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ALKAPTONURIA:
metabolic aspects and new therapeutic approaches
of a rare disease

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ABSTRACT
Alkaptonuria (AKU) is an autosomal recessive disorder, caused by deficiency of the homogentisate 1,2 dioxygenase (HGO), an enzyme which normally catalyses the conversion of homogentisic acid (HGA) into maleylacetoacetic acid, in the tyrosine degradation pathway. AKU is characterised by a plasmatic accumulation and subsequently an increased urinary excretion of HGA. In absence of HGO, HGA is rapidly oxidized to benzoquinoneacetic acid (BQA). BQA polymerizes to a melanin-like pigment, that accumulates in connective tissues (ochronosis). This condition leads to a severe and crippling arthropathy.

AKU is a very rare disease with a prevalence of 1:100,000-250,000 in most ethnic group, but with the highest incidence in East Europe.

To date, there is no effective therapy of AKU, also because AKU is still a disease without a single pathogenic theory.

In these years, we created cells cultures of biological samples from patients affected by AKU. These samples are derived from cells directly involved in clinical expression of the disease, including chondrocytes and synoviocytes type B.

At present, the deposition of ochronotic pigment in the joint is attributed to HGA plasmatic accumulation due to increase of HGA production by liver. We demonstrated the presence of pigment inside the synoviocytes and chondrocytes and, above all, we showed the presence of mRNA of enzyme in such cells, by RT-PCR. These data indicate that AKU osteoarticular cells may able to produce HGA and consequently ochronotic pigment in loco and this may contribute to favorite ochronotic arthropathy.

Moreover, we demonstrated that treatment with antioxidant substances, including ascorbic acid and N-acetyl-cysteine, reduces apoptosis on AKU synoviocytes and chondrocytes.
Finally, an *in vivo* study has been effectuated to demonstrate that N-acetyl-cysteine is able to increase the renal HGA excretion. Our results give new prospective views into AKU pathogenetical mechanism knowledge and encourage the use of the antioxidant agents in clinical therapy.

1. INTRODUCTION

Alkaptonuria (AKU) is a rare metabolic disease, caused by deficiency of homogentisate 1,2-dioxygenase (HGO), an enzyme which catalyses the
conversion of homogentisic acid (HGA) into maleylacetoacetic acid and fumaric acid, in the catabolism of aromatic amino acids, phenylalanine and tyrosine (Figure 1,2).

![Figure 1: metabolic pathway of phenilalanine and tyrosine](image)

In absence of HGO, HGA is rapidly oxidized to benzoquinoneacetic acid (BQA) with $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and OH production. Under alkaline conditions, BQA polymerizes to a melanin-like pigment, that accumulates in connective tissue.

AKU is characterised by accumulation of HGA and of its oxidation product, BQA, which causes dark pigmentation of the urine exposed to air, a grey-blue pigmentation of sclera, ear cartilage, skin (ochronosis) and an invalidating disease of axial and peripheral joints (ochronotic arthropathy).
1.1 HISTORY OF ALKAPTONURIA
The first recorded case of ochronosis was described in 1977, on an Egyptian mummy of 1500 BC; biochemical and radiological examination of intervertebral discs, hips and knees confirmed the diagnosis.

In 1584, Scribonius was the first to describe dark urine, looking at the case of a child, which eliminated the urine "black as ink". In 1859, Boedeker used for the first time the term *alkaptonuria* (from the arabic alkali and the greek καπτεν) to mean the HGA avidity to capture oxygen in alkaline solutions.

In 1866, Virchow described the autopsy features of a man who died of congestive heart failure, probably related to the high degree of atherosclerosis. He noted that the patient’s intervertebral discs, larynx, trachea, menisci and articular cartilage were heavily pigmented, so he coined the term *ochronosis*, because the pigment microscopically appeared as yellow-ocher.

In 1891, Wolkow and Baumann identified the HGA structure, isolating it from the urine of a AKU patient in southern Germany.

In 1902, Albrecht showed the connection between AKU and Ochronotic arthropathy and two years later Osler diagnosed ochronosis for the first time in two alkaptonuric brothers.

AKU occupies an important page in the history of medicine, mainly thanks to English physician, Sir Arcibald Garrod. At the beginning of 1900, he noted that individuals affected by AKU had both normal parents and sons and that they were frequently born of consanguineous parents, so he hypothesized that alkaptonuria follow Mendelian inheritance of the recessive type.

In 1902, Garrod published a book titled "The Incidence of Alkaptonuria: a Study in Chemical Individuality". This is the first published description of a case of recessive inheritance in men. In 1908, for the first time, Garrod
unveiled the concept of inborn error of metabolism; assuming that AKU was caused by a defective enzyme, therefore he postulated the existence of a relationship between genes and body chemicals, defining the disease as an "inborn error of metabolism" (Figure 3). In 1923, his studies about alkaptonuria were published in a book: "Inborn Errors of Metabolism."

Garrod anticipated by several years the meeting between genetics and biochemistry. In fact, only in 1958 evidence was found of biochemical alterations of AKU.

Biochemical evidence of the defect in AKU was provided by La Du and his colleagues who demonstrated the absence of HGO activity in liver homogenate prepared from an AKU patient and ruled that defect was limited to HGO whereas the other enzymes of the same metabolic pathway were present and normally active².
In the early 90's, the gene responsible for the AKU was mapped to chromosome 3q21-q23\(^3,4\).

In 1995, a group of Spanish researchers cloned and characterized the AKU human gene mutation\(^5\). Gene mutations cause the lack of expression of the enzyme that catalyses a crucial step in the metabolism of tyrosine and phenylalanine amino acid.

The starting point was the cloning and characterization of the gene (hmgA) encoding the HGO enzyme in *Aspergillus nidulans*\(^6\). The Fernandez-Canon team decided to use the filamentous fungus because it had a phenylalanine and tyrosine degradation pathway that was remarkably similar to the humans’ one.

In addition, Northern blotting experiments demonstrated that the expression of this gene is limited to the liver, kidney, small intestine, colon and prostate.

AKU is a rare disease, It has a very low prevalence (1:100,000-250,000) in most ethnic groups. Two countries, Slovakia and Dominican republic, exhibit an increase incidence of this disorder up to 1:19,000\(^7\).

1.2 **PATHOGENIC HYPOTHESIS**
AKU is caused by mutations on homogentisate 1,2-dioxygenase gene (HGD). These mutations are responsible for the complete loss of enzyme (HGO) activity, resulting in accumulation of HGA. These reactions occur mainly in the liver. In the liver of healthy subjects, the catabolism of aromatic amino acids, phenylalanine and tyrosine, requires six enzymes (Figure 4,5).

HGO oxidizes HGA in maleylacetoacetic acid. In normal conditions, maleylacetic acid is converted, by an isomerase enzyme, to fumarylacetoacetic acid and the latter is split, by a hydrolase, into fumarate and acetoacetate. In normal liver, these sequential steps proceed very smoothly and efficiently, without the accumulation. In normal liver and in AKU patients' liver, most of the HGA metabolism takes place in the soluble fraction of the hepatocytes, the fraction which also contains the subsequent enzymes.
Figure 4: scheme of tyrosine metabolism steps that lead to inborn defects causing accumulation of toxic metabolites
Figure 5: metabolic blocks in the phenylalanine and tyrosine pathway

The pathogenic mechanism by which HGO deficiency may lead to ochronosis and ochronotic arthropathy is unclear. Over the years, several theories have been developed\textsuperscript{8} (Figure 6).
Figure 6: pathogenic hypothesis of AKU-ochronosis.

The HGA has a serum concentration of 2,4-12 ng/ml, depending on the protein diet of the individual. The HGA is actively excreted by the kidneys, but its oxidized and reduced forms are accumulated by connective tissue. It has been proposed that HGA can act as a chemical irritant, causing inflammation and a rapid degeneration of the joints or it can bind to connective tissues, altering the structure\(^9\).

One of the most credible hypothesis is that it is not HGA itself, but its oxidation products that cause the degenerative processes in this disease. The HGA accumulated both inside and outside the cells is oxidized to benzoquinone-acetic acid (BQA), which polymerizes into melanin-like compounds\(^10\) (Figure 7).

This oxidation occurs spontaneously in urine after exposure to air, while inside the body, this reaction is catalysed by polyphenol homogentisic-oxidase, an enzyme found in skin and in connective tissue cells of mammals, that alters tissue intermolecular structure\(^11\).
In 1987, a third theory was proposed by the group of Martin, which evaluated the ability of HGA to produce active oxygen species through a process of auto-oxidation.

Considering that oxygen radicals (ROS) are involved in arthritis, they studied the role of the species generated by auto-oxidation in the destruction of connective tissue, showing that HGA is subject to spontaneous auto-oxidation and products of this reaction are $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and BQA.

Milch suggested that BQA is responsible for joint damage. It would bind to collagen fibres, leading to an increase in collagen intermolecular cross linking rate, in a process similar to that observed in tanning hides to make leather\textsuperscript{12}.

Murray and his collaborators demonstrated in chicken embryos that HGA inhibits lysyl-hydroxylase, a microsomal enzyme that catalyzes the

\textbf{Figure 7}: scheme of ochronotic pigment formation.
conversion of lysine residues in hydroxylysine. Hydroxylysine is directly involved in intermolecular cross-linking, and his inhibition would lead to a reduction of the collagen crosslink integrity and subsequently to a collagen biomechanical alteration\textsuperscript{13}.

This hypothesis was later confirmed by Andreotti and colleagues in skin collagen from a patient with AKU\textsuperscript{14}.

Biomechanical studies have shown that ochronotic cartilage is weaker, less elastic and therefore more vulnerable to mechanical stress than a normal one\textsuperscript{15}.

The result of this process is the degeneration of the fibers and a weak connective tissue that undergoes alterations for minor injuries.

It was recently published a study, which describes the progression of articular cartilage degeneration in ochronotic arthropathy from initiation of pigmentation to complete joint failure. Pigmentation initially manifests at the boundary of the subchondral bone and calcified cartilage before proceeding toward the articular surface\textsuperscript{16}. 
1.3 HISTOLOGY

The fragility of the cartilage of AKU patients leads to fragmentation of its upper layer, causing the detachment of small fragments (shards) with consequent exposure of the subchondral bone\textsuperscript{17,18}.

Figure 8: macroscopic examination of the femoral head. Black pigmentation of the cartilage is irregular and eroded with exposed subchondral bone.

The shards are included in the synovium, resulting in inflammatory and degenerative aspects\textsuperscript{19}(Figure 9,10).
Figure 9: synovial fluid. Ochronotic cartilage fragments (shards) are evident.

The synovial membrane looks as hypertrophied and brownish. There is a synoviocitic hyperplastic layer, mostly mono stratified, where shards can be found\textsuperscript{20}.
The sub-lining shows variables characteristics with fibrotic aspects and with focal or diffuse limphomonocitar infiltrates, especially in the perivascular zone. In this site, most of the shards have been found with typical brownish colour, often surrounded by inflammatory cells and multinucleated cells with the characteristic appearance of a foreign body. In these cells, fragments of phagocytised ochronotic material are frequently found (Figure 11,12).
Figure 11: knee joint synovium. Numerous multinucleated giant cells close to ochronotic shards embedded in synovium (hematoxylin-eosin; original magnification, 40).

Different aspects were evaluated by immunohistochemistry, such as cell phenotype, vascularization and activation status of inflammatory cells. The major component consists of monocyte-macrophage CD68+ cells, which is mainly available in the vicinity of the shards. These cells also show a strong expression of DR as evidence of their activation status. The vascular bed is particularly well represented in the subsynoviocytic layer. The overall appearance shows both degenerative character and clear inflammatory aspects, that may be related to a foreign body reaction induced by cartilage shards.
Structural alterations were also found in bone but not as severe as in the cartilage.

Ochronotic pigment was found in the bone matrix and in a small number of osteocytes, which are essential in the physiological activity of the bone, resulting in degeneration and death of these cells.

However, the low number of pigmented osteocytes suggests that their impairment or death does not have significant effects on bone. Furthermore, in contrast to cartilage, that is a stable tissue, and therefore is exposed to the harmful effects of the ochronotic pigment for a long time, the bone undergoes continuous remodelling processes, limiting the damage.

Kidney seems to have a key role in the development of ochronotic arthritis. Kidneys are crucial for the elimination of HGA. In AKU patients, plasmatic levels of HGA are around 10µg/mL with a urinary excretion of HGA about 4-8g per day. So the glomerular filtration is accompanied by a mechanism of active tubular secretion.
In fact, in conditions of normal renal function, with a clearance of about 100ml/min, you reach a filtration of HGA about the 1mg/min or 1.44 g per day, which then allows the elimination by tubular secretion of 4-8g/day. AKU patients are asymptomatic until the third-fourth decade, despite the metabolic defect is present from birth. The onset of clinical manifestations and their rapid evolution seem to have a key role in the kidney and particularly the renal tubule, as I have already mentioned, in fact the latter is responsible for active HGA secretion, as it happens for the other organic acids.

The renal HGA clearance is very efficient in the young, preventing early onset of ochronosis manifestations. Over the years, the efficiency of this process is reduced and the plasmatic HGA level increases slowly. This situation accelerates accumulation of plasmatic HGA, resulting in rapid clinical deterioration of patients with AKU. In literature some cases of renal failure are described as associated with AKU in the later stages of the disease.

However, in the few cases mentioned, renal biopsy showed a similar histological picture: glomerular sclerosis, diffuse tubular atrophy, interstitial fibrosis and ochronotic pigment deposition in all kidney structures.

Venkataseshan et al. described the case of a young woman affected by AKU, the first clinical manifestation was a rapidly progressive renal failure; the first signs of ochronotic pigment deposition appeared only after the onset of metabolic acidosis and uremia.

Introne et al. reported the case of three brothers with AKU, one of these presented a diabetic nephropathy. Plasma concentrations of HGA of the latter patients were doubled those of the other two and the clinical manifestations of ochronosis had progressed much more quickly. Patient
underwent a kidney transplant. Following this intervention, plasmatic and urinary levels of HGA were reduced.

These cases demonstrate how an alteration in normal kidney function may be responsible, at least in part, for the rapid and progressive worsening of clinical manifestations of ochronosis.

Some authors ascribe the renal damage in a concomitant but separate process to AKU, such as acute glomerulonephritis, acute tubular necrosis, amyloidosis, diabetic nephropathy; but it is also true that other authors have described histological features, characterized by the deposition of ochronotic pigment, with the term ochronotic nephrosis.

The real role of the kidney in the development and worsening of clinical manifestations of ochronosis are still contradictory.

Clinical manifestations and ochronotic arthropathy development seem to be influenced by liver function also; in fact, the liver is the main organ in which it is present HGO.

In 2005 it was described a case of a patient with AKU undergoing liver transplantation for HBV-related liver cirrhosis. It showed disappearance of HGA production and improvement of the clinical manifestations of ochronosis26.
1.4 CLINICAL FEATURES

The AKU is in itself a condition clinically asymptomatic. Clinical signs and symptoms of the disease begin to appear when the ochronotic pigment is deposited in connective tissue.

AKU can be clinically divided in three stages\textsuperscript{27}.

The first clinical sign is the appearance of dark spots in nappies of the affected children, due to darkening of urine owing to oxidation of the HGA (Figure 13).

![Figure 13: typical coloration of the urine of patients with Alkaptonuria](image)

The accumulation of HGA and its metabolites in tissues inevitably leads to ochronosis, the dark pigmentation of the connective tissues. These events
start to appear towards the 20-30 age and represent the second stage of disease.

Ochronotic pigment may be deposited in any of the outer structures of the eye: sclera, cornea, conjunctiva. Changes in the sclera are clearly visible by the 40s and are quickly recognized. Pigment can be seen in the nasal and temporal aspects of the sclera (Figure 14).

Ear manifestations are characterized by discoloration of the ear cartilage (Figure 15). This is one of the earliest physical sign of ochronosis and usually appears by the third decade often associated with black cerumen. Costal cartilages, laryngeal and tracheal may be heavily pigmented. This pigment is also present in other fibrous tissues: the fibrocartilage, tendons and ligaments.

**Figure 14:** typical ochronotic pigmentation of the sclera

**Figure 15:** black-gray pigmentation of the ear cartilage
Finally, third stage of disease is characterized by ochronotic arthropathy, involving mainly the large joints, including the spine, hips, knees.

![Figure 16](image)

**Figure 16:** radiograph of the left knee. Marked femoral-tibial and femoral-patellar degenerative manifestations are present. Calcifications of the quadriceps femoris tendon are evident.

The first symptoms related to joint involvement usually start to appear in the fourth decade. They are usually referred to the spine, with stiffness and pain in the lumbo-sacral region. There may be a gradual extension of the stiffening process throughout the spine. There may be a scoliosis or lordosis. Lumbar and thoracic spine symptoms precede cervical spine symptoms. The rate of involvement of the whole spine depends on the severity of the disease, but it may be complete within ten years. Associated with the spinal changes there is a diminution of stature. Stiffness may become severe suggesting advanced ankylosing spondylitis. The sacroiliac region is usually spared. Chest expansion may be reduced because of involvement of costal cartilage and costovertebral joints. Peripheral
arthropathy usually affects large weight bearing joints and later the shoulders. The knees usually become troublesome at a somewhat later stage than the back (Figure 16). Others affected joints are hips, shoulders and ankles. Fifty percent of individuals requires at least one joint by age 55\textsuperscript{28}. Small joints involvement are uncommon.

By age 64, 50\% of patients with AKU have a history of renal stones and prostate stones. Bladder stones are less frequently.

The cardiovascular manifestations of AKU relate to deposition of ochronotic pigment within connective tissue which acts as a trigger for dystrophic calcification. Histological analysis has shown such deposits in heart valves, endocardium, aortic intima and coronary arteries. The aortic valve was preferentially affected. Patients with AKU above the age of 40 years should undergo routine echocardiographic surveillance\textsuperscript{29}.

The diagnosis of AKU is based on a determination of HGA in the urine, blood and other tissues, using a method specific enzyme, HPLC (high performance liquid chromatography). In normal subjects, serum levels of HGA are 2.4-12 ng/ml\textsuperscript{30}.

Diagnosis of ochronosis is basically clinical. The radiological is of great help, particularly with the spine. Radiographic changes may precede the onset of symptoms. In the spine the first changes are narrowing of disc spaces, commencing in the thoraco-lumbar region. The disc vertebral surfaces become increasingly radiopaque due to calcification, giving a doubling of the outline (wafer-like), secondary to deposition of calcium pyrophosphate and hydroxyapatite crystals\textsuperscript{31}. The nucleus pulposus is the last part of the disc to become calcified. The vertebral bodies become porotic. Massive osteophyte formation develops at the vertebral margins and intervertebral bony bridging may occur. Association between ankylosing spondylitis and ochronotic spondylopathy was described\textsuperscript{32} (Figure 17,18).
In the knees the first changes are those of calcification of the menisci. There is increased density in the subchondral region with areas of osteoporosis. The articular surfaces become irregular, the joint space narrowing asymmetrically and marginal osteophytes develop.
Figure 17: radiographs of the hips show the progression of ochronotic arthropathy in two years: sclerosis and narrowing of joint space. Marked sclerosis of the lumbar vertebrae.
Figure 18: radiographs of the dorsolumbar spine: narrowing of the intervertebral spaces with mild, non-marginal osteophytosis. Calcification of intervertebral discs (wafer-like) are also evident
1.5 THERAPEUTIC APPROACHES

At present, there is no specific treatment for AKU. The main goal of AKU treatment is to prevent or minimize damage of ochronotic arthropathy. Currently, symptomatic treatment is the only option. Despite ochronotic arthropathy is treated with analgesics and exercises but inevitably progresses; and at the last stage, total joint replacement is often required. Whereas the AKU itself does not lead to a reduction in life expectancy, joint damage and cardiac involvement often leads to a significant deterioration in the quality of life.

The ideal therapy would be replacement of the missing enzyme by use of genetic engineering but this treatment is not available.

Dietary restriction of phenylalanine and tyrosine has been proposed to reduce HGA production. In fact, it was observed that protein restriction resulted in a significantly lower excretion of HGA in the urine of children younger than 12 years but continuation of this regimen to older age seems questionable and not practical.

A potential therapy for AKU could be nitisinone. Nitisinone is a triketone herbicide that inhibits 4-hydroxyphenylpyruvicate oxidase, an enzyme of the tyrosine catabolic pathway responsible for converting hydroxyphenylpyruvicate to HGA, by a rapid but reversible bond (Figure 19). Nitisinone is used to treat Type I tyrosinemia, a rare hereditary pediatric disease causing progressive liver failure and hepatocellular carcinoma in children. Type I tyrosinemia is caused by deficiency of fumarylacetoacetate hydrolase, an enzyme of tyrosine catabolic pathway. Nitisinone reduced urinary HGA excretion. However, nitisinone causes high plasmatic levels of tyrosine, leading to corneal irritation, due to the presence of tyrosine crystals. These manifestations may disappear in about
two days with a significant reduction in tyrosine and phenylalanine in the diet.

In addition, treatment with nitisinone could lead to the development of tyrosinemia type III, which is associated with neurological complications including tremor, ataxia and delayed development or it could lead at 4-hydroxyphenylpyruvate dioxygenase deficiency, an extremely rare disorder.

It has recently published a phase II clinical study, initiated in 2005, to evaluate 40 patients with AKU. 20 patients treated with nitisinone and 20 representatives control group. 20 patients treated with nitisinone have shown 95% reduction of HGA in plasma and urinary compared to the control group. Clinically, this drug did not prove beneficial. Side effects were infrequent. This trial illustrates the remarkable tolerability of nitisinone, its biochemical efficacy, and the need to investigate its use in younger individuals prior to development of debilitating arthritis. Soon, Phase III clinical trial with nitisinone should start. It will be an international study, which will include also Italy.
For several years, ascorbic acid has been considered an effective therapy in the treatment of AKU. Vitamin C inhibits of HGA polyphenol oxidase and it prevents oxidation to BQA and free radical formation (Figure 20). But
results are equivocal because, under certain conditions, it functions as a pro-oxidant rather than an anti-oxidant. Some studies have shown that ascorbic acid reduces urine HGA excretion but not prevent its accumulation and complications. The clinical effects have been minimal. It has been proved that ascorbic acid is co-oxidized during HGA autoxidation by the oxidative action of homogentisic acid semiquinone, BQA or $O_2^-$. Hydrogen peroxide and oxygen radicals are products of HGA-induced ascorbic acid oxidation and these reactive intermediates have toxic and carcinogenic properties.

Furthermore, it appears that vitamin C can promote formation of kidney stones and increases the severity of spontaneous knee osteoarthritis\textsuperscript{36}.

Figure 20: action level of nitisinone and Vitamin C

Considering the contrasting results obtained with ascorbic acid, we studied another molecular with antioxidant propriety, N-acetyl-cysteine (NAC). NAC has an antioxidant action having a free thiolic (-SH) nucleophil group which can interact directly with electrophil groups of oxidant
radicals. NAC works as an analogue and precursor of intracellular L-cysteine and reduced glutathione (GSH). GSH is the main mechanism for intracellular defence against free radicals and citotoxic substances. The safety of this drug in humans is supported by more than 40 years of clinical use, mainly as a mucolytic agent but also as an antidote against acute intoxication. A number of experimental studies in clinical trials provides evidence for the potential ability of this thiol compound to inhibit oxidative, genotoxic and carcinogenic effects.

NAC is used as mucolytic agent in some respiratory conditions and as antidote for the treatment of paracetamol overdose. NAC can neutralize the acetaminophen derivative N-acetylbenzoquinoneimine\textsuperscript{37}, whose chemical structure is similar to that of BQA.

Studies, carried out in Siena Hospital, have demonstrated that NAC is able to inhibit polymerization processes of HGA\textsuperscript{38,39}. 

![Effect of N-acetyli-cysteine on homogentisic acid polymerization](image)
It seems that this antioxidant may interfere with some pathogenetic mechanisms through different ways. Firstly, acting as a scavenger of oxygen free radicals and thereby limiting tissue damage. Secondly, preventing or delaying the accumulation of HGA. Finally, since NAC can neutralize the acetaminophene derivate N-acetylbenzoquinone, whose chemical structure is similar to that of BQA, could be assume a similar action on the BQA, determining cartilage damage.

As previous stated, during HGA autoxidation ROS are produced. When ROS levels exceed the antioxidative ability of the chondrocytes, the latter undergoes oxidative stress. ROS damages DNA and other molecules in the chondrocytes, leading to apoptosis and cartilage degeneration. Because NAC possesses direct (scavenging ROS) and indirect (generating intracellular glutathione) antioxidant proprieties toward ROS, NAC can exert therapeutic effects against some pathological conditions related to ROS, as AKU.

Recently a study was carried out in vitro on primary cultures of chondrocytes treated with HGA, using NAC in combination with ascorbic acid and has been seen that NAC is able to counteract the potential pro-oxidant effect of Vitamin C.
2. RATIONALE OF THE STUDY

To date, AKU is still a disease without a single pathogenic theory and there is not an effective therapy for AKU. This is partly due to the fact that it is an extremely rare disorder and it is particularly difficult to collect enough samples to carry out adequate studies in vitro and in vivo. Furthermore, it is not easy to find funds to advance this specific research.

In the last years, there has been a significant increase attention to rare diseases, including AKU. In fact, the AKU Society was found in United Kingdom and subsequently in other countries, including Italy. The aim of this society is to make aware not only doctors but also patients and their families about the disease and its effects.

One further difficulty to study this rare genetic disease is the lack of reliable experimental models. Therefore, in these years, first, we aimed to create cell cultures obtained from AKU biological samples. These samples have been derived from cells directly involved in the clinical expression of the disease, including chondrocytes and synoviocytes type B.

Homogenate 1,2 dioxigenase enzyme is found in the liver, kidneys, prostate and intestine, whereas according to the current knowledge, such enzyme is not expressed elsewhere, including the joint milieu. Thus, the presence of ochronotic pigment inside the joint structures is actually attributed to the genetic impairment of tyrosine metabolic pathway leading to over-production and HGA plasmatic accumulation.

The main aim of this study is to evaluate whether cells largely involved in this pathology, synoviocytes and chondrocytes, are able to produce HGA and consequently ochronotic pigment.
We also evaluated the influence of antioxidant substances, including ascorbic acid and N-acetyl-cysteine, on alkaptonuric cells viability. The same evaluations have been conducted on control cells, represented by primary cultures of synoviocytes and chondrocytes from patients with osteoarthritis. This in vitro system has given us also the opportunity to conduct a morphological ultra-structural study by transmission electronic microscopy and to evaluate the evolution of ochronotic pigment stored into the cells.

In parallel with these in vitro studies, we conducted a clinical study on 4 patients affected by AKU. All patients were treated with N-acetyl-cysteine to evaluate whether this antioxidant is able to increase the clearance of HGA by renal excretion.
3. MATERIALS AND METHODS

3.1 Patients selection

All patients underwent surgery for total hip replacement. Chondrocytes were obtained from cartilage of femoral heads of three patients with Alkaptonuria-ochronosis and three patients with osteoarthritis (OA). Synoviocytes were obtained from synovial fluid of three patients with Alkaptonuria-ochronosis and three patients with OA, following arthrocentesis of the knee. Patients with OA were used as control. All patients were selected according to medical records, clinical, laboratory and radiology specific. Written informed consent was obtained from all patients in accordance with the Principles of the Declaration of Helsinki and the study was approved by the local ethical committee. All patients affected by Alkaptonuria-ochronosis received treatment with Ascorbic acid, but at the moment of arthrocentesis or hip replacement surgery received discontinued treatment for at least 4 days.

3.2 Isolation and culture of chondrocytes and synoviocytes

Chondrocytes were isolated by enzymatic digestion of cartilage tissue specimens obtained from patients with AKU-ochronosis and OA undergoing total joint replacement surgery. Tissue samples, minced into 2-3 mm pieces, underwent sequential enzymatic digestion as follow described: 30 min with 0.1% hyaluronidase (Sigma), 1 hour with 0.5% pronase (Sigma) and 1 hour with 0.2% collagenase (Sigma) at 37°C in wash solution (Dulbecco’s modified eagle medium (DMEM) +
penicillin/streptomycin solution + Amphoterycin B). Cells were centrifuged for 10 min at 700 x g and pellets were then re-suspended in DMEM containing 10% fetal calf serum (FCS) and expanded in monolayer culture. Finally cells were cultured separately at 37°C in 95% relative humidity and in a 5% CO₂ atmosphere.

Synoviocytes were isolated, in aseptic condition, from synovial fluids obtained by knee arthrocentesis. After centrifugation at 700g for 10’ the cell suspensions were plated in 10 ml of DMEM supplemented with L-glutamine (2mM), 10% FCS, Penicillin (200 U/ml) and Streptomycin (200 U/ml), in 100 mm culture dishes, and incubated in a humidified atmosphere containing 5% CO2.

3.3 Culture stimulation

Chondrocytes and synoviocytes were plated in a six well plate at a concentration of 3x10⁵ cells/well. After an overnight culture in reducing condition (0.5% FCS) cells were incubated with N-acetyl cysteine (NAC) and Ascorbic Acid (VIT C) alone and/or in combination at a concentration of 10 µM for 48 h. We used this concentration because it was the more effective in the experiments performer using the in vitro model.

All experiments were conducted at the third passages in order to avoid changes in the original phenotype.

3.4 Evaluation of cell viability

Chondrocytes and synoviocytes plated in a six well plate at a concentration of 3x10⁵ cells/well were treated with NAC and Ascorbic acid at a
concentration of 10 µM. After 48 hours of treatment cells were used to evaluate apoptosis and necrosis by cytofluorimetric assay (FACS). Apoptotic evaluation was performed according to protocol, using Annexin V and propidium iodide (PI). Cells staining was evaluated with fluorescein isothiocyanate FITC-Annexin V (green fluorescence), and with PI, red fluorescence negative. Staining for FITC-Annexin V binding and for cellular DNA using PI was performed as follows. After washing twice with PBS, cells were resuspended in binding buffer. FITC-Annexin V was added at a final concentration of 1µg/mL Annexin V 0.1 PI/mL cell suspension. The mixture was incubated for 10 min in the dark room temperature and then measured by FCM. In cells with a damage cell membrane PI induces a red fluorescence of DNA, whilst it is excluded by cells with preserved cytoplasmatic membrane. Hence during the initial phase of apoptosis the cells are still able to exclude PI and therefore do not show any red fluorescence signal, similar to that of living cells.

3.5 Evaluation of ochronotic pigment by transmission electron microscope

Chondrocytes and synoviocytes, treated as previously described, were collected in PBS, and centrifuged at 700 g/min. Then, cell suspensions were fixed for 2 hours at 4°C in cold Karnovsky fixative, rinsed overnight in 0.1M pH 7.2 cacodylate buffer, post-fixed for 1 hour at 4°C in 1% buffered OsO4, dehydrated in a graded series of ethanol and embedded in Epon-Araldite. Ultrathin sections cut with an LKB III ultramicrotome were mounted on copper grids, stained with uranyl acetate and lead citrate and then photographed with a Philips CM10 electron microscope. We observed at least 30 synoviocytes and chondrocytes from each patients.
3.6 Homogentisic oxidase enzyme mRNA expression

In order to evaluate homogentisic oxidase enzyme gene (HGD) expression we performed Real Time PCR (RT-PCR) analysis. Total RNA has been isolated from primary cultures of ochronotic synoviocytes and chondrocytes by RNeasy Mini Kit (Quiagen). Briefly, cells were lysed in RLT buffer and after mechanical homogenization RNA was purified by using different columns and finally eluted in DEPC water. Following the evaluation of RNA integrity, concentration and purity it was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen). GAPD, ACTB, HPRT were used as reference genes for normalization. Quantitative PCR (q-PCR) reactions were done on light cycler 1.0 (Roche Molecular Biochemicals) and were performed in triple using QuantiFast SYBR Green PCR Kit (Qiagen). Reaction conditions were: 5’ at 95°C for HotStart polymerase activation, 45 cycles for 15s at 95°C for denaturation and 30s at 60°C for primer annealing and amplification and temperature was raised from 70°C to 95°C at 0.1°C/step. HGD Cq values and efficiencies of primers were calculated with LingReg, converted in relative quantities and normalized (Pfaffl, 2001). Same analyses were performed on OA cells as control.

3.7 Urinary HGA assay

24-hour urine samples were obtained from 4 patients (3 females and 1 male) affected by AKU, with an average age of 59 years. Patients were treated with NAC at a dose of 1.2g/die (600mg twice a day) for 2 months. Assays were performed at T0 and after 2-7-14-30-60 days of treatment with
NAC. In addition, the dosage urinary HGA was also performed after 15 days of discontinuation of therapy.

The urine sample was acidified 1:1 in 4% trichloroacetic acid (p/v), then centrifuged and injected into HPLC (stroke): phase A, 0.05% trifluoroacetic acid (v / v), phase B, acetonitrile. Isocratic separation: 94% phase A, 6% phase B. Flow: 1.2 ml / min. Zorbax Eclipse C18 column 4.6 x 150 mm, Agilent 1100 HPLC.

### 3.8 Statistical analysis

All values presented are expressed as the mean±SD of three separate experiments. Analysis of variance (ANOVA) was used for a comparison of mean values. Differences of p<0.05 were considered significant.
4. RESULTS

4.1 CELL VIABILITY

4.1.1 Synoviocytes

The evaluation of apoptosis on primary cultures of synoviocytes obtained from patients with OA and ochronosis arthropathy was performed by flow cytometry. We observed higher levels of apoptosis and advanced apoptosis in ochronotic synoviocytes than control (mean ± sd: 8,83 ± 1 vs 0,6 ± 0,5), (mean ± sd: 32,3 ± 3,8 vs 8,2 ± 2,1). No differences were observed for necrosis between OA and ochronotic synoviocytes. (mean ± sd: 4,3 ± 1,7 vs 4,5 ± 0,8) (p<0,05).
Figure 21: cells viability in OA and AKU synoviocytes. Cultured AKU synoviocytes showed increase of apoptosis and advanced apoptosis (apoptosis+necrosis) than control. No differences were observed in the number of necrotic cells. * p<0.05

The treatment of ochronotic synoviocytes for 48 h with NAC [10 µM] and vitamin C [10 µM] did not detect significant differences in advanced apoptosis and necrosis compared with AKU cells in basal condition (data not shown). Whereas, it has been highlighted a 21% reduction of apoptotic ochronotic synoviocytes treated with vitamin C (mean ± sd: 8,9 ± 2,3 vs 7,1 ± 1,5) and a 37% reduction of cells treated with NAC (mean ± sd: 8,9 ± 2,3 vs 5,6 ± 1,1). (p<0,05)
Figure 22: apoptosis of AKU synoviocytes before and after treatment with ascorbic acid [10 μM] and NAC [10 μM]. The antioxidant agents, in particular NAC, were able to reduce the number of AKU cells undergoing apoptosis. § represents the difference between AKU cells in basal condition and OA cells. *represents the difference between AKU cells in basal condition and after antioxidants treatment. §, *p<0.05

4.1.2 Chondrocytes

Similar results were obtained in primary cultured of chondrocytes compared to control. In particular, we observed that AKU cells undergo apoptosis and advanced apoptosis more than control cells (apoptosis: mean ± sd 8,34 ± 1,3 vs 3,61 ± 0,7; advanced apoptosis: mean ± sd 27,7 ± 3,1 vs 7,1 ± 2,3). There was no evidence for cell necrosis (mean ± sd: 3,5 ± 1,5 vs 3,8 ± 0,9). (p<0,05)
Figure 23: cells viability in OA and AKU chondrocytes. AKU chondrocytes showed increase of apoptosis and advanced apoptosis than control. No differences were observed in the number of necrotic cells. *p<0.05

Also in ochronotic chondrocytes, the treatment with NAC [10 µM] and vitamin C [10 µM] showed no significant differences in necrosis and advanced apoptosis compared with untreated cells (data not shown). Treatment with NAC and ascorbic acid showed a reduction of apoptotic cells by about 57% in both cases compared to untreated AKU chondrocytes (mean ± sd: 8,6 ± 3,1 vs NAC 3,77 ± 1,5; vs ascorbic acid 3,74 ± 2,3). (p<0,05)
Figure 24: Apoptosis of AKU chondrocytes before and after treatment with ascorbic acid [10 µM] and NAC [10 µM]. The antioxidant agents were able to reduce the number of AKU cells undergoing apoptosis. § represents the difference between AKU cells in basal condition and OA cells. * represents the difference between AKU cells in basal condition and after antioxidants treatment. §,*p<0.05
4.2 TRANSMISSION ELECTRON MICROSCOPY

Electron microscope analysis showed clear pyknosis and nuclear fragmentation phenomena that are suggestive of cellular suffering, both in ochronotic synoviocytes than in ochronotic chondrocytes. It was also possible to observe the presence, in both cells type, vacuoles of dark pigment, probably ochronotic pigment. The deposits within the synoviocytes are larger and more abundant than those found in chondrocytes. The addition of NAC [10 µM] to the cell cultures did not induce detectable morphological changes or of pigment.
Figure 25: AKU synoviocytes (a,b) and AKU chondrocytes (c,d) contain intracellular dark pigment probably ochronotic origin. The pigment deposition within the synoviocytes is larger and more abundant than that found in chondrocytes. (transmission electron microscopy: a 6000X, c 12000X, b and d 20000X)
4.3 RT-PCR

The RT-PCR analysis showed expression of HGD gene in ochronotic and osteoarthrosic chondrocytes and synoviocytes. There were no significant differences between AKU cells and the control group (mean ± sd: 0,9 ± 0,1 for AKU chondrocytes; 0,95 ± 0,15 for AKU synoviocytes).

Figure 26: AKU chondrocytes and synoviocytes express homogentisic oxidase enzyme mRNA. No significant difference was observed between AKU and control cells.
4.4 **HGA URINARY LEVELS**

The study was conducted on 4 patients affected by AKU. The treatment with 1.2 gr/day of NAC suggests that such antioxidant is able to significantly increase the clearance of HGA by renal excretion of almost 100% after 60 days of treatment, from an average of 6.5 ± 1.8 to values 13.0 ± 2.4 (p<0.05). The discontinuation of NAC administration resulted in a restoration of basal levels.

**Figure 27:** After 60 days, treatment with NAC shows a reduction of HGA urinary levels of almost 100%. There was a restoration of the initial values after 60 days NAC withdrawal.
5. DISCUSSION

Ochronotic pigment is widely present in the chondrocytes and osteocytets (19)(20). We demonstrated for the first time, by TEM, the presence of ochronotic pigment also in type B synoviocytes. Both the chondrocytes and the synoviocytes aren't cells with phagocytic activity. Up until now, the presence of ochronotic pigment in the cells was attributed to plasmatic accumulation of HGA. The way by which HGA arrives in the site where the pigment is formed, is still not clear. Therefore the articular damage could be related to an HGA accumulation produced elsewhere. Intracellular pigment, noticed in cultures at third step, points out two hypothesis. As for the first one, the pigment is inherited by progenitor cells and then it is transmitted during the mitotic divisions to daughter cells; as for the second one these cells are able to synthesize the pigment by their own. These data represent an important further step in studying the ochronosis pathogenesis. In fact, as previously noticed, HGA is synthesized mainly by liver and, lesser, by kidneys; we demonstrated not only the presence of pigment inside the syinoviocytes and chondrocytes, but, above all, we pointed out the presence of mRNA of enzyme in such cells, by RT-PCR.

The presence of pigment in cells during the third step and, mainly, the presence of enzyme could indicate that chondrocytes and synoviocytes have an active intracellular tyrosine metabolism, thus able to synthesize HGA.

These data were recently confirmed also by Prof. Santucci's group who demonstrated the mRNA expression encoding for HGD and the related protein in chondrocytes, synoviocytes and osteoblasts\textsuperscript{41}. 
Another aspect that electronic microscope allows us to stretch was the presence of cells suffering of our cultures, as shown by a fragmented nucleus and picnosis phenomena. These aspects of cellular suffering, probably due to apoptotic nature, are coherent with in vitro experiment results; indeed, the evaluation of both ochronotic type cells viability have shown an increment of apoptosis than osteoarthritic cells.

Up until now, there is no effective therapy for AKU but only symptomatic treatment of complications due to ochronotic arthropathy. In fact, it has been demonstrated that diet restrictions of phenilalanine and tyrosine are not practicable for long time and genetic engineering has not yet available effective substitute enzymatic therapy. Nitisinone is approved for tyrosinemia type I therapy and it is under investigation for AKU treatment. As already said, HGA auto-oxydation process causes the formation of oxygen free radicals. When their levels exceed the anti-oxidation capability of chondrocytes, the chondrocytes itself is subjected to oxidation stress. Therefore ROS cause damages on cellular DNA and, then, apoptosis and articular structure alteration. For these reasons, the use of antioxidant substances may result as a fundamental step in AKU therapy.

For several years, the only approved drug for AKU treatment has been ascorbic acid. But as time goes by, the data regarding its efficiency were discordant.

In some studies made in our Institute, N-acetil-cysteine has been used; it is a substance well known for its antioxidant properties and by now in commerce for the last 40 years mainly as a mucolytic. Furthermore, in 2007 was published a study which showed that NAC inhibits chondrocyte hypertrophy in endochondral ossification which
chondrocytes undergo in case of excessive increase of ROS levels by means of inhibition of apoptosis, angiogenesis, and mineralization. We proposed three ways of action of NAC: firstly, it could act as scavengers of oxygen free radicals, thus limiting tissue damage. Secondly, preventing or delaying the oxidation of HGA by increase of glutathione. Finally, since NAC can neutralize the acetaminophen derivateive N-acetylbenzoquinoneimine, whose chemical structure is similar to that of benzoquinoneacetic acid, it would be tempting to speculate that a third mode of action of NAC could be a direct neutralization of benzoquinoneacetic acid.

We demonstrated that NAC can improve cellular viability by reducing apoptosis cells rate, although, no significant cell morphology changes have been observed by TEM. In parallel to NAC add, Vitamin C was used. But this drug doesn’t show significant changes appreciable neither in synoviocytes viability nor in their morphological alterations and pigment. On the other side, C Vitamin induced a significant reduction of apoptosis in AKU chondrocytes.

To support the role of NAC as a potential therapeutic agent for Alkaptonuria-Ochronosis, there are data obtained by HGA urinary levels in AKU patients. NAC add induced a two-fold increase, in two months, of renal HGA excretion. This would suggest that NAC may acts at tubular level, increasing its active secretion.

According to our studies’ results, NAC may perform its therapeutic action either acting at cellular level, reducing cellular oxidative stress and apoptotic process, and enhancing the tubular HGA excretion.

In conclusion this work, is aimed to further explore several aspects of the disease, with the common goal to deep the knowledge of pathogenic and clinical mechanisms, in order to be able to study and apply new therapeutic approaches.
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