aromatic and aliphatic C-H stretches, phenolic C-O stretches, aromatic ring C=C bonds, and C=O stretches due to –COOH groups) characteristic of a pigment structure derived from HGA. X-ray diffraction spectra of the pigment showed similar characteristics to that of melanin, with broad diffraction indicating an 'amorphous' compound structure. The absorbance spectra showed a broad band at wavelengths 280-350 nm, with increased general absorbance across the UV-visual range without distinct peaks. Similar absorbance properties have been reported previously for OP (Roberts et al. 2015; Taylor and Vercruysse 2017) and also melanin (eumelanin derived from 5,6,-dihydroxyindole-2-carboxylic acid; Tran et al. 2006), and are thought to reflect a chemically heterogeneous structure of oligomers formed by a range of different bonding mechanisms, also referred to as 'chemical disorder' (Riesz 2007). These absorbance properties might also account for the physical appearance of some pigmented substances, which in the case of melanin is thought to provide its physiologically important optical characteristics, *i.e.* its ability to absorb UV light (Chen et al. 2014; Roberts et al. 2015). In contrast, specific, well-defined chemical signatures have been obtained for BQA, the proposed low molecular weight oxidation product of HGA. Specific visual-range absorbance peaks corresponding to BQA have been reported in a series of publications by Tokuhara and

colleagues. These peaks were observed at 406 and 430 nm from analysis of solutions of HGA or AKU urine following alkalinisation (Tokuhara et al. 2014, 2018) although other authors have been unable to replicate these findings (Roberts et al. 2015). Specific BQA signals were also recently reported by Tokuhara et al. (2018) from LC-QTOF-MS and NMR analyses.

Computational analyses of eumelanin support a structure formed by stacked eumelanin protomolecules with random-like arrangement; in other words loosely-bound aggregates as opposed to a covalent polymer (Chen et al. 2014). This aggregate structure of melanin is further supported by mass spectrometric and spectrophotometric analyses indicating a possible formation mechanism by self-aggregation of L-dopa by a combination of non-covalent mechanisms including hydrogen bonds, π- π stacking and ionic bonds (Li et al. 2015). The same aggregation was observed for other structurally similar catecholamines which, like HGA, are derived from tyrosine. In support of this data, a recent ultrafiltration study on solutions of pigment derived from synthetic HGA in our laboratory (Norman [unpublished data]) using a 10,000 Da molecular-weight filter suggested a heterogeneous mixture of compounds of varying molecular weight. The filtered solution was still pigmented, although visually lighter in appearance, suggesting presence of some OP molecules >10,000 Da (Figure 5E). Together, these more recent data are inconsistent with the idea that OP or melanin are polymers comprised of regularly repeating units with distinct chemical signatures. The term 'polymer' therefore does not appear to accurately describe the chemical nature of OP or melanin, as currently understood.

Chemical characterisation of OP in human AKU tissue: Another approach employed to study the chemical structure of OP is chemical analysis of heavily pigmented AKU cartilage samples. Solid-state NMR analyses revealed remarkably similar spectra between deeply pigmented and non-pigmented cartilage from AKU patients (Chow et al. 2011; Norman et al. 2018). The spectra were dominated by clear amino acid signals attributable to collagen protein. The absence of a specific NMR signal attributable to OP or related structures could be due to the reduced sensitivity of NMR compared with other analytical platforms such as mass spectrometry. However, with NMR signal enhancement achieved by recent developments using dynamic nuclear polarisation, Norman et al. (2018) observed the first NMR signal attributable to OP in cartilage. A signal at 116.8 ppm in the ^{13}C spectrum (^{1}H-^{13}C FSLG HETCOR 2D NMR experiment) was observed from analysis of pure, dried OP derived from synthetic HGA (3-month incubation at 37°C, as above) and heavily pigmented AKU cartilage. The same signal was observed in non-ochronotic AKU cartilage but much weaker, and even

weaker in non-AKU osteoarthritic cartilage. The 2D NMR spectra were more indicative of a poly-hydroquinone versus poly-benzoquinone structure, suggesting that the final structure of OP may not be formed simply by polymerisation of the BQA intermediate.

Raman spectroscopy has recently been identified as a promising analytical technique for studying the nature of OP. Studies of cartilage samples from AKU patients revealed distinct spectra for ochronotic versus non-ochronotic cartilage. Ochronotic samples were highly fluorescent and, unlike non-ochronotic samples, provided limited to no discernible Raman spectral peaks. A novel peak was obtained from the ochronotic sample that was also observed in the spectra from pigment derived from synthetic HGA (Taylor et al. 2019). The ability of Raman spectroscopy to clearly distinguish between ochronotic and non-ochronotic tissue warrants further study, as the technique appears to have potential to provide fundamental information on the chemical nature of OP. Furthermore, there is potential for the technique to be employed as an *in vivo* tool for measuring and monitoring ochronosis progression in a clinical setting (Taylor et al. 2019).

Distribution of ochronosis is not uniform (Table 1; Figures 3, 4): At post-mortem, pigment is patchy and present in areas of stress/damage in non-cartilaginous tissues, suggesting that 'damaged' tissue undergoes pigmentation whereas 'undamaged' tissue is resistant to pigmentation; all cartilage is more consistently and highly pigmented, but also unevenly (Helliwell et al. 2008). Possible factors involved in 'damaging' tissues and producing pigment are shown in Table 1. It is not known if the OP structure is similar in nature in these diverse areas. Despite the constant exposure of all body tissues to HGA in AKU, most tissues are resistant to pigmentation.

Cartilages are especially affected, and these include highly loaded tissues such as articular cartilage, fibrocartilage (intervertebral discs, pubic symphysis), costochondral, as well as less stressed cartilage in the pinna and nose, and the trachea-bronchial system (Helliwell et al. 2008). Highly stressed tissues such as joints, spine, tendons and ligament are pigmented. OP has been noticed in the skin, especially at the interface of the palmar and dorsal skin of the hands, possibly sites of greater stress (Vijaikumar et al. 2000). Pigmentation of the nails of the hands and feet has been described. Secretory glands in the axilla, groin and eyelids pigment (Srsen et al. 1982). Ocular tissues, the conjunctivum, cornea and sclera, pigment in the second and third decade. Ochronosis has been observed in the tympanic membrane and ear wax. Cardiac valves are affected by OP more in left side of heart, and aortic more than mitral valve

(Helliwell et al. 2008); the pulmonary vascular system and the right side of the heart is much less pigmented. Pigmentation can be observed in the arterial system, mostly around branch points and tributaries, areas of greater stress (Helliwell et al. 2008). There is currently no information on pigmentation in the venous system. Renal parenchyma, especially the medulla and pyramidal tissues, show OP. The periosteum has been shown to pigment but not the bone itself, raising the possibility of bone mineral binding to collagen preventing HGA-derived molecules from binding. Teeth enamel have been said to pigment, but needs more evidence given that bone does not pigment (Siekert and Gibilisco 1970). It is generally held that muscle, liver, lung (excluding the bronchial system) and brain are not affected by OP. There is no convincing description of gastrointestinal and genital pigmentation. There is no description of pigment in pancreas, an organ where alkaline secretions are produced unlike the salivary gland. It is not known if there is biliary excretion of HGA, even though pigmented gall stones make this likely. Paucicellular tissues such as cartilage, tendon and ligament appear to pigment easily compared to highly cellular tissues such as liver.

It is likely that HGA is increased in the CSF, tears and saliva although direct evidence is lacking. Circulating HGA is well characterised but tissue HGA, i.e. intracellular HGA, has not been directly assessed. It is likely HGA (and other metabolite acids) is protein-bound but requires characterisation.

Detection of ochronosis: Gross ochronosis is easily visible as blue-black pigment in tissues such as the cartilage of the ears and sclera of the eyes. Photographs of the eyes and ears have been used to follow the OP process, both in terms of understanding the natural history as well as to study the effect of reducing HGA concentrations on OP using HPPD inhibitors, and may be the most efficacious way to follow pigment change (Ranganath et al. 2018).

However, to detect small amounts of pigment, more sensitive techniques are required. Such techniques can employ the property of HGA as a reducing agent and can be used in tissues *in vitro* and *ex vivo* to more easily detect the OP; one such approach employs Schmorl's stain, a ferricyanide reduction method (Figure 5B) for tissue reducing substances, to detect microscopic OP (Tinti et al. 2011b); this supports the idea that OP originated from HGA. Biopsy of tissues such as ear cartilage reveals OP even when it is not visible externally visually through the intact skin (Vijaikumar et al. 2000).

Investigations such as arthroscopy and bronchoscopy can reveal OP and suggest diagnosis of AKU for the first time. Diagnosis due to black aorta at open heart surgery for aortic valve replacement has been made (Karavaggelis et al. 2017). Direct detection of OP *in vivo* by Raman spectroscopy is possible. Such a technique has been validated in *ex vivo* tissue (Cox et al. [in press]) and is being adapted as an *in vivo* technique using ear cartilage and Achilles tendon, both tissues with little subcutaneous tissue (Taylor et al. 2019).

Ochronosis can be seen in *ex vivo* samples especially from joints and these can vary in extent and degree of pigment. The earliest pigment is articular cartilage is seen in calcified cartilage cells at the junction between the calcified cartilage and subchondral bone (Taylor et al. 2010c). Similarly, OP is found in intervertebral discs and adjacent articular cartilage (Helliwell et al. 2008). Investigations such as arthroscopy and bronchoscopy can reveal OP and suggest diagnosis of AKU for the first time. Diagnosis due to black aorta at open heart surgery for aortic valve replacement has been made (Cox et al. [in press]).

It would be an advantage to quantify the pigmentation *in vivo* but such a technique is not available at present. Availability of techniques to monitor changes in whole body pigment, increase or decrease or no change over time, would be highly informative.

Magnitude of flux in OP: It can be difficult to know how much metabolite flux is taking place in the phenylalanine/tyrosine pathway as these amino acids are normally fully degraded and utilised. Justus Von Liebig's theory of the minimum applied to dietary amino acid consumption suggests that amino acids lysine, threonine, methionine, and tryptophan are limiting in diet (Liebig 1831). Tyrosine and phenylalanine are not limiting and are therefore consumed to excess. Since only 5% of consumed phenylalanine and tyrosine are needed to meet normal needs, these surplus aromatic amino acids require degradation via HGA. An attempt has been made to quantify the flux in the pigment pathway following HPPD inhibitor therapy (Milan et al. [under review]); data suggests a large flux in the ochronotic pathway.

Effect of ochronosis (Table 2): The deposition of OP in tissue including cartilage alters its material properties leading to the tissue becoming hard and brittle. The Young's modulus is altered depending upon the degree of pigmentation in AKU. Our group has proposed a model of joint failure based on initiation of ochronosis in calcified cartilage before progressing to involve the entire cartilage and spiralling into joint failure, requiring joint replacement (Taylor et al. 2011b).

This process of ochronotic stiffening also compromises intervertebral discs and adjoining bone leading to severe spinal disease characterised by severe pain as well as kyphosis and scoliosis; spinal cord compression by involved discs can require decompressive spinal surgery. Spinal fusion can ensue in the latter stages resulting in loss of mobility and flexibility in all parts of the spine, but especially in the cervical and lumbar regions.

Although all cardiac valves can show ochronosis, it is the aortic valve and the aortic root that are subject to more pigmentation resulting in severe aortic stenosis requiring valve replacement surgery. Valve replacement surgery is often hazardous due to the friable ochronotic aortic root and valve.

Scleral ochronosis can distort the corneal curvature and result in astigmatism (Ranganath [unpublished observations]; Lindner and Bertelmann 2014). Ochronotic ear cartilage may be associated with pain in pinna of the ear (Ranganath [unpublished observations]). Hearing loss especially to high frequency is a feature of AKU (Pau 1984; Steven et al. 2015). Rigid articular cartilage can lead to subchondral osteopenia. Generalised osteoporosis is also noted in AKU and associated with increased fracture (Cox and Ranganath 2011). The failure of ochronotic connective tissue results in tendon ruptures, especially of the Achilles tendon, but other tendon ruptures have also been observed such as flexor and extensor foot, patellar, bicipital and gluteal regions (Manoj Kumar and Rajasekaran 2003; Ranganath and Cox 2011). Similarly ruptures of ochronotic ligaments have been described. Muscle rupture has also been reported more frequently in AKU. Stone formation featuring OP has been found in kidney, prostate, gall bladder and salivary gland, resulting in symptomatic obstruction of these organs (Taylor et al. 2010a). Renal failure can ensue both due to obstruction and renal parenchymal ochronosis, sometimes leading to fatal intractable haemolytic anaemia (Mullan et al. 2015; Davison et al. 2016).

Interestingly, the frequency of atheromatous vascular disease is not increased, despite pigmentation of atherosclerosis plaques. Neo-angiogenesis has been reported in the synovia of AKU patients (Millucci et al. 2016). Millucci and colleagues propose that in AKU angiogenesis and inflammation are inter-related pathological manifestations. These authors postulate that newly-formed blood vessels provide inflammatory cells with oxygen and nutrients, resulting in the release of pro-inflammatory cytokines which support angiogenesis. In this way angiogenesis may contribute to the progression from acute to chronic inflammation in AKU, as is the case in the chronic rheumatic disease synovitis (Millucci et al. 2016). In AKU, the exact association between angiogenesis and OP specifically is not

fully understood. However, it is plausible that neo-vascularisation increases access of circulating HGA to the synovium, thereby further propagating ochronosis and associated inflammation.

Despite a sedentary lifestyle imposed by the morbidity of AKU, overweight and obesity is not more common in AKU, and also consequently, there is no increased prevalence of diabetes. It is debatable whether this relates to loss of nutrient, i.e. HGA, in the urine from birth, comparable to the use of inhibitors of renal glucose transport, to induce nutrient loss, in diabetes management.

Mechanism of ochronotic joint and spine disease (Figure 5C): Ageing causes changes in the composition and organization of the extracellular matrix. These include loss of proteoglycans and disruption of collagen fibrils. Trauma can exacerbate these changes. Reactive molecules attack collagen fibres lacking protective proteoglycans. In AKU, homogentisic acid is the culprit leading to ochronosis (Taylor et al. 2010b). HGA-pigment modified collagen fibres become stiffened and less resistant to mechanical loading, leading to a downward spiral of structural damage. In AKU this cascade is initiated in calcified cartilage and spreads throughout the hyaline cartilage to the articular surface. Ochronosis initiates in calcified articular cartilage, beginning with the deposition of pigment in individual chondrocytes and their territorial matrix in calcified cartilage, spreading to other chondrons in the calcified matrix, then proliferating throughout the hyaline cartilage. Ochronotic cartilage shields the underlying bone from normal mechanical loading, leading to aggressive resorption of the subchondral plate, including calcified cartilage and bone, leading to catastrophic failure of the cartilage and joint itself, with fragments of cartilage escaping into the synovial space as well as impacting into underlying trabecular bone and bone marrow (Taylor et al. 2011b).

It is postulated that a similar sequence of events may also take place in the spine with OP build up in the intervertebral disc and adjacent articular cartilage, leading to increased stress in the vertebral bodies and bone loss. Direct experimental evidence for this is at present lacking.

OP and amyloid: Amyloidosis involves the accumulation of normally soluble proteins into insoluble fibrillar aggregate structures. A growing literature suggests that it is a secondary effect of ochronosis *in vitro* (Millucci et al. 2012; Spreafico et al. 2013; Braconi et al. 2017) and *in vivo* (Millucci et al. 2012, 2014, 2016, 2017). A number of observations report presence

of amyloid A protein aggregates and fibrils in AKU serum and ochronotic tissue from a number of locations including cartilage, synovia, heart, periumbrical abdominal, articular fat and labial salivary gland. Amyloidosis is well-recognised in the chronic inflammatory condition rheumatoid arthritis (Obici et al. 2009), and in AKU it is also proposed to result from oxidative protein modification due to presence of reactive oxygen species (Millucci et al. 2015a). The co-localisation of amyloid with shards of OP suggests a close association with ochronosis (Millucci et al. 2015a), although the clinical significance of amyloidosis in AKU remains unknown. In addition, the observation that OP can reverse (Ranganath et al. 2018) is difficult to explain on the basis of amyloid being the major component of pigment.

Linking HGA, ochronosis and damage: The genetic defect and the disease manifestations can be linked as follows. The genetic defect in AKU leads to an increase in HGA. Ochronosis is the result of increased HGA. Ochronotic tissue breaks down causing the multisystem damage in AKU. Conversely, lowering HGA should decrease ochronosis, in turn reducing damage and tissue breakdown.

Incubating osteoblastic (Figure 2D) and chrondrocytic cell lines with HGA leads to development of OP within 3 weeks (Tinti et al. 2011b). OP is seen both within and outside cells. The amount of OP formed is directly proportional to the concentration of HGA in the medium. In the AKU mouse model, OP develops by around 15 weeks increasing progressively in articular joints around the body of the mouse (Taylor et al. 2012; Preston et al. 2014). Decreasing HGA in mice by employing HPPD inhibitors prevents ochronosis when started soon after birth and arrests ochronosis when started later (Taylor et al. 2012; Preston et al. 2014). In humans, the OP process is accelerated when renal failure supervenes and this also leads to more rapid clinical deterioration (Introne et al. 2002).

HGA and causation of Alkaptonuria: Despite the fact that HGA is normal in those without AKU, regulators still consider HGA a biomarker of disease rather than a cause of AKU in their decisions regarding approval of drugs. Debates about causative agents in disease have raged for a long time. In the nineteenth century bacteria were blamed for causing all sorts of diseases, including alkaptonuria. A bacterium in the intestine was blamed as the culprit of the black urine of affected patients that was said to convert tyrosine to homogentisic acid. In the midst of this chaos, Robert Koch established objective rules through which a causative role could be attributed to an organism/agent/factor (Koch 1876; Brown and Goldstein 1992). We have applied Koch's postulates to AKU as seen in Table 3.

Koch's postulates and AKU (Table 3): Over time it came to be recognized that Koch's postulates did not comply with all situations to establish a causal relationship and other criteria have been proposed such as the Bradford-Hill criteria or Hill's criteria for causation (Hill 1965). We have applied Hill's criteria for HGA in AKU also as shown in Table 3. It is clear that HGA conforms to the requirements for a causative molecule.

Lessons for AKU from the cholesterol and atherosclerosis and cardiovascular disease (Table 4): What can AKU learn from other disorders? There are strong similarities between familial hypercholesterolaemia (FH) and AKU; both are inherited diseases that form a template for the more common conditions cardiovascular disease (CVD) and osteoarthritis, respectively. AKU and FH are present from birth but the effects are delayed. HGA is the culprit molecule in AKU, while cholesterol is the molecule in CVD. Ochronosis is the process by which HGA causes the morbidity, while the comparable process in CVD is atherosclerosis. HPPD inhibitors decrease HGA production, while HMG-CoA reductase inhibitors (statins) decrease cholesterol production. Recent data suggests partial reversibility of external ochronosis by HPPD inhibition, while data from imaging studies have shown a similar partial reversal of atherosclerosis by reducing cholesterol (Nissen et al. 1991). A study has been reported showing a slower progression of AKU using HPPD inhibition, similar to statin trials on CVD progression (Ranganath et al. 2018). However it is worth noting differences in the inheritance of AKU and FH; AKU is autosomal recessive with prevalence of 1:250,000 worldwide, whereas FH is autosomal dominant (Nordestgaard et al. 2013) and relatively more common with estimated worldwide prevalence of 1:200-300 (Vallejo-Vaz and Ray 2018). Table 4 shows the comparison between AKU and FH.

It is worth emphasizing that modification of the disease process (atherosclerosis) by imaging has been used as outcomes in a number of studies in CVD. This is consistent with a proposal to similarly approve the use of modification of the OP process in AKU as acceptable outcomes in clinical interventional studies. Statins have revolutionised the management of cardiovascular disease, and in FH it has been shown that earlier use of statins at low dose is superior in prevention terms compared to use of high dose statins later on in the natural history (Nordestgaard et al. 2013). Mouse studies indicate that nitisinone treatment from soon after birth completely prevents ochronosis, the main pathophysiological process in AKU (Preston et al. 2014).

Therapy and ochronosis:

Antioxidant therapy by preventing oxidation of HGA to BQA, is expected to decrease ochronosis but for reasons which are unclear antioxidant ascorbic acid therapy has no clear benefit in AKU, a condition with a proposed oxidant-damage hypothesis in terms of ochronosis formation (Roberts et al. 2015); this is analogous to the lack of efficacy of antioxidant strategies in coronary artery disease studies despite the well-validated oxidised LDL theory of atherogenesis (Ranganath et al. 2013).

Lower protein intakes should also in theory be associated with less ochronosis and less morbidity in AKU. However, except occasional case reports in childhood, there is no evidence that restricting dietary protein decreases ochronosis. Anecdotally vegetarians and vegans are noted to have less ochronosis and lower morbidity but no systematic evidence exists to support low protein diet in AKU (Ranganath [unpublished observations]). All other approved therapies used in clinical practice are supportive and palliative and do not address the HGA and its effects (Ranganath et al. 2013).

The use of HPPD inhibition employing nitisinone to decrease flux in the tyrosine pathway has revolutionised the treatment of hereditary tyrosinaemia (Lindstedt et al. 1992; McKiernan 2013). Nitisinone was first suggested as a treatment for HGA in AKU in 1998 (Anikster et al. 1998), and is now shown to be highly effective in reducing HGA despite not being approved in AKU (Ranganath et al. 2018). Nitisinone not only decreases HGA but also arrests ochronosis in mice (Preston et al. 2014). A recent publication shows photographic evidence of partial reversal of ochronosis in sclera and ear cartilage in AKU patients (Ranganath et al. 2018).

The NIH carried out a nitisinone interventional study in AKU between 2005 and 2009 and showed a sustained and marked decrease in urine HGA over the 3-year duration of the study (Davison et al. 2015). However, their agreement with the regulatory agency, the FDA, to approve nitisinone for AKU, required them to show a difference in range of motion at the hip between the nitisinone-treated and untreated groups; statistical significance was not found for the range of motion comparison and the study reported inconclusive.

The dose of nitisinone used in the NIH study was 2 mg daily. This same dose is being used in the NAC, as mentioned in a recent publication (Ranganath et al. 2018). Further the NAC data in the publication confirmed the biochemical efficacy of nitisinone in AKU in terms of urinary HGA, but also showed slower progression of morbidity. Finally, the NAC data showed overt partial reversal of ochronosis, the primary pathogenetic event in AKU.

It is important to note that nitisinone, while shown to be a highly effective therapy for preventing ochronosis, is not a perfect therapy in AKU. It is well recognised that nitisinone treatment results in siginificant hyper-tyrosinaemia in AKU (Phornphutkul et al. 2002) and hereditary tyrosinaemia type-I (HT-I) (Lindstedt et al. 1992; McKiernan 2013). The consequences of hyper-tyrosinaemia are not fully understood in AKU, but concerns have been raised that it may contribute to the neurodevelopmental delay observed in infants with HT-I on nitisinone therapy (McKiernan 2013). Recent analyses did not find changes to monoamine neurotransmitters in brain tissue from nitisinone-treated mice (Davison et al. 2019), although more data are required to fully ascertain the impact of hyper-tyrosinaemia on central nervous system homoeostasis. Other potential future therapies for AKU may employ approaches to directly restore HGD activity for example by enzyme replacement therapy and gene therapy. However research into these approaches is still in very early stages, and they are also not without their own concerns and challenges, for example potential off-target effects.

We believe that it is time that the scientific community recognised the fundamental role played by pigmentation due to ochronosis in AKU and to take this into consideration when assessing the disease as well as effectiveness of treatments for the disease. The accumulation of HGA in AKU is comparable to the increase in xylulose in pentosuria, but unlike pentosuria where there is no syndrome associated with pentose sugar accumulation, the situation is different in AKU, a condition dominated by ochronosis. While much has been learnt about ochronosis, there still remains unanswered questions.

References

Allcock H, Lampe F, Mark J (2003) Contemporary Polymer Chemistry, 3rd edn. Prentice Hall, New Jersey

Anikster Y, Nyhan WL, Gahl WA (1998) NTBC and Alkaptonuria. Am J Hum Genet 63:920–921. doi: 10.1086/302027

Bernardini G, Leone G, Millucci L, et al (2019) Homogentisic acid induces morphological and mechanical aberration of ochronotic cartilage in alkaptonuria. J Cell Physiol 234:6696–708. doi: 10.1002/jcp.27416

Bhattar P, Zawar V, Godse K, et al (2015) Exogenous Ochronosis. Indian J Dermatol 60:537–42. doi: 10.4103/0019-5154.169122

Braconi D, Bernardini G, Bianchini C, et al (2012) Biochemical and proteomic characterization of alkaptonuric chondrocytes. J Cell Physiol 227:3333–43. doi: 10.1002/jcp.24033

Braconi D, Bianchini C, Bernardini G, et al (2011) Redox-proteomics of the effects of homogentisic acid in an in vitro human serum model of alkaptonuric ochronosis. J Inherit Metab Dis 34:1163–76. doi: 10.1007/s10545-011-9377-6

Braconi D, Laschi M, Amato L, et al (2010a) Evaluation of anti-oxidant treatments in an in vitro model of alkaptonuric ochronosis. Rheumatology 49:1975–83. doi: 10.1093/rheumatology/keq175

Braconi D, Laschi M, Taylor AM, et al (2010b) Proteomic and redox-proteomic evaluation of homogentisic acid and ascorbic acid effects on human articular chondrocytes. J Cell Biochem 111:922–32. doi: 10.1002/jcb.22780

Braconi D, Millucci L, Bernardini G, Santucci A (2015) Oxidative stress and mechanisms of ochronosis in alkaptonuria. Free Radic Biol Med 88(Pt A):70–80. doi: 10.1016/j.freeradbiomed.2015.02.021

Braconi D, Millucci L, Bernini A, et al (2017) Homogentisic acid induces aggregation and fibrillation of amyloidogenic proteins. Biochim Biophys acta Gen Subj 1861:135–146. doi: 10.1016/j.bbagen.2016.11.026

Brown MS, Goldstein JL (1992) Koch's postulates for cholesterol. Cell 71:187–188. doi: 10.1016/0092-8674(92)90346-E

Chen CT, Chuang C, Cao J, et al (2014) Excitonic effects from geometric order and disorder explain broadband optical absorption in eumelanin. Nat Commun 5:3859. doi: 10.1038/ncomms4859

Chow WY, Taylor AM, Reid DG, et al (2011) Collagen atomic scale molecular disorder in ochronotic cartilage from an alkaptonuria patient, observed by solid state NMR. J Inherit Metab Dis 34:1137–40. doi: 10.1007/s10545-011-9373-x

Cox T, Psarelli E, Taylor S, et al Subclinical ochronosis features in Alkaptonuria: a cross-sectional study. BMJ Innov in press:

Cox TF, Ranganath L (2011) A quantitative assessment of alkaptonuria: Testing the reliability of two disease severity scoring systems. J Inherit Metab Dis 34:1153–62. doi: 10.1007/s10545-011-9367-8

Damian LO, Felea I, Boloşiu C, et al (2013) A case of alkaptonuria - ultrasonographic findings. Med Ultrason 15:321–25. doi: 10.11152/mu.2013.2066.154.lod2

Davison AS, Milan AM, Gallagher JA, Ranganath LR (2016) Acute fatal metabolic complications in alkaptonuria. J Inherit Metab Dis 39:203–10. doi: 10.1007/s10545-015-9902-0

Davison AS, Milan AM, Hughes AT, et al (2015) Serum concentrations and urinary excretion of homogentisic acid and tyrosine in normal subjects. Clin Chem Lab Med 53:e81-3. doi: 10.1515/cclm-2014-0668

Davison AS, Strittmatter N, Sutherland H, et al (2019) Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging. Metabolomics 15:81. doi: 10.1007/s11306-019-1531-4

Fisher AA, Davis MW (2004) Alkaptonuric ochronosis with aortic valve and joint replacements and femoral fracture: a case report and literature review. Clin Med Res 2:209–15. doi: 10.3121/cmr.2.4.209

Gaines JJ, Tom GD, Khankhanian N (1987) An ultrastructural and light microscopic study of the synovium in ochronotic arthropathy. Hum Pathol 18:1160–4. doi: 10.1016/S0046-8177(87)80385-4

Galdston M, Steele JM, Dobriner K (1952) Alcaptonuria and ochronosis; with a report of three patients and metabolic studies in two. Am J Med 13:432–52

Gallagher JA, Dillon JP, Sireau N, et al (2016) Alkaptonuria: An example of a "fundamental disease"-A rare disease with important lessons for more common disorders. Semin Cell Dev Biol 52:52–57. doi: 10.1016/j.semcdb.2016.02.020

Garrod AE (1902) The incidence of alkaptonuria: a study in chemical individuality. Lancet 2:1616–20. doi: 10.1016/S0140-6736(01)41972-6

Helliwell TR, Gallagher J a., Ranganath L (2008) Alkaptonuria - A review of surgical and autopsy pathology. Histopathology 53:503–512. doi: 10.1111/j.1365-2559.2008.03000.x

Hill AB (1965) The Environment and Disease: Association or Causation? Proc R Soc Med 58:295

Hunter RC, Newman DK (2010) A putative ABC transporter, hatABCDE, is among molecular determinants of pyomelanin production in Pseudomonas aeruginosa. J Bacteriol 192:5962–71. doi: 10.1128/JB.01021-10

Introne WJ, Perry MB, Troendle J, et al (2011) A 3-year randomized therapeutic trial of nitisinone in alkaptonuria. Mol Genet Metab 103:307–14. doi: 10.1016/j.ymgme.2011.04.016

Introne WJ, Phornphutkul C, Bernardini I, et al (2002) Exacerbation of the ochronosis of alkaptonuria due to renal insufficiency and improvement after renal transplantation. Mol Genet Metab 77:136–142. doi: 10.1016/S1096-7192(02)00121-X

Karavaggelis A, Young C, Attia R (2017) Black Heart at Surgery - Primary Diagnosis of Alkaptonuria at Surgery. J Cardiol Curr Res 9:00335. doi: 10.15406/jccr.2017.09.00336

Keenan CM, Preston AJ, Sutherland H, et al (2015) Nitisinone arrests but does not reverse

ochronosis in alkaptonuric mice. J Inherit Metab Dis 73:284-9.

Knox WE (1958) Sir Archibald Garrod's Inborn Errors of Metabolism. IV. Pentosuria. Am J Hum Genet 10:385–97

Koch R (1876) Investigations into bacteria: V. The etiology of anthrax, based on the ontogenesis of Bacillus anthracis [in German]. Cohns Beitrage zur Biol der Pflanz 2:227–310

La Du BN, Zannoni VG, Laster L, Seegmiller JE (1958) The nature of the defect in tyrosine metabolism in alcaptonuria. J Biol Chem 230:251–60

Levin CY, Maibach H (2001) Exogenous ochronosis: An update on clinical features, causative agents and treatment options. Am J Clin Dermatol 2:213–17. doi: 10.2165/00128071-200102040-00002

Lewis R (2018) Consequences and prevention of elevated circulating tyrosine during nitisinone therapy in alkaptonuria. Liverpool John Moores University

Li Y, Liu J, Wang Y, et al (2015) Mass Spectrometric and Spectrophotometric Analyses Reveal an Alternative Structure and a New Formation Mechanism for Melanin. Anal Chem 87:7958–63. doi: 10.1021/acs.analchem.5b01837

Liebig J (1831) Ueber einen neuen Apparat zur Analyse organischer Körper, und über die Zusammensetzung einiger organischen Substanzen. Ann Phys 21:1–47. doi: 10.1002/andp.18310970102

Lindner M, Bertelmann T (2014) On the ocular findings in ochronosis: a systematic review of literature. BMC Ophthalmol 14:12. doi: 10.1186/1471-2415-14-12

Lindstedt S, Holme E, Lock EA, et al (1992) Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet 340:813–17. doi: 10.1016/0140-6736(92)92685-9

Manoj Kumar R V, Rajasekaran S (2003) Spontaneous tendon ruptures in alkaptonuria. J Bone Joint Surg Br 85:883–6

Martin JP, Batkoff B (1987) Homogentisic acid autoxidation and oxygen radical generation: implications for the etiology of alkaptonuric arthritis. Free Radic Biol Med 3:241–50

McKiernan PJ (2013) Nitisinone for the treatment of hereditary tyrosinemia type I. Expert Opin Orphan Drugs 1:491–7. doi: 10.1517/21678707.2013.800807

McNaught AD, Wilkinson A (2014) IUPAC. Compendium of Chemical Terminology 2nd ed. (the "Gold Book"). Blackwell Scientific Publications, Oxford

Mekala LP, Mohammed M, Chintalapati S, Chintalapati VR (2018) Stable Isotope-Assisted Metabolic Profiling Reveals Growth Mode Dependent Differential Metabolism and Multiple Catabolic Pathways of l -Phenylalanine in Rubrivivax benzoatilyticus JA2. J Proteome Res 17:189–202. doi: 10.1021/acs.jproteome.7b00500

Mekala LP, Mohammed M, Chinthalapati S, Chinthalapati VR (2019) Pyomelanin production: Insights into the incomplete aerobic L-phenylalanine catabolism of a photosynthetic bacterium, Rubrivivax benzoatilyticus JA2. Int J Biol Macromol 126:755–64. doi: 10.1016/j.ijbiomac.2018.12.142

Milan A, Hughes A, Davison A, et al Quantification of tyrosine flux in the ochronotic

660 pathway during nitisinone treatment of Alkaptonuria. (under review)

661 Millucci L, Bernardini G, Marzocchi B, et al (2016) Angiogenesis in alkaptonuria. J Inherit
662 Metab Dis 39:801–06. doi: 10.1007/s10545-016-9976-3

663 Millucci L, Bernardini G, Spreafico A, et al (2017) Histological and Ultrastructural
664 Characterization of Alkaptonuric Tissues. Calcif Tissue Int 101:50–64. doi:
665 10.1007/s00223-017-0260-9

666 Millucci L, Braconi D, Bernardini G, et al (2015a) Amyloidosis in alkaptonuria. J Inherit
667 Metab Dis 38:797–805. doi: 10.1007/s10545-015-9842-8

668 Millucci L, Ghezzi L, Braconi D, et al (2014) Secondary amyloidosis in an alkaptonuric
669 aortic valve. Int J Cardiol 172:e121-3. doi: 10.1016/j.ijcard.2013.12.117

670 Millucci L, Giorgetti G, Viti C, et al (2015b) Chondroptosis in alkaptonuric cartilage. J Cell
671 Physiol 230:1148–57. doi: 10.1002/jcp.24850

672 Millucci L, Spreafico A, Tinti L, et al (2012) Alkaptonuria is a novel human secondary
673 amyloidogenic disease. Biochim Biophys Acta - Mol Basis Dis 1822:1682–91. doi:
674 10.1016/j.bbadis.2012.07.011

675 Mistry JB, Jackson DJ, Bukhari M, Taylor AM (2016) A role for interleukins in ochronosis in
676 a chondrocyte in vitro model of alkaptonuria. Clin Rheumatol 35:1849–56. doi:
677 10.1007/s10067-015-3091-y

678 Mullan A, Cocker D, Taylor G, et al (2015) Fatal oxidative haemolysis and
679 methaemoglobinaemia in a patient with alkaptonuria and acute kidney injury. Clin
680 Kidney J 8:109–12. doi: 10.1093/ckj/sfu121

681 Nissen SE, Gurley JC, Grines CL, et al (1991) Intravascular ultrasound assessment of lumen
682 size and wall morphology in normal subjects and patients with coronary artery disease.
683 Circulation 84:1087–99. doi: 10.1161/01.CIR.84.3.1087

684 Nordestgaard BG, Chapman MJ, Humphries SE, et al (2013) Familial hypercholesterolaemia
685 is underdiagnosed and undertreated in the general population: guidance for clinicians to
686 prevent coronary heart disease: Consensus Statement of the European Atherosclerosis
687 Society. Eur Heart J 34:3478–3490. doi: 10.1093/eurheartj/eht273

688 Norman B Unpublished data

689 Norman BP, Ying CW, Sutherland H, et al (2018) Disruption of collagen triple helix
690 hydrogen bonding in ochronotic human cartilage in alkaptonuria observed by dynamic
691 nuclear polarisation-enhanced solid-state nuclear magnetic resonance spectroscopy.
692 Osteoarthr Cartil 26:S99. doi: 10.1016/j.joca.2018.02.214

693 O'Brien WM, La Du BN, Bunim JJ (1963) Biochemical, pathologic and clinical aspects of
694 alcaptonuria, ochronosis and ochronotic arthropathy - Review of world literature (1584-
695 1962). Am J Med 34:813–38. doi: 10.1385/1-59259-763-7

696 Obici L, Raimondi S, Lavatelli F, et al (2009) Susceptibility to AA amyloidosis in rheumatic
697 diseases: a critical overview. Arthritis Rheum 61:1435–40. doi: 10.1002/art.24735

698 Pau HW (1984) Involvement of the tympanic membrane and ear ossicle system in ochronotic
699 alkaptonuria [German]. Laryngol Rhinol Otol (Stuttg) 63:541–4

700 Phornphutkul C, Introne WJ, Perry MB, et al (2002) Natural History of Alkaptonuria. N Engl

701 J Med 347:2111–21. doi: 10.1056/nejmoa021736

702 Preston AJ, Keenan CM, Sutherland H, et al (2014) Ochronotic osteoarthropathy in a mouse
703 model of alkaptonuria, and its inhibition by nitisinone. Ann Rheum Dis 73:284–9. doi:
704 10.1136/annrheumdis-2012-202878

705 Ranganath L Unpublished observations

706 Ranganath LR, Cox TF (2011) Natural history of alkaptonuria revisited: Analyses based on
707 scoring systems. J Inherit Metab Dis 34:1141–51. doi: 10.1007/s10545-011-9374-9

708 Ranganath LR, Jarvis JC, Gallagher JA (2013) Recent advances in management of
709 alkaptonuria (invited review; best practice article). J Clin Pathol 66:367–373. doi:
710 10.1136/jclinpath-2012-200877

711 Ranganath LR, Khedr M, Milan AM, et al (2018) Nitisinone arrests ochronosis and decreases
712 rate of progression of Alkaptonuria: Evaluation of the effect of nitisinone in the United
713 Kingdom National Alkaptonuria Centre. Mol Genet Metab 125:127–34. doi:
714 10.1016/j.ymgme.2018.07.011

715 Riesz J (2007) The spectroscopic properties of melanin. University of Queensland

716 Roberts NB, Curtis SA, Milan AM, Ranganath LR (2015) The pigment in alkaptonuria
717 relationship to melanin and other coloured substances: A review of metabolism,
718 composition and chemical analysis. JIMD Rep 24:51–66. doi: 10.1007/8904_2015_453

719 Siekert RG, Gibilisco JA (1970) Discoloration of the teeth in alkaptonuria (ochronosis) and
720 parkinsonism. Oral Surgery, Oral Med Oral Pathol 29:197–99. doi: 10.1016/0030-
721 4220(70)90081-2

722 Spreafico A, Millucci L, Ghezzi L, et al (2013) Antioxidants inhibit SAA formation and pro-
723 inflammatory cytokine release in a human cell model of alkaptonuria. Rheumatol
724 (United Kingdom) 52:1667–73. doi: 10.1093/rheumatology/ket185

725 Srsen S, Srsnová K, Lányi A (1982) [Clinical manifestation of alkaptonuria in relation to age
726 (author's transl)]. Bratisl Lek Listy 77:662–9

727 Steven RA, Kinshuck AJ, McCormick MS, Ranganath LR (2015) ENT manifestations of
728 alkaptonuria: report on a case series. J Laryngol Otol 129:1004–8. doi:
729 10.1017/S0022215115002315

730 Taylor AM, Batchelor TJP, Adams VL, et al (2011a) Ochronosis and calcification in the
731 mediastinal mass of a patient with alkaptonuria. J Clin Pathol 64:935–6. doi:
732 10.1136/jcp.2011.090126

733 Taylor AM, Boyde A, Wilson PJM, et al (2011b) The role of calcified cartilage and
734 subchondral bone in the initiation and progression of ochronotic arthropathy in
735 alkaptonuria. Arthritis Rheum 63:3387–96. doi: 10.1002/art.30606

736 Taylor AM, Hsueh MF, Ranganath LR, et al (2017) Cartilage biomarkers in the
737 osteoarthropathy of alkaptonuria reveal low turnover and accelerated ageing.
738 Rheumatology (Oxford) 56:156–64. doi: 10.1093/rheumatology/kew355

739 Taylor AM, Jenks DD, Kammath VD, et al (2019) Raman Spectroscopy identifies differences
740 in ochronotic and non-ochronotic cartilage; a potential novel technique for monitoring
741 ochronosis. Osteoarthr Cartil S1063-4584:. doi: 10.1016/j.joca.2019.04.012

742 Taylor AM, Kammath V, Bleakley A (2016) Tyrosinase, could it be a missing link in
743 ochronosis in alkaptonuria? Med Hypotheses 91:77–80. doi:
744 10.1016/j.mehy.2016.04.001

745 Taylor AM, Preston AJ, Paulk NK, et al (2012) Ochronosis in a murine model of
746 alkaptonuria is synonymous to that in the human condition. Osteoarthr Cartil 20:880–
747 886. doi: 10.1016/j.joca.2012.04.013

748 Taylor AM, Vercruysse KP (2017) Analysis of Melanin-like Pigment Synthesized from
749 Homogentisic Acid, with or without Tyrosine, and Its Implications in Alkaptonuria.
750 JIMD Rep 35:79–85. doi: 10.1007/8904_2016_27

751 Taylor AM, Wilson PJM, Ingrams DR, et al (2010a) Calculi and intracellular ochronosis in
752 the submandibular tissues from a patient with alkaptonuria. J Clin Pathol 63:186–8. doi:
753 10.1136/jcp.2009.071365

754 Taylor AM, Wlodarski B, Prior IA, et al (2010b) Ultrastructural examination of tissue in a
755 patient with alkaptonuric arthropathy reveals a distinct pattern of binding of ochronotic
756 pigment. Rheumatology 49:1412–14. doi: 10.1093/rheumatology/keq027

757 Taylor AM, Wlodarski B, Wilson PJ, et al (2010c) Deposition of ochronotic pigment in
758 articular cartilage in alkaptonuria is initiated near the tidemark and progresses to the
759 articular surface. Bone 47:S79. doi: 10.1016/j.bone.2010.04.156

760 Tinti L, Spreafico A, Braconi D, et al (2010) Evaluation of antioxiodant drugs for the
761 treatment of ochronotic alkaptonuria in an in vitro human cell model. J Cell Physiol
762 225:84–91. doi: 10.1002/jcp.22199

763 Tinti L, Spreafico A, Chellini F, et al (2011a) A novel ex vivo organotypic culture model of
764 alkaptonuria-ochronosis. Clin Exp Rheumatol 29:693–6

765 Tinti L, Taylor AM, Santucci A, et al (2011b) Development of an in vitro model to
766 investigate joint ochronosis in alkaptonuria. Rheumatology 50:271–7. doi:
767 10.1093/rheumatology/keq246

768 Tokuhara Y, Shukuya K, Tanaka M, et al (2014) Detection of Novel Visible-Light Region
769 Absorbance Peaks in the Urine after Alkalization in Patients with Alkaptonuria. PLoS
770 One 9:e86606. doi: 10.1371/journal.pone.0086606

771 Tokuhara Y, Shukuya K, Tanaka M, et al (2018) Absorbance measurements of oxidation of
772 homogentisic acid accelerated by the addition of alkaline solution with sodium
773 hypochlorite pentahydrate. Sci Rep 8:11364. doi: 10.1038/s41598-018-29769-w

774 Tran ML, Powell BJ, Meredith P (2006) Chemical and structural disorder in eumelanins: A
775 possible explanation for broadband absorbance. Biophys J 90:743–52. doi:
776 10.1529/biophysj.105.069096

777 Vallejo-Vaz AJ, Ray KK (2018) Epidemiology of familial hypercholesterolaemia:
778 Community and clinical. Atherosclerosis 277:289–297. doi:
779 10.1016/j.atherosclerosis.2018.06.855

780 Vijaikumar M, Thappa DM, Srikanth S, et al (2000) Alkaptonuric ochronosis presenting as
781 palmoplantar pigmentation. Clin Exp Dermatol 25:305–7. doi: 10.1046/j.1365-
782 2230.2000.00649.x

783 Virchow R (1866) Ein Fall von allgemeiner Ochronose der Knorpel und knorpelähnlichen

784 Theile. Arch für Pathol Anat und Physiol und für Klin Med 37:121–19. doi:
785 10.1007/bf01935634

786 Zannoni VG, Lomtevas N, Goldfinger S (1969) Oxidation of homogentisic acid to ochronotic
787 pigment in connective tissue. Biochim Biophys Acta 177:94–105

788 Zannoni VG, Malawista SE, La Du BN (1962) Studies on ochronosis. II. Studies on
789 benzoquinoneacetic acid, a probable intermediate in the connective tissue pigmentation
790 of alcaptonuria. Arthritis Rheum 5:547–556. doi: 10.1002/art.1780050603

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Tables

Table 1. Factors influencing tissue ochronotic pigmentation

Tissue	Damaging factor
Spine intervertebral discs	Weight bearing stress
Joints	Weight bearing stress, movement damage
Tendons	Tensile stresses
Ligaments	Tensile stresses
Aortic valve	Systemic blood pressure, expansile stress
Aortic root	Systemic blood pressure, expansile stress
Arterial tree branch points	Shear stress, Bernoulli effect
Airways cartilage	Expansion, lengthening and contracting
Ear cartilage	Pressure on ears during sleep
Sclera and conjunctiva	UV light damage, stress arising from muscle contraction

797 Table 2. The effect of ochronosis on tissues

Tissue with ochronosis	Effect
Articular cartilage	Resorption of calcified articular cartilage and subchondral bone and joint failure
Articular cartilage	Osteopenia due to resorption activated by stiff pigment
Intervertebral discs	Spondylosis, fracture, osteopenia, scoliosis, kyphosis, cord compression, radiculopathy
Ligament	Rupture
Aortic valve and root	Aortic stenosis and Aortic rigidity
Tendons (e.g.Achilles, foot flexor and extensor, patellar, gluteal, biceps)	Rupture
Sclera	Distortion in corneal curvature; astigmatism, glaucoma
Ear cartilage	Possible pain in ear
Middle/inner ear	Hearing loss, ear ossicles disorder leading to conductive deafness and high frequency hearing loss

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800 Table 3. Koch's postulates and its application to Alkaptonuria

Koch's postulates rules	**Relevance to AKU**
The agent must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms	HGA found in abundance in AKU but not in non-AKU
The microorganism must be isolated from a diseased organism and grown in pure culture.	HGA can be isolated from AKU patients by preparative HPLC techniques to yield pure HGA.
The cultured microorganism should cause disease when introduced into a healthy organism.	HGA can be incubated with cell cultures and shown to produce the features of AKU namely pigment.
The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.	Producing a mutation of the HGD gene in a normal mouse can produce an AKU mouse showing high HGA.
Hill's Criteria and HGA and cause in AKU	
Bradford-Hill criteria for causation	**How does AKU fit in**
Strength: A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal	Strong relationship between HGA and disease severity.
Consistency: Consistent findings observed by different persons in different places with	Link between HGA in AKU and disease by various dispersed researchers.

different samples strengthens the likelihood of an effect.	
Specificity: Causation is likely if there is a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship	Only HGA causes the disease process characteristic of AKU
Temporality: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay)	In mouse models where the mutation of HGD has been produced, HGA levels increase after the mutation. The disease process however develops after slight delay both in mouse and humans.
Biological Gradient: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence	Places with high frequency of HGD mutations have high HGA and AKU disease.
Plausibility: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge)	HGA can reproduce the disease process ochronosis *in vivo* and is therefore plausible.

Coherence: Coherence between epidemiological and laboratory findings increases the likelihood of an effect.	The relationship between mutations of the HGD gene, high HGA, increasing ochronosis over time and increasing morbidity is coherent.
Experiment: Occasionally it is possible to appeal to experimental evidence	Tissues exposed to HGA produce a similar disease process to naturally occurring condition AKU
Analogy: The effect of similar factors may be considered	HGA in AKU is similar to cholesterol in atheromatous coronary disease.

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803 Table 4. Comparison between Familial Hypercholesterolaemia and Alkaptonuria

	Familial hypercholesterolaemia	**Alkaptonuria**
Frequency	Heterozygote 1 in 500 Homozygote 1 in 1,000,000	Heterozygote 1 in 500 Homozygote 1 in 1,000,000
Causative agent	Cholesterol (LDL)	Homogenstic acid
Condition	Familial hypercholesterolaemia	Alkaptonuria
Genetic defect	LDL receptor, Apo-B defects, PCSK9 mutations	HGD mutations
Disease process	Atherosclerosis	Ochronosis
Main consequence	Atherosclerosis	Ochronosis
Main disease	Myocardial infarction	Spondyloarthropathy
Childhood	Minimal disease	Minimal disease
Latent period	40 -50 years in heterozygous 20 years in homozygous	20-30y
Prevention	Yes, lifestyle and statins	Nitisinone
Lifestyle factors	Yes, hypertension, physical activity, Smoking, diabetes, others	Diet, activity, occupation
Disease modifying therapy	HMG CoA inhibitors (Statins), others	HPPD inhibitors, Nitisinone
Reversibility	Partial	Partial
Earlier treatment beneficial	Yes	Probably yes, mouse data

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Legend to Figures

Figure 1. Tyrosine metabolic pathway – highlighting (1) the metabolic fate of tyrosine in health, (2) site of the enzyme defect observed in Alkaptonuria, *homogentisate dioxygenase* (*HGD* EC 1.13.11.5) and Hereditary Tyrosinaemia type 1, maleylacetoacetate isomerase (*MAI* EC 5.2.1.2), and (3) the site where nitisinone inhibits *4-hydroxyphenylpyruvate dioxygenase* (*HPPD* EC 1.13.11.27) activity

Figure 2. Ochronotic pigmentation features (1). A. Ochre or yellowish discoloration due to ochronotic pigment in the unstained ear cartilage in two patients with alkaptonuria; less in left panel, more in right panel. B. TEM image of ochronotic ligamentous capsule. Collagen fibres in longitudinal section show a distinct electron-dense pigment on their surface. Not all fibres present with pigment deposition. Numerous pigment shards can be seen on single fibres. Gradient of pigmentation can be seen running left (no pigment on fibres) to right (large electron-dense shards replacing fibres). Arrows indicate a distinct periodic binding pattern associated with pigment granules on a single fibre. C. Schematic representation of the development of ochronosis. Genetic lack of homogentisate 1,2-dioxygenase leads to an increase in concentration of homogentisic acid (HGA). HGA, its oxidation product, benzoquinone acetic acid or the final product ochronotic pigment binds to collagenous matrices. Initially, matrix is resistant to pigmentation, but following loss or breakdown of specific constituents, including proteoglycans (PG), HGA-associated compounds access binding sites which are associated with the ultrastructural periodicity of the collagen fibrils. It is proposed that the initial binding event initiates ochronosis and that the process of widespread joint pigmentation occurs over time. Pigmentation increases the stiffness of the collagen fibres, which leads to further biomechanical and biochemical damage and a downward spiral of ochronosis and tissue destruction. D. Schmorl's staining of HGA-derived pigment deposits in cultures of SaOS-2 cells. E. Samples obtained during ultrafiltration of solutions of ochronotic pigment formed via incubation of an aqueous solution of HGA (10 mmol/L) at 37 °C for 3 months. Solutions 1 and 2 are samples pre- and post-centrifugation (10 min at 2500 x g) respectively. The pigment solution was filtered using an Amicon Ultracel 10 K filter (Merck Millipore) by centrifugation (10 min at 2500 x g). Solution 3 is taken from the portion of the solution that had not passed the filter after 10 min centrifugation. Solution 4 is a sample of the filtrate and is visibly lighter in colour, indicating that the 10 K filter had retained some of the pigment.

Figure 3. Ochronotic pigmentation features (2). A. Dark urine of AKU. B. External ear cartilage pigmentation. C. Dark pigmentation of temporal aspect of sclera in right eye with vessels coursing superficial to pigmentation. D. Unstained cut section of femur condyle showing little pigment on left side progressing to full thickness on the right side. E. Longitudinal cut section of abdominal aorta and common iliac bifurcation, showing more pigment at bifurcation and branch point orifices. F. Pigment at junction of palmer and dorsal skin of hands.

Figure 4. Ochronotic pigmentation features (3). A. Markedly ochronotic bulging intervertebral discs and vertebral body seen from within abdominal cavity. B. Spine seen from posterior or dorsal aspect showing ochronotic pigment. C. Dark pigmentation seen in arthroscopy of knee joint showing fibrillar blackened cartilage. D. Appearance of pigmented head of femur showing uniform pigmentation. E. Hip joint showing rim of cartilage and

849 marked cartilage loss with exposure of underlying bone. F. Low pressure pulmonary trunk
850 and valve showing little pigment. G. Aortic root and valve with marked pigmentation.

851 **Figure 5.** Presence of ochronotic pigment in the knee joint and its progression in AKU. A.
852 Ochronotic pigment in the joint capsule of the knee of a patient with AKU. Pigment deposits
853 are seen as brown granules in the ECM and within fibroblasts. Section stained with nuclear
854 fast red. B. Near serial section of the joint capsule with Schmorl's stain. Ochronotic deposits
855 are stained green. Bar: 50 μm. C. Schematic representation of the progression of ochronosis
856 in articular cartilage from initiation in calcified cartilage to eventual destruction of the joint:
857 a) ochronosis begins with the deposition of pigment in individual chondrocytes and their
858 territorial matrix in calcified cartilage. Pigmentation leads to focal increases of stiffness
859 altering the load distribution and inducing stress risers; b) ochronosis spreads to other
860 chondrons in the calcified matrix, then c) proliferates throughout the hyaline cartilage; d)
861 ochronotic cartilage shields the underlying bone from normal mechanical loading, leading to
862 aggressive resorption of the subchondral plate, including calcified cartilage and bone; e)
863 despite the increased stiffness, the pigmented shell of the remaining articular cartilage fails
864 catastrophically. Pigmented cartilage becomes impacted on the underlying trabecular bone
865 and embedded in the marrow space.

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867 Figure 1

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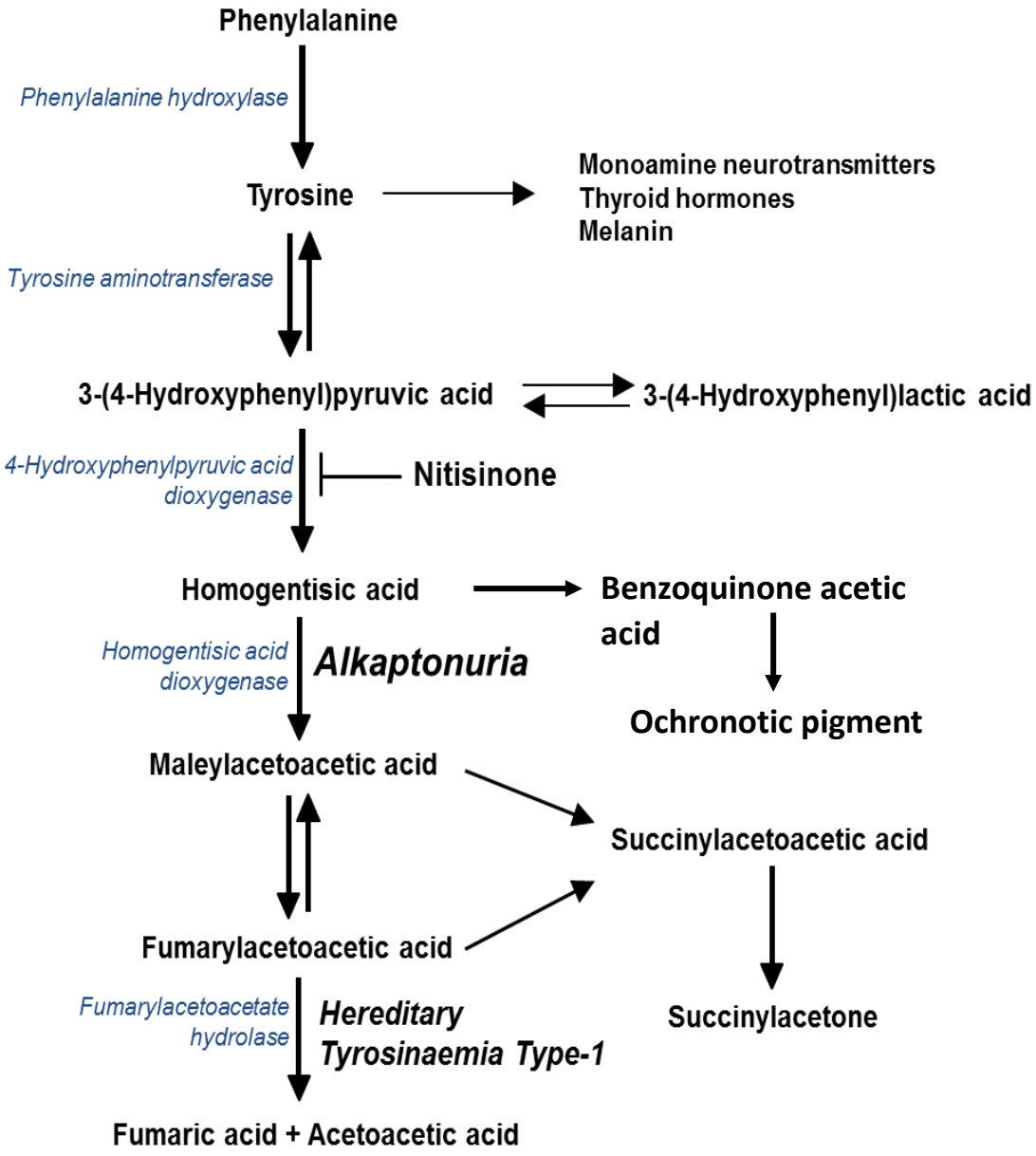
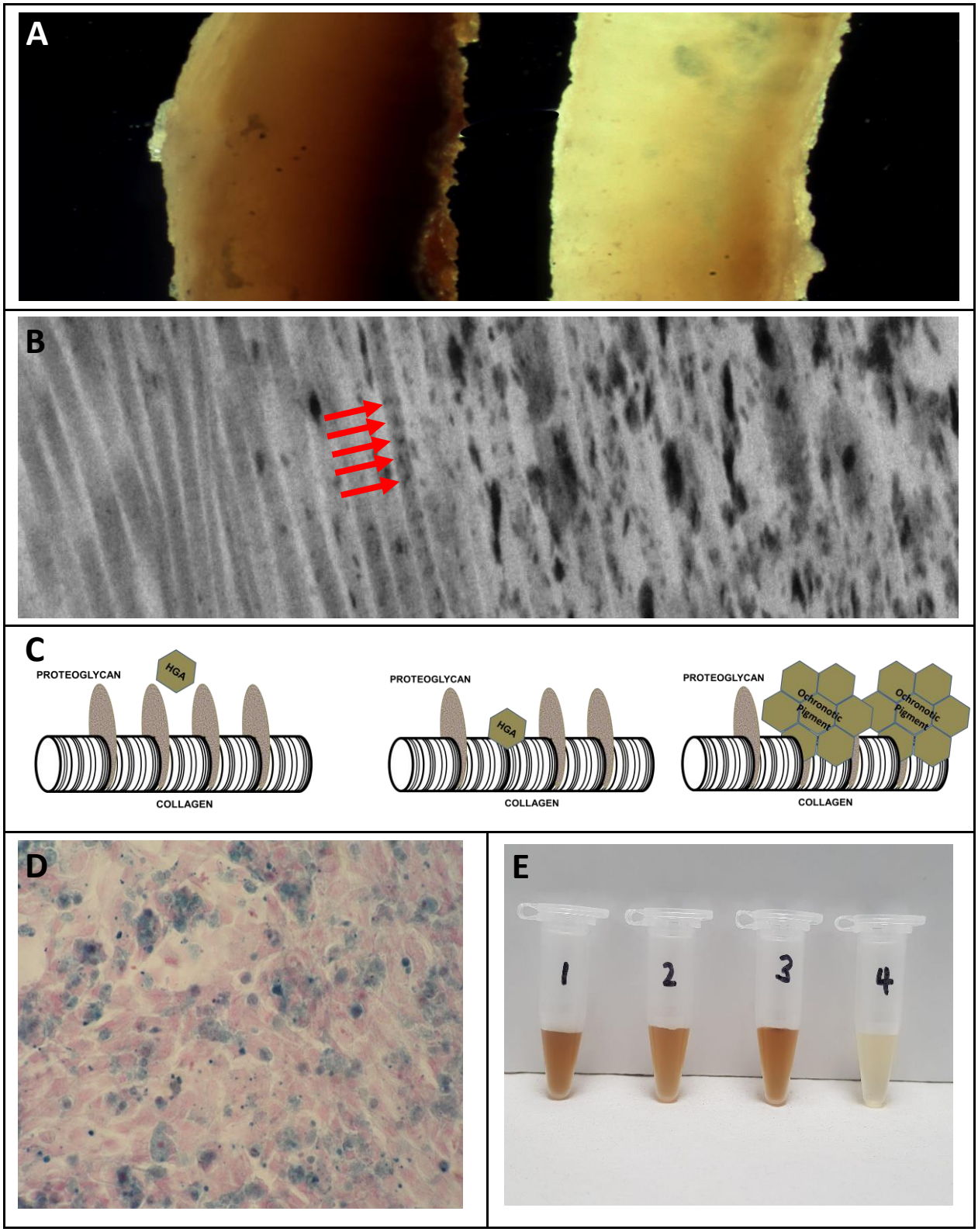


Figure 2


A
B
C
PROTEOGLYCAN
HGA
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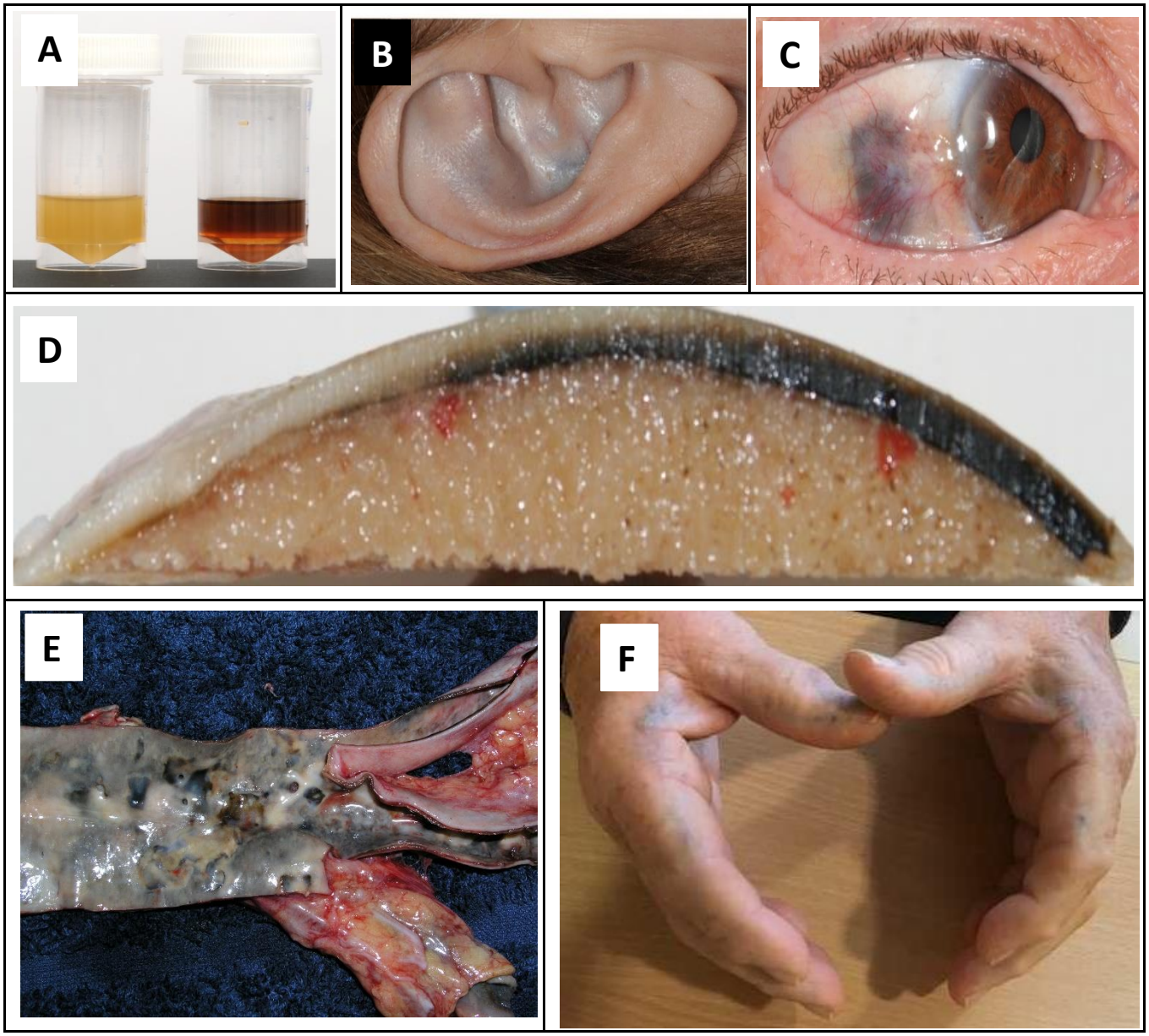
874 Figure 3



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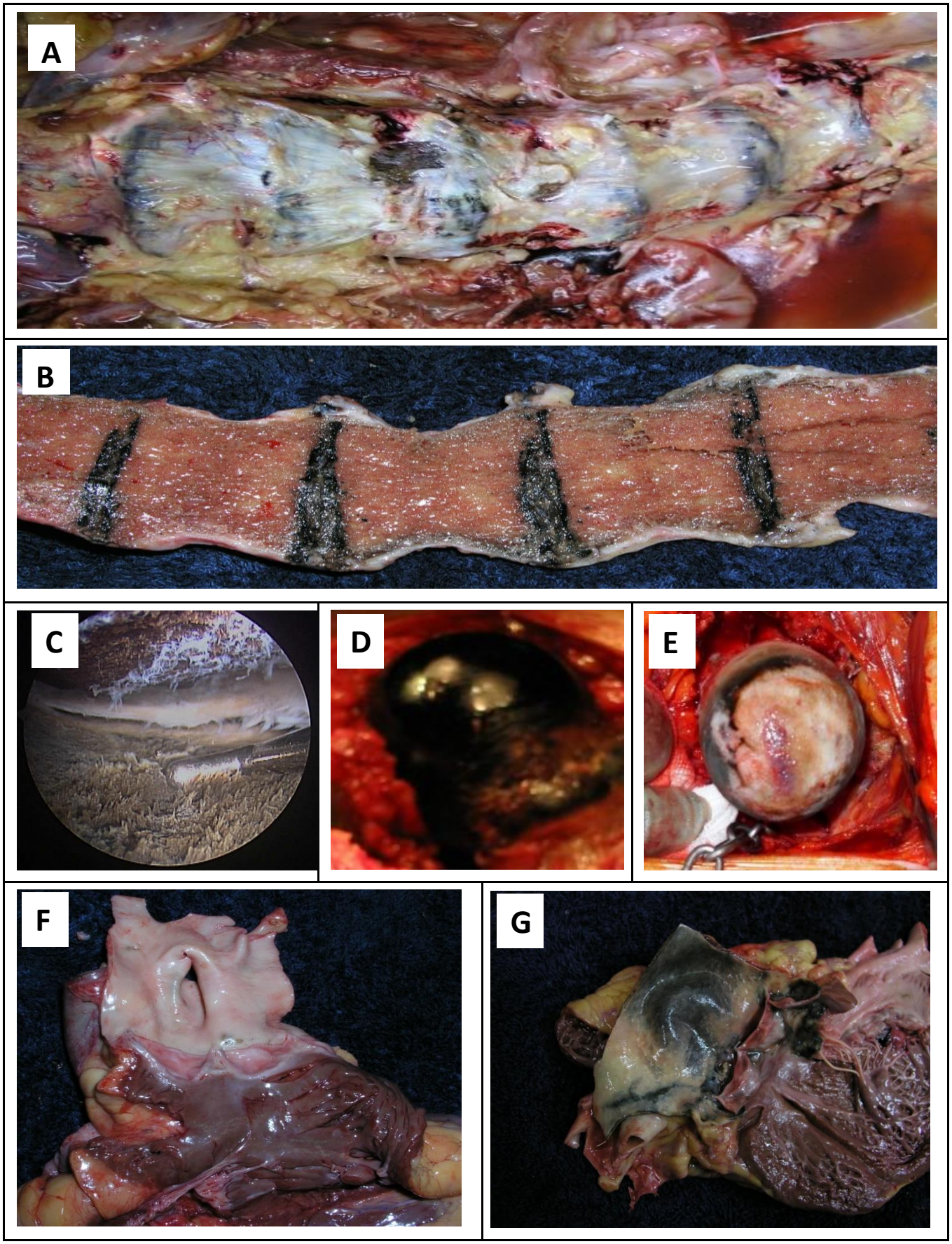
877 Figure 4



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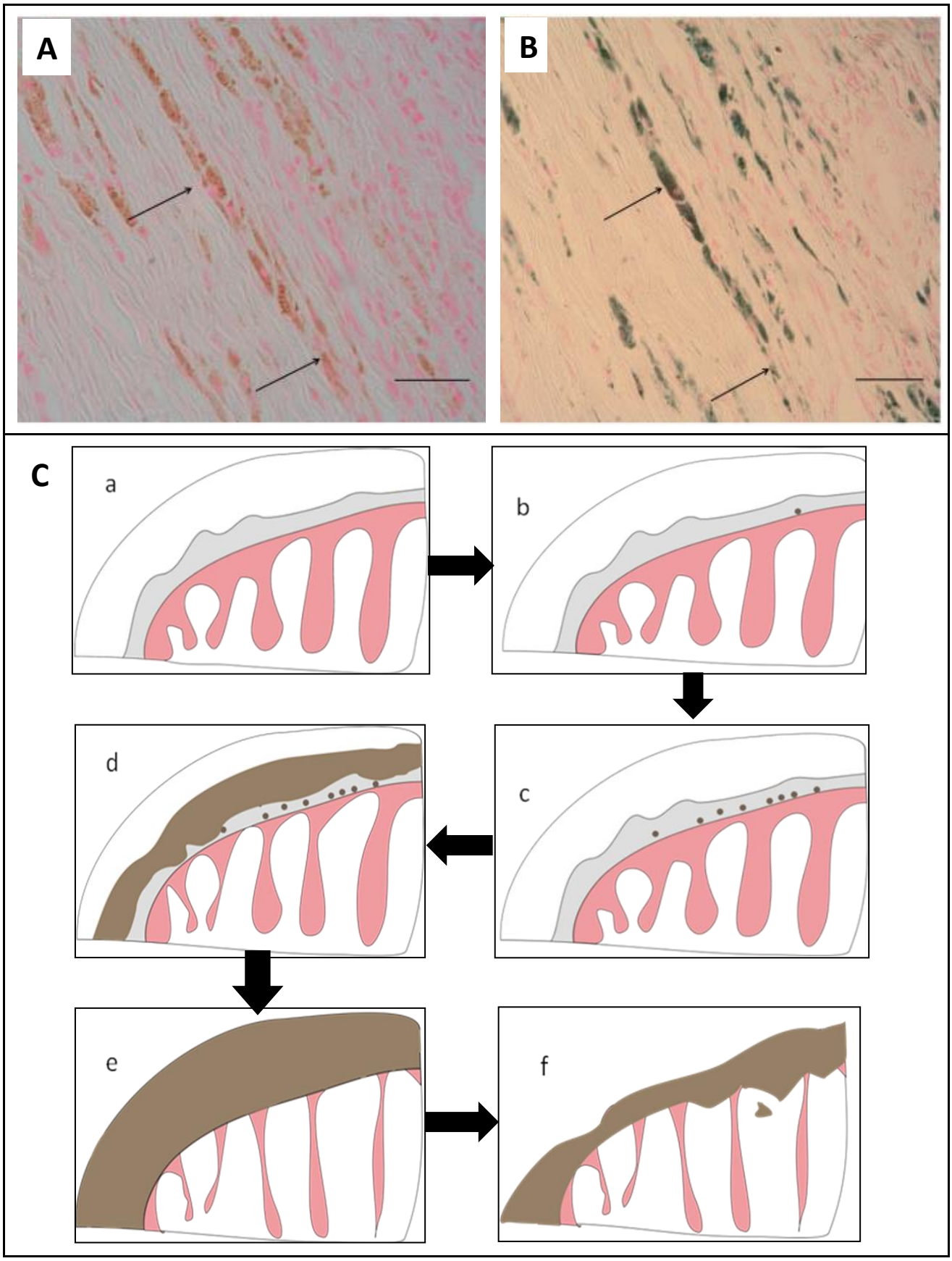
879 Figure 5



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