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A Novel Reproducible Cell-Based Model to Investigate Alkaptonuria: Overcoming Biopsy-Related Challenges and Unravelling Hallmark Cellular Events Implicated in Disease Onset and Progression

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Table of Contents

Thesis abstract *1*

Introduction *3*

Rare diseases **3**

General features 3

Medical and social aspects of rare diseases 3

Alkaptonuria (AKU) **4**

Chronohistory of AKU 5

HGD enzyme 6

Genetic 6

Epidemiology 7

Clinical course of the diseases 7

Psychosocial outcomes 10

Diagnosis 11

Follow-up 11

Therapy and prognosis 12

Emerging Therapeutic Strategies: Pharmacological Chaperones and Gene Therapy 14

Digital Platforms, Machine Learning, and Imaging Innovation in AKU 14

Similarities and correlation with neurodegenerative diseases and processes 15

Molecular aspects of Alkaptonuria **17**

Oxidative stress 17

Inflammation 18

Secondary (AA) amyloidosis 22

Molecular features of Alkaptonuria 24

The ochronotic pigment deposition directly affects the cartilage matrix 31

HGA, oxidative stress, and post-translational protein modifications 35

BQA role in post-translational protein modifications 37

Updated Level of AKU Interpretation **37**

An In Vitro Cell Model for Exploring Inflammatory and Amyloidogenic Events in Alkaptonuria *39*

Abstract **39**

Introduction **40**

Materials and Methods **42**

Cell cultures and treatments 42

Fontana Masson Assay ... 42
Detection of amyloid deposits ... 42
Quantification of ochronotic pigment deposition by Autofluorescence ... 43
Quantification of Intracellular ROS Formation ... 43
Determination of NO production ... 44
Western Blotting ... 44
Immunofluorescence study ... 45
Statistical Analysis ... 45

Results ... 46

A substantial accumulation of ochronotic pigment was observed in the C20/A4 model following HGA treatment ... 46
HGA induced aggregation of amyloidogenic proteins ... 49
Detection of SAA and SAP in HGA-treated C20/A4 cells ... 49
HGA induced pronounced oxidative stress ... 50
HGA-induced inflammatory effects ... 53

Discussion ... 54

HGA triggers SAA aggregation and accelerates fibril formation in the C20/A4 alkaptonuria cell model ... 60

Abstract ... 60

Introduction ... 61

Materials and Methods ... 64

Cells cultures and treatment ... 64
Immunofluorescence study ... 64
In situ proximity ligation assay (PLA) ... 64
Detection of amyloid deposits ... 65
Western Blotting ... 65
Statistical Analysis ... 66

Results ... 67

HGA treatment potentially induced SAA and SAP colocalization ... 67
HGA treatment promoted amyloidogenic protein aggregation. ... 71
HGA treatment triggered SAA aggregation ... 72

Discussion ... 74

Conclusions ... 77

Homogentisic acid induces autophagy alteration leading to disfunction in cartilage cells homeostasis ... 79

Abstract ... 79

Introduction **80**

Materials and Methods **84**

Cells cultures and treatment 84
Immunofluorescence study 84
Live Cell Imaging study 85
Quantification of Intracellular ROS Formation 85
Isolation and culture of human articular cartilage 86
Live AKU cartilage imaging 86
Statistical Analysis 86

Results **87**

HGA induced chronic oxidative stress condition 87
HGA treatment induced alterations of mitochondrial morphology 89
Endoplasmatic reticulum response and mithocondrial ROS generation induced by HGA treatment 93
HGA-induced autophagy flux alteration in the C20/A4 AKU cell model 95
Chronic HGA-derived oxidative stress altered lysosome functionality 99
Lysosome activity is altered in AKU cartilage 101
HGA altered normal autophagic flux 103

Discussion **105**

References ***111***

Thesis abstract

Alkaptonuria (AKU) is an ultra-rare autosomal recessive metabolic disorder caused by loss-of-function mutations in the HGD gene, which encodes homogentisate 1,2-dioxygenase (HGD), a key enzyme of the tyrosine and phenylalanine catabolic pathway. Enzymatic deficiency leads to lifelong accumulation of homogentisic acid (HGA), which undergoes oxidation and polymerization, forming the melanin-like ochronotic pigment that progressively deposits in connective tissues. Ochronosis drives chronic oxidative stress, inflammation, extracellular matrix degradation, mitochondrial dysfunction, and ultimately severe early onset osteoarthropathy. Increasing evidence also implicates impaired autophagy, lysosomal failure, and Endoplasmatic Reticulum (ER) stress in disease progression, but the scarcity of patient-derived chondrocytes severely limits mechanistic studies. The aim of this thesis was to establish and validate a reliable, reproducible, and human-relevant in vitro AKU model using C20/A4 immortalized human chondrocytes exposed to physiologically relevant HGA concentrations (0.066–0.1 mM). The model was used to investigate ochronotic pigmentation, oxidative and inflammatory responses, amyloidogenic processes, autophagy-lysosome dynamics, and mitochondrial and ER response, under HGA treatment. HGA induced time- and dose-dependent accumulation of ochronotic pigment, confirmed by Fontana Masson staining and ochronotic pigment autofluorescence. Oxidative stress was evident from early ROS production and 4-HNE accumulation after prolonged treatment, paralleled by upregulation of inflammatory mediators, including nitric oxide (NO), Nitric oxide synthase (iNOS), and Cycloxygenase 2 (COX-2). Amyloidogenic events were also reproduced and investigated: Serum Amyloid A (SAA) and Serum Amyloid P (SAP) expression were increased and redistributed into intracellular aggregates, with a marked colocalization under HGA treatment, and Congo red staining revealed amyloid-positive birefringent fibrils exclusively in HGA-treated cells, indicating the presence of amyloid deposition. A key finding of this thesis is the detailed characterization of autophagy impairment. After one week of HGA

treatment, LC3, p62, and LAMP1 upregulation, along with increased LC3-LAMP1 and p62-LAMP1 colocalization, indicated active autophagy and efficient autophagosome-lysosome fusion. After two weeks, however, LC3 levels decreased, p62 accumulated, and LC3-LAMP1 colocalization declined sharply, despite persistent LAMP1 upregulation. Live-cell imaging confirmed this biphasic response: LysoTracker and LC3-GFP signals increased at early time points but markedly dropped at two weeks, revealing lysosomal acidification deficiency and defective autophagic flux. These changes were accompanied by progressive mitochondrial dysfunction from early hyperfusion and reduced superoxide production to later fragmentation and elevated mitochondrial ROS, as well as pronounced ER remodeling, with early ER reduction followed by severe ER swelling and structure disorganization. Altogether, these results demonstrate that chronic HGA exposure induces a sequence of pathological events recapitulating AKU progression: pigment formation, oxidative stress, inflammation, amyloidogenesis, and ultimately collapse of cellular quality-control systems due to lysosomal failure, impaired autophagy, mitochondrial dysfunction, and maladaptive ER stress response. This work provides a comprehensive in vitro AKU model and offers an efficient tool for investigating disease mechanisms and testing potential therapeutic strategies.

Introduction

Rare diseases

General features

A disease is called "rare" when its prevalence, meaning the number of cases present in a population, remains below a given threshold. In the EU this threshold is set at 0.05% of the population, that is no more than 1 case per 2,000 people (Decision N. 1295/1999/EC of the European Parliament). To date, the number of known rare diseases is over 7,000, but this value is steadily increasing as science progresses, particularly in genetic research, although not all rare diseases are of genetic origin (DelveInsight). Contrary to what the term "rare" suggests, there are many people suffering from such diseases, 30 million in Europe alone, 2 million of whom, according to Orphanet Italia, live in our country, where approximately 19,000 new diagnoses are reported each year. At least 70% of cases are paediatric patients, and the most common diseases in this age group are congenital malformations, endocrine gland diseases, metabolic diseases, and immune disorders, whereas adult patients are more likely to have diseases affecting their nervous system, sense organs, blood, and hematopoietic organs (Marimpietri and Zuccari, 2023).

Medical and social aspects of rare diseases

Unfortunately, for a long time, rare diseases have historically received very little attention from both the scientific community and institutions. Until a few decades ago, there were no specific scientific projects or appropriate policies whose primary goal was to implement and fund research activities, or to develop efficient models of social integration for people affected by such pathologies (Gammie et al., 2015). Significant progress has been achieved since then, and many patients have experienced a significant improvement in their quality of life, as a result of expanded diagnostic capabilities, improved care, and more social recognition of the disease (Ferreira, 2019). Despite this, there remains

a long way to go. Many people with rare diseases continue to face difficulties in obtaining a diagnosis and accessing qualified professionals. The absence of such forms of assistance inevitably results in a variety of negative effects on the individual's life: access to quality social and medical care, autonomy, and community integration become particularly difficult (Nguengang Wakap et al., 2020). At the same time, previous accomplishments must not be overlooked, no matter how minor they appear in comparison to the work that remains to be done. In fact, they demonstrate that it is possible to improve the quality of life for people suffering because of these diseases and that stepping up research efforts produces concrete results. It is critical that governmental policies move in this direction to help make interest in these diseases a social duty. On 30 November 2009, the European Commission formally established the *Committee of Experts of the European Union on Rare Diseases (EUCERD)* (2009/872/EC) to coordinate and implement community activities in the field of rare diseases, and to encourage the sharing of results in order to tackle the fragmentation of work.

Alkaptonuria (AKU)

AKU (OMIM database, code 203500) is an extremely rare genetic disease with autosomal-recessive transmission caused by the deficiency of the enzyme homogentisate 1,2-dioxygenase (HGD), mainly expressed in the liver, kidneys, colon, prostate, and cells of the osteo-articular structures (Grasko et al., 2009). This enzyme is physiologically involved in the transformation of homogentisic acid (HGA), a product of the catabolism of tyrosine and phenylalanine, into maleylacetoacetate (MAA) (Knox and Edwardst, n.d.). Due to this deficiency, AKU patients experience an accumulation of HGA, which then undergoes initial oxidation followed by additional, not yet fully clari(Knox and Edwardst, 1955)fied, mechanisms of dimerization and polymerization. Eventually, the oxidative process leads to the production of a melanin-like pigment (Braconi et al., 2015). Once the pigment is deposited within the tissues, a condition known as "ochronosis" occurs, which is sometimes visible to the naked eye, such as the formation of a brown-bluish hue in the urine, sclerae or earlobes.

Although the deposition of ochronotic pigment represents the distinctive sign of the disease, a number of other important biochemical and structural effects contributing to the multi-systemic character of this pathology are observed overall: alterations of the cytoskeleton, proteomic alteration, oxidative stress, lipid peroxidation, release of pro-inflammatory cytokines, amyloidosis, and neo angiogenesis (Braconi et al., 2015; Millucci et al., 2015a).

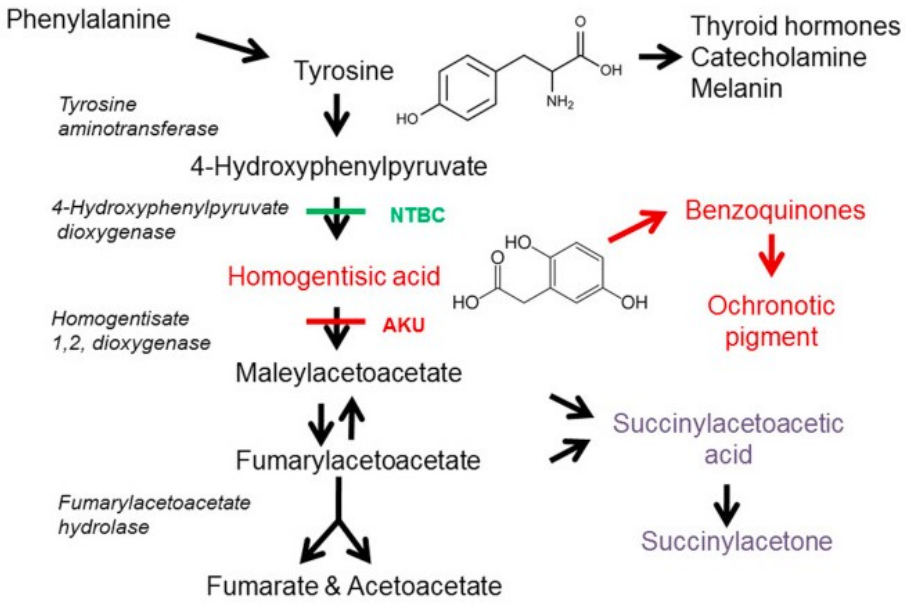

Figure 1. Tyrosine and phenylalanine metabolism, indicating the steps inhibited in AKU and tyrosinemia types I, II, and III, as well as by the drug nitisinone. Enzymes are shown in green, diseases in purple, and toxic products in red. (*Cianflone et al., 2022)*

Chronohistory of AKU

- The first case of AKU, dating back to 1500 b.C. in radiographs of an Egyptian mummy, showed large calcification and narrowing in both hip and knee joints due to the disease (Stenn et al., 1979).
- In 1584 Scribonio first wrote about the most easily observable symptom of AKU, describing a boy whose urine turned black after exposure to air (Gaines, n.d.).
- Over the centuries, there were further descriptions of ochronosis, provided by Zacutus Lusitanus in 1649, Singer in 1775, and Marcet in 1822.
- In 1859, Boedeker first introduced the term "*Alkaptonuria"*, from Arabic *alqualy* (alkaline) and Latin *capere* (to take) (Gutzmer et al., 1997).

- In 1891, Wolkow and Baumann identified HGA as the compound responsible for the disease, although the exact nature of the disorder had not yet been determined (Hanika et al., 2002).
- In 1958, La Du and colleagues published a study based on a liver biopsy performed on a patient suffering from the disease. The activity of other enzymes involved in tyrosine catabolism was evaluated and compared with that observed in non-Alkptonuric patients. This work demonstrated that the aetiology of AKU depends exclusively on the alteration of the activity of the enzyme HGD (Du et al., 1958).

HGD enzyme

The HGD enzyme catalyzes the cleavage of the aromatic ring of HGA, using molecular oxygen as a co-substrate to generate maleylacetoacetate. This reaction represents a crucial step in the catabolic pathway of phenylalanine (Phe) and tyrosine (Tyr) and ultimately leads to the production of fumarate and acetoacetate, metabolites that can enter various metabolic routes, including the tricarboxylic acid cycle and ketone body catabolism. Because of HGD deficiency, circulating concentrations of HGA become markedly elevated, despite its efficient urinary excretion (Bernardini et al., 2024).

Genetic

More than 250 pathogenic variants of the gene encoding HGD have been identified over the years in patients from various populations (Ladjouze-Rezig et al., 2006; Yang et al., 2013), many of which are documented in a dedicated database (http://hgddatabase.cvtisr.sk). Most patients are heterozygous, meaning they carry two different alleles for the HGD gene, both of which are defective, while only a minority are homozygous (having the same allelic variant on both gene copies). Approximately 65% of mutations are missense, characterized by a single aminoacid substitution that alters the final protein structure. Depending on the resulting protein, these mutations can produce a fully or partially non-functional enzyme, leading to reduced or absent activity. Other types of HGD gene mutations include nonsense (4%), frameshift (11%), splicing (14%), and large deletions (5%),

most of which are very severe. To date, a genotype-phenotype correlation has not been established, and even cases of alkaptonuric siblings can show different ages of onset and varying symptom severity, despite having the same mutations and similar diets (Zatkova et al., 2020). The absence of such a correlation, along with the multisystem nature of the disease, complicates early diagnosis (Mathieu et al., 1997).

Epidemiology

The incidence of AKU at birth in most ethnic groups is estimated to be between 1/250,000 and 1/1,000,000 (Tóth et al., 2010). The condition is most common in Slovakia and the Dominican Republic, where it affects approximately 1 in 19,000 newborns (Nemethova et al., 2016; Zatkova et al., 2003). In Italy, the exact prevalence remains unknown.

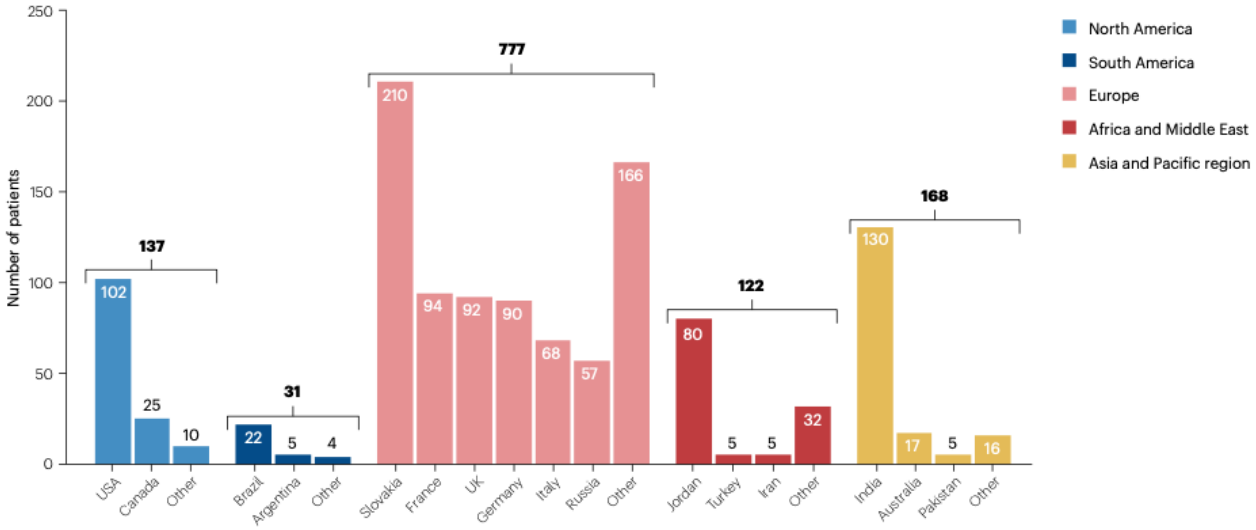

Figure 2. Overview of the number of patients with alkaptonuria (AKU) reported worldwide (Bernardini et al., 2024).

Clinical course of the diseases

The first signs of the disease can be observed already in the first months of the life, with the appearance of dark spots on diapers. These are detected by the presence of HGA in the urine, which, when exposed to air, gradually oxidises to benzoquinone acetate (BQA) (Loyd R Wagner et al., 1960). However, this symptom is found in only 21% of subjects (Mistry et al., 2013), because the slow oxidation process often goes unnoticed; hence the diagnosis typically occurs in adulthood following

more evident manifestations. In the first decades of life, the patient rarely manifests symptoms, other than the presence of small pigment deposits of pigment in the sclera, ear cartilage, or skin of the hands and tendons. The pigment is also excreted through the earwax and sweat glands, which is why stains can be detected on the garments following sweating (De Haas et al., 1998).

From the age of thirty, the first osteo-articular changes visible radiologically begin to manifest. These are associated with inflammation of the large joints and the onset of back pain due to early forms of spinal damage. X-ray images show a reduction in joint spaces, flattening and calcification of the intervertebral discs, followed by fusion, the appearance of osteophytes, and calcification of the intervertebral ligaments. Post-mortem surgical studies have shown that ochronotic pigment is primarily deposited in chondrocytes and in the cartilage matrix (Bernardini et al., 2024), initiating the joint degenerative process, which is the main clinical manifestation of AKU. As the pigment becomes widely deposited, the normal architecture of the joint is altered, and the cartilage becomes brittle and prone to breakage (Shimizu et al., 2007).

In general, arthropathies, chronic and progressive pathologies, lead to disability around the age of sixty (Castagna et al., 2006). In the case of osteoarthritis in AKU, it is often necessary to implant joint prostheses before this age, on average around fifty-five (Hanika et al., 2002). People affected by AKU experience a gradual reduction in motor skills and a severe decrease in tolerance to sitting (Fisher and Davis, 2004). Furthermore, they are frequently prone to tendon ruptures even after minor traumas.

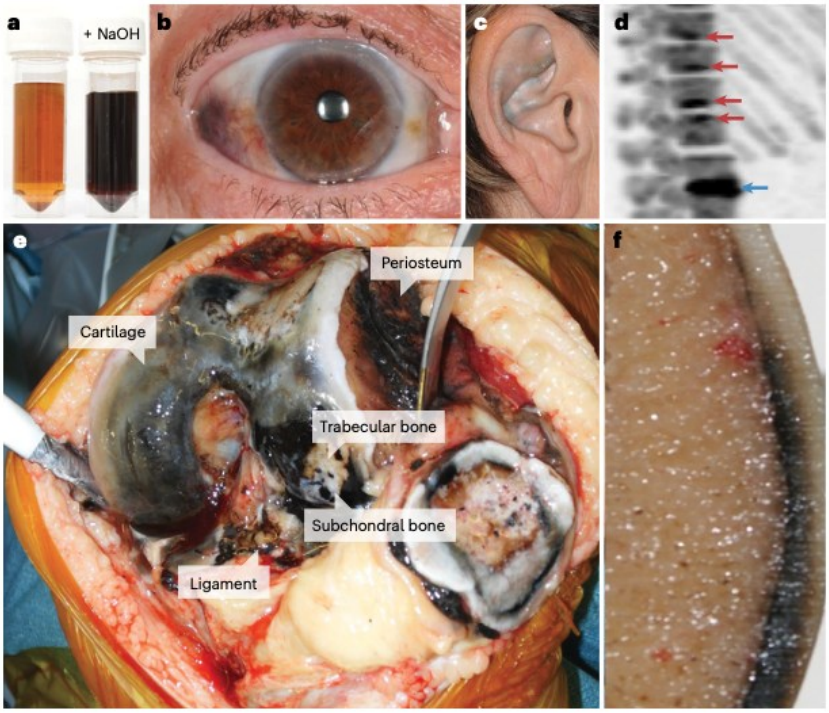

Figure 3. Clinical features of ochronosis (Bernardini et al., 2024). a) The presence of homogentisic acid pigment in urine from patient with alkaptonuria (AKU) is revealed by the addition of sodium chloride (NaOH). b) Ochronosis in sclera and cornea. c) Ochronosis in the ear cartilage. d) spinal ochronosis, which begins in the third decade of life. e) knee ochronosis visible at joint replacement surgery. f) a slide through articular cartilage reveals that ochronosis starts in the deep zone and spreads to the surface.

From the age of fifty, cardiac impairment may occur due to calcification of the coronary arteries or the aortic and/or mitral valves (Introne et al., 2003), sometimes requiring replacement. Kidney stones are frequently present, probably due to the large amount of HGA excreted via the urinary tract, while prostate, bile, and salivary stones occur (Bernardini et al., 2024). Correlations with impaired vision and hearing have also been proposed, but to date, it has not been possible to identify a definitive association between AKU and these symptoms (Shimizu et al., 2007). Overall, life expectancy is not reduced compared to the general population, but the quality of life is significantly compromised (Braconi et al., 2018).

Psychosocial outcomes

AKU can impact the psychosocial wellbeing and overall quality of life of affected individuals. Progressive joint degeneration, leading to osteoarthritis, often compromises mobility and limits personal independence. As a result, patients frequently require support for daily activities such as dressing, personal hygiene, and household tasks. The consequent loss of mobility and autonomy can contribute to social isolation, depression, and reduced self-esteem (Barone et al., 2020). Because symptoms progress gradually, many adults with AKU must transition prematurely from employment to disability status, which can lead to financial instability and emotional strain. Working-age individuals commonly report significant work disruption, whereas younger patients may struggle to complete vocational training or maintain employment (Bernardini et al., 2024). Interpersonal relationships and intimacy are also affected. Patients frequently describe considerable strain within marriages and partnerships, as reduced mobility limits participation in shared activities. Caregiver stress and depression are common when a partner is affected by debilitating AKU (Barone et al., 2020). Concerns about transmitting AKU to offspring further influence family planning decisions (Barone et al., 2020). Additionally, cosmetic manifestations such as skin discoloration negatively affect body image and self-confidence. To help individuals, Support groups play an important role by allowing individuals with AKU to connect, share experiences, and receive guidance from others facing similar challenges. Psychological counselling and pain management programs can facilitate healthier coping strategies, whereas vocational assistance may help patients transition into more suitable forms of employment (Sestini et al., 2021). Mobility aids and home modifications also promote greater independence. Addressing psychosocial health is essential to improving overall wellbeing in individuals living with AKU. However, research aimed at optimizing social, emotional, and functional outcomes remains limited, underscoring the need for further investigation.

Diagnosis

Diagnosis can proceed on three different levels: clinical, biochemical, and molecular. Clinical diagnosis is based on the presence of darkened urine, ochronosis, osteoarthritis, and calculosis. Thanks to technological advances, it is now possible to identify secondary alterations resulting from the deposition of ochronotic pigment even in the early stages of the disease. Asymptomatic thickening and stretching of tendons can be visualized with magnetic resonance imaging (MRI), while calcifications of the coronary arteries or heart valve defects can be detected using echocardiography and computed tomography (CT). The presence of stones in the prostate and kidneys can be identified by X-ray or ultrasound (Ranganath et al., 2019).

Biochemical diagnosis, in the presence of signs and symptoms of AKU, is primarily based on the detection of high levels of HGA in the urine (1-8 grams/day) through gas chromatography and/or mass spectrometry (Introne et al., 2003). Additionally, recent studies have shown that alkaptonuric patients experience increased bone resorption, leading to higher urinary excretion of type I collagen N-telopeptide, which could represent an additional diagnostic method (Aliberti et al., 2003).

Finally, molecular diagnosis is based on the identification of genetic variants responsible for the defective synthesis of the HGD enzyme. Many mutations have been identified in at least 106 of the 114 alleles of the gene encoding HGD (Hanika et al., 2002), and, to date, the development of new techniques has allowed the rapid identification of some of these mutations, enabling early detection as soon as the first symptoms appear (Zatkova et al., 2003).

Follow-up

Clinical monitoring of the disease includes several characteristic stages. During the initial visit, a thorough medical history is compiled, with particular attention to any limitations into spinal and large joint movement. In the early stages of assessment, an abdominal ultrasound is performed to check for the presence of kidney stones, and an X-ray is conducted if prostate stones are suspected. Genetic counselling is also particularly important, as it helps to clarify the nature of the disease and its

mechanism of transmission. Cardiological evaluations begin at the age of forty and consist of an electrocardiogram and echocardiogram every one or two years, along with computed axial tomography to detect possible calcifications in the coronary arteries. Annual measurements of TSH and free thyroxine levels are carried out to assess potential hypothyroidism (Bernardini et al., 2024). The multisystemic nature of AKU makes it difficult to accurately assess the severity of the disease and the effectiveness of treatments. For this reason, a clinical scoring system called AKUSSI (Alkaptonuria Severity Score Index) has been developed, which assigns scores to specific clinical manifestations and to evidence of the disease at the spinal and articular levels (Braconi et al., 2018), thereby allowing the definition of disease severity.

Therapy and prognosis

Currently, treatments for AKU are primarily palliative, focusing on alleviating symptoms rather than addressing the underlying pathophysiological causes of the disease. The standard approach includes pharmacological management of joint pain, physiotherapy, and lifestyle modifications to minimize the impact on the osteoarticular structures. Patients are advised to avoid physically demanding work and refrain from intense sports activities, opting instead for swimming to help maintain joint mobility. In some cases, a diet low in phenylalanine and tyrosine, like the one recommended for individuals with phenylketonuria, may be suggested. However, close monitoring is essential to prevent amino acid deficiencies that could impair optimal growth or lead to related syndromes. From a surgical perspective, procedures may involve the implantation of prosthetic joints (commonly in the knees, hips, and shoulders), heart valve replacements, or the removal of kidney or prostate stones when necessary (De Haas et al., 1998). Recently, the European Medicines Agency (EMA) approved the use of nitisinone following an international study demonstrating its effectiveness in slowing the progression of AKU (L. Ranganath et al., 2020). Nitisinone acts as a competitive inhibitor of 4-hydroxyphenyl pyruvic dioxygenase, an enzyme that precedes HGD in tyrosine metabolism (Lock et al., 2014). There is ongoing debate regarding the appropriate age to start the treatment, with

considerations about whether to base the decision solely on observed severity or to begin early with low doses regardless of symptoms. The potential benefits for patients with advanced pathology are also uncertain. Notably, the use of nitisinone can lead to tyrosine accumulation and associated side effects, like those seen in tyrosinemia, including corneal and skin lesions, visual disturbances, liver damage, and cognitive impairments. Consequently, protein intake must be reduced to prevent plasma tyrosine spikes. Despite the fact that nitisinone has been effective in decreasing plasma HGA levels (Laschi et al., 2016), it can only halt or delay disease progression without correcting the underlying metabolic defect related to HGD. Antioxidant treatments, such as N-acetylcysteine (NAC) and ascorbic acid (ASC), have also been proposed (alone or in combination) to reduce oxidative stress. NAC has been shown to inhibit the dimerization and polymerization of HGA and BQA (Mannoni et al., 2004). Additionally, the combination of NAC with ASC has yielded encouraging results in inhibiting the formation of the ochronotic pigment (Braconi et al., 2010b; Tinti et al., 2010). However, the effectiveness of ASC is controversial, as it has been reported to promote the formation of ROS under certain conditions. In vitro studies on primary cells extracted from AKU biopsies, indicate that ASC can be co-oxidized to dehydroascorbate at pH 7.4, producing increased levels of H_2O_2 through a mechanism involving ASC, ascorbyl radicals, HGA, and superoxide radicals (Martin and Batkoff, 1987). Other antioxidants explored include lipoic acid, taurine, and phytic acid (Spreafico et al., 2013a). Lipoic acid's antioxidant properties stem from its ability to scavenge ROS, regenerate endogenous antioxidants, and chelate metals, thereby reducing the formation of new ROS. Taurine and phytic acid have shown effectiveness in preventing or delaying the production of HGA-induced ochronotic pigment (Braconi et al., 2010a). In cases of hypothyroidism, thyroid hormone replacement therapy may be initiated if clinically indicated. Overall, the life expectancy of patients with AKU is not significantly reduced; however, persistent pain and progressive functional decline can lead to a gradual loss of mobility. As a result, many patients may require walking aids such as crutches or wheelchairs. Additionally, heart complications should not be overlooked, as they can be potentially life-threatening and may worsen the prognosis.

Emerging Therapeutic Strategies: Pharmacological Chaperones and Gene Therapy

Beyond nitisinone, which remains the only approved therapy for AKU despite important limitations, several innovative therapeutic strategies are emerging. A promising avenue involves pharmacological chaperones targeting the misfolded HGD enzyme. Since approximately two-thirds of pathogenic HGD variants are missense mutations that destabilize the oligomeric structure of the enzyme, efforts have focused on restoring residual activity through small molecules capable of stabilizing the native hexameric conformation. Recent high-throughput screens employing *Escherichia coli* models expressing HGD variants have identified multiple FDA-approved compounds that can significantly enhance the activity of mutant HGD, including variants such as G161R, suggesting the feasibility of a mutation-tailored therapeutic approach (Lequeue et al., 2025, 2022). Even more transformative is the progress in gene therapy: a liver-directed adeno-associated virus (AAV) vector delivering wild-type HGD has demonstrated robust metabolic correction in an HGD-deficient mouse model, resulting in marked reduction of circulating HGA and prevention of pigment deposition (Lequeue et al., 2025). These findings position AKU as a strong candidate for future causal therapies, with chaperone-based and gene-replacement approaches offering complementary strategies to address the underlying enzymatic defect and overcome the limitations of chronic HGA-lowering therapy.

Digital Platforms, Machine Learning, and Imaging Innovation in AKU

Recent advances in digital health technologies have begun to transform the clinical and research landscape of AKU. A major development is the creation of ApreciseKUre, an integrated digital platform specifically designed to collect and harmonize multi-layered data from AKU patients, including genetic variants, biochemical profiles, imaging studies, clinical metrics, and patient-reported outcomes, in a unified resource accessible to clinicians and researchers (Milella et al., 2024). This system enables the application of machine learning algorithms to identify clinically meaningful patterns, refine patient stratification, and explore genotype-phenotype relationships that were previously obscured by the rarity of the condition and the fragmentation of available datasets. Parallel

progress has been made in the field of medical imaging: a deep learning model applied to spinal radiographs of AKU patients was recently shown to accurately quantify global and regional disease severity, while also detecting subtle imaging signatures associated with nitisinone treatment that were not identifiable by human observers (Flaharty et al., 2025). These innovations highlight the growing role of artificial intelligence and digital infrastructures in improving diagnostic precision, monitoring disease progression, and supporting personalized management strategies in AKU.

Similarities and correlation with neurodegenerative diseases and processes

Oxidative stress, a central feature in AKU, is also frequently observed in neurodegenerative diseases. This phenomenon is often driven by chronic neuroinflammation, dysregulation of metal homeostasis, and mitochondrial dysfunction (Myhre et al., 2013; Urrutia et al., 2014). Notably, there are interesting links between the pathophysiology of AKU and neurodegenerative conditions, such as the auto-oxidation of the neurotransmitter dopamine in Parkinson's disease. The oxidative transformation of dopamine into black-colored neuromelanin, a pigment similar to the ochronotic pigment seen in AKU, contributes to the characteristic pigmentation of dopaminergic neurons and is one of the factors leading to their progressive degeneration. Recent studies have indicated that oxidation products of catecholamines can significantly alter the surrounding proteome, affecting its functionality and stability (Biosa et al., 2018). As for the direct correlation between AKU and neurodegenerative processes, current information does not provide a comprehensive understanding. However, as shown by Bernardini et al. (2015) (Bernardini et al., 2015), HGD is expressed in the central nervous system, where it is believed that HGA may accumulate. Indeed, deposits of ochronotic pigment have been identified in the central nervous systems of patients with AKU (Bernardini et al., 2024). Furthermore, neurological manifestations such as astrocytomas, pituitary adenomas (Hanika et al., 2002), neuroblastomas (Mathieu et al., 1997), migraines, and depression have been described in association with AKU (Barone et al., 2020).

Even though there is no substantial evidence linking AKU directly to Alzheimer's disease, a correlation with Parkinson's disease has been proposed (Bernardini et al., 2015; Millucci et al., 2015a; Ranganath et al., 2023). In this context, it is hypothesized that the deposition of ochronotic pigment may also play a key role in the neurodegenerative processes. The possibility that HGA may accumulate in the central nervous system, alongside the formation of ochronotic pigment, raises the hypothesis that amyloid protein deposition could also be involved in neurodegenerative processes related to AKU.

Molecular aspects of Alkaptonuria

Oxidative stress

Oxidative stress is a key event in cellular biology and modern medicine. It refers to a condition in which there is an imbalance between the production of ROS and the biological system's ability to neutralize them or repair the resulting damage (Sies, 2015). When this equilibrium is disrupted, a cascade of molecular events can occur that severely compromises the integrity and functionality of cells and tissues.

ROS are unstable and highly reactive oxygen-containing molecules, including the superoxide radical (O_2^{-}•), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$). These molecules are naturally produced as by-products of aerobic cellular metabolism, particularly within the mitochondria during oxidative phosphorylation. In addition to mitochondria, other intracellular sources of ROS include enzymes such as NADPH oxidase, xanthine oxidase, and myeloperoxidase, which are often activated in response to inflammatory stimuli or environmental stressors.

Under physiological conditions, ROS play critical regulatory functions: they modulate gene expression, participate in intracellular signalling, regulate cell proliferation and apoptosis, and play a role in immune defence (Valko et al., 2007). However, when ROS levels exceed the antioxidant capacity of the organism, their accumulation leads to extensive oxidative damage to biomolecules with serious consequences.

Excessive ROS generation can cause oxidative damage to major cellular components and structures:

- Lipids: lipid peroxidation alters cell membrane structure and integrity.
- Proteins: oxidative modification of amino acids may distort protein conformation, impair function, or trigger immune recognition.
- DNA: ROS can cause strand breaks, base modifications, and mutations that increase oncogenic risk.

Numerous studies have linked oxidative stress to a wide array of chronic and degenerative diseases. In the cardiovascular system, it contributes to atherosclerosis by promoting the oxidation of LDL and endothelial dysfunction. In the nervous system, oxidative stress is involved in neuronal death and neuroinflammation as observed in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Amyotrophic Lateral Sclerosis. It is also implicated in tumour progression, facilitating genomic instability, uncontrolled proliferation, and resistance to apoptosis (Liguori et al., 2018). Emerging evidence supports the involvement of oxidative damage in rare genetic diseases, such as AKU.

Inflammation

Inflammation is a complex biological response to cellular damage, infection, or irritation. It represents a natural mechanism that is activated when the body recognizes a harmful agent, such as bacteria, viruses, toxins, or physical trauma, involving the immune system in order to eliminate the threat and begin the repair process (Chen et al., 2018). The characteristics of inflammation were first described in the 1st century A.D. by Cornelio Celso:

- *Calor*: increased temperature in the inflamed area, caused by increased blood flow.
- *Rubor*: due to dilation of blood vessels.
- *Tumor*: fluid accumulation in tissues due to increased permeability of blood vessels.
- *Dolor*: caused by the release of chemicals that stimulate pain receptors.
- *Functio laesa*: possible impairment of the function of the affected area.

Inflammation can be triggered by various factors, which can be categorized according to their nature and sequence:

- Physical factors (such as heat, cold, and injury): these primarily exert their effects indirectly by prompting the release of substances from damaged cells and tissues.

- Chemical factors (including poisons, irritants, and toxins): these factors are relatively straightforward to reproduce in experimental settings.

- Biological factors (such as viruses, bacteria, and parasites): these are the most common triggers, resulting from the action of microorganisms either through their ability to reproduce or through the effects of their metabolic byproducts.

Two main types of inflammation are distinguished, based on a temporal criterion:

- Acute: a rapid and short-term response to an infection or injury (such as cuts, insect bites, or mild infections).

- Chronic: prolonged inflammation that can last for months or years, often resulting from autoimmune diseases, prolonged exposure to irritants, or persistent infections (rheumatoid arthritis is an example of a chronic inflammatory condition).

A variety of molecules play a role in the inflammatory process, such as histamine, serotonin, prostaglandins, ROS, leukotrienes, NO, and cytokines. NO, which is produced by nitric oxide synthase (NOS) from oxygen and L-arginine, is particularly important in inflammation. Its ability to diffuse easily across cell membranes allows it to regulate the recruitment of white blood cells and to act as a powerful vasodilator. However, when present in high amounts, NO can be harmful, especially during intense macrophage activity.

Nitric oxide synthase comes in two main forms:

- eNOS (endothelial NOS): this is the constitutive form found in blood vessels and various tissues, such as the lungs and brain. It helps maintain low blood pressure and prevents white blood cells and platelets from sticking to vessel walls.

- iNOS (inducible NOS): unlike eNOS, this form is not triggered by an increase in calcium ions. It is found in macrophages as well as in liver and cartilage tissues. iNOS is key to the inflammatory

response, as it significantly boosts NO production to help fight off microorganisms that cause inflammation. It is activated in macrophages by cytokines and in microglial cells by lipopolysaccharides (LPS).

ROS are important signalling molecules that contribute significantly to inflammation. They are produced by cells that defend the body and serve as both signalling molecules and mediators of inflammation. However, if ROS levels become too high, they can cause tissue damage and lead to chronic inflammation, contributing to diseases like cardiovascular, neurodegenerative, and metabolic disorders. Moreover, ROS can act as second messengers in signalling pathways, stimulating certain enzymes and affecting the permeability of blood vessels by damaging endothelial cells.

Interestingly, there is growing evidence that ROS also play a role in resolving inflammation. A key event in this process is the large-scale death of neutrophils, which is regulated by ROS-dependent signalling. For instance, studies have shown that H_2O_2 can induce neutrophil apoptosis and help resolve inflammation in models of arthritis.

Arachidonic acid, a polyunsaturated fatty acid found in cell membranes, is crucial for producing a group of mediators known as eicosanoids. Over the past 15 years, research has shown the importance of eicosanoids in inflammation. These molecules are derived from long-chain polyunsaturated fatty acids like arachidonic acid and include various products, such as prostaglandins, thromboxanes, and leukotrienes, all of which act as inflammatory mediators.

There are two main types of cyclooxygenase (COX) enzymes:

- COX-1: this isoform is present in healthy tissues and is vital for maintaining homeostasis. It produces prostaglandins that support normal physiological functions.

- COX-2: this form is more active during inflammation or tumour development.

Prostacyclins (PGI2), a type of prostaglandin, is produced by enzymes in endothelial cells and helps dilate blood vessels while preventing platelet aggregation. Leukotrienes are another group of lipid

molecules derived from arachidonic acid. They enhance the inflammatory response by causing blood vessel constriction and increasing permeability. In contrast, lipoxins help start the resolution phase of acute inflammation by promoting blood vessel dilation and triggering the production of resolvins, which aid in tissue repair. When arachidonic acid is released, it also leads to the formation of platelet-activating factor (PAF). This lipid is essential for platelet aggregation and blood clotting. Studies have shown that PAF not only activates platelets and leukocytes but also affects various biological functions, including increasing blood vessel permeability, reducing heart output, and lowering blood pressure. Since the 1980s, it has been recognized that PAF can relax or contract smooth muscle in different tissues, such as the respiratory and digestive systems, and affects blood vessels. Cytokines are small proteins released by cells that facilitate communication between them. The term "cytokine" is broad and includes different subtypes, such as lymphokines (from lymphocytes), monokines (from monocytes), chemokines (with chemotactic properties), and interleukins (that act on other leukocytes). There's substantial evidence that certain cytokines not only kickstart inflammation but also help sustain pathological pain by directly activating pain-sensing neurons. They also play a role in central sensitization following nerve injury and inflammation. Cytokines can have both pro-inflammatory and anti-inflammatory effects. Pro-inflammatory cytokines are mainly produced by activated macrophages and promote inflammatory responses. Notable examples include IL-1β and TNF-α. IL-1β is produced when cells are damaged or infected and has various roles in inflammation, such as promoting blood vessel dilation, stimulating prostaglandin production, and recruiting immune cells to the infection site. TNF-α, also called cachectin, is another important inflammatory cytokine involved in several pain models. It's primarily produced by monocytes and macrophages, but other cells, such as T and B lymphocytes, can also produce it, although in smaller amounts. Blocking TNF synthesis or action can prevent many secondary inflammatory effects and the release of other cytokines like IL-1, IL-6, and interferon (IFN). TNF-α is also known for its ability to induce cell death in virus-infected cells, especially when combined with IFN. Additionally, it influences lipid metabolism, blood clotting, and cell regulation through apoptosis. As mentioned before, oxidative

stress and inflammation are strictly interconnected: ROS increase activate the inflammatory response, that, on its turn, includes the stimulation of ROS production. Specifically, ROS drive the activation of different transcriptional factors, among which NF-kB. In particular, ROS induce its translocation from the cytosol to the nucleus of cells, where NF-kB promotes the expression of genes coding for inflammatory proteins(Morgan and Liu, 2011). Consequently, different factors are expressed during the oxidative stress condition, as cytokines and chemokines, which take part in the inflammatory signalling and immune cells recruitment. Moreover, inflammatory cells generate ROS, causing a loop mechanism of amplification of the inflammatory signal. Chronic inflammation causes hypo responsiveness of the immune cells, that leads to an higher susceptibility to viral infections (Furman et al., 2019). In several disorders, these pathways are activated for prolonged period. Moreover, oxidative stress contributes to the pathophysiology of many disorders, such as cardiovascular diseases, chronic kidney disease, neurodegenerative diseases, macular degeneration, biliary diseases, and cancer.

Secondary (AA) amyloidosis

Secondary amyloidosis, or AA amyloidosis, constitutes a prototypical systemic disorder of protein misfolding and deposition, arising from the accumulation of extracellular fibrils composed primarily of fragments of serum amyloid A (SAA). SAA is a family of apolipoproteins associated with high-density lipoproteins (HDL), whose expression is markedly upregulated as part of the acute-phase response (Webb, 2021). Under physiological conditions, circulating SAA levels are tightly regulated and rapidly return to baseline once inflammatory stimuli subside. However, during chronic inflammatory disorders, such as rheumatoid arthritis, chronic inflammatory bowel disease, and persistent infections, SAA levels may remain persistently elevated for extended periods (Scarpioni, 2016). This sustained overproduction creates conditions favoring misfolding, proteolytic processing, and deposition of SAA-derived peptides in tissue parenchyma.

The molecular mechanisms underlying fibrillogenesis in AA amyloidosis involve a complex interplay between SAA concentration, proteolytic cleavage, conformational instability, and tissue microenvironment. SAA undergoes partial proteolysis, primarily by lysosomal or extracellular proteases, generating N-terminal fragments with a heightened propensity to form β-sheet–rich, fibrillar aggregates (Tanaka et al., 2023). These peptides can self-assemble into oligomeric intermediates, which serve as nucleation seeds for the elongation of amyloid fibrils. The fibril architecture is characterized by a cross-β structure, with highly ordered, insoluble aggregates that progressively accumulate within the extracellular matrix (Knowles and Mezzenga, 2016). Factors such as pH, oxidative modifications, glycosaminoglycans, and the local inflammation further modulate both nucleation and fibril propagation (Bernardini et al., 2024), accentuating the organ-specific distribution of deposits. A key modulatory component in amyloid deposition is serum amyloid P component (SAP), a highly conserved pentraxin that binds avidly and irreversibly to amyloid fibrils. SAP contributes to amyloid persistence by stabilizing fibrils against proteolytic degradation and cellular clearance, thereby rendering deposits more resistant to physiological removal mechanisms (Millucci et al., 2015a). Moreover, SAP interacts with complement components and cellular receptors, influencing local inflammatory responses and potentially modulating tissue remodelling (Ma and Garred, 2018). Although SAP does not initiate amyloid formation, its presence is essential for the long-term stability of established amyloid masses, making it an important therapeutic target in emerging anti-amyloid strategies. Clinically, AA amyloidosis most frequently manifests with renal involvement, where progressive deposition within the glomeruli leads to proteinuria, nephrotic syndrome, and eventual decline in renal function (Thorne et al., 2022). Extra-renal deposition, involving organs such as liver, spleen, adrenal glands, and gastrointestinal tract, contributes to the multisystem character of the disease and may exacerbate morbidity. Despite recent advances in controlling chronic inflammatory conditions, which have significantly reduced the incidence of AA amyloidosis in many regions (Lachmann et al., 2007), the disease continues to pose a considerable clinical burden, particularly in cases characterized by refractory inflammation or

delayed diagnosis. Early recognition remains challenging, requiring integration of clinical suspicion with histopathological confirmation, Congo red birefringence, immunohistochemical typing, and increasingly, mass spectrometry- proteomic analyses (Kratka et al., 2025; Merlini and Bellotti, 2003). An emerging area of interest is the occurrence of secondary AA amyloidosis in AKU. The persistent inflammatory state associated with severe ochronotic arthropathy can drive sustained elevation of SAA, thereby predisposing individuals with advanced AKU to develop AA amyloidosis. Renal and cardiac involvement have been reported in AKU patients with AA amyloid deposits (L. R. Ranganath et al., 2020b), expanding the disease's phenotypic spectrum beyond its classical metabolic and orthopedic manifestations. The presence of AA amyloidosis in AKU underscores the shared pathogenic link between metabolic dysfunction, chronic inflammation, and secondary amyloid deposition, while highlighting the importance of comprehensive inflammatory monitoring in patients with long-standing ochronosis.

Molecular features of Alkaptonuria

Alkaptonuria (AKU) is an inborn error of metabolism caused by deficiency of the enzyme homogentisate 1,2-dioxygenase (HGD), which catalyses the aromatic ring-cleavage of homogentisic acid (HGA) in the catabolic pathway of phenylalanine and tyrosine. Under physiological conditions, HGD converts HGA into maleylacetoacetate, allowing progression towards the formation of fumarate and acetoacetate, metabolites that feed into central cellular energy pathways. The HGD gene, located on chromosome 3q13.33 (Pollak et al., 1993), comprises 14 exons and encodes a 445-amino-acid protein that assembles into a highly complex homohexamer, organized as a dimer of two disc-like trimers (Ferná Ndez-Cañ and Peñ Alva, 1995; Granadino et al., 1997). This quaternary structure is stabilized by an intricate network of non-covalent interactions and exhibits low tolerance to amino-acid substitutions, explaining why most of the >200 known pathogenic variants are missense mutations that destabilize protomers, disrupt inter-subunit interactions, or alter residues within the active site (Bernini et al., 2020, 2017). Loss of HGD activity, typically reduced to <1% in affected

individuals, results in markedly elevated circulating levels of HGA, only partially cleared by urinary excretion. The excess HGA undergoes oxidation and subsequent radical-mediated polymerization, forming a dark, melanin-like pigment known as poly-HGA. This pigment progressively deposits within collagen-rich connective tissues, giving rise to ochronosis, the hallmark pathological process of AKU (Bernini et al., 2021). Histopathological and biochemical studies demonstrate that early pigmentation emerges in the extracellular matrix (ECM) of chondrocytes, particularly in calcified cartilage and at the osteochondral junction. Mechanical loading facilitates proteoglycan loss and exposure of collagen fibrils, which act as nucleation sites for pigment adhesion. The accumulation of poly-HGA induces oxidative stress, chronic low-grade inflammation, protein misfolding, and impaired autophagy, ultimately contributing to cellular dysfunction (including chondroptosis) and structural degeneration. Notably, secondary AA amyloidosis has been shown to co-localize with ochronotic pigment, suggesting that persistent inflammatory signalling and oxidative pathways participate in the progression of tissue damage (Bernardini et al., 2024). Despite being present from birth, AKU manifests clinically only after years of progressive molecular injury. Attempts to correlate specific HGD variants with disease severity have not provided clear genotype–phenotype associations. Even variants with residual enzymatic activity of 20–30% do not prevent pathological HGA accumulation, indicating that extragenic factors, including dietary tyrosine intake, renal function, and individual connective-tissue characteristics, may modulate clinical progression. Overall, the molecular defect in AKU establishes a self-perpetuating pathogenic cascade: HGA accumulation, poly-HGA formation, collagen pigmentation, oxidative and inflammatory dysregulation leading to progressive tissue degeneration. This biochemical sequence of events underlies the multisystemic manifestations of AKU and represents the principal target for current and emerging therapeutic strategies (Bernardini et al., 2024).

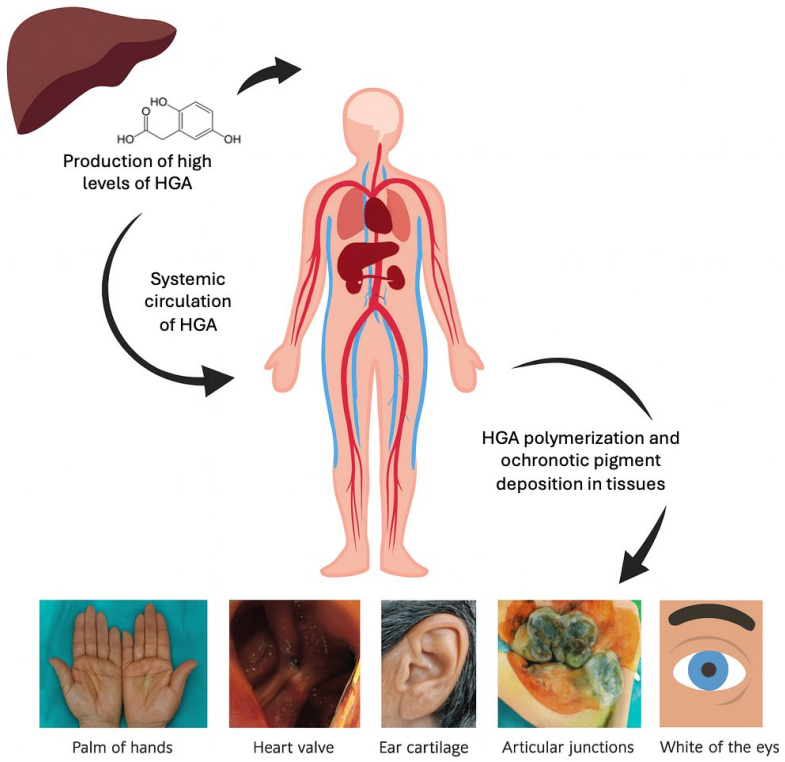

Figure 4. HGA is produced in liver and is spread in the body through systemic circulation. In several tissues, through its auto polymerization, leads the formation of the ochronotic pigment. The pigment settles in specific patients' compartment of the body, as articular joints, heart valves and aortic vessels.

Homogentisic acid (HGA) is highly prone to oxidation, and the reactive intermediates generated during this process trigger extensive radical-mediated reactions. In addition to undergoing autopolymerization into melanin-like poly-HGA pigments, oxidized HGA gives rise to reactive oxygen species (ROS), amplifying oxidative stress and initiating damage to lipids, proteins and nucleic acids. These oxidative modifications compromise the structural integrity and functional capacity of biomolecules, ultimately rendering heavily oxidized substrates incompatible with normal cellular homeostasis. As a consequence, cells activate autophagic and apoptotic pathways in an attempt to remove irreversibly damaged components (Filomeni et al., 2015; Galderisi et al., 2022).

HGA-induced oxidative stress also promotes several post-translational modifications (PTMs), including protein carbonylation, thiol oxidation, 4-hydroxynonenal (4-HNE) adduct formation, glycation and nitration (Braconi et al., 2014, 2010b). Many of these modifications are irreversible

and lead to protein misfolding, increased surface hydrophobicity and a heightened propensity to form aggregates. Both proteins and lipids undergo extensive peroxidation, reflecting a systemic activation of compensatory defense mechanisms against ROS. Importantly, oxidative alterations are not restricted to cells: serum from patients with AKU exhibits significantly elevated levels of protein carbonyls and lipid peroxidation products compared with healthy controls (Albatayneh et al., 2019). Elevated circulating C-reactive protein (CRP) and advanced oxidation protein products (AOPP) have also been reported, further supporting the systemic oxidative burden in AKU (Braconi et al., 2016). In vitro exposure of human serum to HGA produces marked lipid peroxidation, depletion of thiols, reduction of glutathione peroxidase activity, and extensive protein carbonylation (Braconi et al., 2011). Beyond molecular damage, oxidative stress in AKU profoundly alters the proteomic landscape. Proteins involved in stress defense, damage repair, proteostasis, and cell death pathways are upregulated. In AKU serum, altered expression patterns are observed for apolipoproteins, glycoproteins, complement components and protease inhibitors (Braconi et al., 2016). Similar alterations have been identified in AKU chondrocytes, which display dysregulation of proteins involved in protein fate, cytoskeletal organization, cell rescue and stress responses (Braconi et al., 2012). These findings are reproduced in in-vitro HGA-treated cell models, where HGA modulates proteins involved in folding, cytoskeletal integrity and cellular stress signalling (Braconi et al., 2010a).

As in many chronic degenerative diseases, oxidative stress and inflammation are tightly interconnected. Persistent ROS production and cumulative molecular damage activate pro-inflammatory signalling cascades, establishing a state of chronic low-grade inflammation characteristic of AKU. AKU chondrocytes exhibit significantly elevated levels of IL-1β, IL-6, IL-8, IL-10 and TNF-α relative to non-AKU controls (Braconi et al., 2012; Spreafico et al., 2013a). Increased concentrations of pro-inflammatory cytokines have likewise been reported in AKU plasma (Millucci et al., 2014c) and in HGA-treated cell models (Millucci et al., 2012). Circulating IL-6 levels show a positive correlation with patient age and AKU Severity Score, suggesting a contributory role

in disease progression and systemic inflammation. Chronic inflammation in AKU also promotes angiogenic activation, evidenced by increased vascularization of ochronotic tissues and elevated endothelial markers (Braconi et al., 2016).

A major recent advancement in AKU research is the recognition that the disease is frequently associated with secondary AA amyloidosis. This phenomenon is considered a downstream consequence of persistent inflammation and elevated serum amyloid A (SAA) levels. In most AKU patients, SAA concentrations exceed the physiological threshold of 10 mg/L (Braconi et al., 2018, 2016). Amyloid deposition has been documented in multiple tissues, including cartilage, synovium, periumbilical fat, salivary glands and aortic valves (Millucci et al., 2014b),as well as in HGA-treated cellular models (Millucci et al., 2012). Recent studies, including the 2024 Nature Reviews Primer, further confirm the frequent co-localization of AA amyloid with ochronotic pigment, highlighting a mechanistic interplay between poly-HGA deposition, oxidative stress and the amyloidogenic environment characteristic of AKU.

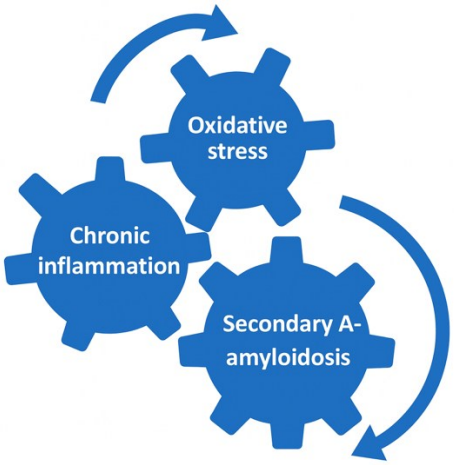

Figure 5. In AKU, oxidative stress, chronic inflammation and secondary A amyloidosis are strictly interconnected, and lead several harmful effects, as proteins post-translational modifications and pro-inflammatory molecules expression.

A peculiar characteristic of AKU is the co-localization of amyloid aggregates with the ochronotic pigment, as demonstrated by the overlap between SAA signal and the pigment staining (Millucci et al., 2012). It is still unclear if the ochronotic pigment accumulation stimulates the amyloid aggregate

formation, as a protective response of the organisma, or, on the opposite, the amyloid fibrils act as a scaffold, increasing the pigment deposition (Figure 6). According to the first hypotheses, amyloid plays a functional role by surrounding the pigment, in order to protect tissues from its toxic and oxidative effects, while the second theory considers amyloid accumulation to be merely a consequence of the SAA high levels in the blood. In this case, the co-localization of pigment deposits with amyloids fibrils could depend on their physical characteristic, which makes them suitable for the pigment deposition. Certainly, the two process are strictly connected, and affect each other. For instance, it was found that, in vitro, HGA acts as an enhancer of SAA aggregation, in a time and dose dependent manner (Braconi et al., 2017). The comprehension of these mechanisms appears fundamental for the study of the molecular mechanism behind the pathology and the research of efficient pharmacological cure.

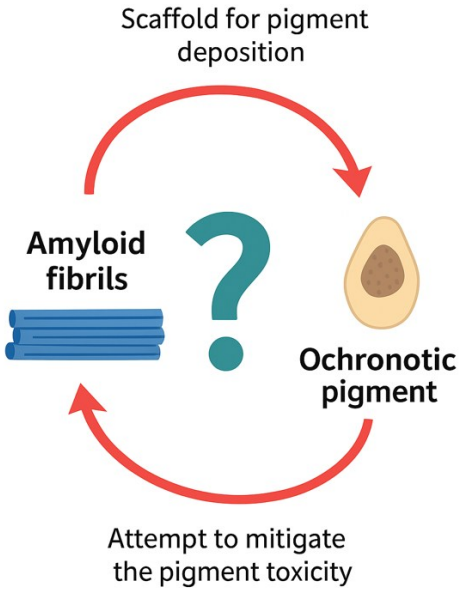

Figure 6. Amyloid fibrils and ochronotic pigment co-localize, but is still not clear if the amyloid accumulation is consequence or cause of ochronotic pigment deposition

A distinctive pathological hallmark of alkaptonuria (AKU) is the co-localization of AA amyloid deposits with ochronotic pigment, as demonstrated by the spatial overlap between serum amyloid A

(SAA) immunostaining and poly-HGA pigmentation in affected tissues (Millucci et al., 2012). Although this association is consistently observed, the causal relationship between pigment accumulation and amyloidogenesis remains unresolved.

Two mechanistic hypotheses have been proposed. The first suggests that amyloid deposition may represent a protective or compensatory response, whereby SAA-derived fibrils surround ochronotic pigment aggregates to limit their pro-oxidant and cytotoxic effects. This interpretation aligns with the notion that chronic oxidative stress and inflammation, both hallmarks of AKU, can induce SAA overexpression and promote its misfolding into amyloid fibrils.

The alternative hypothesis posits that amyloid fibrils themselves act as structural scaffolds, facilitating further pigment accumulation by providing a surface that enhances poly-HGA adhesion and polymerization. In this framework, co-localization arises not from a defensive response but from the physical and biochemical characteristics of amyloid fibrils, which may increase the efficiency of pigment deposition. Recent findings underscore that the two processes are closely intertwined rather than sequential or independent.

Experimental evidence supports this bidirectional interplay: in vitro, HGA has been shown to enhance SAA aggregation in a time- and dose-dependent manner, suggesting that oxidative derivatives of HGA may directly promote amyloid fibrillogenesis (Braconi et al., 2017). Conversely, amyloid-rich microenvironments may favour pigment retention, thereby exacerbating tissue degeneration.

Clarifying the molecular relationship between ochronosis and AA amyloidosis is crucial for understanding AKU pathogenesis. Moreover, unraveling these mechanisms may guide the development of targeted therapeutic strategies, particularly those aimed at mitigating chronic inflammation, oxidative stress, and the pathological cross-talk between pigment deposition and amyloid formation. Recently, it has been reported that poly-HGA deposition, oxidative stress, and SAA overproduction form a pathogenic triad, each amplifying the others and driving progressive tissue damage in AKU (Bernardini et al., 2024).

The ochronotic pigment deposition directly affects the cartilage matrix

Initially, it was thought that HGA could act directly as a chemical irritant and induce tissue degeneration through inflammatory mechanisms (Braconi et al., 2018). However, it was later discovered that HGA primarily undergoes spontaneous oxidation to 1,4-benzoquinone-2-acetic acid (BQA), which then polymerizes into a structure whose composition and properties are not yet fully defined, or dimerizes with BQA (Braconi et al., 2015). Many studies conducted to evaluate the interactions between collagen and HGA or BQA support the theory that BQA, and particularly the polymerization product, is the main cause of osteo-articular degenerative mechanisms. These studies have shown, in vitro, that the interaction between HGA and collagen is mainly reversible and pH-dependent, making it unlikely to have a significant impact in vivo (Braconi et al., 2018; Tinti et al., 2010). In contrast, the interaction with BQA or its polymerized form appears to create, in vitro, a direct and irreversible bond with collagen molecules, independent of pH. This suggests that such a mechanism is the most likely cause of the morphological changes observed in joint tissues in AKU (Tinti et al., 2011, 2010).From a chemical standpoint, these bonds likely result from electrostatic interactions between the carboxylate anion of BQA and ionic groups present in collagen, or coordination bonds between activated Csp2 (-CH=) groups in polymerized BQA and hydroxyl groups in collagen (Braconi et al., 2015). Unfortunately, although understanding the chemical behaviour of HGA and BQA is crucial for understanding the pathophysiological mechanisms in AKU, much remains to be clarified.

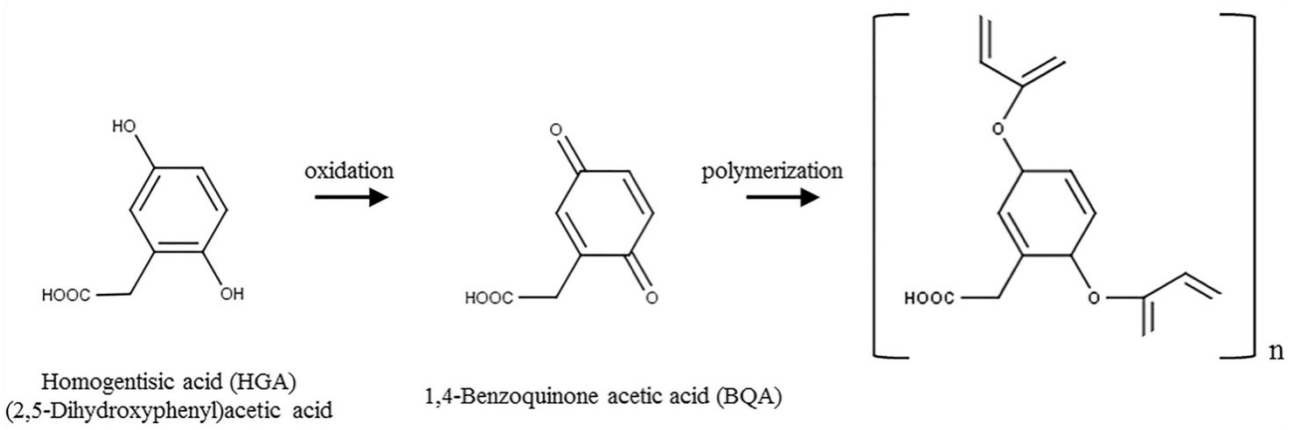

Figure 7. Oxidation of HGA to BQA and subsequent mechanization of polymerization assumed.

Ochronotic pigment is often also called melanin-like pigment, considering that both ochronotic pigment and melanin are derived from tyrosine, and have similar absorbance spectra, with an absorbance peak in the UV-visible range (Ranganath et al., 2019). The development of ochronotic pigment strongly depends on pH, with an acceleration of the process under alkaline condition and in the presence of Fe^{2+}/H_2O_2 (Vercruysse et al., 2017).

In AKU patients, cartilage is one of the most affected tissues. Microscopic observation of AKU cartilage samples reveals that the pigment deposition occurs mostly along collagen fibres. Indeed, the loss of protective molecules such as proteoglycans and glycosaminoglycans, that is a natural aging process increased in AKU patients, exposes binding sites on collagen fibres for HGA (Ranganath et al., 2019). In this way, pigment subunits accumulate on collagen fibres, triggering rapid pigment formation and deposition.

With the progression of the disease, AKU cartilage loses its architecture, and the extracellular matrix (ECM) becomes disorganized, failing to perform its function. Specifically, in AKU there is a reduction in the synthesis of fundamental ECM compounds, such as collagen, glycosaminoglycans (GAGs) and proteoglycans, and other ECM proteins (Galderisi et al., 2021). The reduced quantity of aggrecan, the principal proteoglycan in articular cartilage, leads to a decrease in cartilage resistance, making it brittle and stiff. Moreover, in AKU ECM, the type II collagen fibres break easily and are replaced by type III collagen, thinner and weaker. Therefore, collagen fibres assume a disorganized

arrangement and are scattered between amyloid fibrils (Millucci et al., 2014a). The progressive alteration of the ECM, the reduction of cellular metabolic activity and chondrocyte apoptosis result from the deposition of pigment and amyloid (Millucci et al., 2014c). This process is extremely aggressive and detrimental to joints, causing the resorption of subchondral bone and the calcification of cartilage (Taylor et al., 2011), with the consequent occurrence of arthropathy (Fig. 4). The loss of structure and function in articular joints leads to pain in patients and inability to move, since joints replacement with prothesis is necessary.

Ochronotic pigmentation in AKU arises from the non-enzymatic, radical-mediated oxidative polymerization of HGA, a process that has long been recognized but only recently elucidated in chemical detail. Historically described as a melanin-like substance owing to its broad UV-visible absorbance spectrum and its biochemical origin from tyrosine metabolism (Ranganath et al., 2019), the ochronotic pigment was for decades considered a poorly defined polymer of HGA oxidation products. However, modern analytical techniques have substantially revised this interpretation. Early studies established that the autoxidation of HGA is strongly pH-dependent, accelerating under alkaline conditions and in the presence of transition metals such as Fe^{2+} or H_2O_2, conditions that facilitate Fenton chemistry and generate semiquinone radicals (Vercruysse et al., 2017). These factors mirror the oxidative milieu found in degenerating AKU cartilage, providing a compelling explanation for the accelerated pigmentogenesis in vivo. A decisive breakthrough in understanding ochronotic pigmentation was provided recently (Grasso et al., 2025). Using solid-state NMR, FTIR microspectroscopy, scanning electron microscopy, mass spectrometry, and X-ray absorption spectroscopy, it was demonstrated conclusively that ochronotic pigment is not a linear or homogeneous polymer but rather a heterogeneous, crosslinked, polyphenolic network. The pigment consists of densely packed aggregates of oxidized HGA moieties, including benzoquinone-acetic-acid–like structures, catechols, semiquinones, and a variety of covalent C–C and C–O radical-coupling crosslinks. One of the most significant findings was the demonstration that ochronotic pigment contains metal-binding domains with high affinity for Fe^{3+} and Cu^{2+}, which not only

coordinate to the polymer but also catalyze further oxidation of HGA. This creates a self-amplifying oxidative loop, placing metal dysregulation at the center of pigment maturation.

Among all tissues, articular cartilage is the primary target for ochronotic pigmentation. Pigment deposition begins at the osteochondral junction and in regions subjected to high mechanical load, reflecting both microstructural and biochemical susceptibilities. Healthy cartilage protects collagen fibrils through a dense coating of proteoglycans (PGs) and glycosaminoglycans (GAGs), which prevent direct chemical interactions between collagen and external metabolites. In AKU, however, increased oxidative stress and chronic inflammation accelerate the loss of PGs and GAGs, exposing specific binding sites on the collagen triple helix (Ranganath et al., 2019). HGA quinones show high affinity for these exposed regions, forming both covalent adducts and π–π interactions, effectively anchoring pigment precursors onto collagen fibrils. Chemical mapping confirmed that these initial nuclei are enriched in quinone–collagen adducts, which subsequently act as nucleation centers for further oxidative polymerization (Grasso et al., 2025). This exposed-collagen model explains why the pigment is always deposited in a highly ordered, fibrillar pattern and why cartilage is affected earlier and more severely than other tissues. It also supports the notion that the genesis of the ochronotic pigment is not only a passive precipitation phenomenon, but a chemically targeted process driven by HGA's affinity for partially denatured collagen. As ochronosis progresses, the extracellular matrix (ECM) becomes increasingly compromised. Chondrocytes exposed to HGA and oxidative stress exhibit reduced synthesis of type II collagen, aggrecan, and other essential ECM components (Galderisi et al., 2021). Aggrecan depletion impairs the tissue's ability to bind water, leading to loss of compressive resilience, increased brittleness, and stiffness, hallmark features of AKU cartilage degeneration. In parallel, type II collagen fibrils undergo fragmentation due to oxidative cleavage and crosslinking by quinonoid intermediates. These weakened fibrils are progressively replaced by type III collagen, which is thinner, mechanically weaker, and indicative of tissue repair failure. Compelling evidence indicates that pigment deposition does not occur in isolation: AA amyloid fibrils frequently co-deposit with ochronotic pigment, forming a pathological composite material within the ECM

(Millucci et al., 2015a, 2015b). This colocalization has consistent mechanical and biochemical effects. Amyloid fibrils stiffen the matrix further and create a microenvironment enriched in oxidatively damaged proteins, which, in turn, promotes additional pigment deposition. The interwoven presence of amyloid and pigment is now recognized as a key factor in ECM disorganization and failure, confirming a synergistic pathogenic loop in AKU tissues. The biochemical stress imposed by pigment and amyloid drives progressive chondrocyte dysfunction, including impaired autophagy, mitochondrial dysfunction, and caspase-mediated apoptosis (Galderisi et al., 2022). As chondrocytes die, the ECM loses its primary source of renewal, promoting further degeneration. Ochronotic cartilage often undergoes calcification, driven by disruption of ECM mineralization pathways and chronic inflammation. Simultaneously, subchondral bone undergoes resorption and remodeling, likely due to altered mechanical loading and inflammatory cytokines. Ultimately, the accumulation of pigment, amyloid, oxidative damage, ECM fragmentation, and ossification leads to severe degenerative arthropathy, with structural collapse of major joints such as hips, knees, and spine. This process produces chronic pain, stiffness, and functional limitation, often requiring joint replacement as the only definitive intervention (Tinti et al., 2011).

HGA, oxidative stress, and post-translational protein modifications

Studies on AKU have clearly shown that oxidative stress is one of the most important factors in explaining the toxicity of HGA. The conversion of HGA into BQA is accompanied by the production of ROS such as superoxide anion (O2-), •OH, and H2O2. It has been hypothesized that the free radicals generated during the conversion of HGA to BQA are responsible for the connective tissue alterations observed in AKU, similar to those found in other rheumatic diseases (Kabuyama et al., 2008) and disorders of tyrosine metabolism (De Andrade et al., 2012; Rocha and Martins, 2012). It has been observed that in over two hundred human diseases, oxidative stress, defined as the imbalance between the production of ROS and RNS and the body's ability to neutralize them with antioxidant systems (Fedorova et al., 2014), plays a decisive role, as it causes various forms of post-translational

protein modifications. Given this, the involvement of post-translational modifications related to oxidative stress in AKU is not surprising. Consequently, proteomic studies have been conducted to determine whether HGA can induce redox-related post-translational modifications of proteins. Research has mainly focused on carbonylation and thiolic oxidation, the two most common mechanisms associated with oxidative stress (Dalle-Donne et al., 2006). Protein carbonylation constitutes an irreversible modification, though the exact mechanisms behind it remain unclear (Karsdal et al., 2013). What is certain is that carbonylated proteins cannot be repaired or removed by proteasomal processes. They have an increased tendency to aggregate due to their higher hydrophobicity and reduced recognition by chaperone proteins, which are crucial for protein stabilization and proper folding (Fedorova et al., 2014; Petrov and Zagrovic, 2011; Schaur, 2003). Proteomic studies on human AKU models and ex vivo AKU chondrocyte samples treated with HGA have shown a significant increase in post-translational changes, particularly in carbonyl insertion (Braconi et al., 2010b, 2010a). The primary targets are structural proteins, such as actin, vimentin, and annexin 2, as well as proteins involved in stress responses, including catalase, glutathione S-transferase, heat shock proteins (HSP60 and HSP74), and superoxide dismutase (Braconi et al., 2012). Similarly, increased protein carbonylation was observed in human serum treated with HGA in vitro (Braconi et al., 2010a), with key targets being proteins involved in metal transport or chelation, such as albumin, haptoglobin, ceruloplasmin, and transferrin (Braconi et al., 2011). These proteins are critical effectors of antioxidant activity, so their modification leads to a sharp reduction in the body's ability to defend against oxidative stress. The human serum AKU model treated with HGA has also been successfully used to study the effects of oxidative stress on the thiol groups of proteins. Again, an increase in oxidation products was observed, with a massive depletion of total thiol groups, primarily affecting the same classes of proteins already identified as sensitive to carbonylation (Braconi et al., 2011). Overall, it can be concluded that carbonylation and thiol group depletion in the proteome simultaneously promote aggregation mechanisms, thereby contributing to ochronosis in AKU, and reduce overall plasma antioxidant capacity. This significantly disrupts redox balance,

leaving biological systems more vulnerable to the adverse effects of oxidative stress, which can also lead to cell death. People with AKU experience oxidative stress throughout their lives, and the disease-induced reduction in antioxidant capacity worsens with age, due to the natural decline in defensive capabilities. This could help explain the late onset of joint and cardiac manifestations. Interestingly, a protein thiolation index has recently been proposed to evaluate the impact of the disease on an individual's antioxidant defence capacity (Giustarini et al., 2012).

BQA role in post-translational protein modifications

BQA is a compound with intrinsic cytotoxicity due to its reactivity towards the nucleophilic side chains of certain amino acid residues, primarily cysteine (Cys), through the formation of bonds with aromatic rings (Bernini et al., 2021). It has been discovered that, in vitro, BQA can bind to many human serum proteins, primarily transferrin and haptoglobin (Braconi et al., 2011, 2010b). This suggests that extensive post-translational modifications of the proteome may be induced directly by BQA. These modifications of protein structures may result in functional alterations, reduced stability, and the promotion of pathological aggregation, precipitation, and the formation of ochronotic pigment.

Updated Level of AKU Interpretation

Integrating traditional pathology with advanced spectroscopy, molecular biology, and biomechanics, ochronosis is now understood as a multi-layered pathological cascade, composed of:

- HGA autoxidation and radical generation, accelerated by alkaline microenvironments and metal ions.
- Formation of semiquinones, quinones, and polyphenolic aggregates(Grasso et al., 2025).
- Selective collagen binding, enabled by ECM degradation and exposure of reactive collagen motifs.

- Polymer growth and metal-mediated crosslinking, reinforcing pigment stability and propagating oxidative chemistry.
- ECM disorganization, amyloid co-deposition, and biomechanical failure, culminating in cartilage destruction.
- Cycles of inflammation, oxidative stress, and cell death, which perpetuate and accelerate tissue degeneration.

This refined framework redefines ochronosis not as a simple pigment deposition disorder but as a complex redox-driven, metal-dependent, collagen-targeting, and amyloid-potentiated degenerative process, with multiple therapeutic targets emerging from these new molecular insights, including metal chelators, antioxidants, anti-amyloid therapies, and inhibitors of HGA oxidation.

An In Vitro Cell Model for Exploring Inflammatory and Amyloidogenic Events in Alkaptonuria

Abstract

Alkaptonuria (AKU) is a progressive systemic inherited metabolic disorder primarily affecting the osteoarticular system, characterized by the degeneration of cartilage induced by ochronosis, ultimately leading to early osteoarthritis (OA). However, investigating AKU pathology in human chondrocytes, which is crucial for understanding the disease, encounters challenges due to limited availability and donor variability. To overcome this obstacle, an in vitro model has been established using homogentisic acid (HGA) to simulate AKU conditions. This model employed immortalized C20/A4 human chondrocytes and serves as a dependable platform for studying AKU pathogenesis. Significantly, the model demonstrates the accumulation of ochronotic pigment in HGA-treated cells, consistent with findings from previous studies. Furthermore, investigations into inflammatory processes during HGA exposure revealed notable oxidative stress, as indicated by elevated levels of reactive oxygen species (ROS) and lipid peroxidation. Additionally, the model demonstrated HGA-induced inflammatory responses, evidenced by increased production of nitric oxide (NO), over-expression of inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). These findings underscore the model's utility in studying inflammation associated with AKU. Moreover, analysis of serum amyloid A (SAA) and serum amyloid P (SAP) proteins revealed a potential interaction, corroborating evidence of amyloid fibril formation. This hypothesis was further supported by Congo red staining, which showed fibril formation exclusively in HGA-treated cells. Overall, the C20/A4 cell model provided valuable insights into AKU pathogenesis, emphasizing its potential for facilitating drug development and therapeutic interventions.

Introduction

Alkaptonuria (AKU) is an exceptionally rare autosomal recessive genetic disorder (Bernardini et al., 2024) caused by a deficiency in the enzyme homogentisate 1,2-dioxygenase (HGD). This deficiency leads to the accumulation of homogentisic acid (HGA) due to impaired catabolism of tyrosine and phenylalanine. The oxidized derivative of HGA, benzoquinone acetic acid (BQA), forms polymer deposits that accumulate in connective tissues resulting in a pigmentation condition known as "ochronosis,". This condition causes widespread deposition of ochronotic pigment in various organs, exacerbating organ dysfunction (Hanika et al., 2002) and increasing tissue oxidative stress through the generation of reactive oxygen species (ROS).

Ochronotic arthropathy is the most common clinical manifestation of AKU, characterized by cartilage degeneration, chronic inflammation, and pain (Helliwell et al., 2008). Despite the recent approval of nitisinone therapy (L. R. Ranganath et al., 2020a), treatment remains focused on symptom management, often requiring total joint replacement in advanced stages. Studies have shown that HGA induces significant protein oxidation in serum and chondrocytes (Spreafico et al., 2013a), leading to oxidative stress and chondroptosis, a specific form of apoptosis observed in AKU cartilage (Millucci et al., 2015b). AKU has also been associated with secondary amyloidosis (Millucci et al., 2015a, 2014a, 2014b, 2014c, 2012), particularly AA amyloidosis, where serum amyloid A (SAA) fibrils deposit in tissues (Momohara et al., 2008), potentially leading to organ failure. Amyloid deposits have been identified in alkaptonuric tissue, likely due to localized HGD expression within the osteoarticular system (Bernardini et al., 2012; Laschi et al., 2012). Moreover, pigment deposition in AKU tissues co-localizes with SAA-amyloid (Braconi et al., 2012, 2011, 2010b, 2010a; Tinti et al., 2011, 2010), implicating a role in secondary amyloidosis. Further research into AKU mechanisms has revealed structural and functional alterations in chondrocytes, including abnormal cytoskeletal protein expression (Geminiani et al., 2017), oxidative stress-induced dysfunction (Braconi et al., 2016, 2015, 2010b), reduced primary cilia length, and disrupted Hedgehog signaling (Gambassi et

al., 2017; Thorpe et al., 2017). Autophagy, essential for cellular homeostasis, is progressively impaired by HGA-induced oxidative stress, worsening chondrocyte death (Galderisi et al., 2022). Challenges in developing AKU therapies include the ultra-rare nature of the disease, the lack of severe symptoms in early childhood, and decreasing research investment. Additionally, limited access to biological samples complicates research efforts. To overcome these challenges, in vitro models using human chondrocytes cultured with HGA have been developed (Tinti et al., 2010), aiding in understanding ochronotic pigment deposition mechanisms and facilitating therapeutic strategies. The C20/A4 cell line has emerged as a valuable model for studying AKU in vitro (Braconi et al., 2010a) , providing rapid findings compared to primary cells and eliminating the need for biopsy sample extraction.

The aim of this study was to develop a novel cell model utilizing the C20/A4 human chondrocyte cell line to investigate inflammatory responses in AKU. Our results not only confirmed the reliability of this model for studying AKU but also demonstrated its efficacy in assessing inflammatory processes. Specifically, we observed an increased production of nitric oxide (NO), accompanied by the overexpression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and 4-hydroxynonenal (4-HNE) proteins in C20/A4 cells treated with HGA. Additionally, the investigation of SAA and serum amyloid P (SAP) proteins revealed a potential relationship in HGA-treated C20/A4 cells, further supported by Congo red staining, indicating amyloid fibril production.

Materials and Methods

Cell cultures and treatments

The immortalized human chondrocyte cell line C20/A4 (Sigma-Aldrich, SCC041) was cultured at 37°C in a humidified atmosphere with 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS). A 10 mM stock solution of HGA (Sigma Aldrich) was prepared by dissolving HGA in deionized water. This stock solution was then diluted in the cell culture medium to achieve final concentrations of 0.066 mM and 0.1 mM, reflecting levels observed in AKU patients (Tinti et al. 2011). These concentrations were selected for treatments lasting 1-2 weeks for subsequent experiments.

Fontana Masson Assay

C20/A4 cells were seeded in a 6-well plate at a density of $2x10^5$ cells per well and allowed to grow until reaching 70-80% confluence. The cells were then treated with HGA at concentrations of 0.066 mM and 0.1 mM for 14 days. After treatment, cells were washed, fixed in 75% ethanol, stained with ammoniacal silver solution, fixed again with 2% sodium thiosulfate and finally dehydrated with ethanol. The stained samples were observed under a bright-field microscope. For pigment quantification, ImageJ software was utilized, employing the Measure function. The obtained values were normalized against the area to ensure accurate quantification.

Detection of amyloid deposits

To investigate the distribution of presumptive amyloidosis, C20/A4 cells were seeded into 6-well plates at a density of 2×10^5 cells per well and cultured until reaching sub-confluence (80-85%). Subsequently, the cells were treated with HGA at varying concentrations (0.066 mM, 0.1 mM) for 14 days. Cells were then stained with 1% Congo Red and dehydrated using ethanol, followed by examination under a bright-field microscope. The samples were then further analyzed under polarized light using a Zeiss Axio Lab A1 microscope to detect any birefringence indicative of amyloid fibrils.

Quantification of ochronotic pigment deposition by Autofluorescence

For fluorescence-based pigment detection, C20/A4 cells were seeded in a 24-well plate at a density of $2x10^4$ cells per well for a 1-week treatment, and $1x10^4$ cells per well for a 2-week treatment. The cells were allowed to grow until reaching 70-80% confluence and were then treated with HGA at concentrations of 0.066 mM and 0.1 mM HGA for 7 and 14 days. Following the treatment period, the cells were fixed in 4% formaldehyde. Glass coverslips were then mounted using Fluoroshield mounting medium (Abcam). Images were captured using a Zeiss AxioLabA1 fluorescence microscope equipped with a FITC filter. Fluorescence intensity was quantified using the Measure function in ImageJ software, and the obtained values were normalized against the area to ensure accurate quantification.

Quantification of Intracellular ROS Formation

ROS production in C20/A4 cells was assessed in 96-well plates using 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, Sigma-Aldrich), which is intracellularly deacetylated and oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Ng and Ooi, 2021). The cells were treated with HGA at a concentration of 0.066 mM for 24 hours. $DCFH_2$-DA (5µM), dissolved in Hank's Balanced Salt Solution (HBSS), was then added to the cells and incubated for 10 minutes at 37°C. Fluorescence was measured using an EnVision system (PerkinElmer) with excitation at 485 nm and emission at 535 nm. To visualize DCF fluorescence, samples were observed under a fluorescence microscope with a FITC filter (Zeiss AxioLabA1, Oberkochen, Germany), and images were acquired to confirm the results. The number of cells in each well was then determined using a Crystal Violet assay (Feoktistova et al., 2016). Results were normalized to the relative cell count for each well and expressed as relative ROS production compared to the untreated group.

Determination of NO production

To evaluate NO production in the supernatant of C20/A4 cells, a Griess assay was performed. Cells were seeded in 6-well plates at a density of 2×10^6 cells per well and cultured until they reached sub-confluence (80-85%). The cells were then treated with HGA at different concentrations (0.066 mM, 0.1 mM) for 14 days. Subsequently, 100 µL of conditioned medium from each well was mixed with an equal volume of Griess reagent, consisting of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid. The absorbance was measured at 540 nm using an EnVision system (PerkinElmer). Nitrite concentration was determined by comparing the absorbance values to a sodium nitrite standard curve.

Western Blotting

Cells at 75-80% confluence were subjected to the aforementioned treatment protocol. Following treatment, the cells were washed, and cellular lysates were prepared using RIPA buffer supplemented with phosphate and protease inhibitors. The lysates were then disrupted by sonication in an ice bath. Subsequently, 20 micrograms of protein from each sample were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The primary antibodies utilized were as follows: anti-iNOS (rabbit polyclonal IgG, Sigma-Aldrich), anti-GAPDH HRP-conjugated, anti-SAA (Mouse monoclonal IgG, ThermoFisher Scientific), anti-4-HNE (Mouse monoclonal IgG, Acris), and anti-COX-2 (rabbit polyclonal IgG, Sigma-Aldrich). For secondary antibody detection, anti-rabbit HRP-conjugated antibody and anti-mouse HRP-conjugated antibody (both from Sigma-Aldrich) were used. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) with Luminata Crescendo (Merck Millipore), and images were captured using a LAS4000 machine (GE Healthcare). The optical densities of the immunoreactive bands were analyzed using ImageQuantTL software V 7.0 (GE Healthcare). Protein levels were normalized against GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), which served as a protein-loading control.

Immunofluorescence study

C20/A4 cells were seeded onto glass coverslips at a density of $2x10^4$ cells for 1 week and $1x10^4$ cells for 2 weeks. After treatment, cells were fixed with methanol, permeabilized with pure acetone, and then blocked. The cells were then incubated overnight at 4°C with the following primary antibodies: anti-SAA mouse monoclonal antibody (1:100, Sigma-Aldrich), anti-SAP rabbit monoclonal antibody (1:30, Sigma-Aldrich), and anti-4-HNE mouse monoclonal antibody (1:50, Invitrogen). Afterwards, the cells were incubated with secondary antibodies: Alexa Fluor™ 546 goat anti-mouse Ig (1:100, Thermo Fisher Scientific) and Alexa Fluor™ 488 goat anti-rabbit Ig (1:100, Thermo Fisher Scientific). The cells were then mounted with Fluoroshield mounting medium containing DAPI (Abcam). Images were captured using a fluorescence microscope (Zeiss AxioLabA1, Oberkochen, Germany), and fluorescence intensity was quantified using ImageJ software. Co-localization analysis of SAA and SAP was performed using the JACoP plug-in in ImageJ, which calculates the Manders' coefficient representing the percentage of SAA pixels that overlap with SAP. Co-localization events were visualized as white dots using the Colocalization plug-in.

Statistical Analysis

The experiments were conducted in triplicate. Statistical analyses were carried out using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The data are expressed as mean ± standard deviation (SD) and were compared using either the unpaired t-test or one-way analysis of variance (ANOVA) followed by an appropriate post hoc test. A p-value of 0.05 or less was considered statistically significant.

Results

A substantial accumulation of ochronotic pigment was observed in the C20/A4 model following HGA treatment

A characteristic feature of AKU is the formation of intracellular ochronotic pigment. To track pigment deposition over time, C20/A4 cells treated with HGA were stained using the melanin-specific Fontana Masson staining technique. Concentrations of 0.066mM and 0.1mM HGA were selected to define the C20/A4 AKU model (Galderisi et al., 2022). The ochronotic pigment, identifiable by black-colored spots, was observed in both intracellular and extracellular compartments after 2 weeks of treatment with both concentrations. The results, presented as mean pixel values of pigment deposition relative to the control, revealed a concentration-dependent increase in pigment deposition (Figure 1).

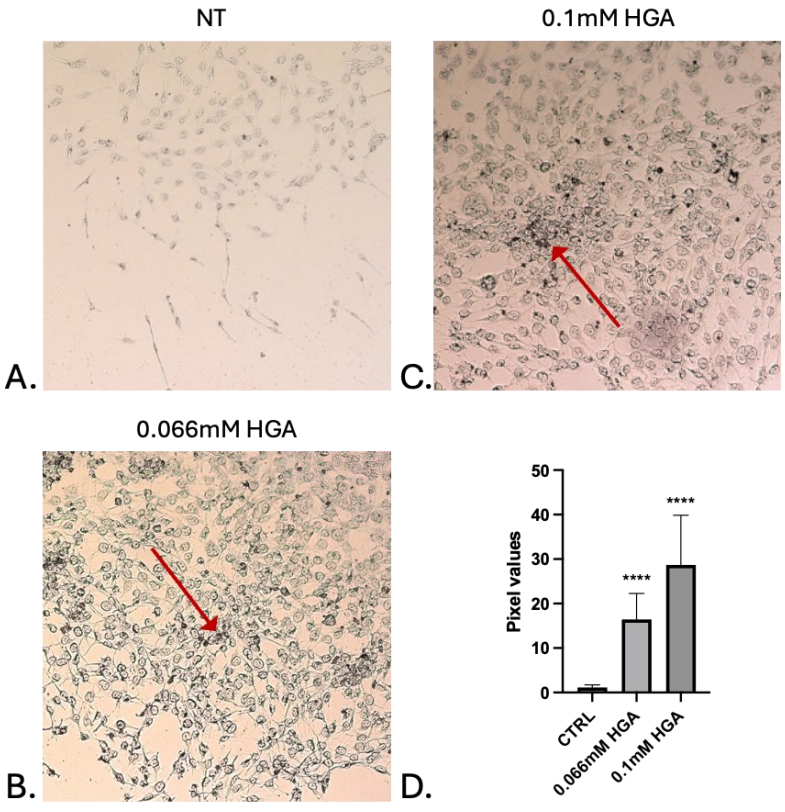

Figure 8. Accumulation of ochronotic pigment (arrow) in C20/A4 cells treated with (b) 0.066 mM and (c) 0.1 mM HGA for 2 weeks. (a) C20/A4 cells without any treatment. Fontana Masson staining revealed black pigmentation indicative of pigment deposition. Cells are shown at 10x magnification. (d) Quantification of black pigmentation intensity was performed using ImageJ software and normalized to the cellular area. Statistically significant differences are denoted by ***p = 0.0003 and ****p < 0.0001 (vs. CTRL). All experiments were performed in triplicate. Data are reported as fold change. p-values were calculated by ordinary one-way ANOVA with Dunnett's post hoc test.

Furthermore, formaldehyde-induced fluorescence was utilized to detect melanin-like ochronotic pigment. Autofluorescence provides a valuable method for studying various pigments, including melanin and melanin-like substances. Melanin, a complex biopolymer responsible for pigmentation in the skin, hair, and eyes, exhibits intrinsic autofluorescence. Notably, this property is enhanced when melanin is exposed to formaldehyde. The reaction between melanin and formaldehyde results in increased autofluorescence, facilitating easier identification and analysis in histological examinations (Hara et al., 2016). Similarly, melanin-like pigments, such as ochronotic pigment, also display autofluorescent properties (Galderisi et al., 2022). Utilizing autofluorescence to study this pigment provides an effective approach for visualizing and evaluating its deposition in cells and tissues, thereby aiding in the diagnosis and understanding of disease progression. Ochronotic pigment autofluorescence, induced by fixation with formaldehyde, became evident in C20/A4 cells containing ochronotic pigment after 1 week of treatment with 0.066mM and 0.1mM HGA and it exhibited significantly higher levels after 2 weeks of treatment at both concentrations (Figure 2).

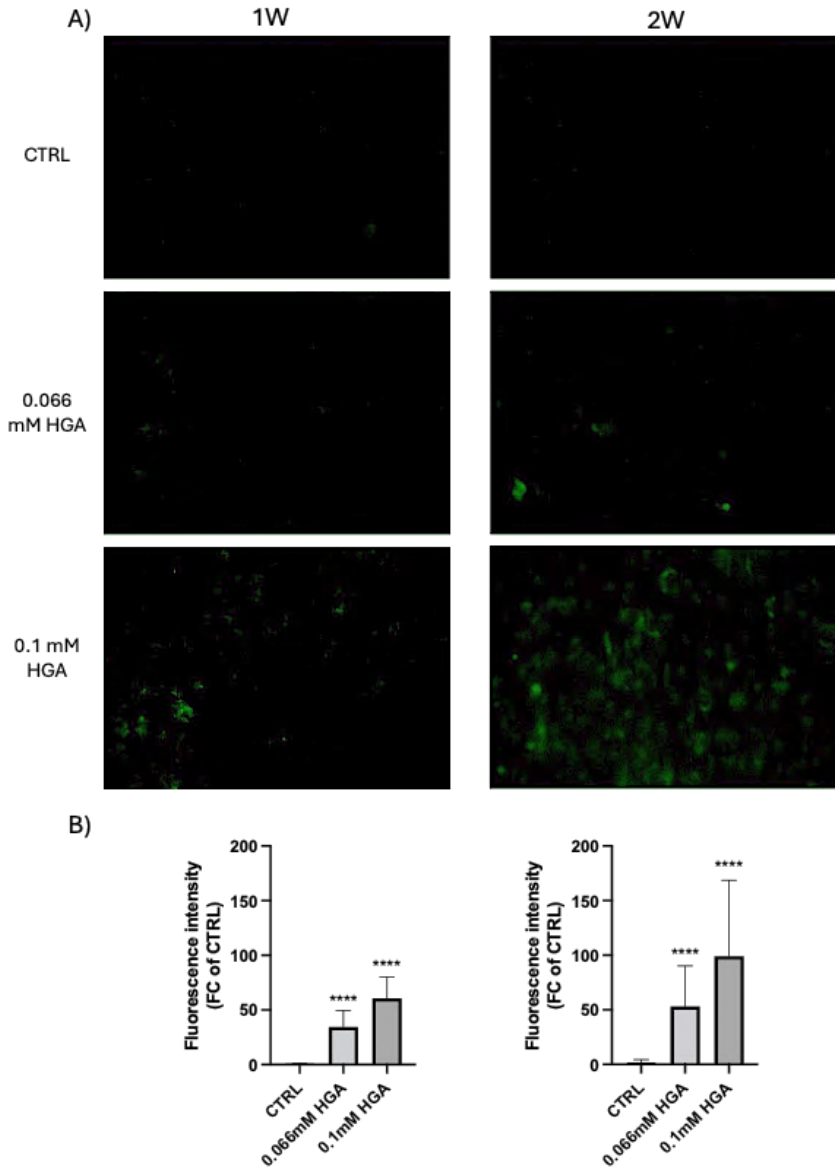

Figure 9. (a) Time-varying ochronotic pigment formation in C20/A4 cells treated with 0.066 and 0.1 mM HGA for one and two weeks, detected by formaldehyde fixation. Cells are shown at 40x magnification. (b) Graphical representation of fluorescence intensity. Statistically significant differences are denoted by **p = 0.002 and ****p < 0.0001 (vs. CTRL). All experiments were performed in triplicate. Data are reported as fold change. p-values were calculated by ordinary one-way ANOVA with Dunnett's post hoc test.

The quantitative analysis of fluorescence intensity revealed an increase in pigmentation that was dependent on both the duration of treatment and the concentrations of HGA. This increase was observed in both intracellular and extracellular compartments after 1 week and, particularly, after 2 weeks of treatment.

HGA induced aggregation of amyloidogenic proteins

Knowledge about SAA and SAP content in AKU has been established, allowing the disease to be classified as a secondary amyloidosis (Millucci et al., 2012). Congo red is an efficient staining method for visualising amyloid components due to its special affinity for amyloid fibrils structures. When bound to amyloid, Congo red exhibits birefringence under polarised light, displaying a characteristic green-yellow colour. This optical property, combined with its high binding affinity, makes Congo Red a reliable and widely used tool in the diagnosis and study of amyloidosis. Under polarized light, the staining in C20/A4 cells treated for 2 weeks with 0.066 mM and 0.1 mM HGA revealed green-yellow birefringence, whereas the control cells did not display such characteristics (Figure 3). The presence of amyloid deposits in HGA-treated cells appeared to be linked to a simulated disease advancement, as untreated cells showed no evidence of deposition.

Detection of SAA and SAP in HGA-treated C20/A4 cells

In order to investigate the presence of SAA and SAP proteins, immunofluorescence staining was conducted on HGA-treated and control C20/A4 cells for 1 and 2 weeks. Both SAA and SAP were detectable in control and treated cells (Figure 4), exhibiting clear and intense red (SAA) and green (SAP) staining.

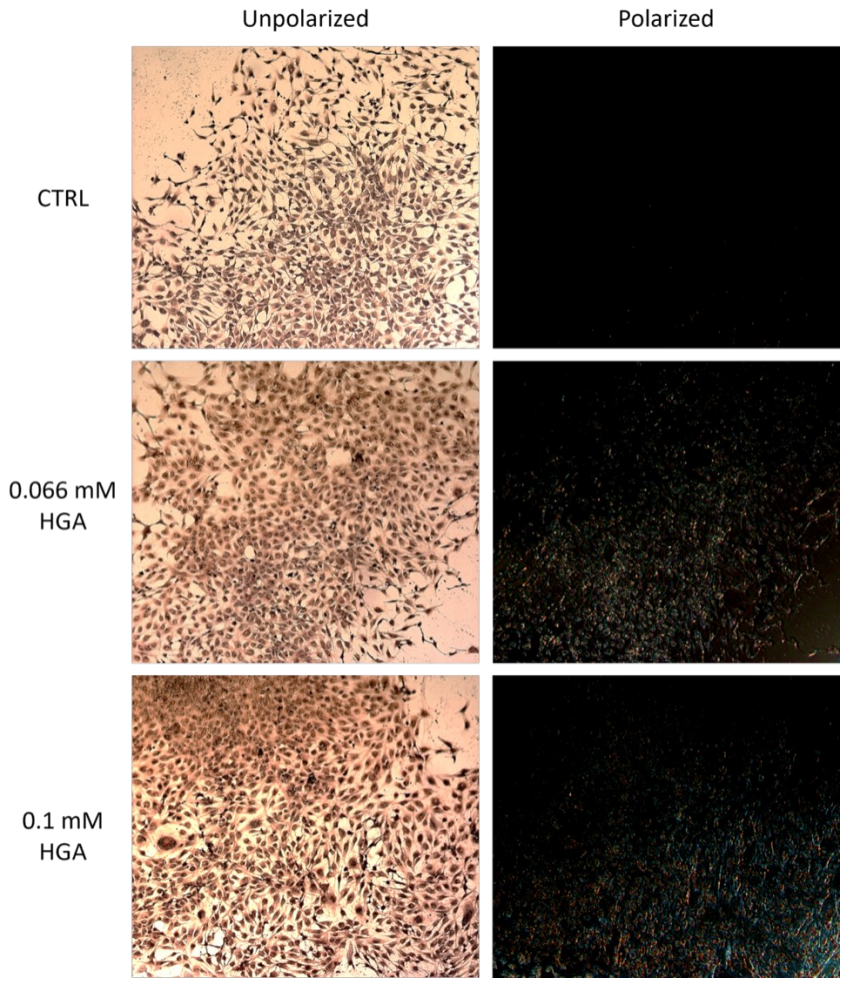

Figure 10. Assessment of amyloid presence in C20/A4 cells treated with 0.066 and 0.1 mM HGA for 2 weeks usingCongo Red staining. Cells are shown at 10x magnification

In untreated cells, the two proteins seemed to be equally distributed. However, in cells treated with 0.066 mM HGA, and particularly at 0.1 mM, both SAA and SAP were primarily localized in the perinuclear zone. After 1 week (Figure 4A) and 2 weeks (Figure 4B) of HGA treatment, C20/A4 cells also began to lose their structure, showing alterations in nuclear shape, with indistinct boundaries and morphology, as well as chromatin condensation. Additionally, observations indicated a tendency for the two signals to overlap in HGA-treated C20/A4 cells, a trend that was less evident in the control samples after 1 or 2 weeks.

HGA induced pronounced oxidative stress

Since HGA is known to cause oxidation in chondrocytes, leading to oxidative stress and chondroptosis (Millucci et al., 2015b), we aimed to investigate its effect during both the acute and

chronic inflammatory phases. To do this, we treated C20/A4 human chondrocytes with 0.066 mM HGA, a concentration consistent with levels observed in alkaptonuric patients (Ranganath et al., 2016). This dosage effectively replicated disease-related events without compromising cell viability (Galderisi et al., 2022).

ROS serve as signaling molecules that play a critical role in the development of inflammatory disorders and are typically produced as part of the early response to stress conditions (Forrester et al., 2018). In chondrocytes, ROS production is also involved in regular processes, such as the regulation of the homeostasis of extracellular matrix (Henrotin et al., 2003). Therefore, we investigated the impact of treating C20/A4 cells with HGA on intracellular ROS production. The DCF fluorescence exhibited a notable increase in HGA-treated cells compared to the control, indicating the induction of oxidative stress even after 24 hours (Figure 5).

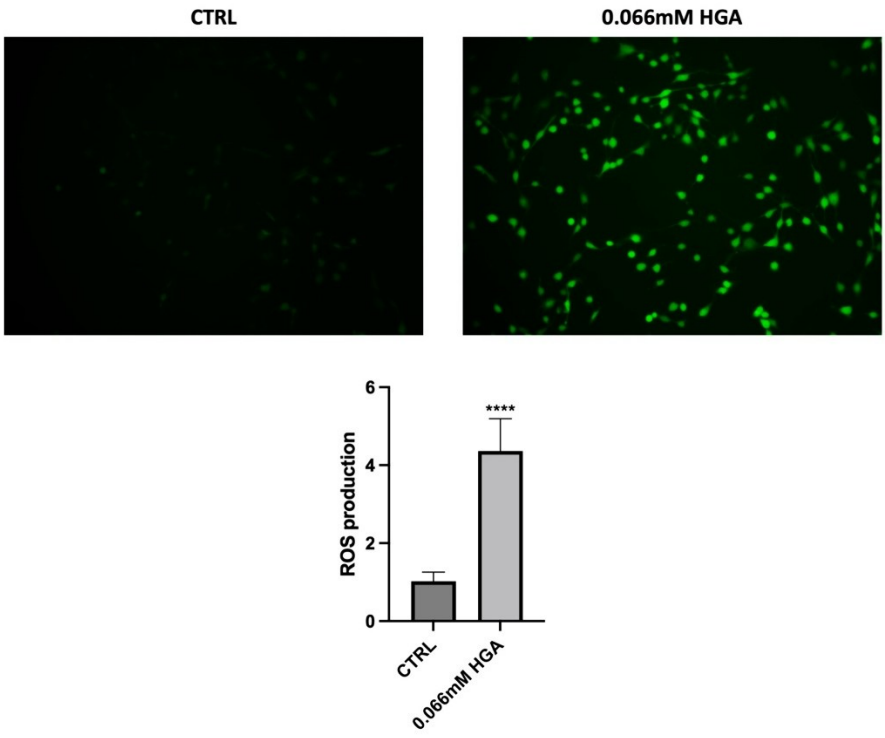

Figure 11. Exploration of ROS generation by labelling with the H_2DCFDA probe (green fluorescence) in C20/A4 control and 0.066 mM HGA treated cells. Data are presented as bar graphs for ROS levels measured from fluorescence intensity normalized to cell count using Crystal Violet assay. All experiments were performed in triplicate. Data are reported as fold change. An unpaired t-test was used to assess statistically significant differences (****$p < 0.0001$). Cells are shown at 20× magnification.

Long-term oxidative stress usually results in chronic disorders characterised by damage to DNA and lipidic structures, resulting in structural changes and loss of stability (Galderisi et al., 2022).To evaluate the presence of major products of lipid peroxidation (LPO), 4-HNE expression was investigated. The fluorescence signal detected in HGA-treated C20/A4 cells (Figure 6A) revealed a significant presence of LPO, contrasting with control cells. The findings are consistent with those observed in Western blot analysis, wherein 4-HNE expression revealed LPO-induced damage to the cellular lipid structures (Figure 6B).

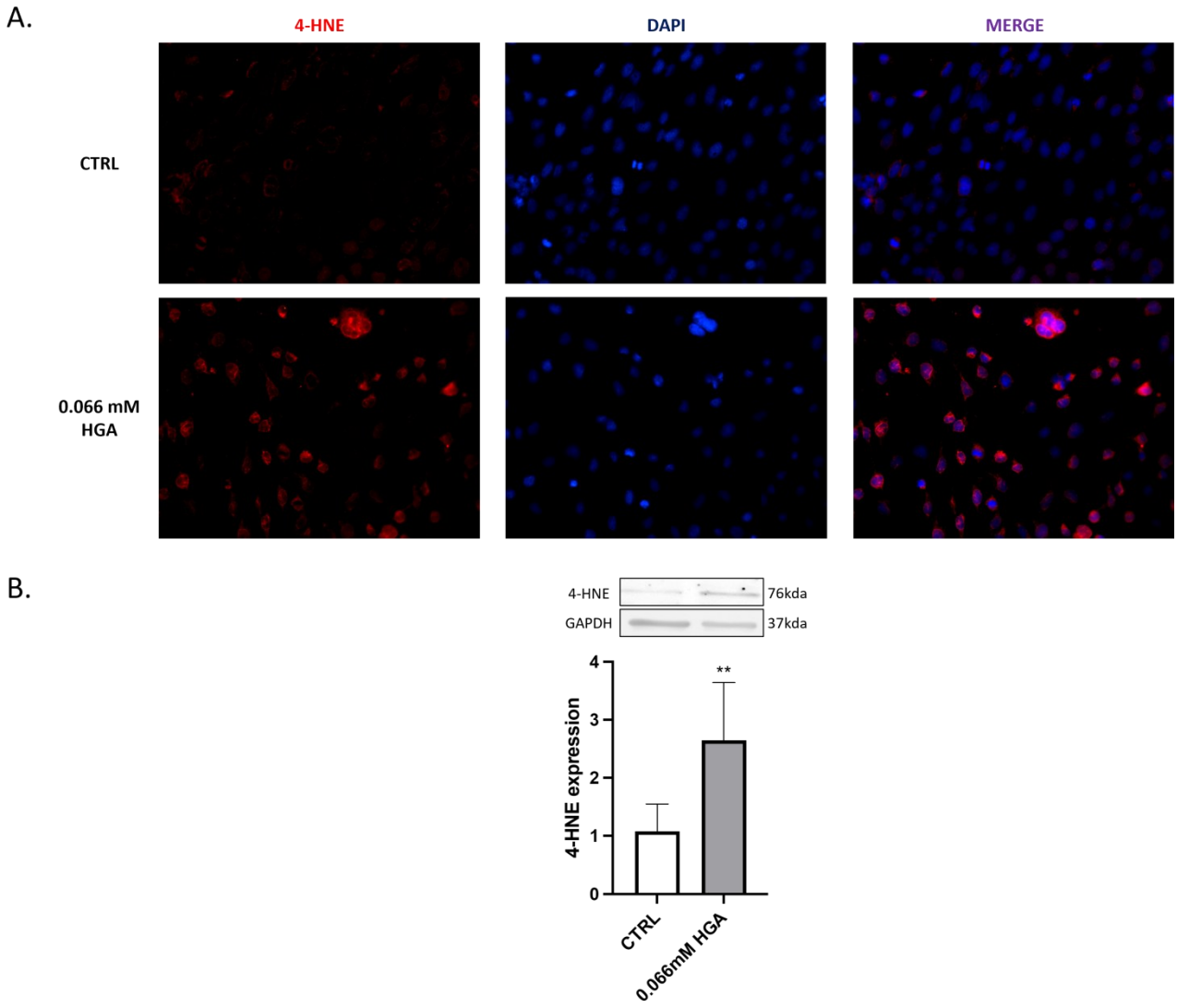

Figure 12. (a) Examination of 4-HNE expression in control and HGA-treated C20/A4 chondrocytes after 2 weeks of treatment with 0.066 mM HGA by immunofluorescence staining. Nuclei were counterstained with DAPI. (b) Detection of 4-HNE in C20/A4 cells byWestern blot analysis. Quantification of relative band intensities was determined from three independent experiments. Data are reported as fold change. An unpaired t-test was used to assess statistically significant differences, (**p = 0.0026). Cells are shown at 40× magnification.

HGA-induced inflammatory effects

According to recent studies, AKU has been linked to a chronic inflammatory state (Braconi et al., 2016). The presence of HGA and its oxidized byproducts in connective tissues, along with a state of oxidative stress and amyloid deposition, promotes an inflammatory response, which contributes to joint and cartilage deterioration, as well as other systemic implications (Braconi et al., 2018). To investigate inflammatory events in AKU, C20/A4 cells were treated with HGA at a concentration of 0.066 mM for 2 weeks and the levels of key pro-inflammatory mediators were quantified. The production of NO, detected using the Griess assay, was significantly higher in HGA-treated cells (Figure 7A) compared to the control.

The protein expression of iNOS and COX-2 was evaluated via Western blotting. Quantitative analysis of immunoreactive bands revealed an upregulation in both iNOS and COX-2 expression in comparison to the control (Figure 7B-C).

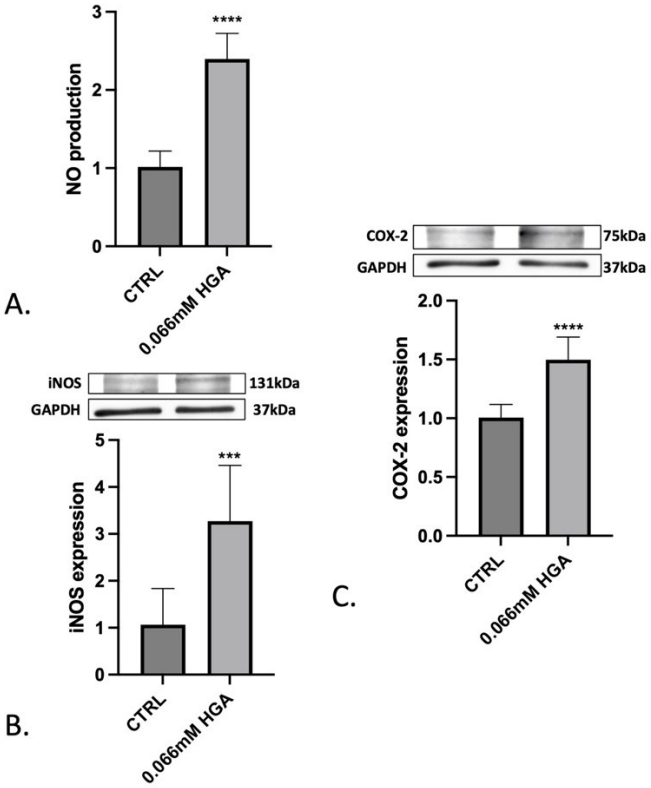

Figure 13. Evaluation of NO production and iNOS and COX-2 expression in C20/A4 chondrocytes treated with 0.066 mM HGA for 2 weeks. (a) Supernatants from cell cultures were analyzed to measure NO levels using the Griess assay. iNOS (b) and COX-2 (c) expression levels were determined by Western blot analysis. Data are reported as fold change. All experiments were performed in triplicate. An unpaired t-test was used to assess statistically significant differences (***p = 0.0006 and ****p < 0.0001).

Discussion

AKU is a complex degenerative disorder that significantly affects multiple organ systems, particularly the osteoarticular system, with cartilage being notably more susceptible to damage compared to bone (Fisher and Davis, 2004). Ochronosis, an essential characteristic of AKU, entails a cascade of effects including weakening of cartilage, increased stiffness, risen susceptibility to fractures, chronic inflammation, and eventual onset of OA (Gallagher et al., 2015). Chondrocytes, the only cells in cartilage, play a pivotal role in orchestrating the synthesis of extracellular matrix components such as collagen and proteoglycans. These components are essential for maintaining the structural integrity and biomechanical resilience of cartilage, which is important for withstanding mechanical stresses. Nevertheless, conducting research on human chondrocytes faces numerous obstacles, primarily due to the difficulties in obtaining a sufficient number of primary cells from a single source. These challenges are further exacerbated by inherent variations among donors, which can be attributed to factors such as age and concurrent medical conditions. Given the scarcity of AKU cases, obtaining biological specimens for research purposes is not only rare but also extremely difficult to get in controlled experimental settings. This limits the ability to compare and apply research findings to a wider population. Generally, the investigation of events related to AKU has been conducted by analyzing cells directly obtained from biopsies of AKU patients or by using cellular models that replicated the disease condition. The use of an immortalized cell line of human chondrocytes as a cellular model for AKU has provided a novel opportunity for investigating this rare disease (Braconi et al., 2010b; Galderisi et al., 2022) eliminating the need for biopsy-derived samples. The aim of this study was to further validate C20/A4 cells as a cellular model for studying AKU *in vitro* and to develop, for the first time, an AKU inflammatory cell model. This model will be used to study the inflammatory processes associated with the disease, delving into the complex mechanisms involved in the formation of ochronotic pigment and its consequential impact on osteoarthropathy.

AKU, classified as a form of OA, implicates inflammatory conditions as pivotal in the clinical manifestation of the disease (Millucci et al., 2017). In our previous research, we demostrated that HGA promotes the production and release of SAA and proinflammatory cytokines, along with oxidative stress, amyloid and ochronotic pigment formation, and cell death in an AKU cell model (Braconi et al., 2011, 2010b; Tinti et al., 2010). However, to the best of our knowledge, this is the first report on the expression of inflammation biomarkers in C20/A4 human chondrocytes treated with HGA. At first, our investigation revealed a notable increase in the production of NO, coupled with a heightened level of iNOS and COX-2 enzymes. These findings highlighted the pro-inflammatory environment induced by exposure to HGA. Articular cartilage is a connective tissue that consists of a single type of cell named chondrocytes. These cells are surrounded by a self-produced extracellular matrix (ECM), that contains water, collagen 2, agglomerated proteoglycans, and hydrophilic biological macromolecules. The ECM of cartilage has a dual function, serving both as a mechanical support and as a lubricant for bones and joints. The disruption of cartilage tissue structure in OA, and AKU, is associated with alterations in the molecular composition of ECM (Martel-Pelletier et al., 2016). This degradation process is controlled by specific enzymes called aggrecanases, including A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and -5, as well as collagenases such as matrix metalloproteases (MMPs). The production of these enzymes is increased by NO (Wu et al., 2019). Additionally, a continuous increase in NO levels significantly inhibits the release of gelatinase and prostaglandins (PGs) in chondrocytes (Sun et al., 2018). Primarily, an excessive release of NO may compromise the structure of cartilage due to its regulatory influence on both proteoglycans and collagen metabolism. As reported by Jiang, H. et al. (Jiang et al., 2023), OA chondrocytes spontaneously secrete various inflammatory mediators such as IL-1β, IL-6, IL-8, TNF-α, and PGE2, whereas healthy chondrocytes do not release these mediators. It is widely recognized that iNOS plays a pivotal role in inflammatory processes. NO, a downstream signaling molecule of iNOS, acts as a potent reactive species at sites of inflammation.

Furthermore, iNOS has been associated with the activity of COX-2, as they both play a role in similar inflammatory pathways.

Several HGA-related oxidative effects have been demonstrated in a wide range of AKU models in recent years. These includes the oxidation of chondrocytic proteins, evidenced by increased carbonylation and thiol oxidation (Braconi et al., 2015, 2012, 2010b, 2010a; Tinti et al., 2010) and the production of a fluorescent melanin-like ochronotic pigment in human serum, which could be partially counteracted by the use of antioxidants (Braconi et al., 2011, 2010a). Additionally, enhanced LPO (Braconi et al., 2011; Millucci et al., 2014c, 2012; Spreafico et al., 2013a) and inflammation, as indicated by the release of pro-inflammatory cytokines (Millucci et al., 2014c; Spreafico et al., 2013b) have also been observed. Although the oxidative stress biomarkers detected in AKU models are not specific to the disease, since these modifications are common in in many other disorders (Dalle-Donne et al., 2006), their presence underscores the role of HGA as a potent pro-oxidant compound. Our study of cell inflammatory status during HGA-induced ochronosis has revealed a marked increase in the generation of ROS, as demonstrated by heightened fluorescence intensity detected in H_2DCFDA assay. This observation resonates with existing literature underscoring the pivotal role of ROS in governing chondrocyte homeostasis and extracellular matrix remodeling dynamics (Shen et al., 2021). NO can react with superoxide anions (O_2^-) to generate peroxynitrite, which is known to trigger inflammation and cell death in cartilage tissues. ROS could drive chondrocytes towards necrosis, while NO specifically induced apoptosis-like morphological alterations (Blanco et al., 1995). Both NO and ROS are implicated in causing DNA damage, as evidenced by Chen's findings (Chen et al., 2008), revealing a significant increase in oxidative DNA damage in chondrocytes affected by OA. The progression of OA predominantly depends on ROS and oxidative stress. ROS are highly reactive molecules often considered harmful byproducts of cellular metabolism. However, recent research has revealed their complex and dual role in manteining and regulating cartilage homeostasis (Henrotin et al., 2003). Physiologically, ROS act as key signaling molecules which regulate chondrocyte function, ECM formation, and cartilage regeneration (Bardaweel et al., 2018).

At low concentrations, ROS contribute to normal cellular activities such as proliferation, differentiation, and mechanical stress response, enabling cartilage to adaptively remodel itself. However, excessive ROS production, which is frequently induced by oxidative stress, might, lead to detrimental effects (Hong et al., 2024). Under oxidative stress, especially during inflammatory conditions, ROS production in cartilage can be significantly elevated. Inflammatory processes activate immune cells, including macrophages, which release pro-inflammatory cytokines such as TNF-α and IL-1β. These cytokines, in turn, stimulate chondrocytes, leading to increased mitochondrial activity and ROS generation (Kan et al., 2021). ROS in cartilage are primarly produced as byproducts of the electron transport chain and through the activities of enzymes like NADPH oxidase (De Almeida et al., 2022). Elevated levels of ROS can cause oxidative damage to critical biological molecules, including DNA, lipids, and proteins, disrupting the delicate balance of cartilage homeostasis by damaging ECM components, such as collagen and proteoglycans (Bardaweel et al., 2018). This oxidative stress is typically associated with the development of degenerative joint conditions like OA, where ROS contribute to chondrocyte death, ECM degradation, and chronic inflammation. Immunofluorescence and Western blot analysis demonstrate that 4-HNE levels increased in C20/A4 human chondrocytes treated with HGA compared to control cells. It has been established that 4-hydroxynonenal (4-HNE), a by-product of LPO, not only plays a crucial role in the degradation of cartilage in OA (Vaillancourt et al., 2008) but was also detected in both AKU cartilage and chondrocytes (Geminiani et al., 2017). These results further strengthen the evidence indicating the presence of oxidative stress in cells treated with HGA.

Due to the established connection between AKU and amyloidosis (Millucci et al., 2015a), the well-known role of HGA in the development of AKU-associated SAA amyloidosis (Braconi et al., 2017), as well as the common production of SAA during inflammatory responses (Sorić Hosman et al., 2021), we have choosen to investigate its expression in C20/A4 human chondrocytes upon stimulation with HGA. Our results revealed that there was an increase of SAA levels in both the control and treated cells. The two proteins were evenly distributed in control C20/A4 human

chondrocytes; prior studies have addressed the intrinsic production of SAA of these cells as a consequence of their tumorigenic characteristics (Lee and Beatty, 2021; Lin et al., 2019; Malle et al., 2009; Moshkovskii, 2012). Nevertheless, when the cells are exposed to HGA, SAA and SAP become mostly concentrated in certain areas, particularly around the nucleus, confirming the role of HGA in the aggregation of SAA, but also indicating a possible physical connection between SAA and SAP. Similarly, we noted a comparable pattern for SAP. These findings were further validated by Congo Red staining, which revealed the presence of amyloid fibril structures exclusively in HGA-treated cells, as indicated by green/yellow staining, while such structures were absent in control cells. Our hypothesis suggests that although SAA and SAP proteins are synthesized in control cells, their interaction, ultimately leading to amyloid formation, does not occur in the absence of HGA-induced effects. This is supported by the lack of staining observed under polarized light by Congo Red staining, indicative of the inability to facilitate aggregation and subsequent amyloid fibril formation in the absence of HGA. In conclusion, this study reveals a novel link between inflammation and AKU progression, providing new insights into the disease's underlying mechanisms. Our findings have significantly advanced our understanding of the pathophysiological processes, particularly HGA-induced ochronosis and its association with inflammatory pathways, using a novel cellular model. This model offers a more efficient and valuable approach for examining disease-related events and developing targeted therapeutic options. Future research should build on these findings to further investigate AKU progression and explore targeted anti-inflammatory therapies that could alleviate or prevent ochronosis-related pain in AKU patients.

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RESEARCH ARTICLE

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An in vitro cell model for exploring inflammatory and amyloidogenic events in alkaptonuria

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Abstract

Alkaptonuria (AKU) is a progressive systemic inherited metabolic disorder primarily affecting the osteoarticular system, characterized by the degeneration of cartilage induced by ochronosis, ultimately leading to early osteoarthritis (OA). However, investigating AKU pathology in human chondrocytes, which is crucial for understanding the disease, encounters challenges due to limited availability and donor variability. To overcome this obstacle, an in vitro model has been established using homogentisic acid (HGA) to simulate AKU conditions. This model employed immortalized C20/A4 human chondrocytes and serves as a dependable platform for studying AKU pathogenesis. Significantly, the model demonstrates the accumulation of ochronotic pigment in HGA-treated cells, consistent with findings from previous studies. Furthermore, investigations into inflammatory processes during HGA exposure revealed notable oxidative stress, as indicated by elevated levels of reactive oxygen species and lipid peroxidation. Additionally, the model demonstrated HGA-induced inflammatory responses, evidenced by increased production of nitric oxide, overexpression of inducible nitric oxide synthase, and cyclooxygenase-2. These findings underscore the model's utility in studying inflammation associated with AKU. Moreover, analysis of serum amyloid A and serum amyloid P proteins revealed a potential interaction, corroborating evidence of amyloid fibril formation. This hypothesis was further supported by Congo red staining, which showed fibril formation exclusively in HGA-treated cells. Overall, the C20/A4 cell model provided valuable insights into AKU pathogenesis, emphasizing its potential for facilitating drug development and therapeutic interventions.

KEYWORDS

alkaptonuria, homogentisic acid, inflammation, metabolic disease, ochronotic pigment, oxidative stress

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HGA triggers SAA aggregation and accelerates fibril formation in the C20/A4 alkaptonuria cell model

Abstract

Alkaptonuria (AKU) is a rare autosomal recessive metabolic disorder caused by mutations in the homogentisate 1,2-dioxygenase (HGD) gene, leading to the accumulation of homogentisic acid (HGA), causing severe chronic inflammatory conditions. Recently, the presence of Serum Amyloid A (SAA) has been reported in AKU tissues, classifying AKU as novel secondary amyloidosis, AA amyloidosis characterized by the extracellular tissue deposition of fibrils composed of fragments of SAA. AA amyloidosis may complicate several chronic inflammatory conditions, like rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, chronic infections, neo-plasms, etc. Treatments of AA amyloidosis relieve the inflammatory disorders by reducing SAA concentrations; however, no definitive therapy is currently available. The SAA regulation is a crucial step to improve AA secondary amyloidosis treatments. Here, applying a comprehensive in vitro approach, was provided evidence that HGA is a disruptor modulator of SAA, able to enhance its polymerization, fibril formation, and aggregation upon SAA/SAP colocalization. The results could represent an important starting point for identifying novel therapeutic strategies in AKU and AA secondary amyloidosis-related diseases.

Introduction

Alkaptonuria (AKU) is a rare genetic disorder that results from a defect in the metabolism of the amino acids phenylalanine and tyrosine (Bernardini et al., 2024). Specifically, AKU is caused by mutations in the homogentisate 1,2-dioxygenase (HGD) gene, leading to a deficiency of the HGD enzyme (Milella et al., 2024). This enzyme deficiency prevents the normal breakdown of homogentisic acid (HGA), causing it to accumulate in the body (Bernardini et al., 2024). The excess HGA is excreted in the urine, which darkens upon exposure to air, a distinctive symptom of the disease (Bernardini et al., 2024).

Beyond the urinary symptoms, the chronic buildup of HGA in connective tissues leads to ochronosis, characterized by a bluish-black pigmentation of cartilage and other tissues (Mistry et al., 2013). This can cause a range of complications, including early-onset osteoarthritis, particularly in the spine and large joints, and potentially heart and kidney issues (Milella et al., 2024). Alkaptonuria is a lifelong condition that typically manifests in early childhood but becomes more problematic with age as the cumulative effects of HGA deposition take their toll (Bernini et al., 2021).

A further complication of AKU is caused by presence of unidentified proteins which are involved in both intra- and extra-cellular ochronotic pigment deposition.

Previous studies showed that the AKU disease is also characterized by the presence of serum amyloid A (SAA), classifying AKU as a secondary (AA) amyloidosis (Millucci et al., 2015a).

Serum amyloid A is a group of apolipoproteins primarily produced by the liver which binding with high-density lipoprotein (HDL) play several critical roles in the immune response. These include facilitating the transport of cholesterol, promoting chemotaxis (the movement of immune cells towards sites of inflammation), and modulating the inflammatory response. These proteins are classified as acute-phase reactants due to their significant upregulation in response to inflammation. During inflammatory events, such as infections, trauma, or acute inflammatory diseases, the

concentration of SAA in the bloodstream can increase dramatically, often by up to 1000-fold (Webb, 2021).

Persistently elevated levels of SAA due to ongoing inflammation led to the misfolding and aggregation of this protein into insoluble amyloid fibrils. These fibrils deposit in various organs and tissues, including the kidneys, liver, spleen, and gastrointestinal tract, impairing their normal function leading to a pathological condition known as AA secondary amyloidosis (Mirioglu et al., 2024).

Secondary (AA) amyloidosis, differently from the primary amyloidosis (which is associated with light chain proteins), is a systemic disorder characterized by the extracellular deposition of amyloid fibrils derived from SAA protein. This condition is a complication of chronic inflammatory diseases, chronic infections, or certain malignancies (Scarpioni, 2016).

The symptoms of secondary amyloidosis are diverse and depend on the organs involved. Common signs include fatigue, weight loss, and swelling due to kidney dysfunction. Gastrointestinal involvement can lead to malabsorption and bleeding, while liver and spleen involvement may cause hepatomegaly and splenomegaly, respectively (Real de Asua et al., 2014).

Currently, the development of the secondary (AA) amyloidosis by persistently high plasma concentrations of SAA is still not clear, however, there is strong evidence that the onset depends on a seeding mechanism exerted by misfolded and aggregated AA protein (Haines et al., 2022; Simons et al., 2013). The full-length SAA1 protein is made of 122 residues where the first 18 residues represent the signal peptide, which following cleavage forms the mature SAA1 (104 residues) (Sun and Ye, 2016). In the onset of AA amyloidosis, the SAA1 is frequently cleaved producing different forms of SAA1 fragments (Sack, 2018).

The SAA1 N-terminal region plays a critical role in the formation of amyloidogenic SAA1, as following a misfolding process, it starts the SAA1 polymerization and the fibril formation. The deletion of a single N-terminal amino acid (Arg-1) showed to trig the formation of SAA1 fibrils and amyloid deposits (Tanaka et al., 2023). Also, the deletion of the C-terminal tail destabilized the SAA1 peptide, triggering the fibril formation (Tanaka et al., 2018).

Several proteolytic enzymes have been implicated both in the formation and degradation of AA amyloid, like the lysosomal proteases cathepsin B produces a plethora of fragments, also the metalloproteinases (MMP1, MMP2, and MMP3) are able to degrade the SAA1 into peptides corresponding to AA protein (Stix et al., 2001).

Despite the numerous SAA1 physiologically/structural information and its involvement in the fibril formation, several issues remain to be addressed.

In this study, using the HGA-supplemented C20/A4 cellular model (human chondrocytes), we analyzed the amyloidogenic mechanisms in AKU. In vitro evidence showed that the SAA/HGA relationship caused the fibril aggregation and formation. In addition, our results suggested an increase in the colocalization between the SAA1 and Serum Amyloid P (SAP) proteins in cells treated with HGA, indicating their involvement in amyloid fibril formation.

Therefore, HGA is proposed as a small molecule disruptor of SAA1 enhancing in vitro amyloid aggregation, showing a key role in AKU amyloidosis.

Understanding SAA's regulations is essential for comprehending its role both in normal immune processes and pathological conditions. Research into SAA1 continues to provide insights regarding its potential role as biomarker for inflammatory diseases and its involvement in disorder progression, like in AKU, offering prospects for a wide range of targeted therapeutic strategies and precision medicine approaches.

Materials and Methods

Cells cultures and treatment

The C20/A4 human chondrocyte cell line (Sigma-Aldrich, SCC041) was grown in Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS) at 37°C in a humidified environment with 5% CO_2. HGA (Sigma Aldrich) was prepared by dissolving it in deionized water to produce a 10 mM stock solution. The initial solution was then diluted in the cell culture medium to achieve concentrations of 0.066 mM and 0.1 mM.

Immunofluorescence study

C20/A4 human chondrocytes were cultured on glass coverslips with a concentration of 2×10^4 cells for one week and 1×10^4 cells for two weeks. Following the treatments with 0.066 and 0.1mM HGA, the cells were fixed with methanol, before being permeabilized using pure acetone and then blocked. Next, the cells were incubated overnight at 4°C with anti-SAA mouse monoclonal antibody (1:100, Sigma-Aldrich), and anti-SAP rabbit monoclonal antibody (1:30, Sigma-Aldrich). Afterwards, the cells were incubated with Alexa Fluor™ 546 goat anti-mouse Ig (1:100, Thermo Fisher Scientific) and Alexa Fluor™ 488 goat anti-rabbit Ig (1:100, Thermo Fisher Scientific). Ultimately, the samples were mounted using a fluoroshield mounting medium containing DAPI (Abcam, Cambridge, UK). Images were captured with a fluorescence microscope (Zeiss AxioLabA1, Oberkochen, Germany), and fluorescence intensity was quantified using ImageJ software. A quantitative colocalization and correlation study of SAP and SAA was conducted using ImageJ and the JACoP plug-in. The Manders' coefficient, which indicates the amount of SAP-overlapping SAA pixels, was calculated. The Colocalization plug-in in ImageJ was used to visualize colocalization occurrences as white dots.

In situ proximity ligation assay (PLA)

To assess the colocalization between SAA and SAP, the in-situ proximity ligation assay (Duolink kit, Sigma-Aldrich) was conducted following the instruction provided by the manufacturer. In brief,

C20/A4 human chondrocytes, were placed in a sterile 24 well plate at $1x10^4$ cells/well. The cells were allowed to grow until they reached subconfluence. Then, they were treated with 0.066mM and 0.1 mM HGA. Following treatment, the cells were fixed, blocked, and incubated with primary antibodies that specifically target the proteins of interest, following the previously described protocol. Next, the cells were incubated with secondary antibodies conjugated with oligonucleotides (mouse PLUS and rabbit MINUS probes). After the oligonucleotides were hybridized and ligated, DNA amplification was carried out by adding an amplification solution along with polymerase. The products of the PLA detection reaction were visualized as red fluorescence dots. Finally, the cells were mounted using Duolink In Situ Mounting with DAPI (Sigma-Aldrich) and examined under a fluorescence microscope (Zeiss AXIO LAB AI).

Detection of amyloid deposits

To investigate the distribution of amyloidosis, C20/A4 human chondrocytes were placed into 6-well plates at a concentration of 2×10^5 cells per well and cultured until they reached 80-85% confluence, before being treated with HGA at varying concentrations (0.066 mM, 0.1 mM) for 14 days. Cells were then stained with 1% Congo Red and dehydrated using ethanol, before being examined under a bright field microscope. A Zeiss Axio Lab A1 microscope was then used to examine the samples under polarized light.

Western Blotting

C20/A4 human chondrocytes at subconfluence were treated with HGA at different concentrations (0.066 mM and 0.1 mM) for two weeks. Following treatment, cells were lysed with RIPA buffer supplemented with phosphate and protease inhibitors and then disrupted by sonication for 5 minutes. Protein concentration was evaluated according to Bradford. 20 ug of cell protein lysate were resolved by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred onto a nitrocellulose membrane (0.45mm pore size; Whatman). After blocking for

2h with 5% not-fat dry milk in Phosphate Buffered Saline buffer (PBS) at room temperature, membranes were incubated with anti-SAA rabbit polyclonal antibody (1:1500, Invitrogen), and anti-actin mouse monoclonal antibody (1:4000, Sigma-Aldrich) at 4°C overnight. The membranes were then incubated with anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (1:80.000, Sigma-Aldrich). Immunoreactive bands were revealed with Luminata Crescendo (Merck Millipore) and images were acquired using Image-Quant LAS4000 (GE Healthcare, Milano, Italy). The optical densities of the immunoreactive bands were analyzed using ImageQuantTL software V 7.0 (GE Healthcare, Milano, Italy). Protein levels were normalized against Actin, which served as a protein loading control.

Statistical Analysis

The results are provided by three independent experiments. Statistical analyses were carried out with GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The data are expressed as mean ± standard deviation (SD) and were compared using one-way analysis of variance (ANOVA) followed by a post hoc test. A p-value of 0.05 or less was considered statistically significant.

Results

HGA treatment potentially induced SAA and SAP colocalization

To investigate the cellular localization of the proteins SAA and SAP, immunofluorescence staining was conducted on C20/A4 human chondrocytes treated with HGA at various concentrations (0.066 mM and 0.1 mM) for one and two weeks. The concentrations used were selected to mimic the HGA serum levels reported in AKU patients (Ranganath et al., 2016) and are consistent with those used in previous AKU cellular models (Galderisi et al., 2022, 2021; Gambassi et al., 2017; Geminiani et al., 2017; Tinti et al., 2011).

The red (SAA) and green (SAP) staining was evident in both control and HGA-treated C20/A4 cells, starting from the first week of treatment. In untreated C20/A4 cells, the two proteins appeared to be distributed homogeneously. However, in cells treated with HGA at a concentration of 0.066 mM, and more notably at 0.1 mM, both SAA and SAP seemed to be concentrated primarily in the perinuclear zone of the cells (Figure 1). Moreover, after 1 week of treatment with 0.1 mM HGA, and more notably after 2 weeks of treatment with 0.066 mM and 0.1 mM HGA, C20/A4 cells lost their regular shape, becoming enlarged and multinuclear. Additionally, the cell nuclei became irregular and began to lose their well-defined boundaries. Previous studies have shown that HGA induces oxidative stress, which over time reduces the protective autophagic processes in cartilage, ultimately leading to chondroptosis, a specific form of apoptosis, in AKU chondrocytes, resulting in morphological changes (Millucci et al., 2015b). In a prior study, DAPI staining revealed apoptotic nuclei in C20/A4 cells after HGA treatment. Similarly, in this study, nuclear shape alterations were most evident after one and two weeks of HGA treatment. These findings confirmed that HGA acts as an inducer of apoptosis (Galderisi et al., 2022).

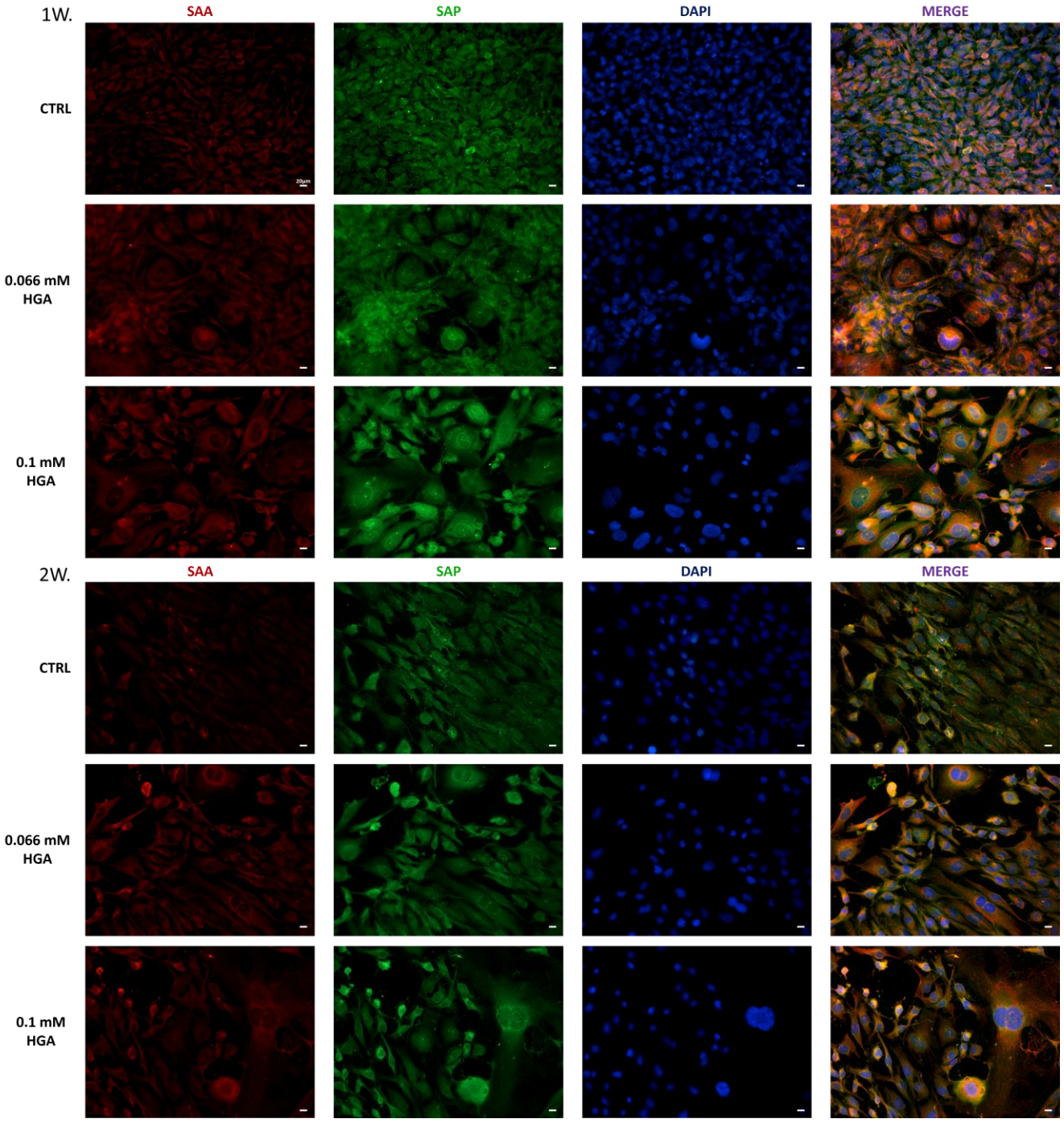

Figure 14. SAA (red) and SAP markers (green) are both double labeled. SAA and SAP staining were visible in both control and 0.066, 0.1 mM HGA-treated cells. After one (1W) and two (2W) weeks of HGA treatment, C20/A4 cells began to lose their organization and exhibit abnormalities in nuclear shapes and morphology. Scale bar = 20 µm

Subsequently, a scatterplot analysis was conducted (Figure 2) to determine the correlation between the red (SAA, x-axis) and green (SAP, y-axis) channels of the immunofluorescence data. A scatterplot is a pixel distribution diagram used to analyze the spatial and functional interactions among several

molecular markers within cells. Colocalization is demonstrated by the presence of data points that cluster together in a diagonal line, indicating that the two markers are present in the same areas (Bolte and Cordelières, 2006). The analysis revealed that the correlation between the two channels, which shows the level of overlap and hence colocalization between the two proteins, became more linear with increasing HGA concentration and extended treatment duration. This suggested a possible interaction between the two proteins due to the HGA treatment.

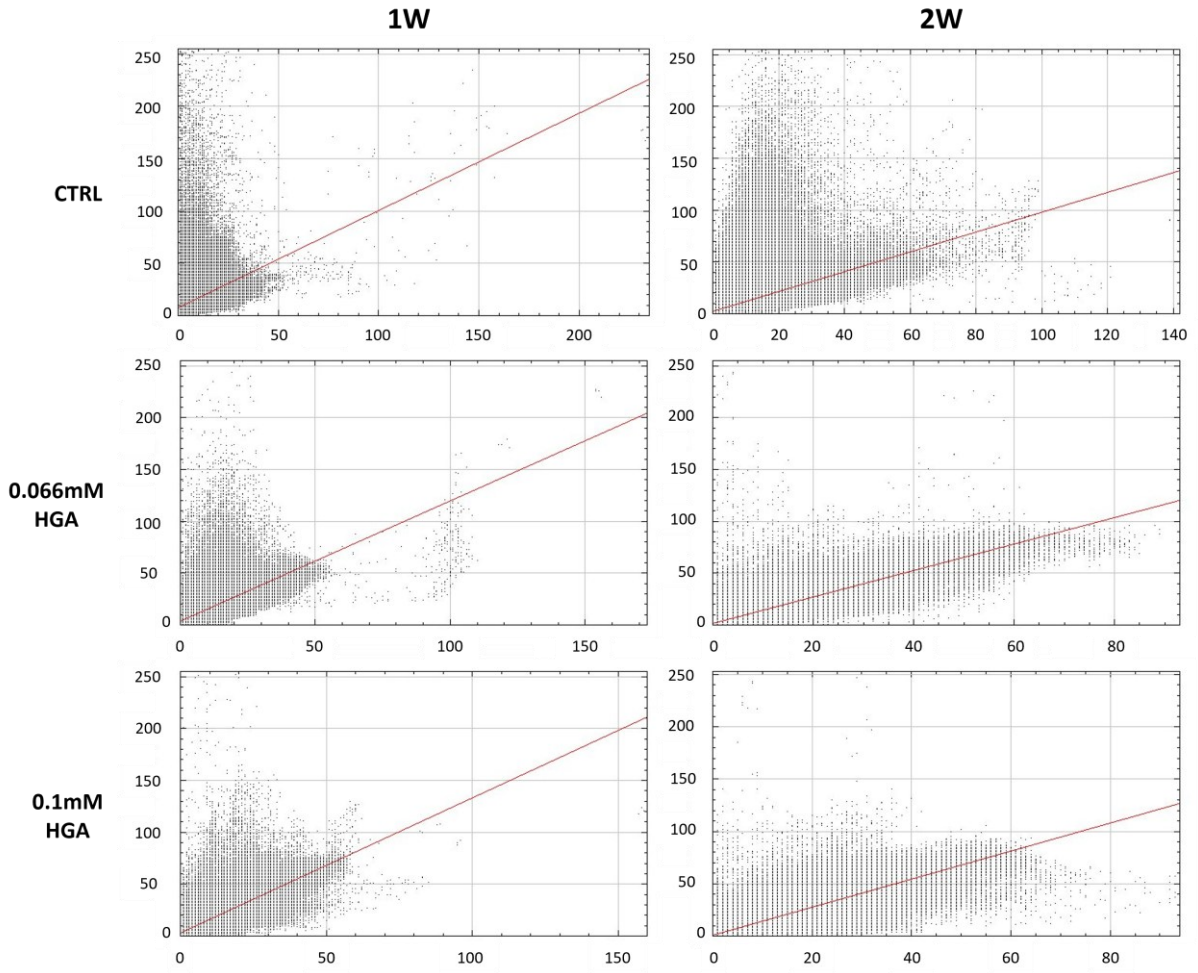

Figure 15. Scatterplot of red (SAA) and green (SAP) pixel intensities of C20/A4 cells following incubation for one (1W) and two (2W) weeks with HGA 0.066 and 0.1mM. As previously stated, the plots of the two proteins indicated a rise in correlation between them, as seen by an increase in linearity that increases with treatment exposure duration and HGA concentration.

White dot colocalization images of SAA and SAP revealed a significant overlap of the two signals (Figure 3). The colocalization was particularly noticeable after two weeks of treatment with HGA, confirming the previously hypothesized interaction between the two proteins. In contrast, untreated

cells showed very low overlap of the two signals, despite the presence of both SAA and SAP, as demonstrated by immunofluorescence analysis.

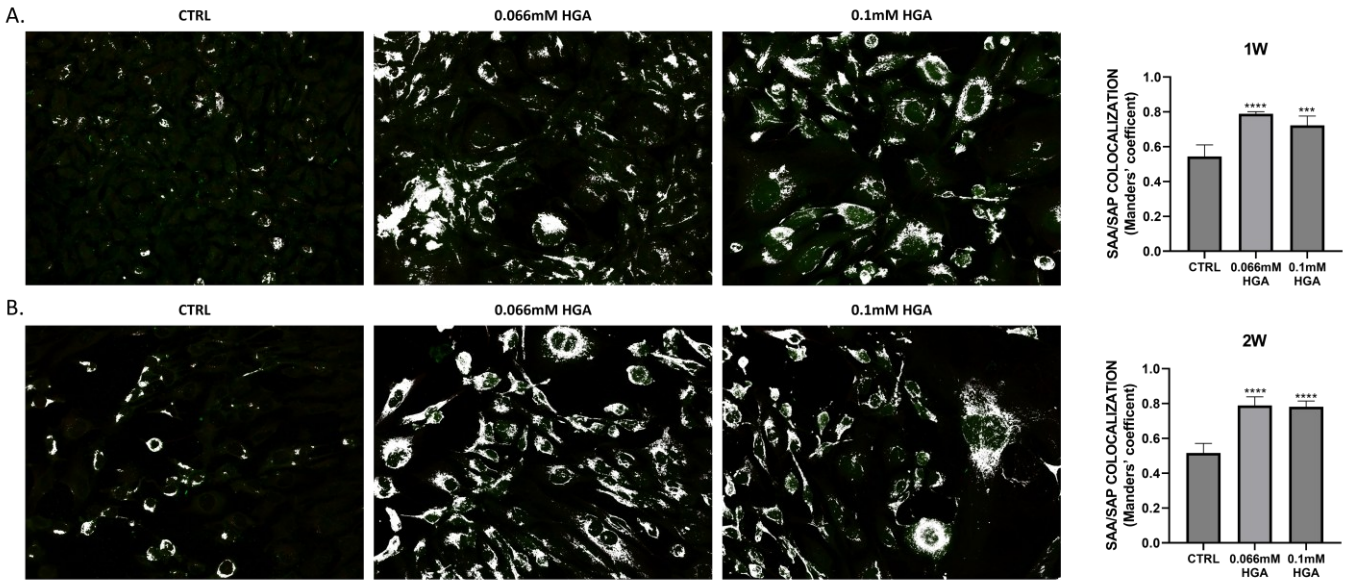

Figure 16. Immunofluorescence analysis was conducted on C20/A4 cells to examine white dot co-localization, employing anti-SAA and anti-SAP antibodies, at (A) 1 and (B) 2 weeks of treatment. Quantitative assessment of SAP colocalization with SAA is depicted in a bar graph utilizing Manders' coefficient (mean ± standard deviation). Statistically significant differences are shown by ***p=0.0002, ****p < 0.0001. Data are displayed as mean ± SD. P-values were calculated by ordinary one-way ANOVA with Dunnett's post hoc test.

Finally, to demonstrate the close proximity of SAA and SAP, the PLA method was performed on untreated and HGA-treated C20/A4 human chondrocytes (Figure 4). The in-situ PLA enables the detection of protein-protein interactions within cells and tissues (Geminiani et al., 2017). This assay reveals colocalization between two proteins separated by less than 30 nm. The strong reaction products, visible as red fluorescent spots, confirmed the colocalization of SAA and SAP. Minimal colocalization was evident throughout the cells in the control group, as indicated by white dot-colocalization. In cells treated with 0.066mM HGA, colocalization was more noticeable, especially around the nuclei. In cells treated with 0.1mM HGA, the red fluorescent spots were predominantly concentrated around the nuclei.

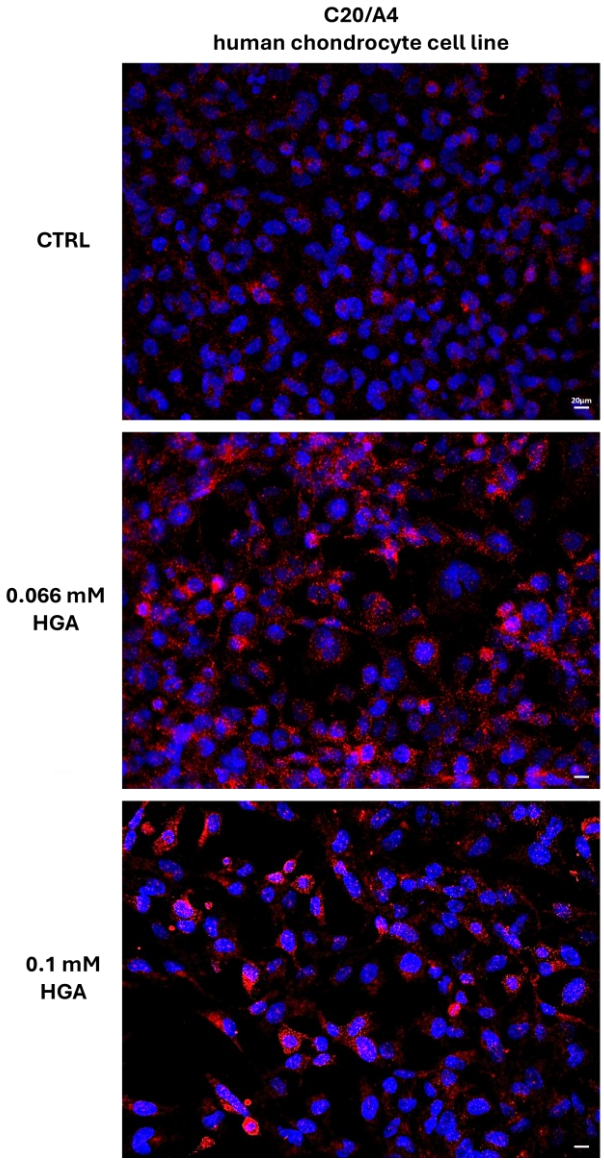

Figure 17. Duolink PLA assay shows colocalization of SAA and SAP. Nuclei are stained with DAPI. Scale bar = 20 µm

HGA treatment promoted amyloidogenic protein aggregation.

The presence of SAA and SAP in AKU has been determined, allowing the condition to be classified as a secondary (AA) amyloidosis (Millucci et al., 2012). SAP is known to bind and stabilize formed amyloid fibrils (Tennent et al., 1995; Westermark et al., 2005). Immunofluorescence and image analysis suggested not only a change in the cellular spatial arrangement of SAA and SAP, but also a potential interaction between the two proteins which became more evident with increasing HGA concentration and time exposure.

Congo Red staining under polarized light showed green-yellow birefringence in C20/A4 cells treated with 0.066 mM HGA, and more noticeably at 0.1 mM HGA after two weeks of treatment, while untreated cells did not display birefringence (Figure 5). This observation might indicate that in HGA-treated C20/A4 cells SAA and SAP interact, and initiate the formation of amyloid fibrils, whereas in untreated cells the interaction between the two proteins is minimal.

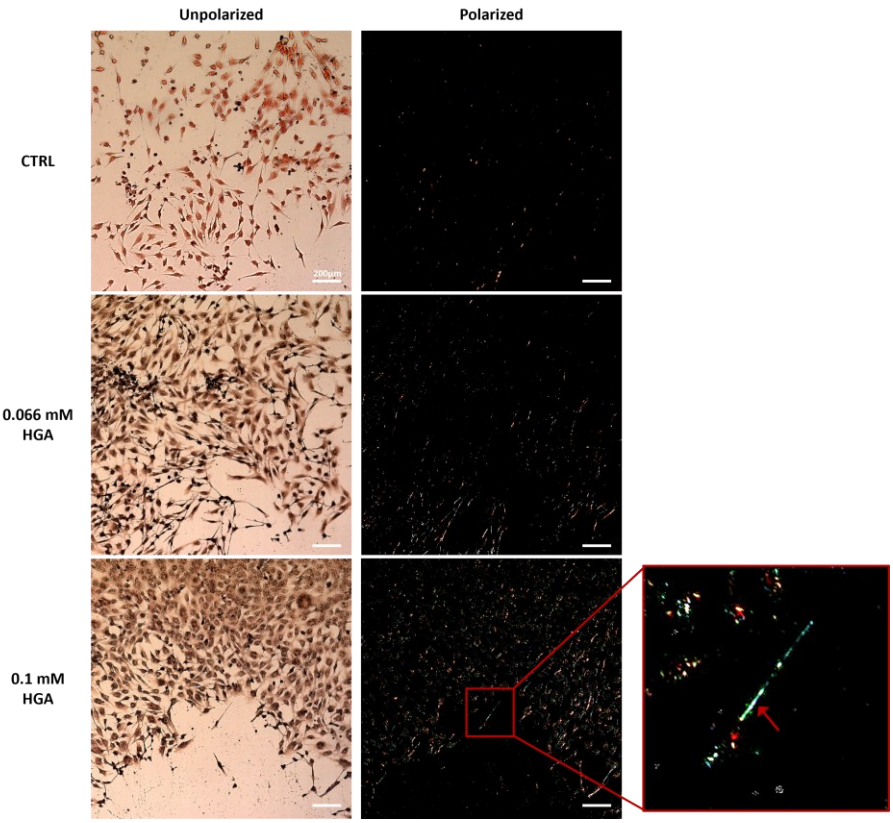

Figure 18. Congo Red staining of untreated (CTRL) and HGA-treated C20/A4 cells (0.066mM and 0.1mM). In the zoomed-in area, green-yellow staining (arrow) highlights amyloid buildup following HGA treatment. Scale bar = 200 µm

HGA treatment triggered SAA aggregation

Secondary amyloidosis is characterized by the production of amyloid fibrils resulting from SAA monomer binding. It is also known that, in AKU, HGA is a potential aggregation enhancer that promotes intermolecular associations (Braconi et al., 2017).

To evaluate the aggregation of SAA in our cellular model, we treated C20/A4 cells with HGA at various concentrations (0.066mM and 0.1mM) for two weeks. The expression levels of SAA were

determined by Western blot, considering the monomer (12.5 kDa), tetramer (50 kDa), and high molecular weight (HMW) aggregates (150 kDa). Analysis of the immunoreactive bands revealed that the SAA monomer was present in both untreated and HGA-treated C20/A4 cells, confirming the results observed with immunofluorescence. Cells treated with 0.1mM HGA showed significantly higher levels of SAA than the control, indicating that HGA might act as a promoter in the synthesis of SAA (Figure 6B). Moreover, treatment with 0.1mM HGA increased the expression of the SAA tetramer by 2-fold compared to the control (Figure 6C). On the contrary, the expression of HMW aggregates was significantly higher in cells treated with 0.066mM HGA (4-fold increase).

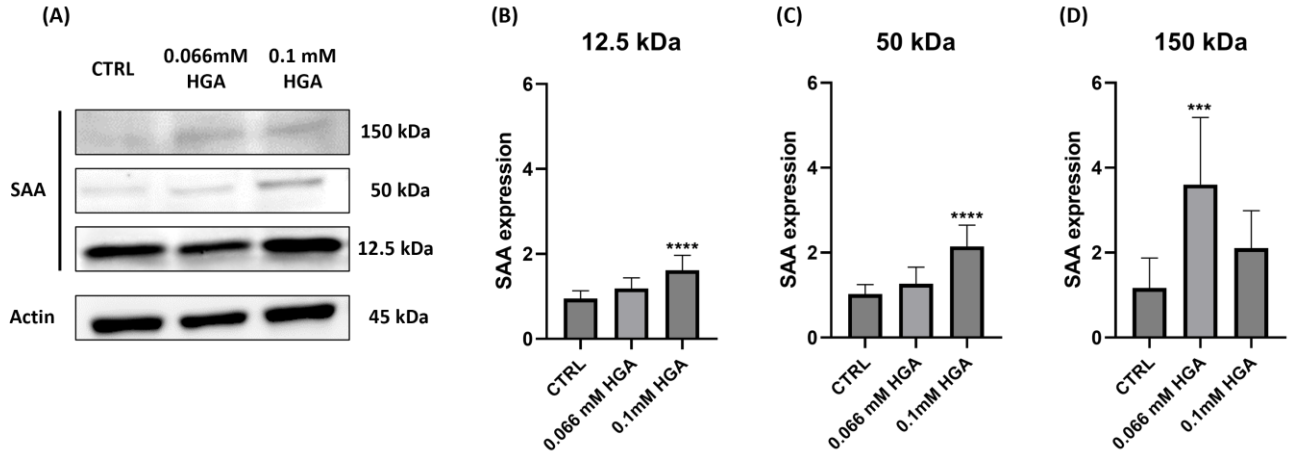

Figure 19. (A) SAA expression levels in C20/A4 cells untreated (CTRL) and treated with 0.066 mM and 0.1 mM HGA for two weeks. (B) SAA expression at 12.5 kDa, (C) SAA expression at 50 kDa, (D) SAA expression at 150 kDa. Data are presented as fold change. Statistically significant differences are indicated by ***p = 0.0002 and ****p < 0.0001. All experiments were performed in triplicate. P-values were calculated using ordinary one-way ANOVA with Dunnett's post hoc test.

Discussion

AKU is a rare autosomal recessive degenerative disease that significantly affects several organ systems, particularly the osteoarticular system, with cartilage being notably more susceptible to damage compared to bone (Fisher and Davis, 2004). Ochronosis, an essential feature of AKU, includes a cascade of effects involving weakening of cartilage, increased stiffness, increased susceptibility to fractures, chronic inflammation, and ultimately the emergence of osteoarthritis (Gallagher et al., 2015). Chondrocytes, cartilage's main cells, play an important role in coordinating the synthesis of extracellular matrix components such as collagen and proteoglycan. These components are critical for cartilage's structural integrity and biomechanical resiliency, which is required to tolerate mechanical stress. Nonetheless, per-forming research on human chondrocytes has various challenges, most notably the difficulty in collecting cells from AKU patients' biopsies. The issues are complicated by variability among donors, which can be related to factors like age and preexisting medical disorders. Scarcity of AKU cases, procuring biological specimens, and other factors make highly challenging in controlled laboratory settings for AKU research, limiting our capacity to compare and apply research findings to a larger population. AKU-related events have been investigated by evaluating cells directly collected from AKU patients' biopsies or by utilizing cellular models that recreate the disease scenario. The adoption of an immortalized cell line of human chondrocytes as a cellular model for AKU has created new perspectives for exploring this rare condition (Braconi et al., 2010b; Millucci et al., 2014c)reducing the necessity for biopsy-derived samples. In recent years, the AKU/amyloidosis relationship has been proposed (Millucci et al., 2015a), as well as the evidence of the presence of SAA and SAP in AKU, which allowed classifying AKU as a secondary (AA) amyloidosis. Amyloidosis is a pathological progression caused by the conversion of normally soluble proteins into insoluble protein fibrillar aggregates, named amyloid, that deposit either systemically or in a specific organ and may be associated with several disorders.

The purpose of this study was to further investigate the AKU-related amyloidosis mechanisms and the development of SAA aggregates and their impact on the formation of amyloid fibrils. Furthermore, the role of HGA in amyloidogenic processes (Braconi et al., 2012; Lee and Beatty, 2021) was also investigated. Here, is suggested new evidence of the HGA amyloidogenic action, confirming the metabolite's preclinical significance in the development of AKU-associated SAA amyloidosis. In addition to modifications caused by HGA, such as changes in cell organization, anomalies in nuclear borders, and cell morphology (Geminiani et al., 2017; Millucci et al., 2014c), our findings from immunofluorescence demonstrated that SAA and SAP were produced in control and treated cells. The two proteins were uniformly distributed in human C20/A4 control chondrocytes; previous studies focused on the intrinsic generation of SAA by these cells caused by their cancerous features (Lin et al., 2019; Malle et al., 2009; Moshkovskii, 2012; Wetzel et al., 2007). Nevertheless, when the cells were exposed to HGA, SAA and SAP become mostly concentrated in certain areas, particularly around the nucleus, as it has already been observed in AKU chondrocytes (Geminiani et al., 2017) confirming the role of HGA in the aggregation of SAA, but also indicating a possible physical connection between SAA and SAP. Similarly, we noted a comparable pattern for SAP. The relationship between the two proteins was further strengthened by the correlation analysis, where the scatterplot (JaCoP plugin, ImageJ software) revealed an increase in linearity between the red and green channels (respectively, SAA and SAP). This observation was substantiated by analysis using ImageJ software (Colocalization plugin), where white dot colocalization indicated a heightened overlap between the two channels, and the Manders' coefficient for SAP overlapping with SAA approached in HGA-treated cells compared to untreated ones. These findings suggest a potential interaction between the two proteins, an interaction seemingly induced by HGA treatment, as evidenced by the persistence of lower white dot colocalization and Manders' coefficient values in untreated cells at both 1 and 2-week intervals. Duolink PLA analysis confirmed the results observed with immunofluorescence and ImageJ analysis. In untreated human C20/A4 chondrocytes, minimal and homogeneously distributed colocalization was observed, indicated by red fluorescent spots

representing a distance of less than 30 nm between the two proteins. Notably, cells treated with 0.066 mM and especially 0.1 mM HGA showed an increase in colocalization, particularly in the area surrounding the nucleus. This suggests a potential role of HGA as an enhancer of SAA and SAP interaction and amyloid fibril formation. These results indicate that HGA may have an essential role in AKU amyloidosis by binding to amyloid proteins and facilitating the onset of amyloid fibrils.

The results were supported by Congo Red staining, which confirmed the presence of amyloid fibril structures only in HGA-treated cells, as evidenced by green/yellow staining, whereas similar structures were absent in controls. Our theory states that, while SAA and SAP proteins are generated in control cells, their interaction, which eventually leads to amyloid formation, does not seem to occur in the absence of HGA-induced effects. This is substantiated by the lack of Congo Red staining under polarized light, indicating the inability to assist aggregation and subsequent amyloid fibril production in the absence of HGA. Our outcomes are consistent with previous studies on the same proteins subjected to various aggregating events (Wetzel et al., 2007). WB analysis after 14 days of HGA treatment revealed minimal aggregation under control conditions. In contrast, cells treated with 0.066 mM and 0.1 mM HGA showed increased levels of SAA monomer, oligomers and HMW, thus leading to the potential generation of amyloid fibrils. The results were consistent with previous in vitro observations (Braconi et al., 2017) which revealed that HGA accelerated the rate of amyloid fibril formation in a time- and dose-dependent manner and that fibrillization of amyloidogenic proteins and peptides incubated with HGA was eventually faster than that of their untreated counterparts.

Conclusions

In the present study, applying comprehensive in vitro approaches were provided new details about the potential role of HGA as an aggregation enhancer for the amyloidogenic process. Findings suggest that HGA contributed to the effectiveness of aggregation events that characterize secondary amyloidosis in AKU, as well as a possible explanation for the clinically confirmed beginning of amyloidosis in AKU patients. The investigation has revealed the links between HGA-induced ochronosis and amyloidogenic processes associated with AKU, offering important insights into the pathophysiological mechanisms underlying AKU progression. The use of various analytical approaches has increased our understanding of AKU pathophysiology, emphasizing the important role of cellular models in comprehending the complexities of rare genetic diseases.

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HGA Triggers SAA Aggregation and Accelerates Fibril Formation in the C20/A4 Alkaptonuria Cell Model

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Abstract: Alkaptonuria (AKU) is a rare autosomal recessive metabolic disorder caused by mutations in the homogentisate 1,2-dioxygenase (HGD) gene, leading to the accumulation of homogentisic acid (HGA), causing severe inflammatory conditions. Recently, the presence of serum amyloid A (SAA) has been reported in AKU tissues, classifying AKU as novel secondary amyloidosis; AA amyloidosis is characterized by the extracellular tissue deposition of fibrils composed of fragments of SAA. AA amyloidosis may complicate several chronic inflammatory conditions, like rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, chronic infections, neoplasms, etc. Treatments of AA amyloidosis relieve inflammatory disorders by reducing SAA concentrations; however, no definitive therapy is currently available. SAA regulation is a crucial step to improve AA secondary amyloidosis treatments. Here, applying a comprehensive in vitro and in silico approach, we provided evidence that HGA is a disruptor modulator of SAA, able to enhance its polymerization, fibril formation, and aggregation upon SAA/SAP colocalization. In silico studies deeply dissected the SAA misfolding molecular pathway and SAA/HGA binding, suggesting novel molecular insights about it. Our results could represent an important starting point for identifying novel therapeutic strategies in AKU and AA secondary amyloidosis-related diseases.

Keywords: alkaptonuria; secondary amyloidosis; amyloid; metabolic disease; serum amyloid A; serum amyloid P; HGA; molecular modeling; docking and molecular dynamics simulation

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1. Introduction

Alkaptonuria (AKU) is a rare genetic disorder that results from a defect in the metabolism of the amino acids phenylalanine and tyrosine [1]. Specifically, AKU is caused by mutations in the homogentisate 1,2-dioxygenase (HGD) gene, leading to a deficiency of the HGD enzyme [2]. This enzyme deficiency prevents the normal breakdown of homogentisic acid (HGA), causing it to accumulate in the body [1]. The excess HGA is excreted in the urine, which darkens upon exposure to air, a distinctive symptom of the disease [3].

Beyond the urinary symptoms, the chronic buildup of HGA in connective tissues leads to ochronosis, characterized by a bluish-black pigmentation of cartilage and other tissues [4]. This can cause a range of complications, including early-onset osteoarthritis, particularly in the spine and large joints, and potentially heart and kidney issues [2]. Alkaptonuria is a lifelong condition that typically manifests in early childhood but becomes more problematic with age as the cumulative effects of HGA deposition take their toll [5].

A further complication of AKU is caused by the presence of unidentified proteins which are involved in both intra and extracellular ochronotic pigment deposition [6].

Homogentisic acid induces autophagy alteration leading to disfunction in cartilage cells homeostasis

Abstract

Alkaptonuria (AKU) is a rare metabolic disorder caused by homogentisate 1,2-dioxygenase deficiency, leading to systemic accumulation of homogentisic acid (HGA) and progressive tissue degeneration characterized by dark urine, ochronosis, and severe osteoarthropathy. Chronic exposure to HGA promotes oxidative stress, chondroptosis, secondary amyloidosis, and impaired autophagy, an essential process for maintaining chondrocyte homeostasis. This study investigated the mechanisms underlying autophagy dysregulation in AKU using the human C20/A4 chondrocyte line treated with 0.1 mM HGA, an established in vitro model of the disease. HGA treatment induced a time-dependent increase in oxidative stress, evidenced by elevated ROS levels and 4-HNE accumulation. Organelle analyses revealed an early adaptive response, characterized by transient reduction in ER activity and enhanced mitochondrial fusion, followed, after two weeks, by ER expansion, maladaptive unfolded protein response activation, mitochondrial dysfunction, and increased mitochondrial ROS production. These findings indicate a shift from compensatory organelle remodeling to irreversible oxidative damage. Autophagy assessment demonstrated initial activation, with increased LC3 and p62 expression and enhanced lysosomal biogenesis (LAMP1). However, prolonged HGA exposure resulted in impaired autophagosome–lysosome fusion, reduced lysosomal activity, and accumulation of inactive autophagosomes, confirming disruption of autophagic flux. Live-cell imaging corroborated a transition from functional autophagy to lysosomal failure under chronic oxidative stress. Overall, this study demonstrates that prolonged HGA exposure disrupts the interplay between oxidative stress, mitochondrial dynamics, ER homeostasis, and autophagic flux. The progressive collapse of these adaptive mechanisms contributes to chondrocyte degeneration and underlies the pathogenesis of cartilage damage in AKU.

Introduction

Alkaptonuria (AKU) is an ultra-rare autosomal recessive metabolic disorder caused by mutations in the homogentisate 1,2-dioxygenase (HGD) gene (Nemethova et al., 2016). These mutations result in the loss of HGD enzymatic function (Bernini et al., 2017), preventing the oxidation of homogentisic acid (HGA) into maleylacetoacetate within the degradative pathway of the amino acids tyrosine and phenylalanine. As a consequence, HGA accumulates systemically and deposits within connective tissues, giving rise to the characteristic dark pigmentation associated with the disease. This pigmentation, called "ochronosis," primarily involves the osteoarticular system, including cartilage, bone, synovia, and tendons; this condition leads to tissue degeneration, chronic inflammation, and ochronotic arthropathy (Millucci et al., 2017). Alkaptonuric cells such as chondrocytes also accumulate intracellular ochronotic pigment (Laschi et al., 2012), sequestered within membrane-bound vesicles in the cytoplasm (Geminiani et al., 2017). Comprehensive analyses have demonstrated that AKU is a multisystemic disorder (Bernardini et al., 2015), with ochronotic deposits detected across multiple tissues and implicated in widespread organ dysfunction (Bernardini et al., 2015; Millucci et al., 2017). Ochronosis is strongly linked to the generation of free radical species, which contribute to oxidative tissue damage and structural degeneration (Braconi et al., 2015, 2011). Cartilage is among the most severely affected tissues in AKU (Braconi et al., 2010b), as AKU chondrocytes display structural and functional abnormalities, including aberrant expression of cytoskeletal proteins (Millucci et al., 2014a), dysregulation driven by HGA-induced oxidative stress (Braconi et al., 2016, 2015, 2010b), reduced primary cilia length, and impairment of Hedgehog signaling (Gambassi et al., 2017; Thorpe et al., 2017). In addition, HGA induces chondroptosis, a specific form of apoptosis in AKU chondrocytes, accompanied by distinct morphological alterations (Millucci et al., 2015b). AKU has further been associated with secondary amyloidosis (Cicaloni et al., 2019; Millucci et al., 2014a) and angiogenesis (Millucci et al., 2016); notably, recent findings show that in AKU and in HGA-treated chondrocytes, ochronotic pigment colocalizes with SAA-

amyloid (Millucci et al., 2012). Autophagy is one of the central processes that preserves articular cartilage homeostasis and maintains chondrocyte functionality (Galderisi et al., 2022). It also contributes to sustaining cellular energy balance during stress conditions, including nutrient deprivation (Glick et al., 2010). Chondrocytes, the only cell type present in articular cartilage, are responsible for extracellular matrix synthesis and turnover. Because cartilage is a post-mitotic and avascular tissue, chondrocyte proliferation is extremely limited and metabolic turnover is slow (Cuervo et al., 2005; Goldring and Goldring, 2007). In this low-nutrient environment, autophagy plays a crucial role by ensuring the intracellular clearance of unnecessary proteins, pathogens, and damaged organelles. Alterations in autophagic flux can therefore compromise cytoprotective mechanisms in cartilage, contributing to disease onset and progression, as exemplified in osteoarthritis (OA), where a marked reduction in autophagy is a characteristic pathological feature (Jeon and Im, 2017). Closely intertwined with autophagy are the functions of mitochondria and the endoplasmic reticulum (ER) (Liu et al., 2022), two organelles strongly affected by HGA-driven oxidative stress. Mitochondria are critical regulators of energy production, calcium homeostasis, apoptosis, and redox balance. Their structural and functional integrity depends on dynamic cycles of fission and fusion and on mitophagy, the selective autophagic clearance of damaged mitochondria (Wang et al., 2024). Mitochondria are also major sources of reactive oxygen species, making them both targets and amplifiers of oxidative damage. In this chronic oxidative condition, mitochondrial dynamics become severely disrupted. The accumulation of oxidative damage impairs mitochondrial respiration, dissipates membrane potential, increases superoxide production, and ultimately compromises cellular energy status. Dysregulated mitochondrial function, in turn, exacerbates the very oxidative environment that initiated damage, creating a self-perpetuating cycle that affects chondrocyte viability (Sun et al., 2021). The ER, another central organelle in cellular homeostasis, is responsible for protein folding, lipid biosynthesis, and calcium regulation (Lu et al., 2024). Oxidative stress and metabolic dysregulation can result in an increase in ER stress, activating the unfolded protein response (UPR). Under moderate stress, the UPR serves adaptive roles by enhancing

chaperone production, reducing protein synthesis, and promoting degradation of misfolded proteins (Lu et al., 2024). However, chronic or excessive ER stress leads to maladaptive UPR signaling, resulting in impaired proteostasis, disruption of lipid metabolism, and activation of cell death pathways (Lu et al., 2024). In connective tissue disorders, including those affecting cartilage, prolonged ER stress contributes to amyloid deposition, matrix degradation, and chondrocyte dysfunction (Lu et al., 2024). Moreover, ER–mitochondria communication via mitochondria-associated membranes is essential for coordinating lipid transfer and initiating autophagosome formation (Liu et al., 2022). Perturbation of this interface by oxidative stress compromises not only the autophagic machinery but also multiple other metabolic and homeostatic processes. Another critical element in this pathophysiological system is lysosomal function. Lysosomes are degradative organelles of autophagy and contain hydrolytic enzymes that require a strongly acidic pH to function optimally (He et al., 2024). Proper lysosomal activity depends on intact membranes, efficient acidification, regulated biogenesis, and precise membrane fusion events (He et al., 2024). Oxidative stress can damage lysosomal membranes, disrupt acidification, and reduce enzymatic activity (Ballabio and Bonifacino, 2020). When lysosomes fail to fuse with autophagosomes or cannot degrade their contents, autophagic flux becomes impaired. The resulting accumulation of autophagosomes and undegraded cargo places further strain on already compromised cellular systems (Galderisi et al., 2022). Lysosomal inefficiency not only affects autophagy but also leads to intracellular accumulation of oxidized proteins and polymerized pigments, conditions conducive to amyloidogenesis and ochronotic pigment formation and accumulation, both of which are observed in AKU-affected tissues and contribute to the disorganization of the extracellular matrix (Braconi et al., 2022). Recent findings have highlighted the relationship between oxidative stress, impaired proteostasis, and abnormal extracellular matrix remodeling in AKU (Milella et al., 2024). Protein misfolding and aggregation, promoted by chronic ROS exposure and ER dysfunction, create an amyloidogenic environment. In various degenerative conditions, amyloid accumulation disrupts tissue structure, promotes inflammation, and contributes to cell death (Mirioglu et al., 2024). The

observation that ochronotic pigment deposition can colocalize with amyloid in AKU cartilage underscores the link between metabolic imbalance, protein aggregation, and structural degeneration (Millucci et al., 2012). Together, these biological processes reveal AKU not simply as a disorder of pigment deposition but as a multifaceted condition involving systemic metabolic dysfunction and widespread failure of cellular quality-control mechanisms. In this intricate biological context, chondrocyte degeneration emerges as the culmination of an escalating series of molecular stresses (Ansari et al., 2021). Chronic exposure to HGA initiates a cascade of oxidative, metabolic, and structural disruptions that gradually erode cellular resilience (Milella et al., 2024). Over time, the combination of mitochondrial dysfunction, ER stress, impaired autophagy, lysosomal insufficiency, and protein aggregation overwhelms the limited compensatory capacity of chondrocytes. The result is progressive loss of cellular integrity, reduced synthetic activity, and eventual cell death (Galderisi et al., 2022). Given the critical role of chondrocytes in maintaining extracellular matrix homeostasis, their decline precipitates cartilage degeneration, joint failure, and the debilitating osteoarthropathy feature of AKU (Milella et al., 2024).

Materials and Methods

Cells cultures and treatment

The C20/A4 human chondrocyte cell line (Sigma-Aldrich, SCC041) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (P/S) and 10% fetal bovine serum (FBS) at 37°C in a humidified environment with 5% CO_2. HGA (Sigma-Aldrich) was prepared by dissolving the compound in deionized water to produce a 10 mM stock solution. The initial solution was then diluted in the cell culture medium to achieve the final concentration of 0.1 mM.

Immunofluorescence study

C20/A4 human chondrocytes were cultured on black 24-well glass-bottom plates at a density of 1×10^4 cells per well for one week and 5×10^3 cells per well for two weeks. Following treatment with 0.1 mM HGA, the cells were fixed with 4% PFA, before being permeabilized using 0.2% Triton X-100 and then blocked with BSA. Next, the cells were incubated overnight at 4°C with: anti-LAMP1 mouse monoclonal antibody (1:50, Invitrogen), anti-p62/SQSTM1 rabbit polyclonal antibody (1:100, Proteintech), anti-LC3B rabbit polyclonal antibody (1:200, Novus Biologicals), and anti-4-HNE mouse monoclonal antibody (1:50, Invitrogen). Afterwards, the cells were incubated with Alexa Fluor™ 568 goat anti-mouse IgG (1:1000, Thermo Fisher Scientific) and Alexa Fluor™ 488 goat anti-rabbit IgG (1:500, Thermo Fisher Scientific). Ultimately, the samples were stained using DAPI. Images were captured with a fluorescence microscope (LEICA DMI8, Wetzlar, Germany), and fluorescence intensity was quantified using ImageJ software. A quantitative colocalization and correlation study of LAMP1 with LC3B and p62/SQSTM1 was conducted using Icy software and the GcoPS plug-in (Lavancier et al., 2019).

Live Cell Imaging study

C20/A4 human chondrocytes were cultured on 96-well slides at a density of 5×10^3 cells per well and treated with 0.1 mM HGA for 24 h, and on black 24-well glass-bottom plates at a density of 1×10^4 cells per well for one week and 5×10^3 cells per well for two weeks. Following the treatment with 0.1 mM HGA, cells were stained with the following dyes:

- Mitotracker Red CMXRos (9082S, Cell Signaling)
- Mitosox Red (M36007, Invitrogen)
- ER-Tracker Green (E34251, Invitrogen)
- LysoTracker Red DND-99 (L7528, Invitrogen)
- Nucblue Live ReadyProbes Reagent (HOESCHT 33342, R37605, Invitrogen)
- BacMam Premo Autophagy Sensor LC3-GFP (P36235, Invitrogen)

Images were captured with a fluorescence microscope (LEICA DMI8, Wetzlar, Germany), and fluorescence intensity was quantified using ImageJ software. A quantitative colocalization and correlation study of LC3B-GFP and LysoTracker Red was conducted using Icy software and the GcoPS plug-in (Lavancier et al., 2019) .

Quantification of Intracellular ROS Formation

ROS production in C20/A4 cells was assessed in 96-well plates using 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA, Sigma-Aldrich), which is intracellularly deacetylated and oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (Ng and Ooi, 2021). The cells were treated with HGA at a concentration of 0.1 mM for 24 hours. $DCFH_2$-DA (5 µM), dissolved in Hank's Balanced Salt Solution (HBSS), was then added to the cells and incubated for 10 minutes at 37°C. Fluorescence was measured using a ClarioStarPlus with excitation at 485 nm and emission at 535 nm. To visualize DCF fluorescence, samples were observed under a fluorescence microscope with a FITC filter (Zeiss AxioLabA1, Oberkochen, Germany), and images were acquired

to confirm the results. The number of cells in each well was then determined using a Crystal Violet assay (Feoktistova et al., 2016). Results were normalized to the relative cell count for each well and expressed as relative ROS production compared to the untreated group.

Isolation and culture of human articular cartilage

Human articular cartilage fragments were isolated, following informed consent, from the humeral head of an AKU patient undergoing joint replacement surgery. The AKU biopsy was washed with phosphate-buffered saline (PBS) to remove residual blood and subsequently and the cartilage was subsequently isolated and cut into smaller pieces using sterile surgical scalpels. The fragments were then transferred into a 100 mm Petri dish containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% penicillin/streptomycin and 0.2% Fungizone (Amphotericin B), and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. After that, the AKU cartilage fragments were transferred to a black 24-well plate and cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS.

Live AKU cartilage imaging

Human cartilage isolated from AKU patients was cultured on Black 24 well plates Glass Bottom, before being incubated with Lysotracker Red DND-99 L7528 (Invitrogen by Thermo Scientific) and Nucblue Live Readyprobes Reagent (HOESCHT 33342) R37605 (Invitrogen by Thermo Scientific). Images were captured with a fluorescence microscope (LEICA DMI8, Wetzlar, Germany).

Statistical Analysis

The results are derived from three independent experiments. Statistical analyses were carried out with GraphPad Prism 10.0 software (GraphPad Software, San Diego, CA, USA). The data are expressed as mean ± standard deviation (SD) and were compared using the unpaired t-test. A p-value of 0.05 or less was considered statistically significant.

Results

HGA induced chronic oxidative stress condition

HGA causes oxidative stress in chondrocytes, ultimately leading to chondroptosis (Bernardini et al., 2024). Its effects have been evaluated under both acute and chronic conditions. C20/A4 human chondrocytes were treated with 0.1 mM HGA, a concentration consistent with levels observed in alkaptonuric patients (Ranganath et al., 2016). This dosage effectively replicated disease-related events without compromising cell viability (Galderisi et al., 2022). Autophagy is a fundamental homeostatic process in chondrocytes, essential for maintaining cartilage integrity by removing damaged organelles and misfolded proteins. It is known to be activated by various cellular stressors, including oxidative stress, and contributes to protecting chondrocytes from degeneration (Galderisi et al., 2022). In this context, ROS can act as upstream signals that modulate autophagic activity, particularly through their impact on mitochondrial quality control (Wang et al., 2024). Thus, changes in intracellular ROS levels may directly influence autophagy-related responses in cartilage. ROS serve as signaling molecules that play a critical role in the development of stress conditions and are typically produced as part of the early response (Forrester et al., 2018). In chondrocytes, ROS production is also involved in regular processes such as the regulation of extracellular matrix homeostasis (Henrotin et al., 2003). Therefore, we investigated the impact of treating C20/A4 cells with HGA on intracellular ROS production. To do this, the cells were seeded (3×10^3 cells/well) in a black 96-well plate before being treated with 0.1 mM HGA for 24 hours. The DCF fluorescence exhibited a notable increase in HGA-treated cells compared to the control, indicating the induction of oxidative stress even after 24 hours (Figure 20).

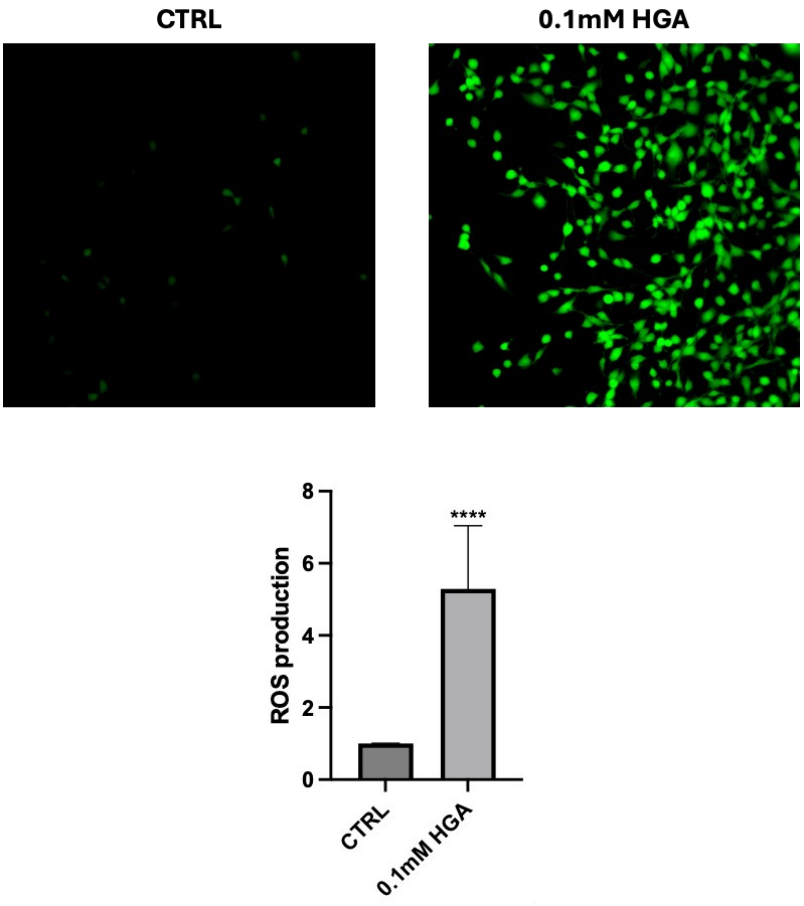

Figure 20. Exploration of ROS generation by labelling with the H_2DCFDA probe (green fluorescence) in C20/A4 control and 0.1 mM HGA treated cells. Data are presented as bar graphs for ROS levels measured from fluorescence intensity normalized to cell count using Crystal Violet assay. All experiments were performed in triplicate. Data are reported as fold change. An unpaired t-test was used to assess statistically significant differences (****$p < 0.0001$). Cells are shown at 20× magnification.

Long-term oxidative stress is commonly associated with chronic pathological conditions, leading to DNA and lipid damage, which in turn results in structural alterations and loss of cellular stability and functionality (Galderisi et al., 2022). Among the major markers of lipid peroxidation (LPO), 4-HNE, a specific aldehyde product, has been extensively studied. Notably, studies have demonstrated that 4-HNE accumulation is particularly prominent in cartilage from AKU patients, especially in regions exhibiting ochronotic pigmentation (Pompella et al., 2002).

Chronic oxidative stress is also known to impair autophagy, a key homeostatic mechanism responsible for removing oxidized or damaged components, such as lipids and organelles (Filomeni et al., 2015). Under prolonged ROS exposure, autophagy initially acts as a protective response; however, sustained oxidative conditions and persistent accumulation of LPO products such as 4-HNE

progressively suppress autophagic flux, contributing to chondrocyte dysfunction and impaired cartilage maintenance (W. Li et al., 2022; Zhang et al., 2023). Thus, evaluating lipid peroxidation provides important insight into how long-term oxidative stress may alter autophagy-dependent processes in HGA-treated chondrocytes. To evaluate LPO, the expression of 4-HNE was therefore investigated. C20/A4 cells were treated with 0.1 mM HGA, and 4-HNE was detected through immunofluorescence. C20/A4 cells were seeded in 24-well plates and once sub-confluence was achieved, the cells were treated for two weeks. The expression of 4-HNE was assessed through ImageJ software, using the number of nuclei to normalize the results. In treated cells, 4-HNE expression revealed LPO-induced damage to the cellular lipid structures (Figure 21).

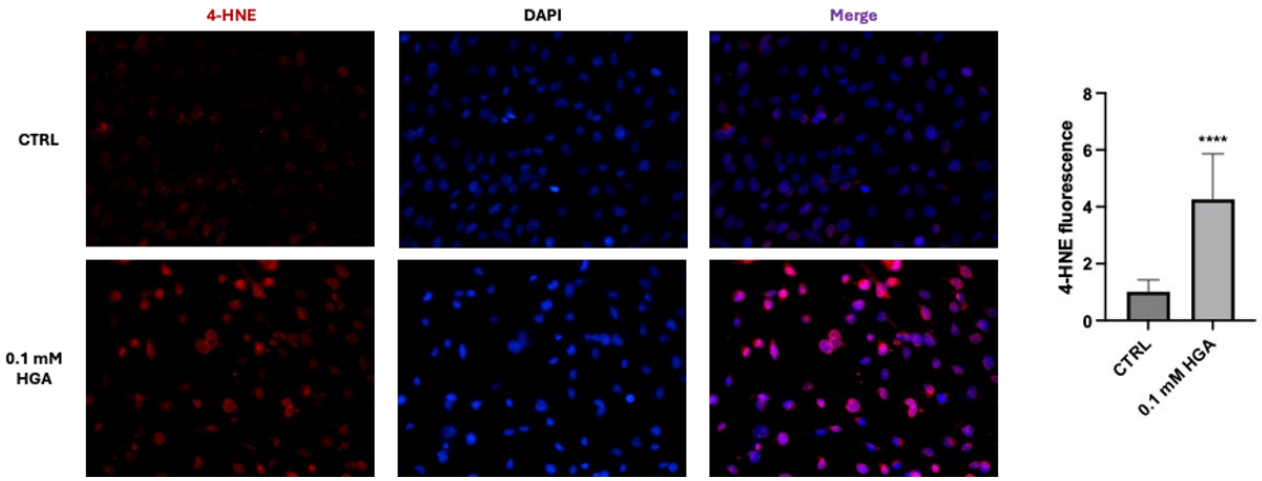

Figure 21. Examination of 4-HNE expression in control and HGA-treated C20/A4 chondrocytes after 2 weeks of treatment with 0.1 mM HGA by immunofluorescence staining. Nuclei were counterstained with DAPI. Data are reported as fold change. An unpaired t-test was used to assess statistically significant differences, (****$p < 0.0001$). Cells are shown at 40× magnification.

HGA treatment induced alterations of mitochondrial morphology

Mitochondria are central regulators of cellular energy metabolism, integrating oxidative phosphorylation with key biosynthetic and signaling pathways. A fundamental requirement for their optimal function is the maintenance of redox homeostasis, which ensures the controlled balance between ROS generation and antioxidant defenses. Under physiological conditions, mitochondria

continuously produce ROS as by-products of electron transport chain (ETC) activity; these species act not only as metabolic intermediates but also as critical second messengers in pathways governing cell survival, proliferation, and adaptation to stress (Sinenko et al., 2021).

Short-term oxidative stress, often triggered by acute metabolic demands or environmental challenges, can transiently disturb mitochondrial redox equilibrium, leading to reversible modifications of mitochondrial proteins, lipids, and DNA. While such changes may initially enhance adaptive responses, sustained or excessive ROS production results in long-term oxidative stress, causing cumulative molecular damage, impairment of ETC complexes, mitochondrial membrane depolarization, and activation of mitophagy or cell death pathways (A. Li et al., 2022). Chronic redox imbalance is therefore closely linked to mitochondrial dysfunction and contributes to the pathogenesis of numerous degenerative, metabolic, and age-related diseases.

Understanding how redox regulation shapes mitochondrial performance is essential for elucidating the mechanisms underlying cellular resilience and for developing strategies aimed at preserving mitochondrial integrity under stress conditions. Thus, oxidative stress is a condition that typically affects mitochondrial function, since mitochondria rely on redox balance for cellular respiration and ATP generation (Xu et al., 2025). Moderate oxidative stress exerts a significant influence on mitochondrial morphology by modulating the dynamic balance between fusion and fission. In the short term, a transient increase in ROS can activate stress-responsive signaling pathways, such as DRP1 phosphorylation or OPA1 processing, leading to rapid changes in mitochondrial shape. These early responses often manifest as increased fission, resulting in a more fragmented network that helps isolate damaged regions and maintain mitochondrial quality control (Fogo et al., 2024). At this stage, morphological changes are frequently reversible and may represent an adaptive mechanism that facilitates metabolic flexibility and rapid removal of impaired mitochondrial segments. However, persistent or repeated episodes of moderate oxidative stress can produce long-term alterations in mitochondrial architecture. Chronic redox imbalance disrupts the coordination of mitochondrial dynamics, impairing fusion capacity, altering cristae organization, and promoting sustained

fragmentation. Over time, these structural abnormalities compromise ATP production, calcium handling, and overall mitochondrial resilience, contributing to cellular dysfunction and increased susceptibility to degeneration (Chen et al., 2024). Thus, while short-term morphological adjustments to oxidative stress may be protective, prolonged exposure can progressively erode the structural integrity of the mitochondrial network.

C20/A4 cells were seeded in black 24-well plates, before being treated with 0.1 mM HGA for 24 h, one week, and two weeks, in order to induce oxidative stress, and stained with MitoTracker Red. Results were evaluated by live-cell imaging using a Cytation 5. Morphology, size, and number of mitochondria changed even after 24 h of treatment (Figure 22),

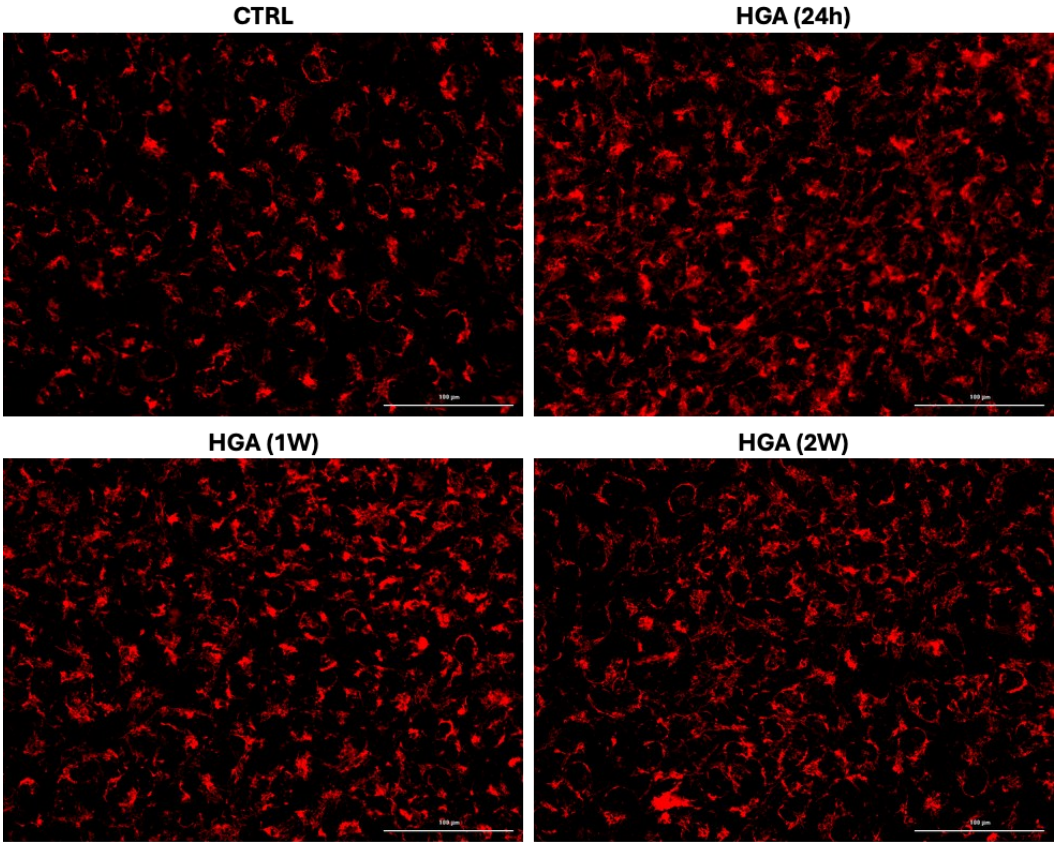

Figure 22. Observation of mitochondrial number, size and morphology in control and 0.1 mM HGA-treated C20/A4 chondrocytes after 24 h, 1W and 2W of treatment by live-cell imaging staining. Scale bar = 100 µm

Mitochondria exhibited a clear adaptive response to HGA treatment, initially undergoing enhanced fusion. This fused network configuration supported a more efficient redox balance and improved cellular respiration. However, after two weeks of prolonged exposure, mitochondria shifted toward pronounced fission and network fragmentation, consistent with a long-term inability to sustain the adaptive fused state under persistent oxidative conditions.

Endoplasmatic reticulum response and mithocondrial ROS generation induced by HGA treatment

Autophagy plays a fundamental role in chondrocytes, where it supports cellular survival under conditions of low vascularization, hypoxia, and chronic mechanical stress (Li et al., 2024). By removing damaged organelles and maintaining metabolic efficiency, autophagy preserves cartilage homeostasis and prevents premature chondrocyte death (Jeon and Im, 2017). The initiation of this process relies on the formation of the phagophore, which originates at specialized contact sites between the endoplasmic reticulum and mitochondria, known as mitochondria-associated membranes (MAMs) (Zwilling and Reggiori, 2022). At these interfaces, the ER provides the membrane platform for phagophore nucleation, while mitochondria contribute essential lipids and local signalling cues, including ROS and calcium fluxes (Liu et al., 2025). For this reason, ER morphology and mitochondrial redox state, particularly superoxide production, are critical determinants of early autophagy activation and strongly influence the cellular capacity to respond to stress. C20/a4 cells were seeded in black 24-well plates, before being treated with 0.1 mM HGA for 24h, 1 week, and 2 weeks, in order to induce oxidative stress, and stained with ER Tracker and Mitosox Red. Results were evaluated by live-cell imaging using Leica DMI8 inverted microscope. C20/A4 cells showed a clear temporal trend in both endoplasmic reticulum fluorescence and mitochondrial ROS levels. At 24 hours and after one week of treatment, a reduction in ER signal intensity and mitochondrial superoxide was detected, indicating a general decrease in fluorescence associated with both organelles. After two weeks of prolonged treatment, both ER fluorescence and mitochondrial ROS increased markedly. At this later time point, notable structural changes in the endoplasmic reticulum were also observed. The ER appeared enlarged, and its membranes lost the typical branched tubular organization, instead displaying a more expanded and swollen morphology. In addition, the intracellular distribution of ER membranes changed, becoming more condensed around the perinuclear region rather than being evenly extended throughout the cytoplasm (Figure 23).

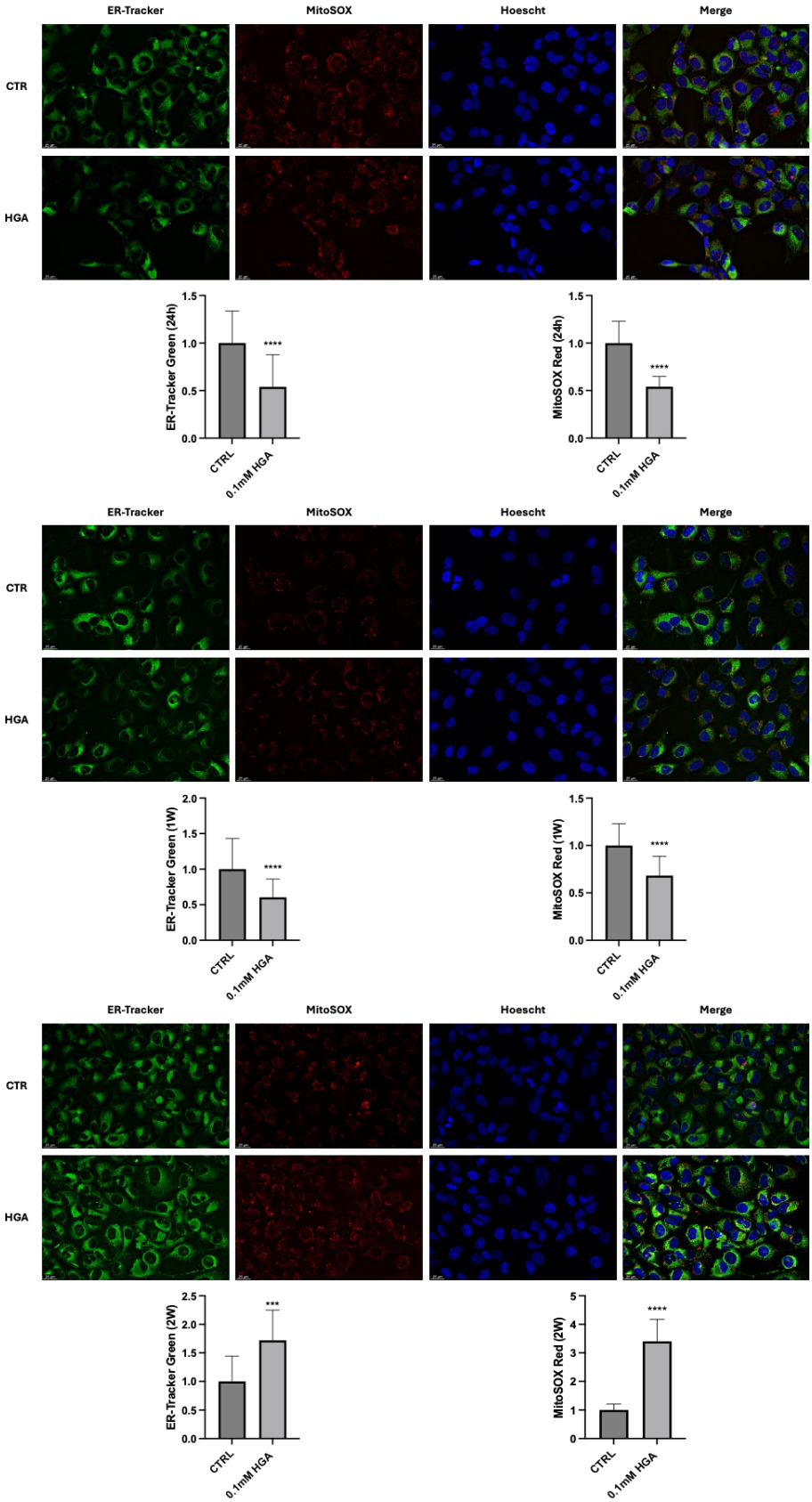

Figure 23. (A) ER (green) and Superoxide (Red) markers are co-stained. After 2 weeks of treatment ER began to lose its organization and showed abnormalities in tubular structure and morphology of membranes. Scale bar = 25 µm (B) Both ER and mitochondrial superoxide trackers showed intensity decrease after 24 h and 1week (1W) of HGA treatment, indicative of adaptive response. After prolonged treatment (2 weeks, 2W), both dyes showed intensity increase, indicating ER shape expansion and generation of mitochondrial superoxide. Statistically significant differences are shown using ***p = 0.0001, ****p < 0.0001. Data are displayed as mean +- SD. p-values were calculated using t-test. Scale bar = 25 µm.

HGA-induced autophagy flux alteration in the C20/A4 AKU cell model

Autophagy consists of a coordinated sequence of events that includes autophagosome formation, lysosomal fusion, and cargo degradation (Dikic and Elazar, 2018). LC3 is a core component of the autophagosomal membrane and plays a central role in autophagosome biogenesis, transitioning from its cytosolic form to the membrane-bound LC3-II that marks autophagic vesicles (Glick et al., 2010). LAMP1 is a major structural protein located on the membrane of lysosomes and late endosomes, and it is essential for preserving lysosomal integrity as well as facilitating their fusion with autophagosomes to form the autophagolysosome (Eskelinen, 2006). p62 (also known as SQSTM1) acts as a selective autophagy receptor that binds ubiquitinated cargo and LC3, enabling targeted sequestration of damaged proteins and organelles; its accumulation typically reflects impaired downstream degradation (Katsuragi et al., 2015). The spatial distribution and expression of these markers provide essential information on the efficiency of autophagosome-lysosome fusion and the formation of mature and functional autophagolysosomes (Mizushima et al., 2010). In this context, it was assessed how prolonged HGA exposure affects these components and the overall progression of the autophagic pathway in C20/A4 cells after one and two weeks of treatment. C20/a4 cells were seeded in black 24-well plates, before being treated with 0.1 mM HGA for 1 week and 2 weeks, to induce oxidative stress, and stained with anti-LC3, anti-p62, anti-LAMP1 antibodies. Results were evaluated through immunofluorescence using a Leica DMI8 inverted microscope (Figure 24). The increase in the amount of LC3 and p62 can be linked either to the formation of autophagosomes or to a block in autophagosomal generation and maturation, and thus to the completion of autophagy pathway (Galderisi et al., 2022). After 1 week of treatment, HGA caused an increase in LC3 and p62 fluorescences compared with untreated samples (Figure 24a). These two events, taken together, and associated with the increase of LAMP1 signal in treated cells, indicated that HGA acted as an inducer of autophagic flux. To further confirm these data, statistical colocalization analysis was performed using Icy software (GcoPS plugin); colocalization increased both between LC3 and LAMP1 and

between p62 and LAMP1, confirming enhanced potential association between autophagosome, ubiquitinated proteins, and lysosomes, thereby proving the ongoing autophagy event.

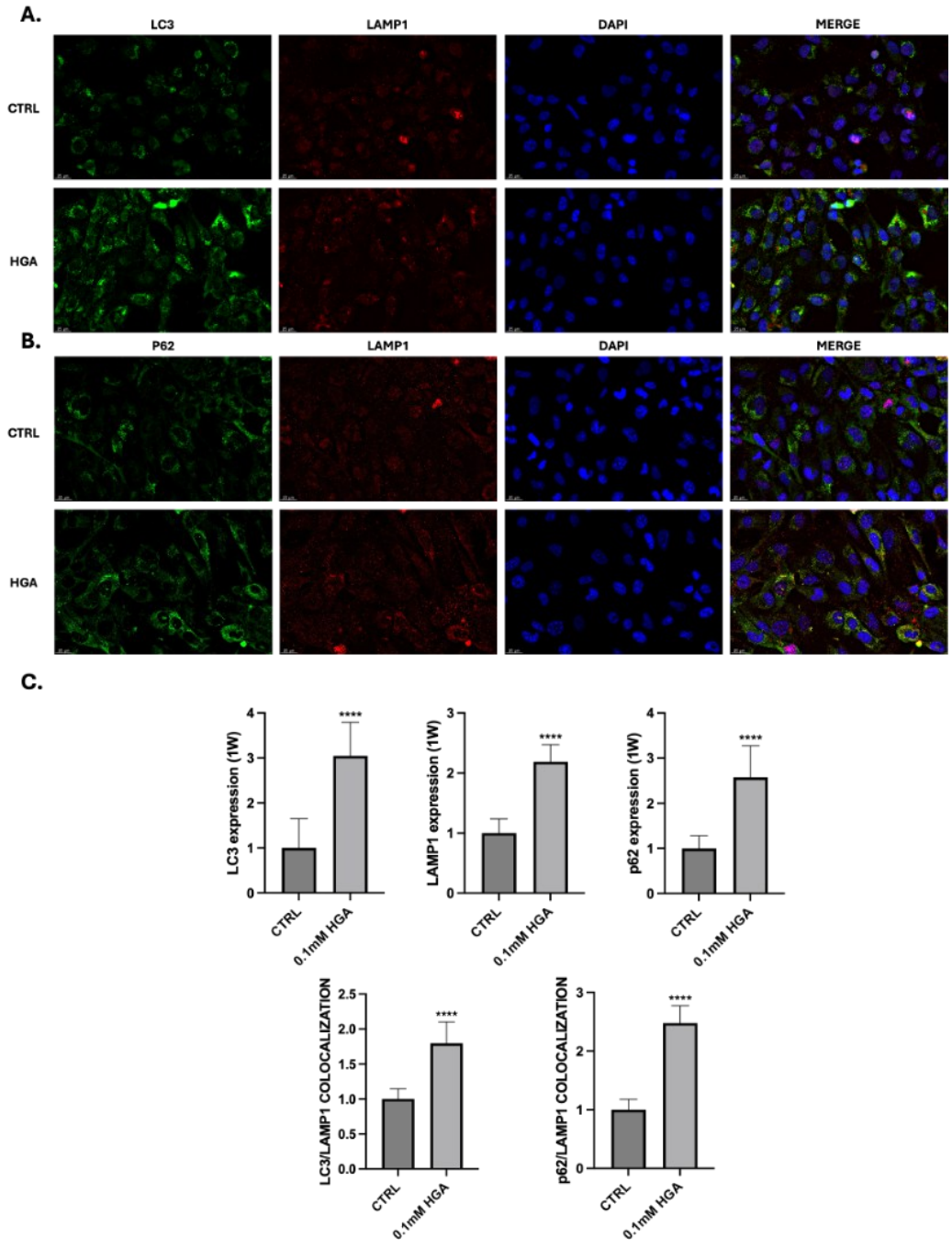

Figure 24. C20/A4 chondrocytes were treated with HGA for one week.(A) LC3 (green) and LAMP1 (red) markers are both double-labeled. LC3 and LAMP1 expression, and their colocalization, increase in HGA treated cells compared to control. (B) p62 (green) and LAMP1 (red) are both double-labeled. P62 and LAMP1 expression, and their colocalization increase in HGA treated cells compared to control. HGA-Treated cells showed more specific and PUNCTA signal. Statistically significant differences are shown using ****$p < 0.0001$. Data are displayed as mean +- SD. p-values were calculated using t-test. Scale bar = 25µm

After confirming the induction of autophagy, the long-term autophagic response of C20/A4 cells to HGA treatment was then evaluated. The increase in p62 levels can be linked to the recognition of waste material and to a block in autophagic clearance. After 2 weeks of treatment, HGA caused a decrease in LC3 expression compared with the untreated samples, confirming an alteration of the autophagic flux (Figure 25). LAMP1 fluorescence increased in treated cells, indicating that HGA acted as an inducer of lysosome formation. The colocalization score (GcoPS) revealed several events: LC3 and LAMP1 showed decreased colocalization, while p62 and LAMP1 maintained a higher colocalization score compared with control cells.

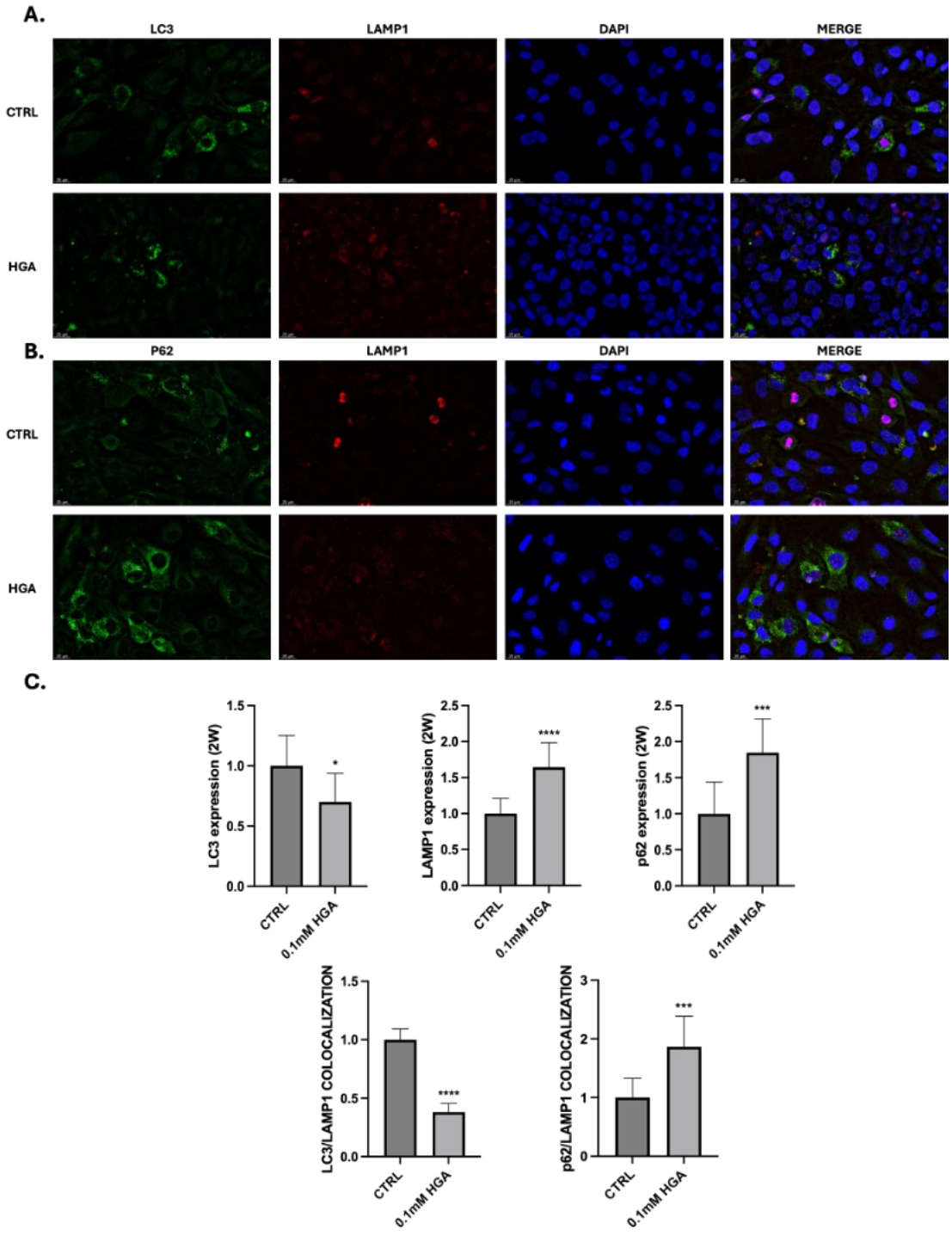

Figure 25. C20/A4 chondrocytes were treated for two weeks. (A) LC3 (green) and LAMP1 (red) markers are both double-labelled. LAMP1 expression resulted increased, while decreased the expression of LC3 and its colocalization with LAMP1, in HGA treated cells compared to control. (B) p62 (green) and LAMP1 (red) are both double-labeled. p62 and LAMP1 expression, and their colocalization increase in HGA treated cells compared to control. Statistically significant differences are shown using *p = 0.0106, ***p < 0.001, ****p < 0.0001. Data are displayed as mean +- SD. p-values were calculated using t-test. Scale bar = 25µm

Chronic HGA-derived oxidative stress altered lysosome functionality

Lysosomes are intracellular membrane-bound organelles with a key role in degrading and recycling cellular waste, a function enabled by their hydrolytic enzymes and the characteristically low luminal pH (4.5-5) (Saftig and Klumperman, 2009). In addition to the breakdown of macromolecules and polymers, including proteins, carbohydrates, and lipids (Ballabio and Bonifacino, 2020), lysosomes also degrade pathogens and cellular debris. However, these organelles are not limited to waste isolation and degradation; they also play a key regulatory role in the maintenance of cell homeostasis at multiple levels (Perera and Zoncu, 2016). Alterations in lysosomal function can result in several consequences, including impaired clearance of potentially toxic cellular waste, induction of inflammation, apoptotic cell death, and dysregulation of cellular signalling pathways (Lie and Nixon, 2019). To evaluate lysosomal activity and functionality, and to monitor changes over time, C20/A4 cells were seeded in black 24-well plates, and then treated with 0.1 mM HGA for 24 h, 1 week, and 2 weeks to induce oxidative stress. Cells were subsequently stained with LysoTracker Red. Results were obtained through live-cell imaging using a Leica DMI8 inverted microscope. LysoTracker Red staining progressively increased after 24 h of HGA treatment, reaching its maximum intensity at 1 week, indicating enhanced lysosomal activation, consistent with the dye's capacity to label acidic compartments and increased lysosomal activity (Figure 26A). These observations were further supported by an interactive 3D plot, which confirmed increased fluorescence intensity and a more pronounced distribution of active lysosomes (Figure 26B). However, after 2 weeks of treatment, HGA-treated cells exhibited a marked reduction in LysoTracker staining, lower even than in control cells, suggesting impaired lysosomal functionality. This reduction likely reflects altered lysosomal pH and the potential inactivation of lysosomal enzymes under prolonged oxidative stress conditions.

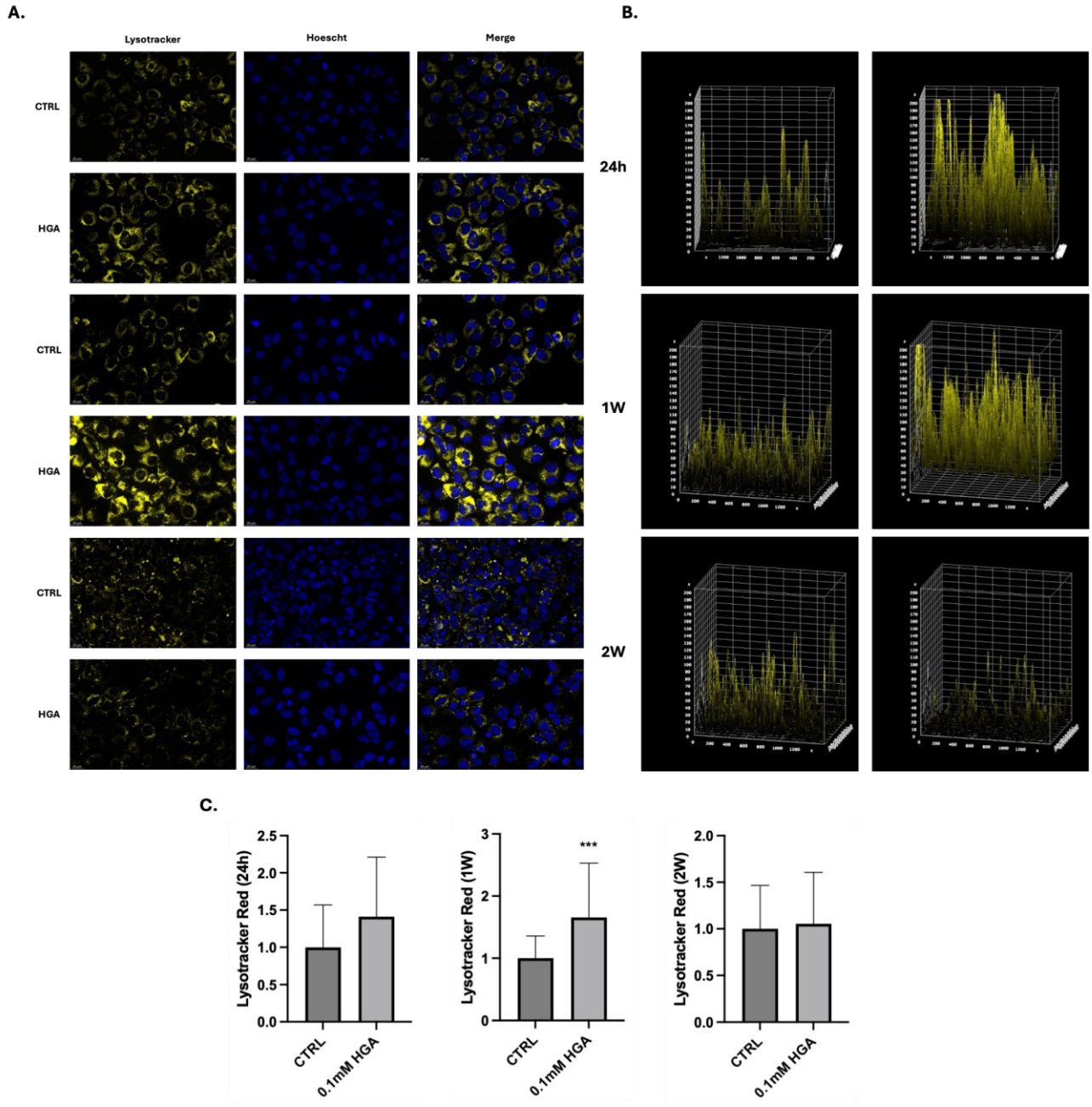

Figure 26. (A) Observation and distribution of active lysosomes. (B) Interactive 3D plot showed increased lysosome intensity and distribution. (C) Quantification of Lysotracker fluorescence showed increased lysosome intensity and distribution after 1W of HGA treatment, and an inverted trend after long term exposure to HGA, evidencing an alteration to the acid lysosomal compartment. Nuclei were counterstained with Hoechst. Statistically significant differences are shown using ***$p < 0.001$. Data are displayed as mean ± SD. p-values were calculated using t-test. Scale bar = 25 µm

Lysosome activity is altered in AKU cartilage

Over time, due to the inefficiency of the HGD enzyme, ochronotic pigment accumulates within connective tissues, particularly cartilage, compromising tissue structure, altering extracellular matrix (ECM) integrity, reducing tissue plasticity, and ultimately affecting cell viability (Milella et al., 2024). Pigment deposition is often heterogeneous, and in AKU patients its accumulation typically progresses over many years (Bernardini et al., 2024). From the biopsy samples, different stages of AKU cartilage were identifiable based on the variable presence and extent of ochronotic pigment deposition. Since chondrocytes are the only resident cell type in cartilage, to validate the results observed in our C20/A4 AKU model, lysosomal functionality was directly assessed in living cartilage obtained from AKU patients (Figure 27). After biopsy collection, cartilage samples were isolated and cultured in black 24-well plates, followed by incubation with LysoTracker and Hoechst nuclear staining. Overall, an alteration in fluorescence intensity and distribution was observed, with areas displaying positive nuclear staining but no detectable Lysotracker signal, thus suggesting impaired lysosomal functionality in AKU-affected tissue consistent with chronic HGA-derived oxidative stress.

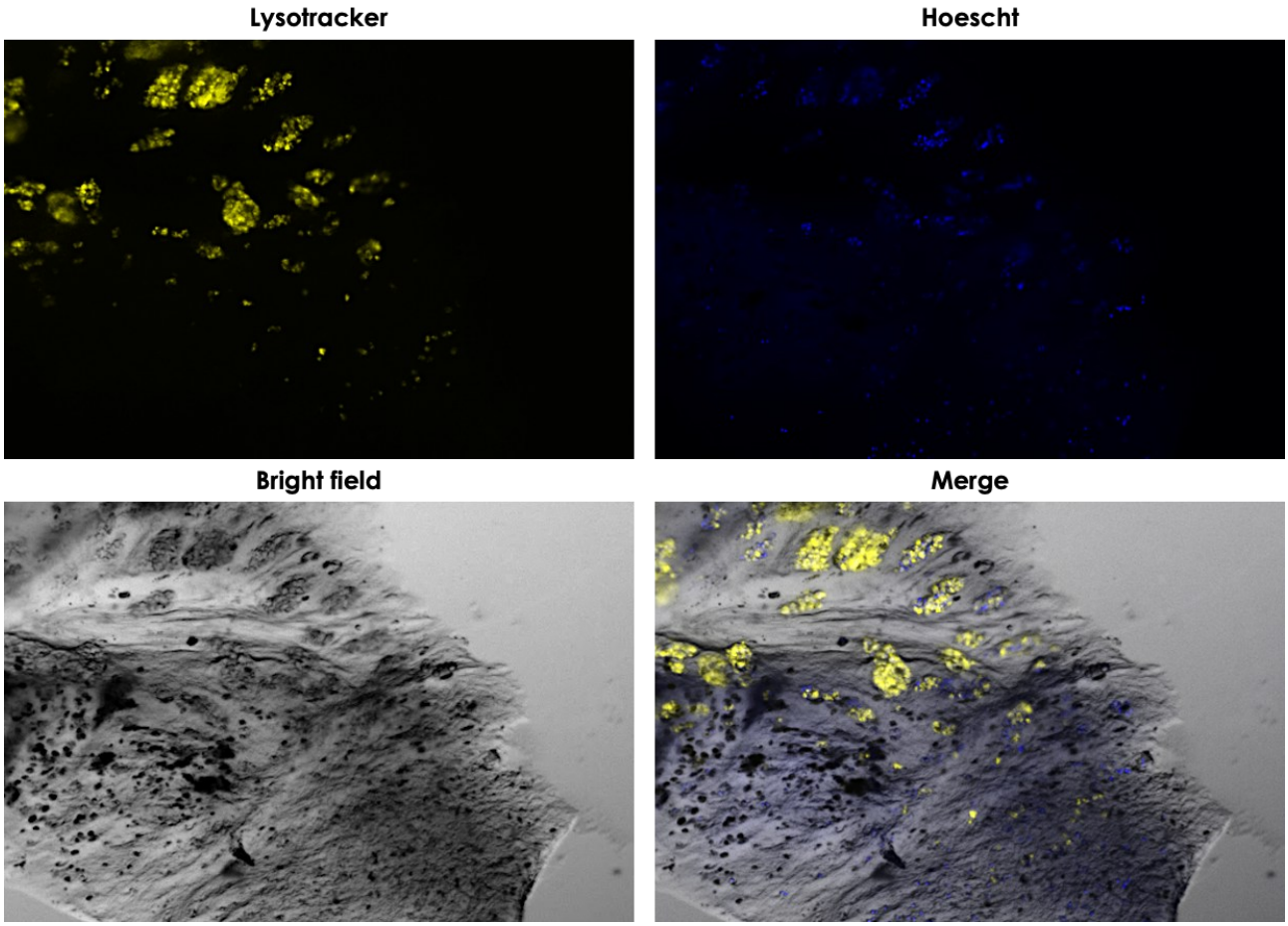

Figure 27. Live observation of cultured AKU cartilage. Lysotracker (yellow) and Hoescht (blue) dyes are both double-labelled. Active lysosomes, marked by Lysotracker staining. Images are shown at 4x magnification.

HGA altered normal autophagic flux

The autophagy process culminates in the fusion of autophagosomes with lysosomes (Mizushima et al., 2010), generating mature autolysosomes in which waste material, previously recognized and sequestered by p62 (Katsuragi et al., 2015), is degraded through the acidic lysosomal pH and proteolytic enzymes (Ballabio and Bonifacino, 2020). To investigate the interaction between these two autophagy components, LysoTracker was used in combination with LC3-GFP–transfected cells to monitor fluorescence intensity, spatial distribution, and their potential colocalization. C20/A4 cells were seeded in black 24-well plates, and then treated with 0.1 mM HGA for 24 h, 1 week, and 2 weeks to induce oxidative stress. Subsequently, cells were transfected with Baculovirus (LC3-GFP) and stained with LysoTracker Red. Quantification of fluorescence intensity, performed using ImageJ, revealed an increase in the fluorescence of both dyes and a decrease in their colocalization after 1 week of treatment (Figure 28A, B). This pattern is consistent with GFP quenching following autophagosome–lysosome fusion. However, after 2 weeks of HGA exposure, autophagy appeared significantly impaired (Figure 28C). No statistically significant differences were detected between control and treated cells regarding LC3 expression (autophagosome formation), LysoTracker fluorescence (lysosomal functionality), or LC3–lysosome colocalization, indicating a deficiency in both autophagosome generation and lysosomal activity under prolonged exposure conditions.

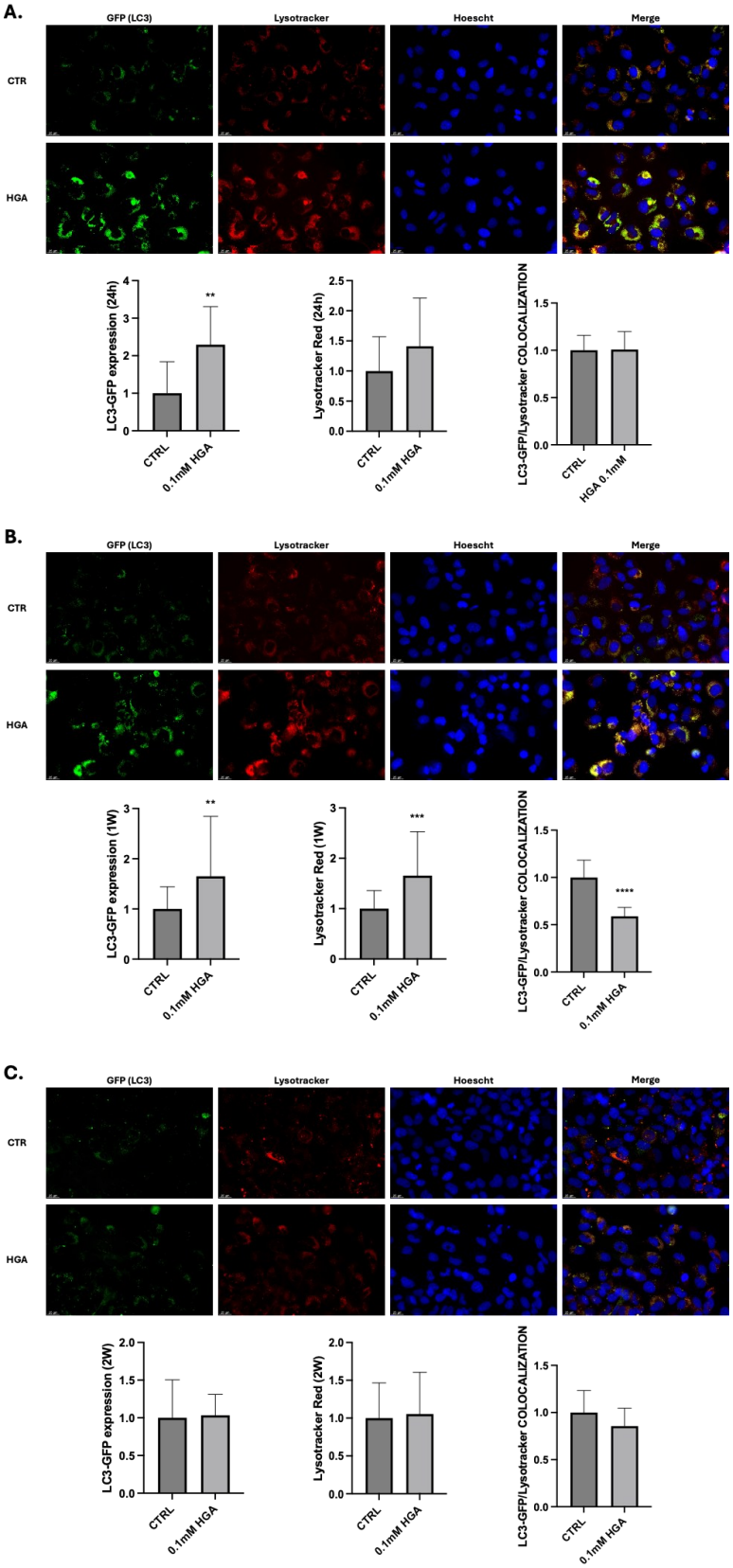

Figure 28. LC3-GFP (green) and Lysotracker (red) dyes are both double-labelled. (A)Lysotracker and LC3-GFP fluorescence resulted increased in HGA-treated cells after 24h, and colocalization resulted similar to that of control. (B)Their expression, after 1W HGA-treatment, increased notably, while their colocalization decreased in HGA treated cells compared to control. (C)After 2W HGA-treatment, their expression and colocalization didn't show any difference between HGA-treated and control cells. Statistically significant differences are shown using **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are displayed as mean ± SD. p-values were calculated using t-test. Scale bar = 25µm

Discussion

Alkaptonuria (AKU) is a rare inborn error of metabolism caused by the deficiency of homogentisate 1,2-dioxygenase activity. The consequent homogentisic acid (HGA) accumulation in body fluids and tissues leads to a multisystemic and highly debilitating disease whose main features are dark urine, ochronosis (HGA-derived pigment in collagen-rich connective tissues), and a painful and severe form of osteoarthropathy (Bernardini et al., 2024). HGA induces chondroptosis, a distinct form of apoptosis in chondrocytes, resulting in characteristic morphological alterations (Millucci et al., 2015b). AKU is also associated with secondary amyloidosis and angiogenesis, as ochronotic pigment has been found to colocalize with SAA-derived amyloid in both AKU and HGA-treated chondrocytes. Autophagy is one of the main processes that maintain the homeostasis of articular cartilage and consequently its functionality. Autophagy also allows to balance energy during critical periods of stress and damage, such as during nutrient deprivation (Galderisi et al., 2022; Glick et al., 2010). Chondrocytes are the only resident cells in the articular cartilage, and they are necessary for the synthesis and the turnover of extracellular matrix (ECM). Since articular cartilage is a post-mitotic tissue, its cell turnover is extremely limited. Moreover, cartilage is an avascular tissue; therefore, low levels of nutrients are available for chondrocytes (Goldring and Goldring, 2007). For this reason, autophagy plays a crucial role in cartilage by maintaining cellular homeostasis through the clearance of unnecessary proteins, pathogens and damaged organelles (Jeon and Im, 2017). Altered autophagy can lead to the loss of cytoprotective mechanisms, contributing to cartilage-related diseases such as osteoarthritis (OA). In AKU, chronic oxidative stress induced by HGA progressively impaired autophagy, leading to chondroptosis and ochronotic pigment accumulation. It has previously been observed a concomitant HGA-induced alteration of autophagy and accumulation of ochronotic pigment, which recapitulates AKU physiopathology features (Galderisi et al., 2022). This study aimed to investigate the mechanisms underlying AKU-related autophagy alterations using the human C20/A4 chondrocyte line, which is well established as an in vitro AKU model. As already assessed,

a concentration of 0.1 mM HGA was selected as optimal to induce AKU-like alterations without compromising cell viability.

First, cronic oxidative stress was confirmed by assessing ROS production and 4-HNE expression. Intracellular ROS levels were evaluated by monitoring the oxidation of H_2DCF-DA after 24h of HGA treatment. Fluorescence was evaluated through fluorescence microscopy using a FITC filter and quantified using a plate reader. The results showed a marked increase in ROS generation in treated cells compared to control. Long-term oxidative stress was further assessed by immunofluorescence analysis, which revealed increased 4-HNE expression in cells treated with HGA for 2 weeks, with a characteristic distribution associated with lipidic membranes, confirming chronic oxidative stress condition. The initiation of autophagy is closely linked to specialized contact sites between the endoplasmic reticulum (ER) and mitochondria, known as mitochondria-associated membranes (MAMs). At these sites, key signaling proteins and lipids accumulate to form the the phagophore, the initial membrane structure of autophagy (Liu et al., 2025). The ER acts as a scaffold, while mitochondria contribute essential lipids, supporting membrane expansion. As the phagophore grows, it gradually curves and closes to form the autophagosome, a double-membrane vesicle that sequesters damaged organelles or misfolded proteins for degradation (Hurley and Young, 2017). In this way, MAMs function as strategic platforms that coordinate metabolic signals and membrane dynamics required for the initiation of autophagy (Liu et al., 2022). ER integrity and morphology were assessed by live-cell imaging at 24h, 1 week, and 2 weeks of treatment, using an ER-Tracker dye. The ER consists of membranes, and its mass is indicative of cellular metabolic activity (Schwarz and Blower, 2016). 24h and 1 week HGA treatment showed reduced fluorescence intensity compared to controls, indicating a reduction in ER mass/structure and biosynthetic activity, as well as activation of the unfolded protein response (UPR). The results were consistent with the AKU literature, which reports the accumulation of misfolded proteins and metabolic impairment under condition of oxidative stress induced by HGA (Bernardini et al., 2024). Reduction of ER mass is crucial to avoid the accumulation of misfolded proteins (Hetz and Papa, 2018). After 2 weeks of treatment, an inverted trend was

observed. ER-tracker stained HGA-treated cells more intensely than controls, showing expansion of the ER lumen and tubular structure, consistent with the accumulation of unfolded protein (as observed in secondary amyloidosis), altered cell structure and morphology, and activation of maladaptive UPR pathways (Hetz and Papa, 2018). Autophagy modulation is crucial for protein and organelle recycling, energy homeostasis, and protection against oxidative stress; moreover, it is heavily affected by ATP availability, especially in chondrocytes, due to the avascular nature of cartilage and the lack of nutrients (Li et al., 2024). Mitochondria are essential organelles for providing energy (ATP) to the cell (Nunnari and Suomalainen, 2012). They are also involved in the regulation of programmed cell death, the apoptosis, which ensures proper development and removal of damaged or harmful cells (Green and Kroemer, 2004). Oxidative stress negatively affects mitochondrial function through excessive production of superoxide, a highly reactive species generated as a natural byproduct of cellular respiration (Murphy, 2009). Under normal conditions, mitochondria maintain a balance between ROS production and antioxidant defenses (Sies and Jones, 2020). However, when ROS levels become too high, this balance is disrupted (Schieber and Chandel, 2014). Excess ROS can damage mitochondrial membranes, proteins, and mitochondrial DNA (mtDNA), thereby impairing the efficiency of the electron transport chain and reducing ATP production (Zorov et al., 2014). As mitochondrial components become damaged, energy production declines, and the organelle's ability to regulate cellular metabolism is compromised. Thus, oxidative stress undermines mitochondrial function by disrupting both structural integrity and energy-producing capacity (Chen et al., 2024). HGA treatment-induced effects on mithocondrial morphology and functionality were investigated by live-cell imaging, through staining with fluorescent dyes: Mytotracker Red CMXRos and MitoSOX Red Mitochondrial Superoxide Indicator. Live-cell imaging revealed a biphasic mitochondrial response to oxidative stress. In the early stages (24 hours to 1 week), cells displayed a more fused mitochondrial network, as revealed by MitoTracker Red CMXRos staining. This shift toward fusion is consistent with stress-induced mitochondrial hyperfusion, a protective response that promotes metabolic efficiency and preserves mitochondrial function (Tondera et al., 2009). During the same

time interval, MitoSOX Red fluorescence decreased, suggesting either reduced mitochondrial superoxide production or enhanced antioxidant buffering, consistent with an adaptive state that maintains redox homeostasis. However, after two weeks of treatment, MitoSOX Red signal markedly increased, indicating elevated mitochondrial ROS levels. This suggests the failure of the initial compensatory response to counteract prolonged HGA-induced stress. At this later stage, mitochondria appear to undergo functional decline, potentially involving impaired electron transport, reduced mitochondrial membrane potential ($\Delta\Psi m$) stability, and/or exhaustion of antioxidant capacity (Zorov et al., 2014). Overall, these findings support a model in which moderate oxidative stress initially induces a protective fusion-mediated adaptation, followed by progressive mitochondrial dysfunction and increased oxidative damage during prolonged exposure. The data support the hypothesis of an alteration in the autophagic flux. Previous research showed a time-dependent effect induced by HGA on autophagy regulatory mechanisms in AKU (Braconi et al., 2015). At first, HGA stimulates autophagy, which appears to act as a protective response against cellular stress. However, under prolonged exposure, HGA induces oxidative stress and mitochondrial damage, leading to a reduction in autophagic efficiency (Milella et al., 2024). As a result, cells are no longer able to maintain homeostasis and progressively undergo chondroptosis, a specialized form of programmed cell death observed in cartilage (Roach et al., 2004). This process is accompanied by the accumulation of ochronotic pigment, the hallmark of AKU. Overall, the findings demonstrate that chronic exposure to HGA disrupts autophagy, promotes oxidative damage, and contributes directly to cartilage degeneration in AKU.

Autophagy alterations were further investigated by immunofluorescence following treatment with 0.1mM HGA for 1 and 2 weeks. As autophagy markers, LC3 was selected as an indicator of autophagosome formation (Glick et al., 2010), while p62 was used as key adaptor protein involved in the recognition and sequestration of cellular waste (Lamark and Johansen, 2012). Their expression and colocalization were analyzed together with LAMP1. LAMP1 is a protein located on the lysosomal membrane, essential organelles that, thanks to their acid pH and to their proteolic enzymes, fuse with

the autophagosome allowing the enzymatic digestion (Eskelinen, 2006). Immunofluorescence analysis of fixed cells showed increased levels of LC3 and p62 in HGA-treated cells, confirming the induction of autophagy under HGA treatment. An increase in LAMP1 expression was observed after 1 week of treatment compared to control cells, indicating enhanced lysosomal activity, and remained elevated after 2 weeks of HGA treatment. Additionally, after 2 weeks, LC3 expression was reduced, while p62 accumulated, showing an alteration of the autophagic flux. Colocalization between LC3 and LAMP1, as well as p62 and LAMP1, was analyzed at both time points using Icy (GcoPS plugin) and ImageJ (Colocalization plugin). Increased colocalization between p62 and LAMP1 was observed in HGA-treated cells, as expected. However, even if colocalization between LC3 and LAMP1 increased after 1 week of HGA treatment, consistent with enhanced autophagic activity, it markedly decreased after 2 weeks of treatment. This reduction indicates impaired fusion between autophagosomes and lysosomes, potentially due to a reduced generation of lysosomes as suggested by LAMP1 expression, or or to defects in autophagosome maturation (Mizushima et al., 2010).

To further investigate this aspect, time-course analysis of HGA-treated cells was performed by fluorescent staining of functional lysosomes with Lysotracker Red DND-99, at 24h, 1week and 2 weeks of treatment with 0.1mM HGA. To assess lysosomal activity, fluorescence intensity and distribution were analyzed using interactive 3D surface plot analysis. An increase in fluorescence intensity and lysosomal distribution was observed in early time points, with a peak at 1 week of treatment. After 2 weeks of HGA exposure, Lysotracker staining significantly decrease, reaching levels even lower compared to the control, indicative of a reduction in lysosomal activity induced by the chronic oxidative stress condition.

This result was directly confirmed in living cartilage isolated from an AKU patient biopsy directly after surgery. Different regions of AKU cartilage were examined using Lysotracker and Hoechst staining. Areas with positive nuclear staining, but markedly reduced or absent LysoTracker fluorescence were identified, reflecting impaired lysosomal activity (Millucci et al., 2012). To assess whether this event was linked to other alterations, cells were analyzed by live-cell imaging, using the

lysotracker in combination with cell transfection with Baculovirus for LC3-GFP, to evaluate autophagosome formation and fusion with lysosomes. In HGA-treated C20/A4 cells, an increase in LC3-GFP expression was observed at both 24h and 1 week of treatment, confirming the induction of autophagy. However, after 2 weeks of treatment, LC3-GFP expression was comparable to that of control cells. Colocalization between LC3-GFP and LysoTracker was analyzed; after 24 h of treatment, the colocalization score between the two markers resulted similar in control and HGA-treated cells, whereas it drastically decreased after 1 week of HGA treatment. GFP is sensitive to lysosomal pH (Pankiv et al., 2007), and its signal decreases or disappears during the fusion between autophagosome and lysosome (Kimura et al., 2009), coherently with the observed results. After 2 weeks of treatment, the colocalization between the 2 dyes appeared to be again similar, due to impossibility of the GFP to be spent by lysosomal pH, showing alteraction of lysosome functionality and accumulation of inactive mature autophagosome, ultimately leading to autophagy inefficiency (Yoshii and Mizushima, 2017). Overall, these results show evidence that chronic HGA exposure disrupts the balance between oxidative stress, mitochondrial activity, and autophagic flux. The progression from adaptive stress responses to organelle dysfunction and impaired autophagy contributes directly to chondrocyte degeneration and cartilage damage characteristic of AKU.

References

Albatayneh, E.M., Al-Sbou, M.S., Mahgoub, S.S., Mwafi, N.R., Alnawaiseh, N.A., 2019. Serum Oxidative-Antioxidative Status in Patients With Alkaptonuria. J Clin Med Res 11, 337–344. https://doi.org/10.14740/jocmr3801

Aliberti, G., Pulignano, I., Schiappoli, A., Minisola, S., Romagnoli, E., Proietta, M., 2003. Bone metabolism in ochronotic patients. J Intern Med 254, 296–300. https://doi.org/10.1046/j.1365-2796.2003.01145.x

Ansari, M.Y., Ball, H.C., Wase, S.J., Novak, K., Haqqi, T.M., 2021. Lysosomal dysfunction in osteoarthritis and aged cartilage triggers apoptosis in chondrocytes through BAX mediated release of Cytochrome c. Osteoarthritis Cartilage 29, 100–112. https://doi.org/10.1016/j.joca.2020.08.014

Ballabio, A., Bonifacino, J.S., 2020. Lysosomes as dynamic regulators of cell and organismal homeostasis. Nat Rev Mol Cell Biol. https://doi.org/10.1038/s41580-019-0185-4

Bardaweel, S.K., Gul, M., Alzweiri, M., Ishaqat, A., Alsalamat, H.A., Bashatwah, R.M., 2018. Reactive oxygen species: The dual role in physiological and pathological conditions of the human body. Eurasian Journal of Medicine. https://doi.org/10.5152/eurasianjmed.2018.17397

Barone, H., Bliksrud, Y.T., Elgen, I.B., Szigetvari, P.D., Kleppe, R., Ghorbani, S., Hansen, E. V., Haavik, J., 2020. Tyrosinemia Type 1 and symptoms of ADHD: Biochemical mechanisms and implications for treatment and prognosis. American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics 183, 95–105. https://doi.org/10.1002/ajmg.b.32764

Bernardini, G., Braconi, D., Spreafico, A., Santucci, A., 2012. Post-genomics of bone metabolic dysfunctions and neoplasias. Proteomics. https://doi.org/10.1002/pmic.201100358

Bernardini, G., Braconi, D., Zatkova, A., Sireau, N., Kujawa, M.J., Introne, W.J., Spiga, O., Geminiani, M., Gallagher, J.A., Ranganath, L.R., Santucci, A., 2024. Alkaptonuria. Nat Rev Dis Primers 10. https://doi.org/10.1038/s41572-024-00498-x

Bernardini, G., Laschi, M., Geminiani, M., Braconi, D., Vannuccini, E., Lupetti, P., Manetti, F., Millucci, L., Santucci, A., 2015. Homogentisate 1,2 dioxygenase is expressed in brain: implications in alkaptonuria. J Inherit Metab Dis 38, 807–814. https://doi.org/10.1007/s10545-015-9829-5

Bernini, A., Galderisi, S., Spiga, O., Amarabom, C.O., Santucci, A., 2020. Transient pockets as mediators of gas molecules routes inside proteins: The case study of dioxygen pathway in homogentisate 1,2-dioxygenase and its implication in Alkaptonuria development. Comput Biol Chem 88. https://doi.org/10.1016/j.compbiolchem.2020.107356

Bernini, A., Galderisi, S., Spiga, O., Bernardini, G., Niccolai, N., Manetti, F., Santucci, A., 2017. Toward a generalized computational workflow for exploiting transient pockets as new targets for small molecule stabilizers: Application to the homogentisate 1,2-dioxygenase mutants at the base of rare disease Alkaptonuria. Comput Biol Chem 70, 133–141. https://doi.org/10.1016/j.compbiolchem.2017.08.008

Bernini, A., Petricci, E., Atrei, A., Baratto, M.C., Manetti, F., Santucci, A., 2021. A molecular spectroscopy approach for the investigation of early phase ochronotic pigment development in Alkaptonuria. Scientific Reports 2021 11:1 11, 1–14. https://doi.org/10.1038/s41598-021-01670-z

Biosa, A., Arduini, I., Soriano, M.E., Giorgio, V., Bernardi, P., Bisaglia, M., Bubacco, L., 2018. Dopamine Oxidation Products as Mitochondrial Endotoxins, a Potential Molecular Mechanism for Preferential Neurodegeneration in Parkinson's Disease. ACS Chem Neurosci 9, 2849–2858. https://doi.org/10.1021/acschemneuro.8b00276

Blanco, F.J., Ochs, R.L., Schwarz, H., Lotz, M., 1995. Chondrocyte Apoptosis Induced by Nitric Oxide Materials and Methods Chondrocyte Isolation and Culture, American journal of Pathology.

Bolte, S., Cordelières, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc. https://doi.org/10.1111/j.1365-2818.2006.01706.x

Braconi, D., Bernardini, G., Bianchini, C., Laschi, M., Millucci, L., Amato, L., Tinti, L., Serchi, T., Chellini, F., Spreafico, A., Santucci, A., 2012. Biochemical and proteomic characterization of alkaptonuric chondrocytes. J Cell Physiol 227, 3333–3343. https://doi.org/10.1002/jcp.24033

Braconi, D., Bernardini, G., Paffetti, A., Millucci, L., Geminiani, M., Laschi, M., Frediani, B., Marzocchi, B., Santucci, A., 2016. Comparative proteomics in alkaptonuria provides insights into inflammation and oxidative stress. Int J Biochem Cell Biol 81, 271–280. https://doi.org/10.1016/J.BIOCEL.2016.08.016

Braconi, D., Bianchini, C., Bernardini, G., Laschi, M., Millucci, L., Spreafico, A., Santucci, A., 2011. Redox-proteomics of the effects of homogentisic acid in an in vitro human serum model of alkaptonuric ochronosis. J Inherit Metab Dis 34, 1163–1176. https://doi.org/10.1007/s10545-011-9377-6

Braconi, D., Geminiani, M., Psarelli, E.E., Giustarini, D., Marzocchi, B., Rossi, R., Bernardini, G., Spiga, O., Gallagher, J.A., Sang, K.H.L.Q., Arnoux, J.B., Imrich, R., Al-Sbou, M.S., Gornall, M., Jackson, R., Ranganath, L.R., Santucci, A., 2022. Effects of Nitisinone on Oxidative and Inflammatory Markers in Alkaptonuria: Results from SONIA1 and SONIA2 Studies. Cells 11, 3668. https://doi.org/10.3390/CELLS11223668/S1

Braconi, D., Giustarini, D., Marzocchi, B., Peruzzi, L., Margollicci, M., Rossi, R., Bernardini, G., Millucci, L., Gallagher, J.A., Le Quan Sang, K.H., Imrich, R., Rovensky, J., Al-Sbou, M., Ranganath, L.R., Santucci, A., 2018. Inflammatory and oxidative stress biomarkers in alkaptonuria: data from the DevelopAKUre project. Osteoarthritis Cartilage 26, 1078–1086. https://doi.org/10.1016/j.joca.2018.05.017

Braconi, D., Laschi, M., Amato, L., Bernardini, G., Millucci, L., Marcolongo, R., Cavallo, G., Spreafico, A., Santucci, A., 2010a. Evaluation of anti-oxidant treatments in an in vitro model of alkaptonuric ochronosis. Rheumatology 49, 1975–1983. https://doi.org/10.1093/rheumatology/keq175

Braconi, D., Laschi, M., Taylor, A.M., Bernardini, G., Spreafico, A., Tinti, L., Gallagher, J.A., Santucci, A., 2010b. Proteomic and redox-proteomic evaluation of homogentisic acid and ascorbic acid effects on human articular chondrocytes. J Cell Biochem 111, 922–932. https://doi.org/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. https://doi.org/10.1016/j.freeradbiomed.2015.02.021

Braconi, D., Millucci, L., Bernini, A., Spiga, O., Lupetti, P., Marzocchi, B., Niccolai, N., Bernardini, G., Santucci, A., 2017. Homogentisic acid induces aggregation and fibrillation of amyloidogenic proteins. Biochim Biophys Acta Gen Subj 1861, 135–146. https://doi.org/10.1016/j.bbagen.2016.11.026

Braconi, D., Millucci, L., Ghezzi, L., Santucci, A., 2014. Redox proteomics gives insights into the role of oxidative stress in alkaptonuria. http://dx.doi.org/10.1586/14789450.2013.858020 10, 521–535. https://doi.org/10.1586/14789450.2013.858020

Castagna, A., Giombini, A., Vinanti, G., Massazza, G., Pigozzi, F., 2006. Arthroscopic treatment of shoulder ochronotic arthropathy: A case report and review of literature. Knee Surgery, Sports Traumatology, Arthroscopy 14, 176–181. https://doi.org/10.1007/s00167-005-0657-2

Chen, A.F., Davies, C.M., De Lin, M., Fermor, B., 2008. Oxidative DNA damage in osteoarthritic porcine articular cartilage. J Cell Physiol 217, 828–833. https://doi.org/10.1002/jcp.21562

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., Zhao, L., 2018. Oncotarget 7204 www.impactjournals.com/oncotarget Inflammatory responses and inflammation-associated diseases in organs, Oncotarget.

Chen, S., Li, Q., Shi, H., Li, F., Duan, Y., Guo, Q., 2024. New insights into the role of mitochondrial dynamics in oxidative stress-induced diseases. Biomedicine and Pharmacotherapy. https://doi.org/10.1016/j.biopha.2024.117084

Cicaloni, V., Spiga, O., Dimitri, G.M., Maiocchi, R., Millucci, L., Giustarini, D., Bernardini, G., Bernini, A., Marzocchi, B., Braconi, D., Santucci, A., 2019. Interactive alkaptonuria database: investigating clinical data to improve patient care in a rare disease. FASEB Journal 33, 12696–12703. https://doi.org/10.1096/fj.201901529R

Cuervo, A.M., Bergamini, E., Brunk, U.T., Dröge, W., Ffrench, M., Terman, A., 2005. Autophagy and aging: the importance of maintaining "clean" cells. Autophagy. https://doi.org/10.4161/auto.1.3.2017

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., Milzani, A., 2006. Biomarkers of oxidative damage in human disease. Clin Chem. https://doi.org/10.1373/clinchem.2005.061408

De Almeida, A.J.P.O., De Oliveira, J.C.P.L., Da Silva Pontes, L.V., De Souza Júnior, J.F., Gonçalves, T.A.F., Dantas, S.H., De Almeida Feitosa, M.S., Silva, A.O., De Medeiros, I.A., 2022. ROS: Basic Concepts, Sources, Cellular Signaling, and its Implications in Aging Pathways. Oxid Med Cell Longev. https://doi.org/10.1155/2022/1225578

De Andrade, R.B., Gemelli, T., Rojas, D.B., Funchal, C., Dutra-Filho, C.S., Wannmacher, C.M.D., 2012. Tyrosine impairs enzymes of energy metabolism in cerebral cortex of rats. Mol Cell Biochem 364, 253–261. https://doi.org/10.1007/s11010-012-1225-y

De Haas, V., Carbasius Weber, E.C., De Klerk, J.B.C., Bakker, H.D., Smit, G.P.A., Huijbers, W.A.R., Duran, M., Poll-The, B.T., 1998. The success of dietary protein restriction in alkaptonuria patients is age-dependent. J Inherit Metab Dis 21, 791–798. https://doi.org/10.1023/A:1005410416482

Dikic, I., Elazar, Z., 2018. Mechanism and medical implications of mammalian autophagy. Nat Rev Mol Cell Biol. https://doi.org/10.1038/s41580-018-0003-4

Du, B.N. La, Zannoni, V.G., Laster, L., Seegmiller, J.E., n.d. THE NATURE OF THE DEFECT IN TYROSINE METABOLISM IN ALCAPTONURIA.

Eskelinen, E.L., 2006. Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Mol Aspects Med. https://doi.org/10.1016/j.mam.2006.08.005

Fedorova, M., Bollineni, R.C., Hoffmann, R., 2014. Protein carbonylation as a major hallmark of oxidative damage: Update of analytical strategies. Mass Spectrom Rev. https://doi.org/10.1002/mas.21381

Feoktistova, M., Geserick, P., Leverkus, M., 2016. Crystal Violet Assay for Determining Viability of Cultured Cells. Cold Spring Harb Protoc 2016, pdb.prot087379. https://doi.org/10.1101/pdb.prot087379

Ferná Ndez-Cañ, J.M., Peñ Alva, M.A., 1995. Molecular Characterization of a Gene Encoding a Homogentisate Dioxygenase from Aspergillus nidulans and Identification of Its Human and Plant Homologues*.

Ferreira, C.R., 2019. The burden of rare diseases. Am J Med Genet A. https://doi.org/10.1002/ajmg.a.61124

Filomeni, G., De Zio, D., Cecconi, F., 2015. Oxidative stress and autophagy: The clash between damage and metabolic needs. Cell Death Differ. https://doi.org/10.1038/cdd.2014.150

Fisher, A.A., Davis, M.W., 2004. Alkaptonuric Ochronosis with Aortic Valve and Joint Replacements and Femoral Fracture: A Case Report and Literature Review. Clin Med Res 2, 209–215. https://doi.org/10.3121/cmr.2.4.209

Flaharty, K.A., Chandrasekar, V., Castillo, I.J., Duong, D., Ferreira, C.R., Hanchard, S.L., Hu, P., Waikel, R.L., Rossignol, F., Introne, W.J., Solomon, B.D., 2025. Deep Learning Study of Alkaptonuria Spinal Disease Assesses Global and Regional Severity and Detects Occult Treatment Status. https://doi.org/10.1101/2025.03.11.25323762

Fogo, G.M., Raghunayakula, S., Emaus, K.J., Torres Torres, F.J., Wider, J.M., Sanderson, T.H., 2024. Mitochondrial membrane potential and oxidative stress interact to regulate Oma1-dependent processing of Opa1 and mitochondrial dynamics. FASEB Journal 38. https://doi.org/10.1096/fj.202400313R

Forrester, S.J., Kikuchi, D.S., Hernandes, M.S., Xu, Q., Griendling, K.K., 2018. Reactive oxygen species in metabolic and inflammatory signaling. Circ Res. https://doi.org/10.1161/CIRCRESAHA.117.311401

Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., Ferrucci, L., Gilroy, D.W., Fasano, A., Miller, G.W., Miller, A.H., Mantovani, A., Weyand, C.M., Barzilai, N., Goronzy, J.J., Rando, T.A., Effros, R.B., Lucia, A., Kleinstreuer, N., Slavich, G.M., 2019. Chronic inflammation in the etiology of disease across the life span. Nat Med 25, 1822–1832. https://doi.org/10.1038/s41591-019-0675-0

Gaines, J.J., n.d. The Pathology of Alkaptonuric Ochronosis.

Galderisi, S., Cicaloni, V., Milella, M.S., Millucci, L., Geminiani, M., Salvini, L., Tinti, L., Tinti, C., Vieira, O. V, Alves, L.S., Crevenna, A.H., Spiga, O., Santucci, A., 2021. Homogentisic acid induces cytoskeleton and extracellular matrix alteration in alkaptonuric cartilage. J Cell Physiol 236, 6011–6024. https://doi.org/10.1002/JCP.30284

Galderisi, S., Milella, M.S., Rossi, M., Cicaloni, V., Rossi, R., Giustarini, D., Spiga, O., Tinti, L., Salvini, L., Tinti, C., Braconi, D., Millucci, L., Lupetti, P., Prischi, F., Bernardini, G., Santucci, A., 2022. Homogentisic acid induces autophagy alterations leading to chondroptosis in human chondrocytes: Implications in Alkaptonuria. Arch Biochem Biophys 717. https://doi.org/10.1016/j.abb.2022.109137

Gallagher, J.A., Ranganath, L.R., Boyde, A., 2015. Lessons from rare diseases of cartilage and bone. Curr Opin Pharmacol. https://doi.org/10.1016/j.coph.2015.04.002

Gambassi, S., Geminiani, M., Thorpe, S.D., Bernardini, G., Millucci, L., Braconi, D., Orlandini, M., Thompson, C.L., Petricci, E., Manetti, F., Taddei, M., Knight, M.M., Santucci, A., 2017. Smoothened-antagonists reverse homogentisic acid-induced alterations of Hedgehog signaling and primary cilium length in alkaptonuria. J Cell Physiol 232, 3103–3111. https://doi.org/10.1002/JCP.25761

Gammie, T., Lu, C.Y., Ud-Din Babar, Z., 2015. Access to orphan drugs: A comprehensive review of legislations, regulations and policies in 35 countries. PLoS One. https://doi.org/10.1371/journal.pone.0140002

Geminiani, M., Gambassi, S., Millucci, L., Lupetti, P., Collodel, G., Mazzi, L., Frediani, B., Braconi, D., Marzocchi, B., Laschi, M., Bernardini, G., Santucci, A., 2017. Cytoskeleton Aberrations in Alkaptonuric Chondrocytes. J Cell Physiol. https://doi.org/10.1002/jcp.25500

Giustarini, D., Dalle-Donne, I., Lorenzini, S., Selvi, E., Colombo, G., Milzani, A., Fanti, P., Rossi, R., 2012. Protein thiolation index (PTI) as a biomarker of oxidative stress. Free Radic Biol Med 53, 907–915. https://doi.org/10.1016/j.freeradbiomed.2012.06.022

Glick, D., Barth, S., Macleod, K.F., 2010. Autophagy: Cellular and molecular mechanisms. Journal of Pathology. https://doi.org/10.1002/path.2697

Goldring, M.B., Goldring, S.R., 2007. Osteoarthritis. J Cell Physiol. https://doi.org/10.1002/jcp.21258

Granadino, B., Beltrá N-Valero De Bernabé, D., Ferná Ndez-Cañ, J.M., Peñ Alva, M.A., Rodríguez De Có, S., 1997. The Human Homogentisate 1,2-Dioxygenase (HGO) Gene, GENOMICS.

Grasso, D., Balloni, V., Baratto, M.C., Mucci, A., Santucci, A., Bernini, A., 2025. Ochronotic Deposition in Alkaptonuria: Semiquinone-Mediated Oxidative Coupling and Metabolic Drivers of Homogentisic Acid Accumulation. Int J Mol Sci 26. https://doi.org/10.3390/ijms26199674

Green, D.R., Kroemer, G., 2004. The pathophysiology of mitochondrial cell death. Science (1979). https://doi.org/10.1126/science.1099320

Gutzmer, R., Herbst, R.A., Kiehl, P., Kapp, A., Weiss, J., 1997. Alkaptonuric ochronosis: brothers Report of two affected.

Haines, M.S., Ramirez, E., Moore, K.B.E., Fortin, J.S., 2022. Revisiting misfolding propensity of serum amyloid A1: Special focus on the signal peptide region. Biochem Biophys Rep 31. https://doi.org/10.1016/j.bbrep.2022.101284

Hanika, C., Hornphutkul, P., Ntrone, E.J.I., Onique, M., Erry, B.P., Sa, I., Ernardini, B., Ark, M., Urphey, D.M., Itzpatrick, I.L.F., Nderson, A.D.A., Arjan, M., Uizing, H., Nikster, A.A., Erber, Y.H.G., Ahl, I.A.G., 2002. NATURAL HISTORY OF ALKAPTONURIA A BSTRACT Background Alkaptonuria, caused by mutations, N Engl J Med.

Hara, H., Naito, M., Harada, T., Tsuboi, I., Terui, T., Aizawa, S., 2016. Quantitative analysis of formaldehyde-induced fluorescence in paraffin-embedded specimens of malignant melanomas and other melanocytic lesions. Acta Derm Venereol 96, 309–313. https://doi.org/10.2340/00015555-2238

He, Y., Fan, Y., Ahmadpoor, X., Wang, Y., Li, Z.A., Zhu, W., Lin, H., 2024. Targeting lysosomal quality control as a therapeutic strategy against aging and diseases. Med Res Rev. https://doi.org/10.1002/med.22047

Helliwell, T.R., Gallagher, J.A., Ranganath, L., 2008. Alkaptonuria - A review of surgical and autopsy pathology. Histopathology. https://doi.org/10.1111/j.1365-2559.2008.03000.x

Henrotin, Y.E., Bruckner, P., Pujol, J.P.L., 2003. The role of reactive oxygen species in homeostasis and degradation of cartilage. Osteoarthritis Cartilage 11, 747–755. https://doi.org/10.1016/S1063-4584(03)00150-X

Hetz, C., Papa, F.R., 2018. The Unfolded Protein Response and Cell Fate Control. Mol Cell. https://doi.org/10.1016/j.molcel.2017.06.017

Hong, Y., Boiti, A., Vallone, D., Foulkes, N.S., 2024. Reactive Oxygen Species Signaling and Oxidative Stress: Transcriptional Regulation and Evolution. Antioxidants. https://doi.org/10.3390/antiox13030312

Hurley, J.H., Young, L.N., 2017. Mechanisms of autophagy initiation. Annu Rev Biochem 86, 225–244. https://doi.org/10.1146/annurev-biochem-061516-044820

Introne, W.J., Perry, M., Chen, M., 2003. Alkaptonuria Synonym: Alcaptonuria.

Jeon, H., Im, G. Il, 2017. Autophagy in osteoarthritis. Connect Tissue Res. https://doi.org/10.1080/03008207.2016.1240790

Jiang, H., Ji, P., Shang, X., Zhou, Y., 2023. Connection between Osteoarthritis and Nitric Oxide: From Pathophysiology to Therapeutic Target. Molecules. https://doi.org/10.3390/molecules28041683

Kabuyama, Y., Kitamura, T., Yamaki, J., Homma, M.K., Kikuchi, S. ichi, Homma, Y., 2008. Involvement of thioredoxin reductase 1 in the regulation of redox balance and viability of rheumatoid synovial cells. Biochem Biophys Res Commun 367, 491–496. https://doi.org/10.1016/j.bbrc.2007.12.178

Kan, S., Duan, M., Liu, Y., Wang, C., Xie, J., 2021. Role of Mitochondria in Physiology of Chondrocytes and Diseases of Osteoarthritis and Rheumatoid Arthritis. Cartilage. https://doi.org/10.1177/19476035211063858

Karsdal, M.A., Nielsen, M.J., Sand, J.M., Henriksen, K., Genovese, F., Bay-Jensen, A.C., Smith, V., Adamkewicz, J.I., Christiansen, C., Leeming, D.J., 2013. Extracellular matrix remodeling: The common denominator in connective tissue diseases possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. Assay Drug Dev Technol. https://doi.org/10.1089/adt.2012.474

Katsuragi, Y., Ichimura, Y., Komatsu, M., 2015. P62/SQSTM1 functions as a signaling hub and an autophagy adaptor. FEBS Journal. https://doi.org/10.1111/febs.13540

Kimura, S., Fujita, N., Noda, T., Yoshimori, T., 2009. Chapter 1 Monitoring Autophagy in Mammalian Cultured Cells through the Dynamics of LC3. Methods Enzymol. https://doi.org/10.1016/s0076-6879(08)03601-x

Knowles, T.P.J., Mezzenga, R., 2016. Amyloid fibrils as building blocks for natural and artificial functional materials. Advanced Materials. https://doi.org/10.1002/adma.201505961

Knox, W.E., Edwardst, S.W., n.d. THE PROPERTIES OF MALEYLACETOACETATE, THE INITIAL PRODUCT OF HOMOGENTISATE OXIDATION IN LIVER*.

Kratka, K., Sistik, P., Olivkova, I., Kusnierova, P., Svagera, Z., Stejskal, D., 2025. Mass Spectrometry–Based Proteomics in Clinical Diagnosis of Amyloidosis and Multiple Myeloma: A Review (2012–2024). Journal of Mass Spectrometry. https://doi.org/10.1002/jms.5116

Lachmann, H.J., Goodman, H.J., Gilbertson, J.A., Ruth Gallimore, J., Sabin, C.A., Gillmore, J.D., Hawkins, P.N., MedSci, F., 2007. Natural History and Outcome in Systemic AA Amyloidosis, N Engl J Med.

Ladjouze-Rezig, A., Rodriguez de Cordoba, S., Aquaron, R., 2006. Ochronotic rheumatism in Algeria: clinical, radiological, biological and molecular studies-a case study of 14 patients in 11 families. Joint Bone Spine 73, 284–292. https://doi.org/10.1016/j.jbspin.2005.03.010

Lamark, T., Johansen, T., 2012. Aggrephagy: Selective disposal of protein aggregates by macroautophagy. Int J Cell Biol. https://doi.org/10.1155/2012/736905

Laschi, M., Bernardini, G., Dreassi, E., Millucci, L., Geminiani, M., Braconi, D., Marzocchi, B., Botta, M., Manetti, F., Santucci, A., 2016. Inhibition of para-Hydroxyphenylpyruvate Dioxygenase by Analogues of the Herbicide Nitisinone As a Strategy to Decrease Homogentisic Acid Levels, the Causative Agent of Alkaptonuria. ChemMedChem 11, 674–678. https://doi.org/10.1002/cmdc.201500578

Laschi, M., Tinti, L., Braconi, D., Millucci, L., Ghezzi, L., Amato, L., Selvi, E., Spreafico, A., Bernardini, G., Santucci, A., 2012. Homogentisate 1,2 dioxygenase is expressed in human osteoarticular cells: Implications in alkaptonuria. J Cell Physiol 227, 3254–3257. https://doi.org/10.1002/jcp.24018

Lavancier, F., Pécot, T., Zengzhen, L., Kervrann, C., 2019. Testing independence between two random sets for the analysis of colocalization in bio-imaging.

Lee, J., Beatty, G.L., 2021. Serum amyloid A proteins and their impact on metastasis and immune biology in cancer. Cancers (Basel). https://doi.org/10.3390/cancers13133179

Lequeue, S., Allach, H., Nulmans, I., Desmet, L., Salvi, N.S., Rombaut, M., Neuckermans, J., Schwaneberg, U., Vanhaecke, T., De Kock, J., 2025. A robust bacterial high-throughput

screening assay to identify pharmacological chaperones targeting human homogentisate 1,2-dioxygenase missense variants in alkaptonuria. Eur J Pharmacol 1005. https://doi.org/10.1016/j.ejphar.2025.178048

Lequeue, S., Neuckermans, J., Nulmans, I., Schwaneberg, U., Vanhaecke, T., De Kock, J., 2022. A robust bacterial high-throughput screening system to evaluate single nucleotide polymorphisms of human homogentisate 1,2-dioxygenase in the context of alkaptonuria. Sci Rep 12. https://doi.org/10.1038/s41598-022-23702-y

Li, A., Gao, M., Liu, B., Qin, Y., chen, L., Liu, H., Wu, H., Gong, G., 2022. Mitochondrial autophagy: molecular mechanisms and implications for cardiovascular disease. Cell Death Dis. https://doi.org/10.1038/s41419-022-04906-6

Li, L., Li, J., Li, J.J., Zhou, H., Zhu, X.W., Zhang, P.H., Huang, B., Zhao, W.T., Zhao, X.F., Chen, E.S., 2024. Chondrocyte autophagy mechanism and therapeutic prospects in osteoarthritis. Front Cell Dev Biol. https://doi.org/10.3389/fcell.2024.1472613

Li, W., Luo, L.X., Zhou, Q.Q., Gong, H.B., Fu, Y.Y., Yan, C.Y., Li, E., Sun, J., Luo, Z., Ding, Z.J., Zhang, Q.Y., Mu, H.L., Cao, Y.F., Ouyang, S.H., Kurihara, H., Li, Y.F., Sun, W.Y., Li, M., He, R.R., 2022. Phospholipid peroxidation inhibits autophagy via stimulating the delipidation of oxidized LC3-PE. Redox Biol 55. https://doi.org/10.1016/j.redox.2022.102421

Lie, P.P.Y., Nixon, R.A., 2019. Lysosome trafficking and signaling in health and neurodegenerative diseases. Neurobiol Dis. https://doi.org/10.1016/j.nbd.2018.05.015

Liguori, I., Russo, G., Curcio, F., Bulli, G., Aran, L., Della-Morte, D., Gargiulo, G., Testa, G., Cacciatore, F., Bonaduce, D., Abete, P., 2018. Oxidative stress, aging, and diseases. Clin Interv Aging. https://doi.org/10.2147/CIA.S158513

Lin, H.Y., Tan, G.Q., Liu, Y., Lin, S.Q., 2019. The prognostic value of serum amyloid A in solid tumors: A meta-analysis. Cancer Cell Int 19. https://doi.org/10.1186/s12935-019-0783-4

Liu, X., Hussain, R., Mehmood, K., Tang, Z., Zhang, H., Li, Y., 2022. Mitochondrial-Endoplasmic Reticulum Communication-Mediated Oxidative Stress and Autophagy. Biomed Res Int. https://doi.org/10.1155/2022/6459585

Liu, Y., Mao, Z.H., Huang, J., Wang, H., Zhang, X., Zhou, X., Xu, Y., Pan, S., Liu, D., Liu, Z., Feng, Q., 2025. Mitochondria-Associated Endoplasmic Reticulum Membranes in Human Health and Diseases. MedComm (Beijing). https://doi.org/10.1002/mco2.70259

Lock, E., Ranganath, L.R., Timmis, O., 2014. The Role of Nitisinone in Tyrosine Pathway Disorders. Curr Rheumatol Rep. https://doi.org/10.1007/s11926-014-0457-0

Loyd R Wagner, -ochronosis BY, Knott, J.L., Machaffie, R.A., Walsh, J.R., 1960. CLINICAL AND PATHOLOGICAL FINDINGS IN, J. clin. Path.

Lu, Y., Zhou, J., Wang, H., Gao, H., Ning, E., Shao, Z., Hao, Y., Yang, X., 2024. Endoplasmic reticulum stress-mediated apoptosis and autophagy in osteoarthritis: From molecular mechanisms to therapeutic applications. Cell Stress Chaperones. https://doi.org/10.1016/j.cstres.2024.11.005

Ma, Y.J., Garred, P., 2018. Pentraxins in Complement Activation and Regulation. Front Immunol. https://doi.org/10.3389/fimmu.2018.03046

Malle, E., Sodin-Semrl, S., Wcislo-Dziadecka, A., 2009. Serum amyloid A: An acute-phase protein involved in tumour pathogenesis. Cellular and Molecular Life Sciences. https://doi.org/10.1007/s00018-008-8321-x

Mannoni, A., Selvi, E., Lorenzini, S., Giorgi, M., Airò, P., Cammelli, D., Andreotti, L., Marcolongo, R., Porfirio, B., 2004. Alkaptonuria, Ochronosis, and Ochronotic Arthropathy. Semin Arthritis Rheum 33, 239–248. https://doi.org/10.1016/S0049-0172(03)00080-5

Marimpietri, D., Zuccari, G., 2023. Development of Medicines for Rare Pediatric Diseases. Pharmaceuticals. https://doi.org/10.3390/ph16040513

Martel-Pelletier, J., Barr, A.J., Cicuttini, F.M., Conaghan, P.G., Cooper, C., Goldring, M.B., Goldring, S.R., Jones, G., Teichtahl, A.J., Pelletier, J.P., 2016. Osteoarthritis. Nat Rev Dis Primers. https://doi.org/10.1038/nrdp.2016.72

Martin, J.P., Batkoff, B., n.d. HOMOGENTISIC ACID AUTOXIDATION AND OXYGEN RADICAL GENERATION: IMPLICATIONS FOR THE ETIOLOGY OF ALKAPTONURIC ARTHRITIS, Free Radical Biology & Medicine.

Mathieu, P., Prevel, A., David, L., Bergeron, C., Bory, C., Chauvin, F., Dia, S., Frappaz, D., Greffe, J., Mathieu, M., Philip, T., 1997. Screening infants for neuroblastoma: discovery of alkaptonuria in one case, Clinica Chimica Acta.

Merlini, G., Bellotti, V., 2003. Molecular Mechanisms of Amyloidosis, n engl j med.

Milella, M.S., Geminiani, M., Trezza, A., Visibelli, A., Braconi, D., Santucci, A., 2024. Alkaptonuria: From Molecular Insights to a Dedicated Digital Platform. Cells 13, 1072. https://doi.org/10.3390/cells13121072

Millucci, L., Bernardini, G., Marzocchi, B., Braconi, D., Geminiani, M., Gambassi, S., Laschi, M., Frediani, B., Galvagni, F., Orlandini, M., Santucci, A., 2016. Angiogenesis in alkaptonuria. J Inherit Metab Dis 39, 801–806. https://doi.org/10.1007/s10545-016-9976-3

Millucci, L., Bernardini, G., Spreafico, A., Orlandini, M., Braconi, D., Laschi, M., Geminiani, M., Lupetti, P., Giorgetti, G., Viti, C., Frediani, B., Marzocchi, B., Santucci, A., 2017. Histological and Ultrastructural Characterization of Alkaptonuric Tissues. Calcif Tissue Int 101, 50–64. https://doi.org/10.1007/S00223-017-0260-9/FIGURES/8

Millucci, L., Braconi, D., Bernardini, G., Lupetti, P., Rovensky, J., Ranganath, L., Santucci, A., 2015a. Amyloidosis in alkaptonuria. J Inherit Metab Dis 38, 797–805. https://doi.org/10.1007/S10545-015-9842-8

Millucci, L., Ghezzi, L., Bernardini, G., Braconi, D., Lupetti, P., Perfetto, F., Orlandini, M., Santucci, A., 2014a. Diagnosis of secondary amyloidosis in alkaptonuria. Diagn Pathol 9. https://doi.org/10.1186/s13000-014-0185-9

Millucci, L., Ghezzi, L., Braconi, D., Laschi, M., Geminiani, M., Amato, L., Orlandini, M., Benvenuti, C., Bernardini, G., Santucci, A., 2014b. Secondary amyloidosis in an alkaptonuric aortic valve. Int J Cardiol 172, e121. https://doi.org/10.1016/J.IJCARD.2013.12.117

Millucci, L., Ghezzi, L., Paccagnini, E., Giorgetti, G., Viti, C., Braconi, D., Laschi, M., Geminiani, M., Soldani, P., Lupetti, P., Orlandini, M., Benvenuti, C., Perfetto, F., Spreafico, A., Bernardini, G., Santucci, A., 2014c. Amyloidosis, inflammation, and oxidative stress in the heart of an alkaptonuric patient. Mediators Inflamm 2014. https://doi.org/10.1155/2014/258471

Millucci, L., Giorgetti, G., Viti, C., Ghezzi, L., Gambassi, S., Braconi, D., Marzocchi, B., Paffetti, A., Lupetti, P., Bernardini, G., Orlandini, M., Santucci, A., 2015b. Chondroptosis in alkaptonuric cartilage. J Cell Physiol 230, 1148–1157. https://doi.org/10.1002/jcp.24850

Millucci, L., Spreafico, A., Tinti, L., Braconi, D., Ghezzi, L., Paccagnini, E., Bernardini, G., Amato, L., Laschi, M., Selvi, E., Galeazzi, M., Mannoni, A., Benucci, M., Lupetti, P., Chellini, F., Orlandini, M., Santucci, A., 2012. Alkaptonuria is a novel human secondary amyloidogenic disease. Biochim Biophys Acta Mol Basis Dis 1822, 1682–1691. https://doi.org/10.1016/j.bbadis.2012.07.011

Mirioglu, S., Uludag, O., Hurdogan, O., Kumru, G., Berke, I., Doumas, S.A., Frangou, E., Gul, A., 2024. AA Amyloidosis: A Contemporary View. Curr Rheumatol Rep. https://doi.org/10.1007/s11926-024-01147-8

Mistry, J.B., Bukhari, M., Taylor, A.M., 2013. Alkaptonuria. Rare Diseases 1, e27475. https://doi.org/10.4161/rdis.27475

Mizushima, N., Yoshimori, T., Levine, B., 2010. Methods in Mammalian Autophagy Research. Cell. https://doi.org/10.1016/j.cell.2010.01.028

Momohara, S., Okamoto, H., Yamanaka, H., 2008. Chondrocyte of rheumatoid arthritis serve as a source of intra-articular acute-phase serum amyloid A protein. Clinica Chimica Acta. https://doi.org/10.1016/j.cca.2008.07.034

Morgan, M.J., Liu, Z.G., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res. https://doi.org/10.1038/cr.2010.178

Moshkovskii, S.A., 2012. Why do cancer cells produce serum amyloid a acute-phase protein? Biochemistry (Moscow). https://doi.org/10.1134/S0006297912040037

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochemical Journal. https://doi.org/10.1042/BJ20081386

Myhre, O., Utkilen, H., Duale, N., Brunborg, G., Hofer, T., 2013. Metal dyshomeostasis and inflammation in Alzheimer's and Parkinson's diseases: Possible impact of environmental exposures. Oxid Med Cell Longev. https://doi.org/10.1155/2013/726954

Nemethova, M., Radvanszky, J., Kadasi, L., Ascher, D.B., Pires, D.E.V., Blundell, T.L., Porfirio, B., Mannoni, A., Santucci, A., Milucci, L., Sestini, S., Biolcati, G., Sorge, F., Aurizi, C., Aquaron, R., Alsbou, M., Lourenço, C.M., Ramadevi, K., Ranganath, L.R., Gallagher, J.A., Van Kan, C., Hall, A.K., Olsson, B., Sireau, N., Ayoob, H., Timmis, O.G., Le Quan Sang, K.H., Genovese, F., Imrich, R., Rovensky, J., Srinivasaraghavan, R., Bharadwaj, S.K., Spiegel, R., Zatkova, A., 2016. Twelve novel HGD gene variants identified in 99 alkaptonuria patients: Focus on "black bone disease" in Italy. European Journal of Human Genetics 24, 66–72. https://doi.org/10.1038/ejhg.2015.60

Ng, N.S., Ooi, L., 2021. A simple microplate assay for reactive oxygen species generation and rapid cellular protein normalization. Bio Protoc 11. https://doi.org/10.21769/BioProtoc.3877

Nguengang Wakap, S., Lambert, D.M., Olry, A., Rodwell, C., Gueydan, C., Lanneau, V., Murphy, D., Le Cam, Y., Rath, A., 2020. Estimating cumulative point prevalence of rare diseases: analysis of the Orphanet database. European Journal of Human Genetics 28, 165–173. https://doi.org/10.1038/s41431-019-0508-0

Nunnari, J., Suomalainen, A., 2012. Mitochondria: In sickness and in health. Cell. https://doi.org/10.1016/j.cell.2012.02.035

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Øvervatn, A., Bjørkøy, G., Johansen, T., 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy*[S]. Journal of Biological Chemistry 282, 24131–24145. https://doi.org/10.1074/jbc.M702824200

Perera, R.M., Zoncu, R., 2016. The Lysosome as a Regulatory Hub. Annu Rev Cell Dev Biol 32, 223–253. https://doi.org/10.1146/annurev-cellbio-111315-125125

Petrov, D., Zagrovic, B., 2011. Microscopic analysis of protein oxidative damage: Effect of carbonylation on structure, dynamics, and aggregability of villin headpiece. J Am Chem Soc 133, 7016–7024. https://doi.org/10.1021/ja110577e

Pollak, M.R., Chou2, Y.-H.W., Cerda, J.J., Steinmann\, B., Du, B.N. La, Seidman, J.G., Seidman, C.E., 1993. Homozygosity mapping of the gene for alkaptonuria to chromosome 3q2.

Pompella, A., Dominici, S., Frank, J., Biesalski, H.K., n.d. Protein-Bound 4-HNE 41 41 6 Indirect Immunofl uorescence Detection of Protein-Bound 4-Hydroxynonenal in Tissue Sections and Isolated Cells.

Ranganath, L., Khedr, M., Milan, A.M., Davison, A.S., Norman, B.P., Janssen, M.C.H., Lock, E., Bou-Gharios, G., Gallagher, J.A., 2023. Increased prevalence of Parkinson's disease in alkaptonuria. JIMD Rep 64, 282–292. https://doi.org/10.1002/jmd2.12367

Ranganath, L., Psarelli, E., Arnoux, J.-B., Braconi, D., Briggs, M., Bröijersén, A., Loftus, N., Bygott, H., Cox, T., Davison, A., Dillon, J., Fisher, M., FitzGerald, R., Genovese, F., Glasova, H., Hall, A., Hughes, A., Hughes, J., Imrich, R., Gallagher, J., 2020. Efficacy and safety of once-daily nitisinone for patients with alkaptonuria (SONIA 2): an international, multicentre, open-label, randomised controlled trial. Lancet Diabetes Endocrinol 8, 762–772. https://doi.org/10.1016/S2213-8587(20)30228-X

Ranganath, L.R., Milan, A.M., Hughes, A.T., Dutton, J.J., Fitzgerald, R., Briggs, M.C., Bygott, H., Psarelli, E.E., Cox, T.F., Gallagher, J.A., Jarvis, J.C., van Kan, C., Hall, A.K., Laan, D., Olsson, B., Szamosi, J., Rudebeck, M., Kullenberg, T., Cronlund, A., Svensson, L., Junestrand, C.,

Ayoob, H., Timmis, O.G., Sireau, N., Le Quan Sang, K.-H., Genovese, F., Braconi, D., Santucci, A., Nemethova, M., Zatkova, A., McCaffrey, J., Christensen, P., Ross, G., Imrich, R., Rovensky, J., 2016. Suitability Of Nitisinone In Alkaptonuria 1 (SONIA 1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-h urinary homogentisic aci. Ann Rheum Dis 75, 362–367. https://doi.org/10.1136/annrheumdis-2014-206033

Ranganath, L.R., Milan, A.M., Hughes, A.T., Khedr, M., Davison, A.S., Shweihdi, E., Norman, B.P., Hughes, J.H., Bygott, H., Luangrath, E., Fitzgerald, R., Psarelli, E.E., van Kan, C., Laan, D., Olsson, B., Rudebeck, M., Mankowitz, L., Sireau, N., Arnoux, J.B., Le Quan Sang, K.H., Jarvis, J.C., Genovese, F., Braconi, D., Santucci, A., Zatkova, A., Glasova, H., Stančík, R., Imrich, R., Rhodes, N.P., Gallagher, J.A., 2020a. Homogentisic acid is not only eliminated by glomerular filtration and tubular secretion but also produced in the kidney in alkaptonuria. J Inherit Metab Dis 43, 737–747. https://doi.org/10.1002/jimd.12181

Ranganath, L.R., Milan, A.M., Hughes, A.T., Khedr, M., Davison, A.S., Wilson, P.J., Dillon, J.P., West, E., Gallagher, J.A., 2020b. Reversal of ochronotic pigmentation in alkaptonuria following nitisinone therapy: Analysis of data from the United Kingdom National Alkaptonuria Centre. JIMD Rep 55, 75–87. https://doi.org/10.1002/jmd2.12137

Ranganath, L.R., Norman, B.P., Gallagher, J.A., 2019. Ochronotic pigmentation is caused by homogentisic acid and is the key event in alkaptonuria leading to the destructive consequences of the disease—A review. J Inherit Metab Dis. https://doi.org/10.1002/jimd.12152

Real de Asua, D., Galvan, J.M., Filigghedu, M.T., Trujillo, D., Costa, R., Cadinanos, J., 2014. Systemic AA amyloidosis: epidemiology, diagnosis, and management. Clin Epidemiol 369. https://doi.org/10.2147/clep.s39981

Roach, H.I., Aigner, T., Kouri, J.B., 2004. Chondroptosis: A variant of apoptotic cell death in chondrocytes?, Apoptosis. Kluwer Academic Publishers.

Rocha, J.C., Martins, M.J., 2012. Oxidative stress in Phenylketonuria: Future directions. J Inherit Metab Dis. https://doi.org/10.1007/s10545-011-9417-2

Sack, G.H., 2018. Serum amyloid A - A review. Molecular Medicine. https://doi.org/10.1186/s10020-018-0047-0

Saftig, P., Klumperman, J., 2009. Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function. Nat Rev Mol Cell Biol. https://doi.org/10.1038/nrm2745

Scarpioni, R., 2016. Secondary amyloidosis in autoinflammatory diseases and the role of inflammation in renal damage. World J Nephrol 5, 66. https://doi.org/10.5527/wjn.v5.i1.66

Schaur, R.J., 2003. Basic aspects of the biochemical reactivity of 4-hydroxynonenal, in: Molecular Aspects of Medicine. Elsevier Ltd, pp. 149–159. https://doi.org/10.1016/S0098-2997(03)00009-8

Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. Current Biology. https://doi.org/10.1016/j.cub.2014.03.034

Schwarz, D.S., Blower, M.D., 2016. The endoplasmic reticulum: Structure, function and response to cellular signaling. Cellular and Molecular Life Sciences. https://doi.org/10.1007/s00018-015-2052-6

Sestini, S., Paneghetti, L., Lampe, C., Betti, G., Bond, S., Bellettato, C.M., Maurizio, S., 2021. Social and medical needs of rare metabolic patients: results from a MetabERN survey. Orphanet J Rare Dis 16. https://doi.org/10.1186/s13023-021-01948-5

Shen, P.C., Chou, S.H., Lu, C.C., Huang, H.T., Chien, S.H., Huang, P.J., Liu, Z.M., Shih, C.L., Su, S.J., Chen, L.M., Tien, Y.C., 2021. Shockwave Treatment Enhanced Extracellular Matrix Production in Articular Chondrocytes Through Activation of the ROS/MAPK/Nrf2 Signaling Pathway. Cartilage 13, 238S-253S. https://doi.org/10.1177/19476035211012465

Shimizu, I., Hamada, T., Khalpey, Z., Miyanishi, K., Hara, T., 2007. Ochronotic arthropathy: Pathological evidence of acute destruction of the hip joint. Clin Rheumatol 26, 1189–1191. https://doi.org/10.1007/s10067-006-0326-y

Sies, H., 2015. Oxidative stress: A concept in redox biology and medicine. Redox Biol. https://doi.org/10.1016/j.redox.2015.01.002

Sies, H., Jones, D.P., 2020. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat Rev Mol Cell Biol. https://doi.org/10.1038/s41580-020-0230-3

Simons, J.P., Al-Shawi, R., Ellmerich, S., Speck, I., Aslam, S., Hutchinson, W.L., Mangione, P.P., Disterer, P., Gilbertson, J.A., Hunt, T., Millar, D.J., Minogue, S., Bodin, K., Pepys, M.B., Hawkins, P.N., 2013. Pathogenetic mechanisms of amyloid A amyloidosis. Proc Natl Acad Sci U S A 110, 16115–16120. https://doi.org/10.1073/pnas.1306621110

Sinenko, S.A., Starkova, T.Y., Kuzmin, A.A., Tomilin, A.N., 2021. Physiological Signaling Functions of Reactive Oxygen Species in Stem Cells: From Flies to Man. Front Cell Dev Biol. https://doi.org/10.3389/fcell.2021.714370

Sorić Hosman, I., Kos, I., Lamot, L., 2021. Serum Amyloid A in Inflammatory Rheumatic Diseases: A Compendious Review of a Renowned Biomarker. Front Immunol. https://doi.org/10.3389/fimmu.2020.631299

Spreafico, A., Millucci, L., Ghezzi, L., Geminiani, M., Braconi, D., Amato, L., Chellini, F., Frediani, B., Moretti, E., Collodel, G., Bernardini, G., Santucci, A., 2013a. Antioxidants inhibit SAA formation and pro-inflammatory cytokine release in a human cell model of alkaptonuria. Rheumatology (Oxford) 52, 1667. https://doi.org/10.1093/RHEUMATOLOGY/KET185

Spreafico, A., Millucci, L., Ghezzi, L., Geminiani, M., Braconi, D., Amato, L., Chellini, F., Frediani, B., Moretti, E., Collodel, G., Bernardini, G., Santucci, A., 2013b. Antioxidants inhibit SAA formation and pro-inflammatory cytokine release in a human cell model of alkaptonuria. Rheumatology (United Kingdom) 52, 1667–1673. https://doi.org/10.1093/rheumatology/ket185

Stenn, F.F., Milgram, J.W., Lee, S.L., Weigand, R.J., Veis, A., Rogers, F., 1979. Biochemical Discovery of Homogentisic Acid Pigment in an Ochronotic Egyptian Mummy, Hosp Med Journal.

Stix, B., Kä, T., Sletten, K., Raynes, J., Roessner, A., Rö, C., 2001. Proteolysis of AA Amyloid Fibril Proteins by Matrix Metalloproteinases-1,-2, and-3, American Journal of Pathology.

Sun, E.Y., Fleck, A.K.M., Abu-Hakmeh, A.E., Kotsakis, A., Leonard, G.R., Wan, L.Q., 2018. Cartilage Metabolism is Modulated by Synovial Fluid Through Metalloproteinase Activity. Ann Biomed Eng 46, 810–818. https://doi.org/10.1007/s10439-018-2010-1

Sun, K., Jing, X., Guo, J., Yao, X., Guo, F., 2021. Mitophagy in degenerative joint diseases. Autophagy. https://doi.org/10.1080/15548627.2020.1822097

Sun, L., Ye, R.D., 2016. Serum amyloid A1: Structure, function and gene polymorphism. Gene. https://doi.org/10.1016/j.gene.2016.02.044

Tanaka, M., Kawakami, T., Okino, N., Sasaki, K., Nakanishi, K., Takase, H., Yamada, T., Mukai, T., 2018. Acceleration of amyloid fibril formation by carboxyl-terminal truncation of human serum amyloid A. Arch Biochem Biophys 639, 9–15. https://doi.org/10.1016/j.abb.2017.12.016

Tanaka, M., Takarada, T., Nadanaka, S., Kojima, R., Hosoi, K., Machiba, Y., Kitagawa, H., Yamada, T., 2023. Influences of amino-terminal modifications on amyloid fibril formation of human serum amyloid A. Arch Biochem Biophys 742. https://doi.org/10.1016/j.abb.2023.109615

Taylor, A.M., Boyde, A., Wilson, P.J.M., Jarvis, J.C., Davidson, J.S., Hunt, J.A., Ranganath, L.R., Gallagher, J.A., 2011. The role of calcified cartilage and subchondral bone in the initiation and progression of ochronotic arthropathy in alkaptonuria. Arthritis Rheum 63. https://doi.org/10.1002/art.30606

Tennent, G.A., Lovat, L.B., Pepys, M.B., 1995. Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis, Medical Sciences.

Thorne, J., Clark, D., Geldenhuys, L., More, K., Vinson, A., Tennankore, K., 2022. Serum Amyloid A Protein–Associated Kidney Disease: Presentation, Diagnosis, and Management. Kidney Med. https://doi.org/10.1016/j.xkme.2022.100504

Thorpe, S.D., Gambassi, S., Thompson, C.L., Chandrakumar, C., Santucci, A., Knight, M.M., 2017. Reduced primary cilia length and altered Arl13b expression are associated with deregulated

chondrocyte Hedgehog signaling in alkaptonuria. J Cell Physiol 232, 2407–2417. https://doi.org/10.1002/jcp.25839

Tinti, L., Spreafico, A., Braconi, D., Millucci, L., Bernardini, G., Chellini, F., Cavallo, G., Selvi, E., Galeazzi, M., Marcolongo, R., Gallagher, J.A., Santucci, A., 2010. Evaluation of antioxiodant drugs for the treatment of ochronotic alkaptonuria in an in vitro human cell model. J Cell Physiol 225, 84–91. https://doi.org/10.1002/JCP.22199

Tinti, L., Taylor, A.M., Santucci, A., Wlodarski, B., Wilson, P.J., Jarvis, J.C., Fraser, W.D., Davidson, J.S., Ranganath, L.R., Gallagher, J.A., 2011. Development of an in vitro model to investigate joint ochronosis in alkaptonuria. Rheumatology 50, 271–277. https://doi.org/10.1093/rheumatology/keq246

Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., Da Cruz, S., Clerc, P., Raschke, I., Merkwirth, C., Ehses, S., Krause, F., Chan, D.C., Alexander, C., Bauer, C., Youle, R., Langer, T., Martinou, J.C., 2009. SlP-2 is required for stress-induced mitochondrial hyperfusion. EMBO Journal 28, 1589–1600. https://doi.org/10.1038/emboj.2009.89

Tóth, K., Kiss-Láaszló, Z., Lénárt, E., Juhász, K., Takács, K., Bender, T., Szabó, J., 2010. Familiar ochronotic arthropathy-caused by a gene mutation traced three hundred years. Joint Bone Spine 77, 355–357. https://doi.org/10.1016/j.jbspin.2010.03.007

Urrutia, P.J., Mena, N.P., Núñez, M.T., 2014. The interplay between iron accumulation, mitochondrial dysfunction, and inflammation during the execution step of neurodegenerative disorders. Front Pharmacol. https://doi.org/10.3389/fphar.2014.00038

Vaillancourt, F., Fahmi, H., Shi, Q., Lavigne, P., Ranger, P., Fernandes, J.C., Benderdour, M., 2008. 4-Hydroxynonenal induces apoptosis in human osteoarthritic chondrocytes: The protective role of glutathione-S-transferase. Arthritis Res Ther 10. https://doi.org/10.1186/ar2503

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. International Journal of Biochemistry and Cell Biology. https://doi.org/10.1016/j.biocel.2006.07.001

Vercruysse, K.P., Taylor, A.M., Knight, J., 2017. Fe 2+ /H 2 O 2 -mediated oxidation of homogentisic acid indicates the production of ochronotic and non-ochronotic pigments. Implications in Alkaptonuria and beyond. https://doi.org/10.1101/223099

Wang, G., Zhang, X., Xu, J., Hou, L., Guo, Z., Sun, K., Guo, F., 2024. The role of mitochondrial autophagy in osteoarthritis. iScience 27. https://doi.org/10.1016/j.isci.2024.110741

Webb, N.R., 2021. High-Density Lipoproteins and Serum Amyloid A (SAA). Curr Atheroscler Rep. https://doi.org/10.1007/s11883-020-00901-4

Westermark, P., Benson, M.D., Buxbaum, J.N., Cohen, A.S., Frangione, B., Ikeda, S.I., Masters, C.L., Merlini, G., Saraiva, M.J., Sipe, J.D., 2005. Amyloid: Toward terminology clarification: Report from the Nomenclature Committee of the International Society Of Amyloidosis. Amyloid 12, 1–4. https://doi.org/10.1080/13506120500032196

Wetzel, R., Shivaprasad, S., Williams, A.D., 2007. Plasticity of amyloid fibrils. Biochemistry 46, 1–10. https://doi.org/10.1021/bi0620959

Wu, Y., Lin, Z., Yan, Z., Wang, Z., Fu, X., Yu, K., 2019. Sinomenine contributes to the inhibition of the inflammatory response and the improvement of osteoarthritis in mouse-cartilage cells by acting on the Nrf2/HO-1 and NF-κB signaling pathways. Int Immunopharmacol 75. https://doi.org/10.1016/j.intimp.2019.105715

Xu, X., Pang, Y., Fan, X., 2025. Mitochondria in oxidative stress, inflammation and aging: from mechanisms to therapeutic advances. Signal Transduct Target Ther. https://doi.org/10.1038/s41392-025-02253-4

Yang, Y.J., Guo, J.H., Chen, W.J., Zhao, R., Tang, J.S., Meng, X.H., Zhao, L., Tu, M., He, X.Y., Wu, L.Q., Zhu, Y.M., 2013. First report of HGD mutations in a Chinese with alkaptonuria. Gene 518, 467–469. https://doi.org/10.1016/j.gene.2013.01.020

Yoshii, S.R., Mizushima, N., 2017. Monitoring and measuring autophagy. Int J Mol Sci. https://doi.org/10.3390/ijms18091865

Zatkova, A., Chmelikova, A., Polakova, H., Ferakova, E., Kadasi, L., 2003. Rapid detection methods for five HGO gene mutations causing alkaptonuria. Clin Genet 63, 145–149. https://doi.org/10.1034/j.1399-0004.2003.00027.x

Zatkova, A., Ranganath, L., Kadasi, L., 2020. Alkaptonuria: Current perspectives. Application of Clinical Genetics. https://doi.org/10.2147/TACG.S186773

Zhang, X., Hou, L., Guo, Z., Wang, G., Xu, J., Zheng, Z., Sun, K., Guo, F., 2023. Lipid peroxidation in osteoarthritis: focusing on 4-hydroxynonenal, malondialdehyde, and ferroptosis. Cell Death Discov. https://doi.org/10.1038/s41420-023-01613-9

Zorov, D.B., Juhaszova, M., Sollott, S.J., 2014. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiol Rev 94, 909–950. https://doi.org/10.1152/physrev.00026.2013.-Byproducts

Zwilling, E., Reggiori, F., 2022. Membrane Contact Sites in Autophagy. Cells. https://doi.org/10.3390/cells11233813