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Study of molecular mechanisms and pharmacological approaches of Alkaptonuria's disease

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ABSTRACT

Alkaptonuria (AKU) is an autosomal recessive disorder, called also Black Bone Disease, for the characteristic dark coloration that some tissues and parts of the body assumed. The pathology is caused by the failure of the enzyme homogentisate 1,2- dioxygenase (HGD), that leads the accumulation of the metabolic intermediate homogentisic acid (HGA), derived by tyrosine. HGA highly reactivity triggers the formation of HGA-derived oxidized products, that react with cellular macromolecules, causing a significant generation of ROS and occurrence of oxidative stress. The ongoing oxidative stress status induces the expression of pro-inflammatory cytokines and the activation of immune cell system, with the consequently occurrence in patients of chronic inflammation and secondary AA amyloidosis. Moreover, HGA molecules bonds generate a dark polymer, called ochronotic pigment, that sticks on several organs, particularly on articular joints. Ochronosis triggers detrimental effects on tissues, as cellular death, extracellular matrix destruction, collagen fibrils rupture, since organs lose their function. Tyrosine and phenylalanine are daily taken by the body with diet, and catabolized by the HGD pathway. Thus, in AKU patients, HGA production is constant, since HGA levels in blood and urine are always elevated. Differently, the ochronotic pigment formation and deposit require more time, indeed symptoms in patients generally appear after the fourth decade of life.

The rarity of AKU implies important challenges for its study. Actually, some aspects of the disease are still unexplored, despite it represents the first genetic disorder discovered that follows the principles of Mendelian recessive inheritance. In particular, one of the principal obstacles is the retrieval of AKU samples, that are scarce and generally in bad condition, for the nature of the disease. For this reason, the first step of this thesis project, described in Chapter 3, focused on the set up of *in vitro* model that allowed to reproduce, in a simple and cheap manner, all the characteristics of pathology. Specifically, it was set up an AKU model based on human primary chondrocytes, that allowed to study the most affected compartment in the disorder. Moreover, it was showed a preliminary study on the development of an *in vivo* Zebrafish model, with the objective of overcome the numerous limitations related to AKU mouse model.

The aim of the present thesis work was dual: explore the molecular characteristics still unknown in AKU, and propose an innovative therapeutic approach, that could be extended to all the chronic inflammatory pathologies (Fig.1).

In our lab, it was already showed that HGA administration to cells led the activation of autophagic process. Following this observation, it was explored in Chapter 4 the role of lysosomes in AKU.

Indeed, is known that a dysregulation of these organelles frequently occurs in different kind of pathologies, as autoimmune or neurodegenerative disorders. Actually, an increase in lysosomes' number had been detected in both AKU samples and model. Moreover, AKU lysosomes were localized in the periphery of cells, that represent a not physiologic conformation suggesting a decrease in their activity. Despite the ochronotic pigment deposition on cartilage and collagen fibrils was deeply studied, its formation and intracellular localization was never explicated. Thus, considering lysosomes' role in the storage and degradation of toxic compounds, it was demonstrated in this work, for the first time, that, when cells were exposed to HGA, the ochronotic pigment was developed intracellularly and concentrated in lysosomes. Obviously, this observation could have enormous impact for the treatment of the disease and the counteraction of ochronotic pigment accumulation.

Oxidative detrimental effects of HGA had been already described. Cellular macromolecules, as proteins and lipids, but also organelles, as mitochondria, undergo oxidative reactions, with the occurrence of damages often irreversible. Is known that oxidative stress and ROS target also DNA, with possible deleterious effects for cells and for all the body, considering the potential development of mutations that lead tumors onset. Thus, this aspect needs to be monitored in the progression of disorders. Therefore, the effect of HGA on the genome integrity was studied for the first time, as shown in Chapter 5. It was highlighted that HGA indirectly affected DNA, causing strand breaks and nucleolar stress. This induced the activation of repair mechanisms, on which depended cells destiny.

In addition, Chapter 8 was dedicated to the study of inflammatory signal activated in AKU, a crucial characteristic of the disease. For the first time, the disorder was modeled on immune cells, in order to analyze the pattern of cytokines stimulated by HGA. It was demonstrated that the molecule was able to directly induce pro-inflammatory cytokines expression in different immune cell types. This preliminary study provides the basis for deeply understand the key issue of inflammation and immune cells activation in AKU patients.

In scientific research, the molecular understanding of biological mechanisms and pathways involved in disorders results fundamental to improve their knowledge. This, beside its crucial significance, provides also the theoretical starting point for the research of possible therapeutic cure. Therefore, frequently these approaches are developed together and strictly connected. Until few years ago, AKU patients were treated only with palliative cure. Recently, EMA (European Medicines Agency) extended the application of the drug nitisinone for the treatment of AKU in adult patients. Despite this represents an important progress for the patients' care, the drug carries some collateral effects, due to the induction of tyrosinemia, and its inability to counteract inflammation. Hence, in the present project it was studied a new therapeutic approach, described in Chapters 6 and 7, based on the

combination of low-doses methotrexate (MTX), a widely used anti-inflammatory drug, with antioxidant molecules. In this way, it was obtained a formulation that combined anti-inflammatory and antioxidants properties, with a stronger effect in the counteraction of these conditions, compared to the effect of single treatments. Moreover, it was proved that the co-administration of drugs allowed to use a low concentration of MTX, with the consequent decrease of its adverse effects, and beneficial impact on patients' health. The effectiveness of the proposed treatment was tested against typical markers of inflammation, oxidative stress and amyloidosis, proving that its application could be extended to different kind of inflammatory disorders (Chapter 6). It was also studied specifically its effect on AKU model, and demonstrated that the combination of MTX and antioxidants successfully reduced ochronotic pigment and amyloid fibrils (Chapter 7).

In summary, the present thesis work gives new insight into molecular mechanisms of AKU and presented a new potential formulate for its treatment.

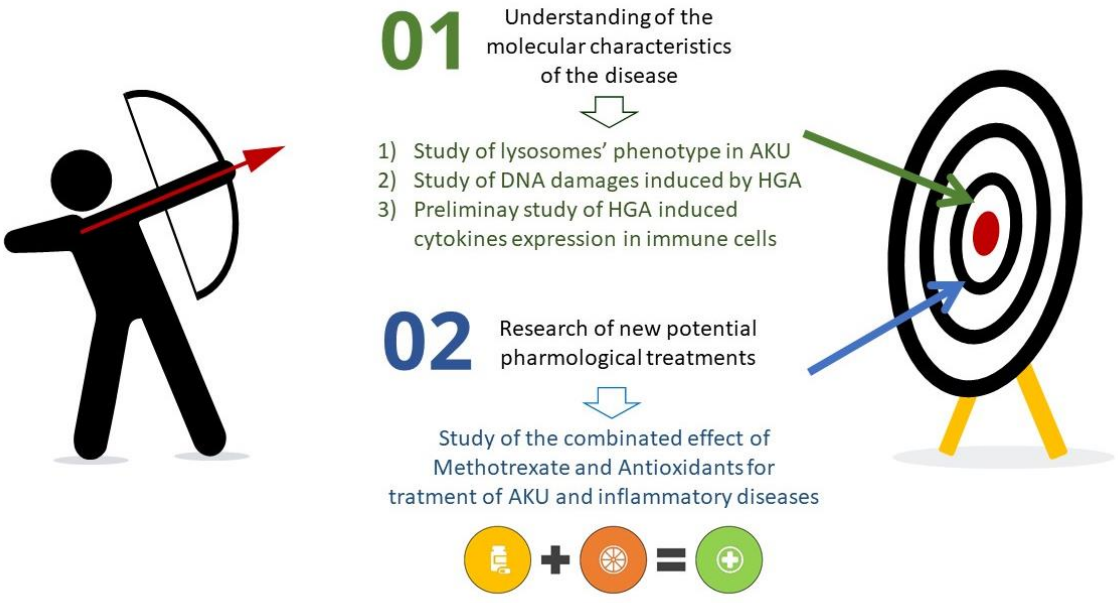


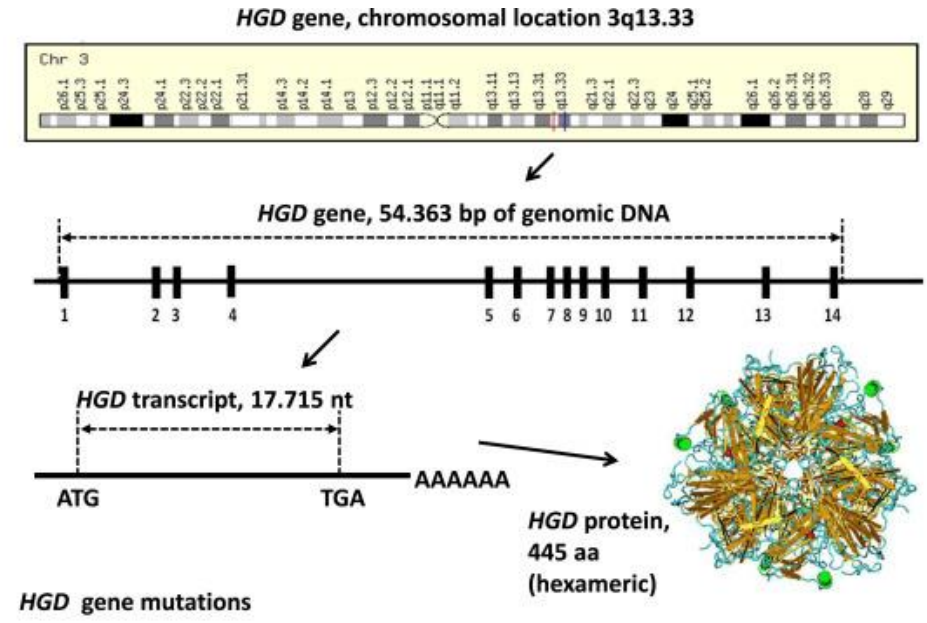
Figure 1: Schematic representation of the workflow of the present thesis.

CHAPTER 1:

Introduction: The ultra-rare disease Alkaptonuria

1.1 Alkaptonuria

Alkaptonuria (AKU) is an ultra-rare autosomal recessive disorder [OMIM #203500], with worldwide prevalence. It was the first genetic disorder subjected to mendelian rules described, by Archibald Garrod in 1902 (Scriver 2008), who defined it an "inborn error of metabolism". Despite this, the mutations that lead the disease were identified only one century later. The gene carrying mutations responsible for AKU is *hgd* (Fernandez-Canòn et al. 1996), located on the chromosome 3q21-q23 (Pollak et al. 1993) and coding for the enzyme homogentisate 1,2-dioxygenase (HGD). *Hgd* is a 54363 bp gene, composed of 14 exons and coding for a protein of 445 amino acids (Fig. 1). While AKU is not very common, a wide variety of causative mutations have been reported: nowadays have been identified 250 variants, collected in the Leiden Open Variation Database (LOVD) (Zatkova et al. 2012). The disease occurs where there is a certain minimum reduction in enzyme activity, specifically a loss of more than 99% of the enzyme activity is required (Vilboux et al. 2009). Most AKU mutations are missense, this is in part due to the complexity of HGD structure, consisting of a dimer of trimers. Indeed, a single residue substitution could easily modify the subunits folding or their interactions, fundamental for the hexamer stability (Bernini et al. 2020; Rodríguez et al. 2000).



HGD gene mutations

EUROPE

Czech republic (4p)		France (23p)		United Kingdom (45p)		Finland (5p)	
unknown	1	unknown	6	unknown	4	S59fs (R58fs)	2
ivs1-1G>A	2	S59fs (R58fs)	1	E42A	11	R330S	2
ivs5+1G>A	1	Y62C	2	S59fs (R58fs)	8	M368V	4
G161R	4	W97G	2	ivs5+1G>T	1	H371R	1
Poland (5p)		D153fs (G152fs)	3	ivs7+2T>C	1	**Germany (9p)**	
ivs1-1G>A	2	D153G	2	C120F	4	L25P	4
G161R	3	ivs7+2T>C	2	G123R	2	G161R	2
ivs13+1G>T	1	V157fs	1	G115R	2	V300G	2
W97C	2	G161R	4	G161R	5	M368V	10
R63fs	1	V181F	2	F169L	1		
G372_P373delinsAla	1	G361R	1	V157fs	1	**REST OF THE WORLD**	
Slovakia (60p)		G270R	1	G217W	1		
ivs1-1G>A	7	H292R	1	R197G	1	**Australia (1p)**	
S47L	1	V300G	1	R225H	1	K57N	1
S59fs (R58fs)	3	G360A	2	G270R	1	G360R	1
ivs5+1G>A	4	M368V	6	K276N	3	**Japan (1p)**	
D153fs (G152fs)	17	P230S	2	V300G	4	E168K	2
G161R	50	P332R	1	G361R	1	**South Korea (1p)**	
E178G	1	G205V	1	G362E	1	Q33R	1
P230S	5	F147S	1	M368V	4	G152A	1
G270R	10	G185R	1	D374H	1	**UAE (1p)**	
V300G	4	A267V	2	K431fs	1	S59fs (R58fs)	2
M368V	2	I346T	2	X446ext	2	**China (2p)**	
H371fs (P370fs)	15	**Italy (37p)**		P359L	1	E42fs	1
T167I	1	unknown	12	N219S	2	F329C	1
Austria (1p)		W60*	2	D18N	1	ivs5+3A>C	1
G161R	1	D153fs (G152fs)	3	D226fs	1	ivs7+1G>C	1
G152R	1	ivs7+5G>A	5	E13K	1	**Turkey (9p)**	
The Netherlands (3p)		G198D	2	K171N	2	S59fs (R58fs)	6
ivs5+1G>T	2	ivs9-56G>A	2	ivs1-1G>A	1	N219S	2
A122D	1	ivs9-17G>A	1	L116P	1	R225H	6
M368V	1	K248R	1	M172T	1	P230S	2
P274L	2	G270R	2	N337D	2	G270R	2
Belgium (1p)		G360R	3	P230S	3	**India (21p)**	
ivs10+1G>T	1	E401Q	5	R225P	5	S59fs (R58fs)	1
P332R	1	G11fs (F10fs)	3	R53Q	3	L116P	2
Macedonia (2p)	1	G161R	3	V245F	1	A122V	8
P158L	1	M368V	5	C120F	1	E168D	4
P274L	1	P230S	6	G115R	1	G360R	1
D153fs (G152fs)	2	V300G	1	G360R	1	R53Q	2
Switzerland (2p)		R53Q	2	C361R	1	ivs2+1G>A	16
unknown	1	K248E	2	**Spain (10p)**		ivs7+6T>C	4
W97G	1	G309V	2	unknown	4	ivs9-85A>G	2
R321X	1	R330S	1	I216T	1	unknown	2
M368V	1	F227S	2	R225H	2	**Jordan (5p)**	
Sweeden (1p)		L44F	1	F227S	2	ex2 deletion	2
G161R	1	S59fs (R58fs)	2	P230S	3	A122V	6
S150L	1	V324fs	1	P230T	1	ivs1-1G>A	1
Lithuania (2p)		G205D	1	D291E	1	unknown	1
G161R	4	Y40S	2	V300G	1	**Lebanon (1p)**	
Portugal (5p)		G251D	1	W322R	2	ex2 deletion	2
R197fs (T196fs)	2	K253Q	1	M368V	3	**Israel (1p)**	
D291E	2	ivs7+2T>C	1			ex2 deletion	2
V300G	2						
M368V	4						

NORTH AMERICA

USA (90p)		USA (90p) cont	
unknown	5	G217W	1
E3A	1	R225L	1
L4S	2	P230S	2
L4*	1	Q258P	2
ivs1-1G>A	2	H269R	1
E42A	6	G270R	2
R53W	1	V300G	4
S59fs (R58fs)	11	S305F	1
S59*	1	R321Q	1
ivs3-2A>G	1	ivs12+2T>A	1
W60G	3	ivs12-2A>T	2
L61P	2	M339fs	1
Y62C	1	M339fs	5
F73L	1	P359L	1
P92T	1	G360R	7
W97R	2	G362E	1
ivs5+1G>T	6	M368V	28
ivs5+1G>A	1	H371fs (P370fs)	2
C120fs	1	P373L	1
C120F	10	E401Q	1
C120W	2	X446ext	2
G123R	2	**Canada (2p)**	
K126fs	2	M368V	1
F136Y	1	S59fs (R58fs)	1
L137P	3	P158L	1
G115fs	1	ivs12-2A>T	1
E143D	1		
R145*	1	**AFRICA**	
N149K	1		
D153fs (G152fs)	3	**Canary islands (1p)**	
ivs7+2T>C	1	P230S	2
P158R	1	**Algeria (14p)**	
P158L	1	unknown	2
Q159H	1	ivs1-1G>A	2
G161R	11	L44F	1
E168K	5	G123A	10
E168*	1	S189I	2
K171N	1	A218fs (G217fs)	2
E178D	1	S287*	3
Q183R	1	A407A	1
ivs8+1G>A	1	D153fs (G152fs)	1
ivs8-2A>C	1	Q29fs	2
R187G	1	I216T	1
C138fs	1	G170A	1
N337fs	1	**La Reunion (2p)**	
V324fs	1	S59fs (R58fs)	1
		V300G	3
LATIN AMERICA		**Mali (1p)**	
		ivs5-11G>A	2
Dominican Rep (8p)		ivs9-13T>G	2
C120W	14		
G270R	2		
Brasil (1p)			
G270R	1		
M186K	1		

Figure 1: Representation of the chromosomal localization of the *hgd* gene, its structure and the hexameric conformation of the protein. In the table are reported *hgd* gene mutations detected in patients in different countries (Gallagherand, JA, Ranganath, LR, Zatkova 2017).

HGD is one of the six enzymes involved in the catabolism of the aromatic amino acids phenylalanine (Phe) and tyrosine (Tyr). These amino acids are essential for human, who takes them principally through high-protein food. Human body could not synthetize Phe its own, but could convert Phe into Tyr thanks to the enzyme phenylalanine hydroxylase. Tyr is involved in several pathways, since can be metabolized to produce hormones, neurotransmitters such as dopamine, adrenaline, or noradrenaline, and the melanin pigment. Despite the numerous functions, the majority of Tyr in the body follow the catabolic pathway, with the final production of fumarate and acetoacetate (https://pubchem.ncbi.nlm.nih.gov/pathway/PathBank:SMP0000006). The pathway (Fig. 2) starts with a reaction of transamination of tyrosine, that generates p-hydroxyphenylpyruvate. The aromatic acid is then substrate of the enzyme p-hydroxylphenylpyruvate-dioxygenase, which generates the compound known as homogentisic acid (HGA). To split the aromatic ring of HGA the enzyme HGD is required. In AKU condition, the loss of function of HGD results in the accumulation HGA, that cannot be converted in maleylacetoacetate. Another inherited severe condition that affect this pathway is Phenylketonuria (PKU) (Cleary and Skeath 2019), in which the body is not able to convert Phe in Tyr, causing problems as intellectual disabilities, behavioural or psychiatric issues, arm and leg jerking.

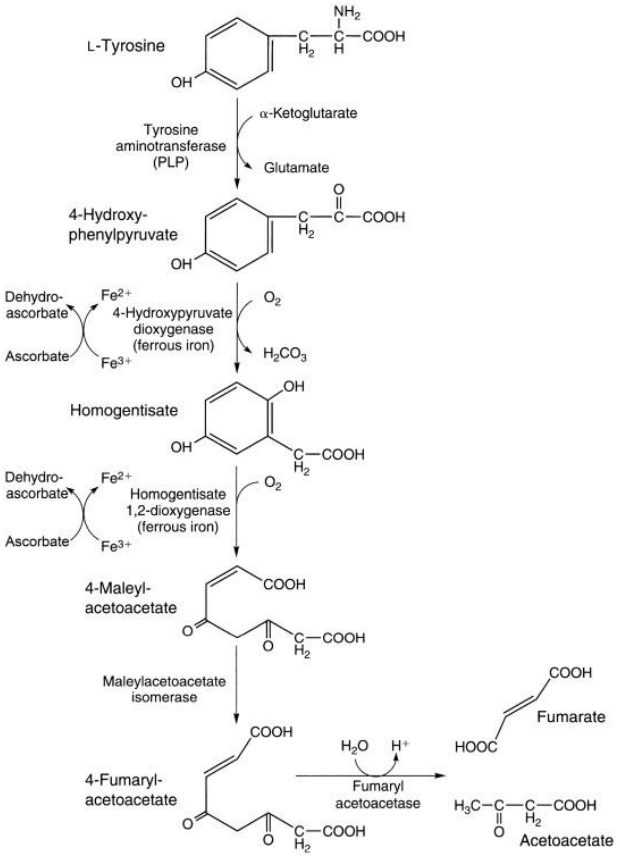


Figure 2: the enzyme homogentisate 1,2-dioxygenase takes part in the pathway of catabolism of tyrosine (Kohlmeier 2015). The deficiency of the enzyme causes Alkaptonuria.

The HGD deficiency causes in AKU constant high levels of systemic HGA. It has been reported that HGD is expressed in liver, kidney, prostate, small intestine and colon, but it has also been detected in osteoarticular cells (Laschi et al. 2012) and in brain (Bernardini et al. 2015). HGA is produced mostly in the liver, circulate in the blood and is partially excreted through urine (Suwannarat et al. 2005), while the remaining accumulates in joins and organs. In AKU patients, the total daily HGA excreted is between 0.4 and 12.4 g, and the plasma HGA level ranges between 3.0 to 27.8 μg per ml, although in normal subjects is undetectable (Phornphutkul et al. 2002). HGA is a highly reactive molecule, that undergoes reactions of autoxidation (Martin and Batkoff 1987) and autopolymerisation, producing a dark-brown melanin-like pigment called ochronotic (Braconi et al. 2015). The reaction occurs spontaneously in presence of oxygen, and is accelerated by the addition of alkali substances. For this reason, AKU patients' urine assumes spontaneously a dark colour (Fig. 3). Adding alkali, the blackening reaction is instantaneous, while acidifying again urine, it doesn't turn back to the normal colour. This is one of the evidence supporting the irreversible nature of the pigment (Mannoni et al. 2004). The molecular structure of the pigment is still unknown, as well as the reactions behind its generation.

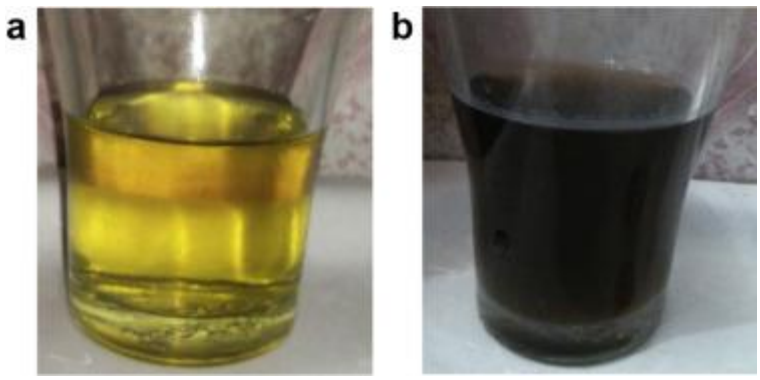


Figure 3: Colour of urine fresh (a) and when was left standing (b) of a 5-year-old boy affected by AKU (Sethi et al. 2020).

The HGA secretion through urines is not sufficient for its elimination, and the molecule accumulates in different districts of the body, causing the ochronotic condition (Millucci et al. 2012). Interestingly, the deposits of the pigment in the body is not uniform: most tissues are resistant to the pigmentation, while HGA is concentrated mostly in connective tissues, as had been showed with the first studies on animals (Zannoni, Malawista, and La Du 1962). Wherefore, articular cartilage, joints, spine, tendons, ligament in AKU are strongly affected. Pigmentation is also visible in the eye sclera, ear cartilage

and wax (Fig. 4), aortic roots and valves, glands, skin and sweat. Pigmented tissues are typically significantly damaged, with in some case the complete loss of function.

Figure 4: Ochronosis development in eye sclera and ear cartilage in different stages of age (Phornphutkul et al. 2002).

1.2 Epidemiology

AKU has a worldwide diffusion of 1 case every 250.000-1.000.000 individuals. However, in some country the prevalence is higher: in Slovakia and Dominican Republique up to 1 in 19 000 individuals are affected. In particular, in Slovakia there are 10 different mutations circulating, 6 of those originated in a small region of the country (Zatková et al. 2000). Because of the recessive genetic nature of the disease, higher incidence appears in isolated geographic regions, in which the genetic isolation is easier, or in small close community. This is the case of the Narikuravar, an Indian nomads group showing a higher incidence of the pathology (Sakthivel et al. 2014).

1.3 Clinical characteristics

The accumulation of HGA and ochronotic pigment causes slow and progressive multi - systemic damages. Tissues as cartilage act as a scaffold for pigment deposition, taking the typical dark coloration. Ochronotic cartilage become thin, stiff and brittle, fragile, susceptible to fracture and splinter. Through radiological analysis, some degenerative changes as joint space narrowing, cartilage irregularities, subchondral sclerosis or peripheral osteophytes cold be detected (Doganavsargil et al.

2015). Moreover, ochronotic pigment accumulation determinate the tendons and ligament spontaneous rupture (Manoj Kumar and Rajasekaran 2003). At microscopically level, cartilage is characterized by dystrophic lacunae formation and by the substitution of collagen type II with the type III, that takes a disorganized arrangement in the tissue (Millucci et al. 2017). Consequently, AKU patients are usually affected by ochronotic arthropathy, that causes pain and loss of function of large joins (Harun et al. 2014). Because of the irreversible destruction of the joins, patients commonly need total replacement surgery of knee, hip and shoulder. Also spinal cordon is impaired, in particular AKU clinical manifestations include the degeneration and calcification of the intervertebral discs, the narrowing of intervertebral spaces and the formation of bony bridges between vertebrae (Gil, Wawrzynski, and Waryasz 2016). AKU patients have difficulty in walking and in movements, with a reduction in the range of the possible motions (Perry et al. 2006). Moreover, the disease is associated to the outcome of scoliosis and kyphosis. Ochronotic spondylosis causes a mean decrement in patient height of 7.9 cm (Phornphutkul et al. 2002). Thus make AKU a debilitating illness, even though fortunately is not fatal.

As mentioned before, in AKU the cardiovascular system is also impacted. Cardiac valves, vessels, endocardium and intima are strongly pigmented, and the aortic valves appear thickened and calcified (Putz et al. 2021). Over time, valves lose their functionality, becoming particularly damaged, since patients need surgical valves replacement. The aortic extensibility is impaired by hypertension, hyperlipidemia, and artery calcification (Thimmapuram et al. 2020). Data obtained from a study related to the cardiovascular manifestations on AKU patients (Pettit et al. 2011) revealed that all patients older than 65 years had aortic stenosis, while in the group of individuals over 50 years, 10% had aortic sclerosis and 40% had aortic stenosis.

Less common manifestations in AKU include renal, urethral, and prostate calculi. Indeed, renal parenchyma is constantly exposed to high amount of HGA, filtrated from the blood and conveyed to the urine. For this reason, in the later stages of the disease, AKU patients are at higher risk of renal and prostate stones (Wolff et al. 2015). It has been shown in AKU patients with renal failure that renal transplantation restores the daily urinary excretion of HGA, allowing its decrease in blood, and provides the enzymatic activity for the metabolism of HGA (Introne et al. 2002).

1.4 Diagnosis

Generally, AKU is diagnosed during the fourth-fifth decade of life, when the symptoms appear. Before this period, the disease is mostly asymptomatic: the only evidence is due to the homogentisic

aciduria, that occurs from birth. However, the dark colour of urine is one of the warning signals also in adult patients, along with the arthritis. Diagnostic tests consist in the measure of HGA level in urine and blood, that allows distinguishing AKU from osteoarthritis and ankylosing spondylitis. Detection of HGA is made with gas chromatography (Oláh et al. 2003), HPLC (Jacomelli et al. 2017), reverse phase of LC-MS/MS (Hughes et al. 2014) or capillary electrophoresis (Öztekin, Balta, and Cansever 2018) analysis. Moreover, recently it has been reported the development of a whole-cell biosensor based on the amount of expression of green fluorescence protein for detect HGA (Navani et al. 2021).

Together with the biochemical diagnosis, it could be carried out the molecular test, sequencing the *hgd* gene, to detect the variant implied but also to provide a genetic counselling to family members (Kaurah and Huntsman 2019).

1.5 Current therapies and patient assistance

Until short time ago, AKU were treated exclusively with palliative cures. A strategy applied in the past was to subject patients to a low-protein diet, considering that the Tyr taken with food is mostly conveyed to the HGD pathway. Protein restriction gave good results in the reduction of HGA excretion in child, but, with increasing age, the effects were less evident (De Haas et al. 1998). Moreover, this kind of diet was difficult to follow and caused complications, so is not a solution suitable for adults.

Pain is specifically treated with not steroidal anti-inflammatory (i.e. FANS) and analgesic, and with physical therapy programs (Dergisi 2005). These limit momentarily the symptoms occurrence, but not the disorder progression. Therefore, antioxidants and vitamin C administration (Morava et al. 2003) allows the reduction of some clinical symptoms and limit the pigment formation, since it is an oxidative process.

Nitisinone is the only drug that has been officially approved by the European Medicines Agency (EMA) in September 2020, for the treatment of adult patients with AKU, with a dose of 10 mg per day. Nitisinone is already used to treat a recessive genetic condition called hereditary tyrosinemia type 1. Its activity derives from the ability to inhibit the enzyme 4-hydroxyphenylpyruvate dioxygenase in the HGD pathway. Thus, the block prevents the HGA formation (Wolffenbuttel, Heiner-Fokkema, and van Spronsen 2021). The effects on AKU patients have been studied through years with different clinical trials. DevelopAKUre is a consortium involving 12 European partners, with the aim of assess the efficacy of the drug for AKU treatment. Three international clinical trial

were conducted to analyse the efficacy of nitisinone in AKU disease (Fig. 5). In a first one, SONIA1 (Suitability Of Nitisinone In Alkaptonuria), different concentrations of the drug were administrated for 4 weeks to randomised groups of patients. Results confirmed that nitisinone decrease the urinary excretion of HGA in a dose-dependent way, resulting in a urinary HGA reduction of 98.8% with the dose of 8 mg daily (Ranganath et al. 2016). The SONIA2 trials involved 2 groups of patients, one taking nitisinone and the other without treatment, for 4 years. Patients were screened for the levels of urinary HGA excretion, the severity of the disease, and the occurrence of adverse events. Results showed the effectiveness of nitisinone in reducing urinary excretion of HGA and ochronosis, and in slowing the disease progression (Ranganath et al. 2020). Despite this, in the nitisinone group still occurred adverse events, and the drug could not mitigate all the characteristics of the disease. It was performed a third clinical trial, SOFIA (Subclinical Ochronotic Features In Alkaptonuria), in which patients were divided in ranges of age, with the aim of understand at what age the ochronosis and joint damages start. Nitisinone administration has some collateral effects, as the induction of high level of plasmatic Tyr. For this reason, is fundamental to deeply understand at what age the therapy could start, in order to reduce the time of drug administration, and consequently the adverse effects. The study revealed that pigmentation could start at the age of 20, while quality of life, pain and HGA levels increase with the age (Cox et al. 2019).

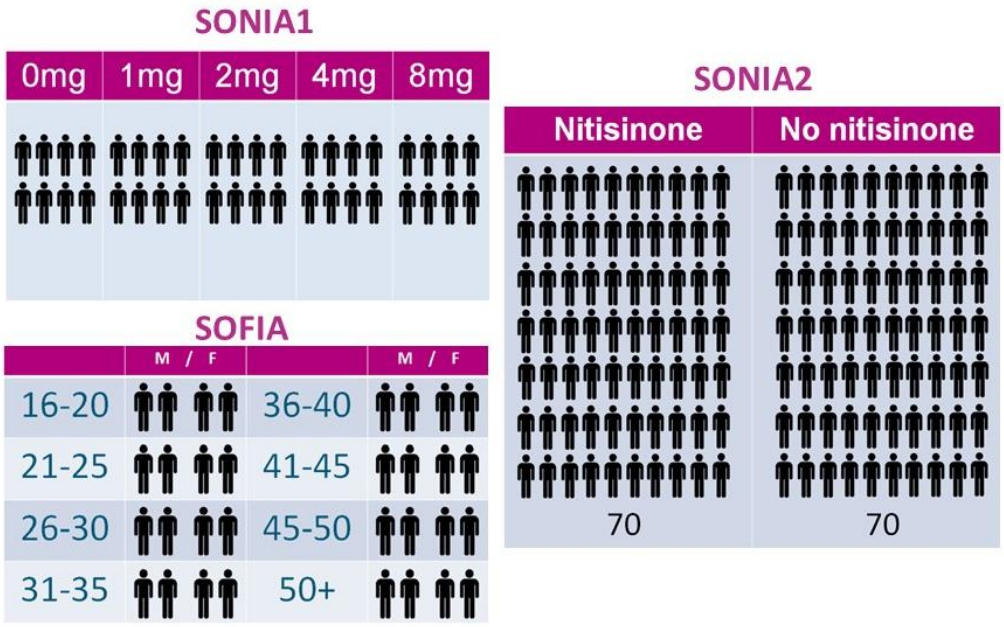


Figure 5: Schematic representation of three clinical trials: SONIA1, SONIA2 and SOFIA.

It has been also created a database, ApreciseKUre, that collect genetic, biochemical and clinical data from all the patients, and can be shared among researchers. This allows to better understand the prognostic biomarker of the disease, and to develop a Precise Medicine approach for personalized management of AKU (Spiga et al. 2017).

In Italy, the association aimAKU represent a landmark for Italian AKU patients and their families, providing information, medical assistance and yearly screening. On the same time, the association supports the scientific research and international collaborations, and is also involved in the bureaucratic requirements.

CHAPTER 2:

Introduction: Molecular aspects of Alkaptonuria

2.1 Oxidative stress and chronic inflammation in diseases

Reactive oxygen species (ROS) are a class of highly reactive molecules derived from O_2, sometimes characterized by unpaired electrons in their external shell. In human body, ROS are either produced naturally from cells, as during the oxidative phosphorylation, and up-taken from exogenous sources, including pollution, cigarette smoke and irradiation (Liguori et al. 2018). Because of their high reactivity, our organism is provided with antioxidant defence systems, that include enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic scavengers (vitamin C and E, carotenoids, glutathione) (Birben et al. 2012). The condition that onset when the balance between antioxidants and ROS is lost, because of the increase of ROS or depletion of antioxidants, is called oxidative stress. Obviously, it is an extremely dangerous situation for cells. Indeed, ROS accumulated react with several macromolecules, such as carbohydrates and proteins, altering their structure and leading their loss of functions and activity, with the consequent production of aberrant signalling. ROS also affect lipids, causing their peroxidation, that determines severe cellular damages to structures and membranes, until the activation of the apoptotic pathway. Finally, ROS interact with nucleic acids and could cause the occurrence of mutations, with catastrophic consequences, as the onset of cancer (Pizzino et al. 2017). For these reasons, during oxidative stress, cells attempt to counteract the harmful ROS effects by inducing the transcription of defensive enzymes, structural proteins, but also inflammatory cytokines, since a protracted oxidative stress condition can causes the occurrence of inflammation.

Inflammation is a defence mechanism that takes place in response to pathogens infection or to endogenous signals that could be dangerous. In some situations, when the body is not able to repair the damage, or the stimulus persists, the inflammation is prolonged for months or years, and the condition assumes the name of chronic inflammation. Numerous are the diseases associated to chronic inflammation, as autoimmune diseases, metabolic disorders such as atherosclerosis, diabetes and cardiovascular diseases, allergies and cancer. Overall, it was estimated that, in all the world, 3 out of 5 person die for chronic inflammatory diseases (Pahwa and Jialal 2019). Chronic inflammation is characterized by accumulation of monocyte-derived macrophages and lymphocytes, by high levels of circulating cytokines and chemokines, and by proliferation of fibroblasts and small blood vessels (Wolff et al. 2015).

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 (Chatterjee 2016). 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.

2.2 Secondary A Amyloidosis

Secondary A amyloidosis is a systemic complication that occurs in several chronic inflammatory disorders, such as rheumatoid arthritis, juvenile idiopathic arthritis, ankylosing spondylitis (Ivanova et al. 2016). It consists in the deposition of amyloid fibrils, derived from serum amyloid A (SAA) proteins, in different tissues, particularly in kidney, gastrointestinal tract and heart (Papa and Lachmann 2018). SAA is an acute phase plasma protein, expressed during inflammation, following TNF-α and IL-1β induction. During chronic inflammation SAA is overexpressed, resulting in constant high systemic levels of the protein. Then, SAA is internalized by cells and accumulated in vesicles, where spontaneously undergoes to self-assembly process. The aggregates formed could affect cellular survival, and are then released in the extracellular space. Here, amyloid deposits grow, with the final formations of amyloidosis fibrils (Claus et al. 2017). AA amyloidosis is progressive, and causes the target-organs failure, since the patients' life expectancy is of 2-5 years (Tanaka et al. 2003).

2.3 Molecular characteristics of Alkaptonuria

Alkaptonuria (AKU) is a genetic disease characterized by a condition of permanent oxidative stress, caused by the progressive accumulation of homogentisic acid (HGA) in the body. HGA is produced mostly in liver, where the catabolic pathway of tyrosine take place. In case of homogentisate 1,2-

dioxygenase (HGD) deficiency, as happens in AKU patients, the molecule could not be converted in 4-maleyl acetoacetate, and is accumulated in the body, carried through systemic circulation. HGA is a highly reducing agent that spontaneously undergoes auto oxidation and polymerization reactions (Bernini et al. 2021), producing a black pigment, called ochronotic pigment. In addition to the pigment formation, HGA oxidation is also evident in the darkening of urine exposed to oxygen (Gallagherand et al, 2017). In AKU patients' body, the pigment is accumulated over years and deposits in different districts, until, after the fourth decade of life, some tissues appear totally pigmented and strongly damaged (Fig. 1).

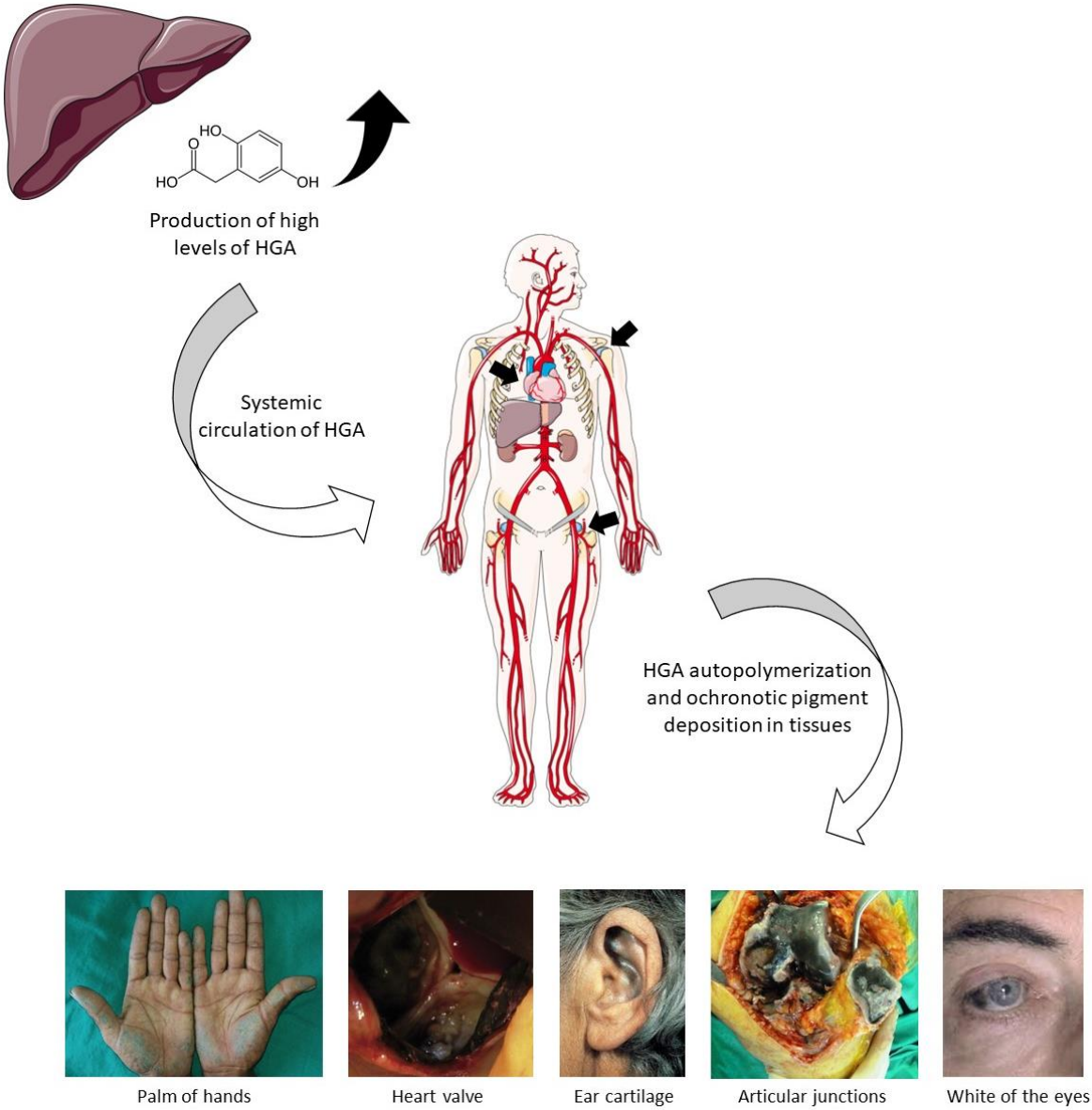


Figure 1: HGA is produced in liver and is spread in the body through systemic circulation. In several tissues, through its auto polymerization, leads the formation of the ochronotic pigment. The pigment settles in specific patients' compartment of the body, as articular joints, heart valves and aortic vessels.

HGA is easily oxidized, and the radical species derived, extremely reactive, besides the auto polymerization, trigger a chain of oxidative reactions, with the consequent formation of ROS. This determines an amplification of the number of oxidative reactive molecules, whose impair several cellular compounds, targeting proteins, lipids and DNA. Consequently, the oxidized biological macromolecules result damaged, losing their structure and, most of the time, their function. High damaged compounds become incompatible with cellular life, leading the activation of autophagy and apoptotic pathway. Moreover, the oxidative stress induced by HGA determines several post-translation modifications in proteins, such as carbonylation, thiol-oxidation, 4-HNE modification, glycated and nitrate addiction (Braconi et al., 2013). These modifications, in some cases irreversible, cause proteins alteration and loss of function, along with structural changes. So, proteins could lose their folding, with an increase of the hydrophobic exposed surface, that makes them prone to form aggregates. In addition, in AKU, both proteins and lipids are peroxidised, as a reaction of the body that implements defence mechanisms against oxidative stress. Oxidative stress affects not only cells, but also serum, in which the levels of protein carbonylation and lipid damages have been found significantly higher than in control serum (Albatayneh et al. 2019). Moreover, in some cases, high levels of C-reactive protein (CRP) and Advanced Oxidation Protein Products (AOPP) had been detected in AKU blood (Braconi et al., 2016). When serum is treated *in vitro* with HGA, it had been observed an increase in lipid peroxidation, a decrease of glutathione peroxidase activity, a massive depletion of thiol groups and the carbonylation of serum proteins (Braconi et al., 2011).

The oxidative stress condition determines not only modifications to macromolecules, but also affects the levels of proteins expression, determining changes in the whole proteome. In particular, in AKU are mostly higher expressed proteins implied in the defence mechanisms against stress, damages repair and pathways involved in programmed death. In AKU serum is modified the expression of apolipoproteins, glycoproteins, complement factors and protease inhibitors (Braconi et al., 2016). Moreover, in AKU chondrocytes are differently expressed proteins involved in: protein fate; cell structure and organization; cell rescue, defence, and stress response (Braconi et al., 2012). These observations were confirmed by *in vitro* AKU model, in which the HGA administration affects the levels of proteins with a role in assisting protein folding, cell organization, cell defence and stress response (Braconi et al., 2010).

As previously discussed, the oxidative stress is strictly bounded to the occurrence of inflammation. As happens in several diseases, also in AKU the persistence of ROS and oxidants molecules, and the resulting damages, determinate the activation of inflammatory signals, that, for the nature of the pathology, persist. For this reason, AKU is characterized always by a chronic inflammatory condition. In AKU chondrocytes had been detected significant higher levels of IL-1β, IL-6, IL-8, IL-10, and

TNFα compared to the non AKU cells (Braconi et al., 2012; Spreafico et al., 2013), supporting the inflammatory nature of the disease. The proinflammatory cytokines were also more concentrated in AKU plasma (Millucci, Ghezzi, Paccagnini, et al. 2014) and in HGA-treated AKU cell model (Millucci et al. 2012) compared to healthy control samples. Moreover, it had been found a positive correlation between the levels of IL-6 in AKU serum, the age and the AKU Severity Score of patients, suggesting a possible role of the cytokines in the pathogenesis of inflammation (Albataineh et al. 2014). In addition, in AKU it had been highlighted also the activation of the angiogenic pathway, feature related to chronic inflammation, demonstrated by the high vascularization of AKU tissues and the expression of endothelial markers (Millucci et al. 2016).

Interestingly, it had been discovered that AKU is associated to the occurrence of secondary AA amyloidosis, whose development is presumably an indirect consequence of inflammation. Indeed, in the majority of AKU patients, the SAA level in serum is out of the reference threshold (10 mg/L) (Braconi et al. 2016, 2018). SAA formations and the consequent amyloid deposits had been found in different patients tissues, as cartilage, synovia, periumbilical fat, salivary gland, aortic valves (Millucci, Ghezzi, Braconi, et al. 2014), as well as in the *in vitro* HGA-treated AKU model (Millucci et al. 2012).

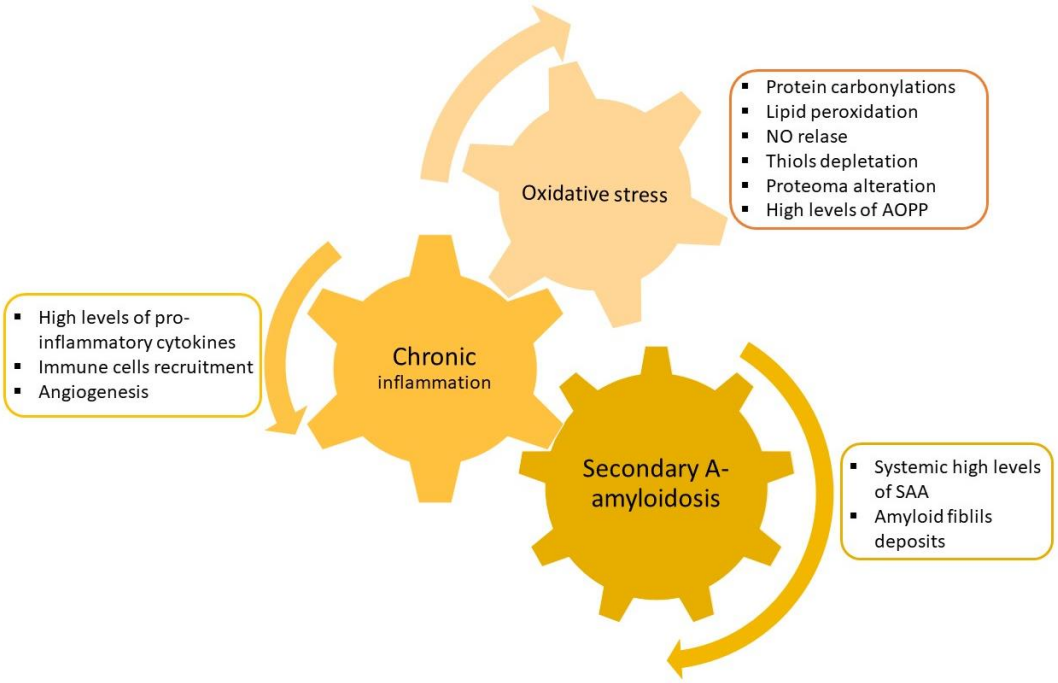


Figure 2: In AKU, oxidative stress, chronic inflammation and secondary A amyloidosis are strictly interconnected, and lead several harmful effects, as proteins post-translational modifications and pro-inflammatory molecules expression.

A peculiar characteristic found in AKU is the amyloid aggregates co-localization with the ochronotic pigment, as it was shown from the overlapping of the SAA signal with the pigment staining (Millucci et al. 2012). Is still not clear if the ochronotic pigment accumulation stimulates the amyloid aggregates formation, as a defence mechanism implemented by the body, or, on the opposite, the amyloid fibrils act as a scaffold, increasing the pigment deposition (Fig. 3). According to the first hypotheses, amyloid plays a functional role, surrounding the pigment in order to protect the body from its toxic and oxidative effects, while in the second theory amyloid accumulation is merely a consequence of the SAA high levels in the blood. In this case, the pigment deposits co-localization with amyloids could depend on its physical characteristic, which makes it suitable for the pigment deposition. Certainly, the two process are strictly connected, and affects 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.

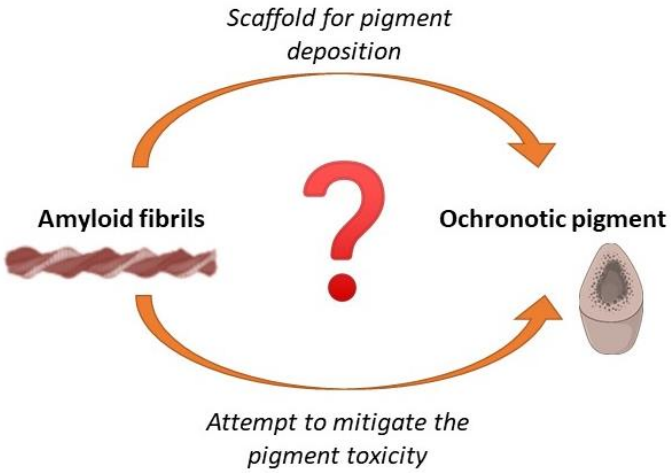


Figure 3: Amyloid fibrils and ochronotic pigment co-localize, but is still not clear if the amyloid accumulation is consequence or cause of ochronotic pigment deposits.

2.3.1 The ochronotic pigment deposition on joints

The ochronotic pigment is considered a polymer, derived from auto polymerization of HGA and its products of oxidations, despite its chemical structure is still unknown. Is called also melanin-like pigment, considering that both ochronotic pigment and melanin derive from tyrosine, and have a similar absorbance spectra, with an absorbance peak in the UV-visible range (Ranganath, Norman,

and Gallagher 2019). The ochronotic pigment development strongly depends from pH, with an acceleration of the process on alkali condition and in presence of Fe^{2+}/H_2O_2 (Vercruysse, Taylor, and Knight 2017).

In AKU patients the cartilage is one of the most affected tissues. Through microscopically observation of AKU cartilage samples, it could be noticed that the pigment settles mostly in correspondence of collagen fibres. Indeed, the loss of protective molecules such as proteoglycans and glycosaminoglycan, that is a natural aging process increased in AKU patients, makes available, in the collagen fibres, binding sites for HGA (Ranganath et al. 2019). In this way, pigment subunits accumulate in the fibres, triggering its rapid formation and deposition.

With the progression of the disease, AKU cartilage loses its architecture, and the extracellular matrix (ECM) become disorganized, failing in its function. Specifically, in AKU there is a reduction in the synthesis of fundamental ECM compounds, as collagen, glycosaminoglycan (GAGs) and proteoglycans, and proteins (Galderisi et al. 2021). The low quantity of aggrecan, the principal proteoglycan in articular cartilage, allows a decrease in cartilage resistance, that gets brittle and stiff. Moreover, in AKU ECM, the type II collagen fibres break easily, and are substituted with the type III, tinier and weaker. So, collagen fibres assumes a disorganized arrangement, and are scattered between amyloid fibrils (Millucci, Ghezzi, Bernardini, et al. 2014). The progressive destruction of ECM, the reduction of cellular metabolic activity and chondrocytes apoptosis results from the seat of the pigment and amyloid (Akasaki et al. 2015). This process is extremely aggressive and detrimental for 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 in patients pain and inability to move, since is necessary their substitution with prosthesis.

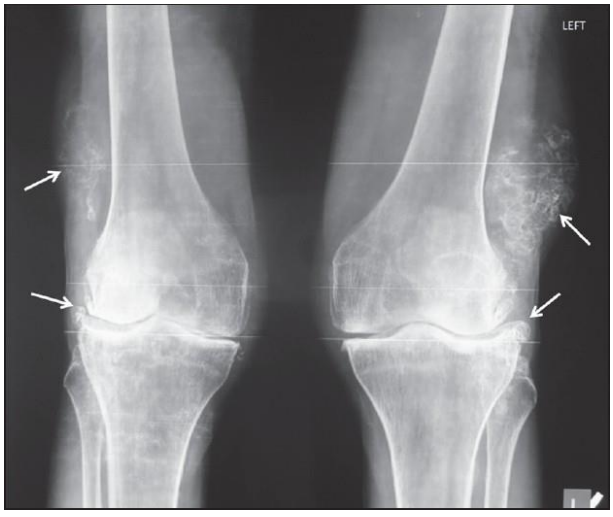


Figure 4: X ray of both knee joints of AKU patient shows degenerative changes, as joint space narrowing and soft tissues calcifications (Reddy et al. 2014)

CHAPTER 3:

Models of Alkaptonuria

3.1 Introduction

Due to the extreme rarity of Alkaptonuria (AKU), added to the frequent late diagnosis of the disease, is extremely challenging to obtain periodically samples from patients. Moreover, samples from most affected tissues, as cartilage, ligaments and heart valves, could be get only with invasive techniques, including surgical joint and valve replacement. After that, biopsies from AKU patients are often strongly damaged and brittle, that makes difficult to isolate cells and to obtain good slides for histological analysis. For these reasons, the necessity to have standardized AKU models for study the disorder's characteristics and drugs effects is evident. In recent years, different models, from *in vitro*, to *ex vivo* or *in vivo*, had been studied, with the purpose to obtain a system that could accurately reproduce the characteristic of the disease, and at the same time could be easy to set up and manipulate.

3.1.1 *In vitro* and *ex vivo* AKU models

As previously said, the most immediate way for study AKU features is by using histological samples and cells obtained from AKU patients. Human primary chondrocytes isolated from patients had been already deeply characterized. Compared to non AKU cells, AKU chondrocytes had higher levels of nitric oxide (NO) and pro-inflammatory cytokines, together with the intracellular ochronotic pigment deposition. Further, a substantial number of cells incur to apoptosis. So these cells brought all the inflammatory characteristic typical of the disorder. Through a proteomic analysis, it was also shown that, in AKU chondrocytes, there is a profound alteration in expression of proteins involved in cell defense, protein folding, and cell organization (Braconi et al. 2012).

To reproduce the characteristics conferred by HGA accumulation, AKU models had been created by treating cells, serum and histological samples with HGA. This reproduces in vivo condition, in which blood, tissues and cells are constantly exposed to high levels of HGA, carried through blood. Then, HGA settles in tissues and is adsorbed by cells. It was shown that the accumulation of the molecule allows the development of oxidative stress, that triggers on its turn a variety of defense reactions. *In vitro,* the response that occurs against HGA is identical to what happens in patients' body. In fact, HGA treatment induces in chondrocytes the expression of SAA, pro inflammatory cytokines, and

lipids peroxidation (Spreafico et al. 2013).

It had been studied the effect of the addition in serum of HGA 0.33 mM, concentration in the range of circulating HGA detected in patients' serum. HGA in serum enhances the production of ochronotic pigment, but also significantly oxidizes proteins, inducing their carbonylation and oxidation of thiol residues. The model is extremely easy and it had been used to test the efficiency of antioxidants in counteracting damages induced by HGA (Braconi, Laschi, Amato, et al. 2010). Obviously, for study the pathways and mechanisms involved in the disorder development is necessary a more complex model. Thereby, it had been set up AKU models based on the incubation of primary articular or immortalized cells with HGA. Osteosarcoma cell lines MG63, SaOS-2 and TE85 had been cultured with different concentration of HGA, ranging from 0.1 mM to 1 mM. After two weeks treatment, it appeared a pigment deposition in a dose-dependent way, with a parallel reduction of vitality. Higher HGA concentration determined severe cell toxicity and cellular death (Tinti, Taylor, et al. 2011). Considering that the articular compartment of the body is the most affected in the disease, appear convenient employ human primary articular cells, in order to study the pathology mechanisms preserving all the biological characteristics that take place in patients. Consequently, it had been developed *in vitro* models using human chondrocytes and osteoblast, isolated from cartilage and sponge bone fragments, obtained from patients undergoing surgery for total hip or knee replacement. Also in this case, HGA induced the ochronotic pigment formation, apoptosis and oxidative stress related features, with dose and time correlation (Galderisi et al. 2021; Schiavone et al. 2020; Tinti et al. 2010). In addition, it had been set up an *ex vivo* model, culturing slides of cartilage with HGA for two months. This allowed to obtain the ochronotic pigmentation, with a more concentrated deposition around the lacunae, as it could be observed in the AKU samples (Tinti, Spreafico, et al. 2011). In agreement with these observations, the AKU models set up appear perfect for the study of the disease, carrying all the molecular modifications induced by HGA, with the advantage of being easy and reproducible.

3.1.2 *In vivo* models

Despite AKU was the first inborn disease identified, the gene *hgd* was characterized for the first time only in 1995 by Fernandez et al. (Fernandez-Canon and Penalva 1995) in the filamentous ascomycete *Aspergillus nidulans,* in which the phenylalanine (Phe) pathway is well conserved and similar to the higher eukaryotic one. The *hmgA* gene (correspondent in *A. nidulans* to human *hgd*) was disrupted in this model, with a consequent secretion of HGA and formation of a red pigment, that turned in brown when the fungus was grown in media containing Phe. Moreover, the gene was efficiency cloned and

expressed in *E.Coli*.

The first study of AKU on animals was conducted on Guinea pigs (La Du, O'Brien, and Zannoni 1962): after HGA introduction in animals through intraperitoneal injection, it was studied its physiological distribution in plasma and tissues. HGA after 30 and 60 min of the injection was mainly localized in skin and cartilage, as happened also in AKU subjects.

Montagutelli et al. created the first mouse with homologous recessive mutation of a gene, in chromosome 16, equivalent to the human gene *hgd* (Montagutelli et al. 1994). The model set up was confirmed by the detection of HGA in urine, that naturally assumed a gradually dark coloration, accelerated by the addition of alkali. Moreover, it was measured the activity of the HGD enzyme in liver, that was 6% in homozygotes mutant mice and 50% in heterozygotes. Despite the model was characterized by the enzyme silencing and HGA production, in mouse it was not visible the ochronosis and arthritis development. Indeed, AKU mouse model exhibited only high HGA levels in plasma, black urine and the pigmentation of chondrocytes. This could depend by the high antioxidative defense mechanisms in mice, considering their endogenous production of vitamin C, by their high capacity of excrete HGA or by their short lifespan. However, the *hgd-/-* mouse model was successfully used to test the nitisinone effect in the reduction of HGA levels in plasma and chondrocytes pigmentation (Keenan et al. 2015; Preston et al. 2014). Differently by *hgd-/-* mouse model, pigmentation could be observed in the kidneys and knee joints of mouse model with the tyrosinemia type 1 mutation (*fah*−/−) combined with AKU heterozygosity (*hgd*+/−), following the spontaneous loss of function of *hgd* (Manning et al. 1999; Taylor et al. 2012). The mutations allowed mice to survive, giving them a phenotype AKU like. However, this model is not much practicable, and mice organs resulted already compromised by the tyrosinemia. Recently, it was generated for the first time an AKU mouse model with conditional deletion, through the Cre-Lox system, instead of the chemical mutagenesis, to overcome the problems of generation of other uncharacterized mutations (Hughes et al. 2018).

Beside all this progresses, mouse is a model that allows to analyze only partially the AKU characteristic and drugs effects, considering the absence of osteoarthritis and joins failure. For this reason, the necessity to create a new model for a more complete study of the disease is evident. In this discussed scenery, zebrafish could be an appropriate *in vivo* model. The versatility, low maintenance costs, rapid external development and simple genetic manipulation have enabled zebrafish to succeed as model organisms in developmental biology first, and then in several fields of research. In particular, the translucency of the larvae, and the development of adult transparent fish, called Casper (White et al. 2008), allow to detailed observe organs development and their features in

living animals. Specifically, zebrafish model is particularly appropriate for the study of articular and skeletal disorders (Carnovali, Banfi, and Mariotti 2019). Transgenic lines, expressing fluorescent proteins in specific organs or conditions, permit to investigate, through *in vivo* imaging, bone and cartilage characteristics. Cartilage and bone could also be simultaneously stained in larvae, in order to investigate the levels of skeleton mineralization (Walker and Kimmel 2007). Moreover, skeletal phenotyping techniques, such as x-ray, microCT imaging and histological stains can be easily performed (Tonelli et al. 2020). At molecular level, is known that many signaling pathways involved in the osteoarticular system are highly conserved in teleost, and mammalian genes have orthologues in zebrafish (Valenti et al. 2020). For all this reasons, zebrafish is an extreme powerful model to investigate AKU disease from the molecular and physiological viewpoint. It could lead a deeper study of the pathology, focusing on the cartilage progressive degeneration, and it could be used to perform drug screening.

3.2 Aim of the study

The aim of the present study was to set up a primary human chondrocyte *in vitro* AKU model, employed in the following experiments of this thesis project. It was assessed the right concentration of HGA that led ochronosis formation with concomitantly fewer negative effects on cellular viability.

Moreover, in this chapter were shown the preliminary data obtained from the set up of zebrafish *in vivo* model. The aim was to develop, for the first time, a zebrafish based AKU model, that could overcome the limits of the mouse model.

3.3 Material and methods

3.3.1 Isolation and culture of primary human chondrocytes

Human articular cartilage fragments were obtained, after informed consent, from femoral heads and knees of patients undergoing joint replacement surgery. Cartilage samples were rinsed with phosphate buffered saline (PBS) and cut with sterile surgical scalpels. The fragments were placed in a Petri dish with 15 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% penicillin /streptomycin (10.000 U/ml penicillin–10.000 mg/ml streptomycin; P/S) and 1% fungizone (Amphotericin B) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 h. Chondrocytes isolation was performed from cartilage fragments through three sequential enzymatic digestions: with hyaluronidase 1 mg/ml for 30 min, with pronase 5 mg/ml for 1 h, and finally with

collagenase (type I) 2 mg/ml in agitation for 45 min, all incubated at 37°C. The cell suspension obtained was filtered twice using 70 mm nylon meshes, washed, and centrifuged for 10 min at 1500 rpm. Finally, the cellular pellet was suspended and seeded in 10 cm-Petri dishes, with complete culture medium (DMEM high glucose supplemented with 1% P/S and 10% fetal bovine serum (FBS)). Chondrocytes were grown at 37°C in humidified atmosphere of 5% CO_2 and used until reached the third passage.

3.3.2 HGA administration on cells

Human primary chondrocytes from different donors were plated in multi-well plates, obtaining a confluence of 80% cells/well. The HGA administration began the day after the seeding. To prepare the stock, HGA power was dissolved in milliQ water with the final concentration of 10 mM and filtered. Then, the fresh dilutions of HGA were prepared in the complete culture medium and added to cells, while in the control conditions was only changed the medium. Medium was replaced twice per week. Cells were photographed in vivo, after 1 week and 2 weeks of culture, to analyze their shape and growth.

3.3.3 Fontana Masson staining

Chondrocytes were seeded at 30.000 cells/well density into 24-well plates, on 13-mm diameter sterile coverslips. The control conditions were grown with the complete medium, while the AKU model was developed treating cells with HGA at different doses (0.036 mM, 0.046 mM and 0.066 mM). After 2 weeks of treatment, coverslips were washed briefly in PBS, fixed in 4% paraformaldehyde for 15 min at RT, washed again and stained with Fontana-Masson stain, consisting in the incubation with 5% ammoniacal silver nitrate solution for 3 h. After that, samples were rinsed in milliQ water 3 times and incubated for 2 min in 2% sodium thiosulfate solution. Nuclei were counterstained with nuclear fast red solution for 5 min. Lastly, slides were dehydrated with increasing concentrations of ethanol and xylene, and fixed on coverslips. The images were acquired using Zeiss AxioLabA1 microscope at 10X magnification.

3.3.4 Cells viability assay

Chondrocytes were seeded into 96-well plates at a concentration of 10.000 cells/well. Cells were treated for 7 days with HGA at concentrations ranging from 0.02 mM to 0.06 mM. Viability was

evaluated using the MTT technique. The assay is based on the reaction catalyzed by the enzyme mitochondrial succinate dehydrogenase, that reduce the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (yellow) to MTT-formazan (purple). Hence, the amount of MTT-formazan formed, obtained spectrometrically, depends on mitochondrial respiration and indirectly on the amount of cells metabolically actives (Chacon, Acosta, and Lemasters 1996). Results were expressed as the average of triplicates, statistical significance was indicated with asterisks (***p<0,01, ****p<0,005).

3.3.5 Maintenance of zebrafish lines

The zebrafish facility of the IFC Institute in CNR (Pisa, Italy) obtained the authorization n°297/2012-A since 12/21/2012. All animal procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament regarding the protection of animals used for scientific purposes. Zebrafish were raised in Tecniplast housing systems, at the constant temperature of 28°C, with 14 h of light and 10 h of dark. Fishes were fed with SDS (Dietex, France) in the morning and in the afternoon, and with Artemia magnetica at noon. Wild type zebrafish line (AB strain) was used in this study.

3.3.6 HGA treatments of zebrafish

Zebrafish embryos at 3 dpf were put in a 6 well plate, with 5 ml/well of E3 medium solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$; pH=7.2). Three conditions, in triplicate, were set up: control, embryos exposed to HGA 0.2 mM and to HGA 0.5 mM. Medium was changed every 3-4 days. At 13 dpf, larvae were transferred in 10 cm Petri dishes, with 50 ml of medium each. At 20 dpf, lavrae were sacrificed.

3.3.7 Zebrafish fixation and staining

At 20 dpf, after 17 days of treatment with HGA, larvae were fixed with paraformaldehyde 4% at 4°C ON. The day after, larvae were raised 3 times with PBS and stained with Schmorl's Stain (1% ferric chloride and 1% potassium ferricyanide in milliQ water) for 10 min. After the staining, they were washed well in tap water and photographed under Leica M80 microscope with Nikon DS-Fi1 camera.

3.4 Results

3.4.1 Establishment of an *in vitro* AKU cells model

To set up an AKU *in vitro* model, human primary chondrocytes were treated with different concentrations of HGA, in order to find a dose not toxic for cells, but also that allows the ochronotic pigment formation. Indeed, high HGA concentrations cause cells death, while low concentrations are not enough for induced a visible pigment formation.

The shape and vitality of cells were analyzed in live (Fig. 1A), during their culture. In the HGA treated conditions, chondrocytes grew slower that the non-treated cells, that rapidly achieved the confluence. In particular, the number of cells and their ability to replicate decreased with the exposition to increasing doses of HGA, until the higher concentration had strong toxic effects and wasn't compatible with cellular life. Moreover, cells treated with HGA 0.046 mM and 0.066 mM assumed globular or elongated shapes, signals that clearly indicated their sufferance. Detrimental effects of high doses of HGA appeared more severe over time of incubation, since cells exposed to 0.066 mM HGA for 2 weeks were few, extremely small and spherical.

At the end of the HGA administration, the pigmentation, revealed through Fontana Masson staining, was evident in the 0.066 mM and 0.046 mM conditions, while it was less present in the 0.036 mM condition (Fig. 1B). However, through observation of slides was confirmed that the higher HGA dose was not compatible with cellular viability, considering the sufferance phenotype of cells. On the opposite, the concentration of 0.046 mM allowed chondrocytes to survive and to develop the pigmentation.

The previous observations on the viability were confirmed with the MTT assay, that showed a statistically significant decrease of the viability in cells treated with HGA up to 0.05 mM (Fig. 1C).

The pigment development in HGA treated cells necessitates 2-3 weeks, that is not surprising considering that pigment evidence in patients emerges after the fourth decade of life. For that, is fundamental to set up a model in which cells could survive for several week, also if continuously exposed to HGA. Therefore, the HGA dose chosen to set up AKU model using human primary chondrocytes, that fulfilled the requirement previously descripted, was 0.046 mM.

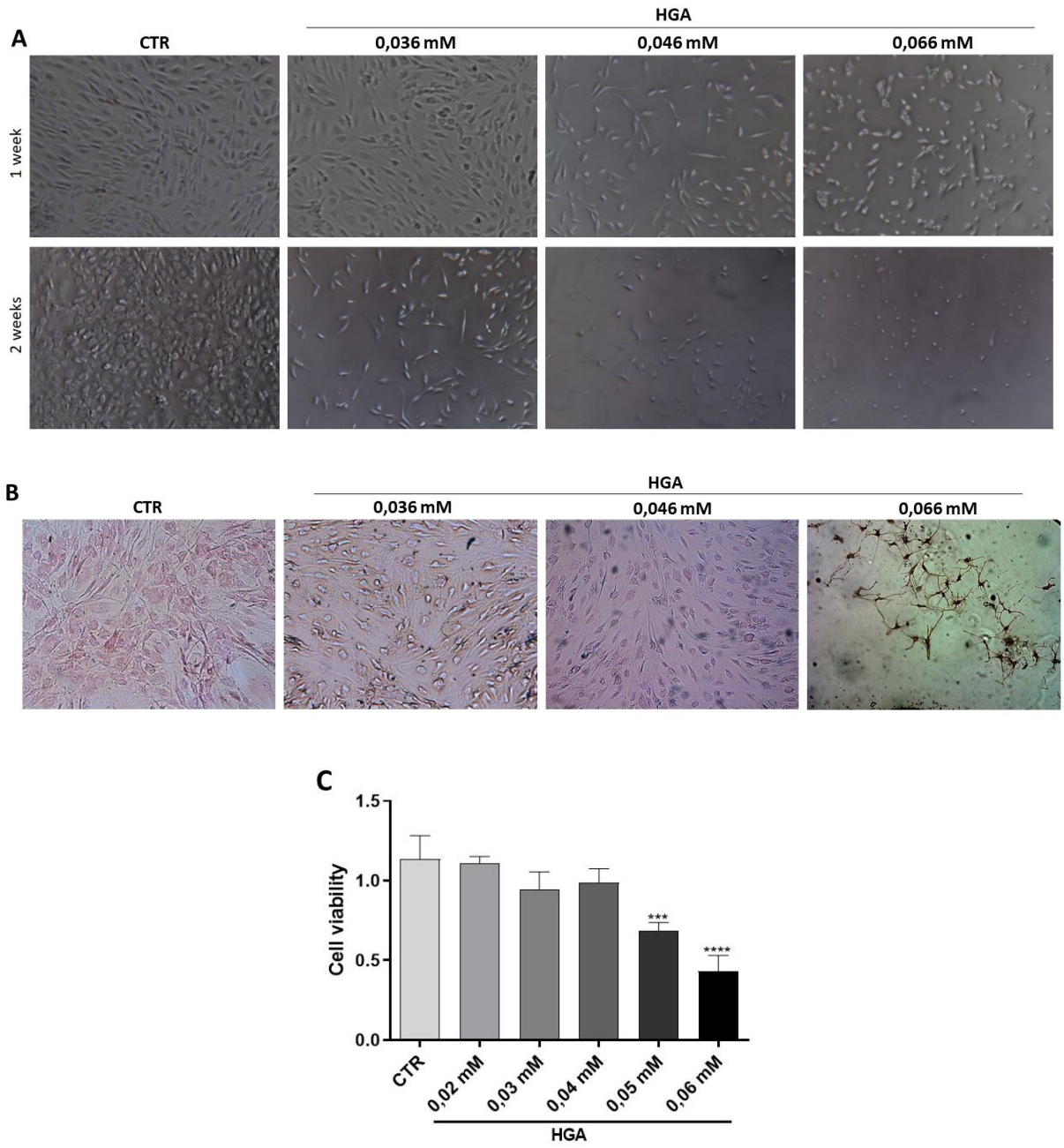


Figure 1: A) Images of alive cells, control (CTR) and treated with 3 different concentrations of HGA, at 2 time points (1 week and 2 weeks). The number of cells and their shape depended on the effects of HGA. B) Fontana Masson staining of CTR and HGA-treated cells after 2 weeks of culture. C) MTT assay of chondrocytes exposed to different doses of HGA for 1 week.

3.4.2 Effect of HGA treatment on zebrafish survival and ochronotic pigment development

To set up an *in vivo* model, fishes were grown with 0.2 and 0.5 mM HGA in plate for 17 days, and compared to the correspondent control condition. At the end of the treatments, in both the HGA

conditions, fishes did not show evident physical and structural changes. Despite this, the vitality of zebrafish grown in presence of HGA was reduced by 50% compared to the control. To analyze if the HGA was internalize in Zebrafish, and underwent auto oxidation and auto polymerization reactions, fixed fishes were treated with Schmorl's staining. The method relies on the reducing properties of melanin and melanin-like molecules, including the ochronotic pigment. So, in presence of melanin, ferric chloride and potassium ferricyanide react to form blue-green granules. In HGA treated Zebrafish, Schmorl's staining conferred a blue coloration, revealing that the continuously exposure to HGA determined the ochronotic pigment development. Thanks to the transparency of the larvae body, was possible to appreciate the blue spot already in the uncut fish (Fig.1 A). Moreover, the coloration could be easily recognized with the smaller magnification of the microscope. Therefore, HGA contained in the water was passively absorbed by the larvae body, as happened with cells, and was then accumulated in tissues. There, molecules naturally auto oxidized and auto polymerized, leading the classical pigment formation. More in detail, the pigment accumulated in the bottom part of torso, in the junction of the fin and along the length of the tail (Fig.1 B). The ochronosis appeared to be HGA dose-dependent, as happened in cellular model. This suggested that 0.5 mM could be the elective HGA dose for the model set up, because it induced the strongest pigmentation.

These preliminary data are extremely encouraging on the use of zebrafish as animal AKU model. Indeed, the fish characteristic to accumulate pigmentation could allow to perform *in vivo* study that are not possible with the mouse model. The animal pigmentation as a naturally consequence of the exposition with only HGA, makes it an extremely simple model to set up. Moreover, the time needed for the pigment formation in zebrafish is extremely low, comparable with the one in the cells. Finally, zebrafish model carries several advantages connected with the rapidity of grow, the external development and the easy manipulation.

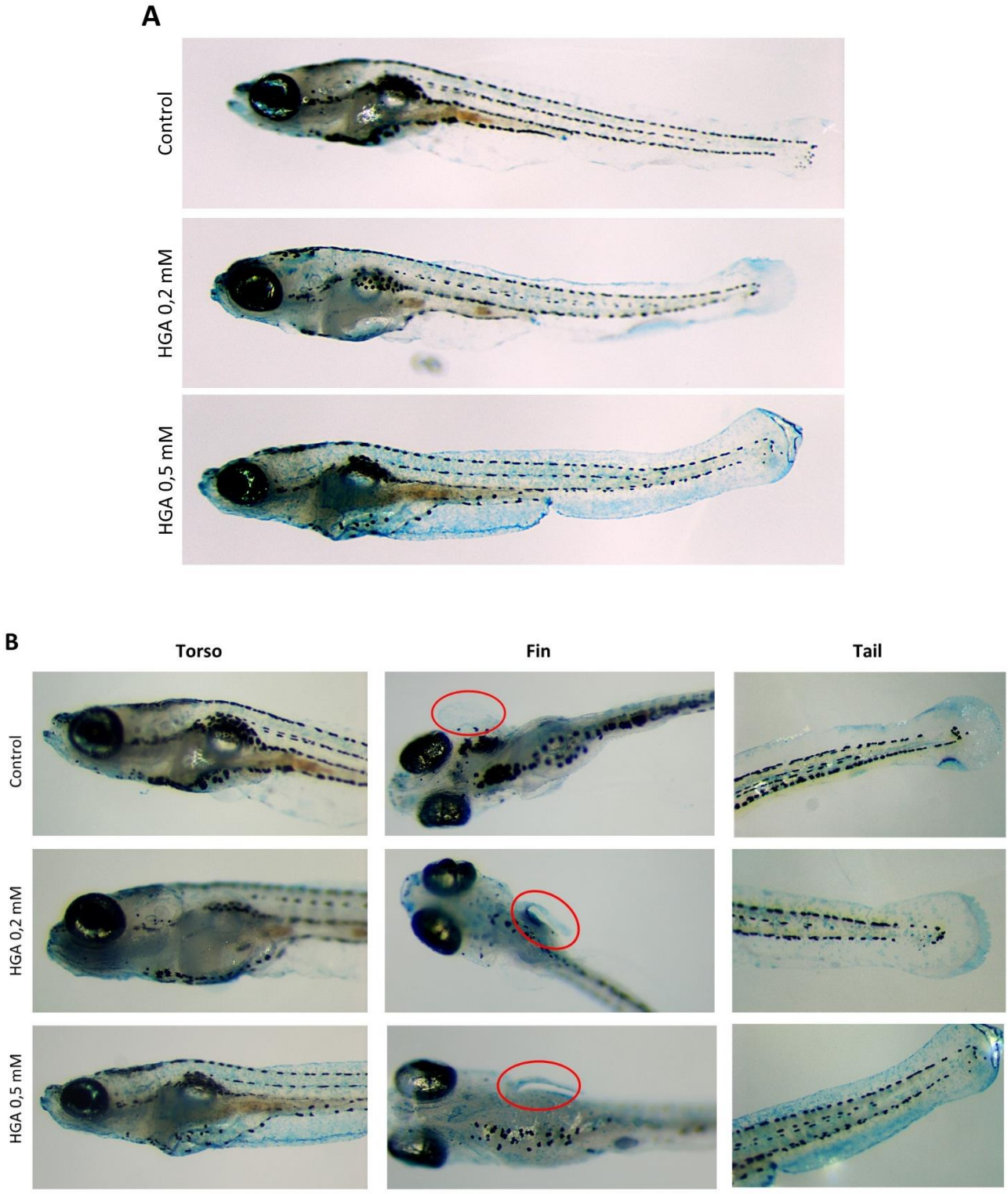


Figure 2: 20 dpf larvae treated or not with HGA for 17 days, and stained with Schmorl's stain. Pictures showed the whole body (A) and some details (B). In the HGA treated conditions, the blue staining revealed the presence of the ochronotic pigment.

3.5 Discussion and conclusion

In the present study, *in vitro* and *ex vivo* models were analyzed, with the aim to obtain simple and reproducible models, that could overcome the limitation due to AKU rarity.

Primary human chondrocytes had been chosen as elective cells for the set up of *in vitro* model, considering that cartilage represents the most affected compartment in AKU patients. Cells were treated with different amount of HGA, and the 0.046 mM dose was selected. Indeed, this concentration did not strongly affect cells viability, but allowed ochronosis development. From previously studies, is known that ochronotic cells carry also all the other molecular characteristics of the disease (Spreafico et al. 2013; Tinti et al. 2010). For this reason, cellular *in vitro* model is extremely convenient, because allows to easily recreate AKU features. The model led, in the project of the present thesis, to investigate the effect of HGA in cells. In particular, it allowed to easy investigate molecular changes induced by HGA, with a particular attention on effects on organelles (Chapter 4) and DNA damages (Chapter 5). In addition, the model set up made possible to test the effect of drugs in different combinations and concentrations (Chapter 7).

AKU had been modeled *in vivo* using *hgd* deficient mice, but the model presented some limits, related to the absence of pigment development and joints failure in mouse. For this reason, it had been studied the effect of HGA to zebrafish, in order to create a new animal model that could carries AKU pathological features. Zebrafish model, indeed, is becoming increasingly popular for the study of arthritic disorder, leading numerous advantages compared to other animal models (Dietrich et al. 2021). Moreover, thanks to the transparence of larvae skin, is widely used for study melanin pigment development and connected human diseases (Cooper 2017). Zebrafish had been exposed to HGA, and, after 2 weeks treatment, stained with Schmorl's staining. Results revealed that HGA induced the ochronotic pigment development, that was accumulated particularly in some parts of the animal body. The discovery of the pigment development opens intriguing future perspectives for the study of multisystem pathogenesis of the disease and the response of organisms to possible pharmacological treatments.

Rapidity and reproducibility in laboratory study are fundamental. Both models proposed in this study result extremely simple, because are based on the simple adding of a defined HGA amount in cells medium or fishes' water. Therefore, they are easy to generate, cheap, and allow to solve the problem related to the few quantities of patients derived samples. Moreover, the disorder could be modeled in a relative short time, comparing to the time of onset symptoms in patients.

CHAPTER 4:

Lysosomes' alteration in the ultra-rare disease Alkaptonuria

Manuscript in preparation

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ABSTRACT

In the last few years, it has been ascribed to lysosomes new critical roles, related to the control of cellular homeostasis and stress response. Particularly, the physiology of these organelles changes in several disease conditions. In this work, we investigated how lysosomes were affected in the ultra-rare disease Alkaptonuria (AKU). The ochronotic pigment is the major manifestations of the disease, and its formation and accumulation cause to patients dramatic effects, as the articular joins failure and the cartilage destruction. For this reason, is fundamental understand its destiny in the cells, during the progression of its polymerization. Results showed different alterations, that involved the lysosomal number, higher in AKU cells and models compared to the control, and the organelles disposition. Indeed, in AKU conditions lysosomes assumed a peripheral position in the cells, instead of their perinuclear physiological localization. Furthermore, in AKU cells the ochronotic pigment was found to be collected inside lysosomes. Therefore, the involvement of lysosomes in the course of the disease could have crucial therapeutic implications.

4.1 Introduction

Lysosomes are intracellular membrane-bound organelles with a fundamental role in the degradation and recycling of cellular waste, implemented by their hydrolytic enzymes and the characteristic low lumen pH (4.5-5). Together with the degradation of cellular macromolecules and polymers, such as proteins, carbohydrates and lipids (Coutinho, et al. 2012; Winchester 2005), lysosomes break down pathogens and cellular debris. However, these organelles do not merely have a function in isolation and degradation of cellular waste and pathogens, but play a key role in the regulation of cell homeostasis at different levels (Carmona-Gutierrez et al. 2016). Lysosomes are strongly related with the surrounding environment and are involved in processes of secretion, plasma membrane repair, signaling and energy metabolism (Settembre et al. 2013a). Indeed, they act as cellular nutrient levels

sensors and take part in the starvation response, inducing upregulation of genes involved in the activation of autophagy, lysosomal biogenesis and lipid catabolism (Settembre et al. 2013b). Additionally, lysosomes contribute to endocytic and phagocytic processes (Luzio et al., 2007), and are involved in the autophagic pathway, that occurs via their fusion with autophagosomes (Wen-You Yim and Mizushima 2020). Accordingly, in recent years the vision of lysosomal roles has drastically changed, recognizing their ability to respond to different stimuli and their crucial function in cellular adaptation to a variety of stress conditions (Raben and Puertollano 2016).

Alterations in lysosomal functions can cause several deleterious effects including: failure to clean potentially toxic cellular waste, development of inflammation, apoptotic cell death and dysregulation of cellular signaling (Bonam et al., 2019). Lysosomal defects, besides their implications in rare lysosomal storage disorders (LSDs) (Marques and Saftig 2019), underlie also common diseases, including inflammatory and autoimmune disorders, such as rheumatoid arthritis (Weissmann 1966), systemic lupus erythematosus (Monteith et al. 2016), cancer and neurodegenerative condition, such as Alzheimer's, Parkinson's, and Huntington's disorders (Fraldi et al. 2016).

Among chronic inflammatory diseases there is Alkaptonuria (AKU), an ultra-rare autosomal recessive pathology caused by the deficiency of the enzyme homogentisate 1,2-dioxygenase, that takes part in the tyrosine and phenylalanine pathway, and results in accumulations of homogentisic acid (HGA), a highly reactive molecule that undergoes reactions of auto polymerization (Bernini et al., 2021), leading to the development of a dark pigment, called ochronotic (Braconi et al. 2015). The pigment settles in several tissues, particularly in the cartilage and connective tissues, causing resorption of calcified articular cartilage, tendon ruptures and joint failure (Ranganath, Norman, and Gallagher 2019). For this reason, AKU is considered a disabling disease. AKU cartilage becomes stiff and brittle, due to a disorganized collagen fibrils arrangement (Rossi et al., 2020). Moreover, the tissue is characterized by the presence of foci of mineralization, the proteoglycans depauperation and the accumulation of amyloid fibrils (Millucci et al. 2017). AKU chondrocytes and osteoblasts have an altered redox homeostasis and produce increasing levels of pro-inflammatory cytokines (Braconi et al. 2012; Schiavone et al. 2020), until these lead to the activation of apoptotic pathway and to cells death. Additionally, the ochronotic pigment, which accumulates during patients' life, deposits on the collagen fibers and in correspondence of the amyloid fibrils (Millucci et al. 2012). The pigment shows characteristic similar to the melanin: both are fluorescent macromolecules with a similar Xray diffraction spectrum (Ranganath et al. 2019). However, the pigment molecular structure is still unknown, and it is also not clear the mechanism of his accumulation and deposition.

In this work, we focused our attention on the study of the peculiar characteristics of lysosomes in

AKU, to investigate the crucial issue of cells homeostasis, that could lead to the identification of new potential pharmacological targets. Lysosomal associated membrane protein 1 (LAMP1), the major constituent of the lysosomal membrane, was used as a lysosome marker. Experiments were performed on AKU articular primary cells and on the cellular model, that was already set up, treating primary chondrocytes and osteoblasts (Schiavone et al., 2020) with a concentration of HGA that allowed the ochronotic pigment formation, without causing cellular death. Secondly, we tried to better understand the pigment destiny in the cells, by investigating the hypothesis that the pigment is accumulated in lysosomes, since it is recognized by cells as a toxic compound.

4.2 Material and Methods

4.2.1 Materials

The reagents used to perform experiments were: DMEM High Glucose (HG) and Low Glucose (LG) w/o Sodium Pyruvate w L-Glutamine, Euroclone; Solution 1X of Penicillin (10.000U)/Streptavidin (10mg/ml) (P/S), Sigma-Aldrich; Amphotericin B (250 μg/ml), Sigma-Aldrich; Fetal Bovin Serum (FBS) and FBS Ultra Low (UL), Euroclone; Hyaluronidase lyophilized powder, Sigma-Aldrich; Pronase from Streptomyces griseus lyophilized powder, Sigma-Aldrich; Collagenase type IV from Clostridium histolyticum powder, Sigma-Aldrich; Protease inhibitor cocktail P8340, Sigma-Aldrich; Phosphatase inhibitor cocktail P5726, Sigma-Aldrich; Immobilon Crescendo Western HRP substrate, Merk-Millipore; anti LAMP1 polyclonal antibody (PA1-654), Invitrogen; anti rabbit IgG (whole molecule) Peroxidase antibody (A0545), Sigma-Aldrich; anti GAPDH Peroxidase monoclonal antibody (G9295), Sigma-Aldrich; anti rabbit IgG cross adsorbed secondary antibody, Alexa Fluor 488 (A-11008), Thermo Fisher; Fluoresceine conjugated Dextran (3000 MW), Thermo Fisher.

4.2.2 Cells culture

Human primary chondrocytes and osteoblasts were extracted from cartilage and spongious bone, obtained from donors or AKU patients who underwent total hip or knee replacement. All the procedures were in accordance with the ethical standards of the responsible committee on human experimentation (Comitato Etico Policlinico Universitario di Siena) and with the Declaration of Helsinki of 1975, as revised in 2000. Informed consent was obtained from all patients. Samples were firstly incubated 72h in DMEM with 2% P/S and Amphotericin B diluted 1:400, to prevent potentials bacterial or fungal infections. Then, biopsies were cut in 2 mm^2 slices. Spongious bone samples were

incubated in DMEM LG + 1% P/S + 10% FBS-UL medium, so osteoblasts could directly spread in the plate, whereas, for cartilage samples digestion steps were required for cells extraction. Therefore, cartilage biopsies were incubated at 37°C in the dark in 0,1% Hyaluronidase solution for 30 min, then in 0,5% Pronase solution for 1h, and in 0,2% Collagenase solution for 45 min in agitation. Thereafter, chondrocytes were isolated from the cartilage employing a 70 μm filter, collected through centrifugation and plated in DMEM + 1%P/S + 10% FBS medium. Cells were cultured at 37°C in a humidified atmosphere of 5 % CO_2, and were used until their third passage, to avoid the loss of the characteristic phenotype.

4.2.3 Fontana Masson staining

To highlight the ochronotic pigment development, primary chondrocytes and osteoblasts were stained with Fontana Masson method. Cells, seeded at 30.000 cells/well density on sterilized 13 mm glass coverslips in 24 well plate, were incubated with medium supplemented or not with 46 μm of HGA for 3 weeks. Culture medium was changed two times per week. Then, medium was removed, and samples were washed in phosphate buffered saline (PBS), fixed by 15 min incubation in a solution of 4% formaldehyde (PFA) in PBS, and washed again. To stain the pigment, cells were incubated for 2 h in a 5% ammoniacal silver solution, made the day before and stored in the dark. After the incubation, slides were rinsed with mQ water, fixed with 2% sodium thiosulphate, and washed. After dehydration through exposition to increasing concentrations of ethanol and to xylene as last step, slides were stuck to the coverslips using Eukitt mounting medium and photographed under a light microscope using Zeiss camera.

4.2.4 Western blot

Chondrocytes and osteoblasts were seeded at 150.000 cells/well density in 6 well plates, medium was changed twice in a week, with or without the addition of HGA 46 μm. After 7, 14 and 21 days, total proteins were extracted using RIPA lysis buffer with 1% protease inhibitor cocktail and phosphatase inhibitors (NaF 4,2 g/l + Na_3VO_4 18,4 g/l). Moreover, AKU and control cells were cultured in 6 well plate in medium without HGA, and after 1 week proteins were extracted. Protein concentration was determined by bicinchoninic acid analytical method, and a 30 μg protein sample for each condition was denatured by adding Laemmli Sample Buffer and heating for 5 min at 95°C. Samples were separated in 8% sodium dodecyl sulphate-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked with 5% Bovine Serum Albumin (BSA) in Tris buffered saline-Tween 0,05%

(TBS-T). Then membranes were incubated overnight at 4°C in primary antibody anti LAMP1 or anti GAPDH, diluted in a solution of TBS-T 1% BSA. The day after, membranes were washed 3 times with TBS-T. For LAMP-1 detection, membrane was incubated 1 h with secondary anti-rabbit antibody solution and washed again. The signal was detected using Crescendo substrate and the ChemiDoc™ MP Imaging System instrument. Analysis of band intensities was performed by Image-Quant TM TL analysis software.

4.2.5 Immunofluorescence

30.000 cells/well of chondrocytes or osteoblasts were plated on sterilized glass coverslips in 24 well plate. Negative control samples and AKU cells were cultured only with medium, while to achieve AKU model, cells were treated with 46 µm HGA for 21 days. At the end of the treatment, samples were fixed in 4% PFA, permeabilized for 1 h with a solution of BSA 1% and TritonX100 0,02% in PBS, and incubated with primary antibody anti LAMP1 overnight at 4°C. The day after, coverslips were washed in PBS three times, incubated with secondary antibody anti-rabbit conjugated with Alexa Fluor 488 for 1 h, and washed again. Coverslips were mounted on glass slides using Mowiol Mounting Medium containing 4',6-Diamidino-2-phenylindole (DAPI), that bounded the genomic DNA, and photographed with Zeiss Axio Lab.A1 fluorescent microscope at 63X and 40X magnifications. The fluorescence of each cell was quantified with the function Measure of ImageJ software.

4.2.6 Intracellular vesicles analysis

Chondrocytes were seeded at 30.000 cells/well density on sterilized 13 mm glass coverslips in 24 well plate and treated with 46 µm HGA for 7, 14 and 21 days. The day before the end of the treatment, cells were incubated with Dextran 0,8 mg/ml dissolved in DMEM for 24 h, to allow its uptake in the vesicles. Then, coverslips were fixed in a solution of PFA 3% and Glutaraldehyde 2,5 % for 15 min, washed in PBS and mounted on glass slides. Samples were observed at fluorescent microscope using the FICT filter for excitation.

4.2.7 Lysosomal fraction extraction and Dot blot

Human primary chondrocytes were grown in eight 10 cm Petri dishes, replacing the medium twice in a week. Four of them received the medium supplemented with HGA 46 µm. At the end of the

treatment, cells were harvested with Trypsin 1X-EDTA, washed in PBS, and the isolation of the lysosomal fraction was performed through ultracentrifugation in a self-generated Percoll gradient, following Graham's protocol (Graham 2000). The effective isolation of lysosomes was demonstrated trough Dot blots against all the fractions obtained at the end of the centrifugation. The technique confirmed that lysosomes were concentrated in the opaque layer formed close to the bottom of the gradient. Consequently, the layer of each condition was collected, tested again trough Dot blot, and used for the subsequent analysis. The Dot Blot was performed on nitrocellulose membrane: after spotted 5 µl of samples, the membrane was blocked for 1 h in agitation, incubated O.N at 4°C with the LAMP-1 primary antibody, and after 3 wash, for 1 h with the secondary antibody. A total protein extract was used as positive control, while BSA solution (2 mg/ml) was used as negative control.

Before doing the following experiment, the lysosomal fractions were concentrated with Amicon Ultra Centrifugal Filter 3KDa (Millipore).

4.2.8 Analysis of the pigment fluorescence

The lysosomal fractions isolated from control and HGA treated cells were run in 8% polyacrylamide gel. Samples were loaded with Laemmli Sample Buffer. After sample running, the fluorescent signal of the pigment was revealed at ChemiDoc™ MP Imaging System instrument, exposing the gel at 30 sec UV light exposition.

4.2.9 Statistical analysis

Each experiment was performed in triplicate, and the samples were prepared using cells isolated from different donors. Data were presented as mean ±standard deviation. The statistical significance was tested with ANOVA test, and indicated with *$p<0,05$.

4.3 Results

4.3.1 AKU cells model presented the characteristic ochronotic pigment

AKU in vitro model was reproduced adding HGA in the culture medium, with the aim of mime the condition of articular cells in patients, that are in constant contact with the high concentration of HGA, carried from the blood. Therefore, after 3 weeks of treatment with HGA, chondrocytes and

osteoblasts were fixed and stained with Fontana Masson, that enhances the presence of ochronotic pigment through its specific affinity with the Ag^{+} ions. In cells incubated with HGA the presence of the ochronotic pigment was evident (Fig. 1A): cells contained the characteristic black spots, while in the controls there were no traces of ochronosis. We had previously showed that in HGA-treated AKU cell models, concurrent with the pigmentation, cells acquired all the characteristics connected with the AKU disease, as inflammation and oxidative stress (Spreafico et al. 2013). For this reason, the model is reliable for the study of the pathology. Moreover, previous observations through TEM microscopy showed that HGA-treated AKU cell models were also characterized by the presence of several vacuoles, of various sizes, in the cytosol (Millucci et al. 2015). Cellular vacuolization occurred as a consequence of oxidative stress and cell damage is the sign of the cellular degeneration in several pathologies (Henicsa & Wheatleyb, 1999). When cells were stained with Fontana Masson staining and labelled with LAMP1 antibody, HGA-treated chondrocytes and osteoblasts showed, in contrast to the control, a large cytosolic vacuolization (Fig. 1B), because of the damage induced by the pigment.

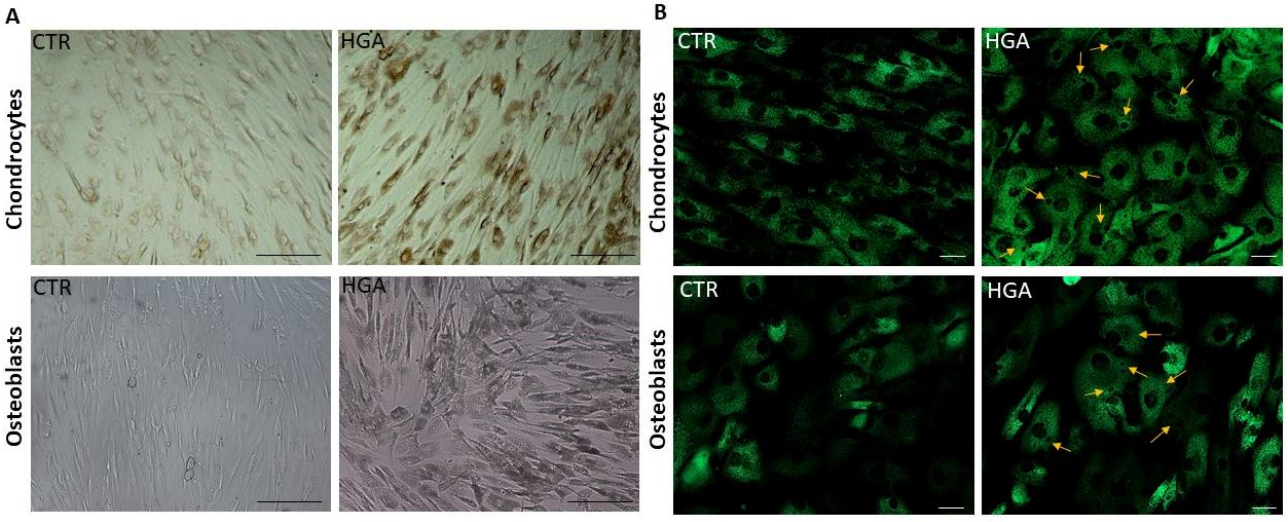


Figure 1: A) Chondrocytes and osteoblasts stained with Fontana Masson, after 21 days of treatment with HGA or without (CTR, control). In HGA-treated cells the ochronotic pigment accumulation was clearly visible. Scale bar = 100 µm. B) HGA-treated cells were incubated with anti LAMP1 antibody and stained with Fontana Masson. The high number of cytosolic vesicles (indicated by yellow arrows) was evident only in the HGA-treated cells. Scale bar = 30 µm.

4.3.2 Lysosomes in AKU cells and in AKU cell models were more numerous than the control and were distributed in the peripheral region of the cytoplasm

The number of lysosomes was quantified through Western blot, by comparing the levels of LAMP1 in control and in AKU cells, as well as in AKU cell model. Lysosome amount was detected in different time points, to evaluate the dynamic of their biogenesis. The results showed that in AKU chondrocytes and osteoblasts the number of lysosomes was doubled compared to the control. Moreover, there was a higher quantity of lysosomes also in HGA-treated AKU cell models, in which LAMP1 expression raised over time, until, after 21 days of treatment, it reached, in chondrocytes and osteoblasts respectively, 5-fold and 2-fold higher level than the controls (Fig. 2). These results underlined that the process involved was not a rapid reaction of the cells to the HGA incubation. The growing accumulation of HGA in cells, with their consequent suffering, acted as a signal for the increment of lysosomes. Indeed, the lysosomal amount increase followed the same timing of the ochronotic pigment development in the cells.

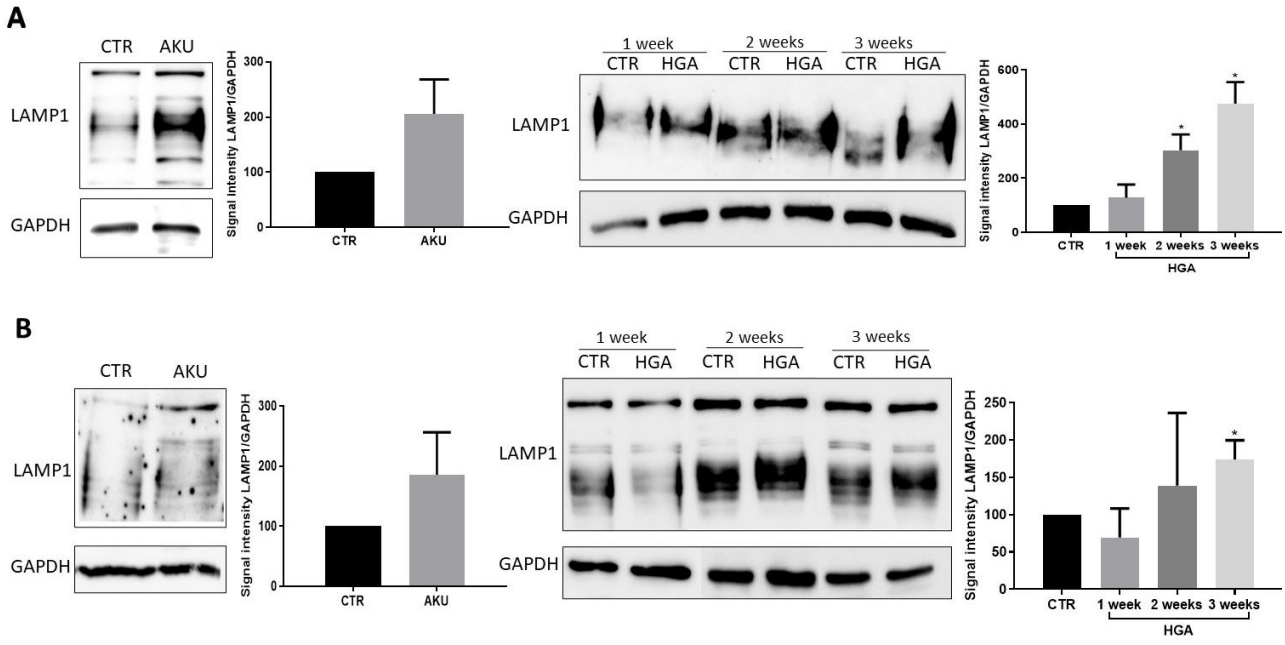
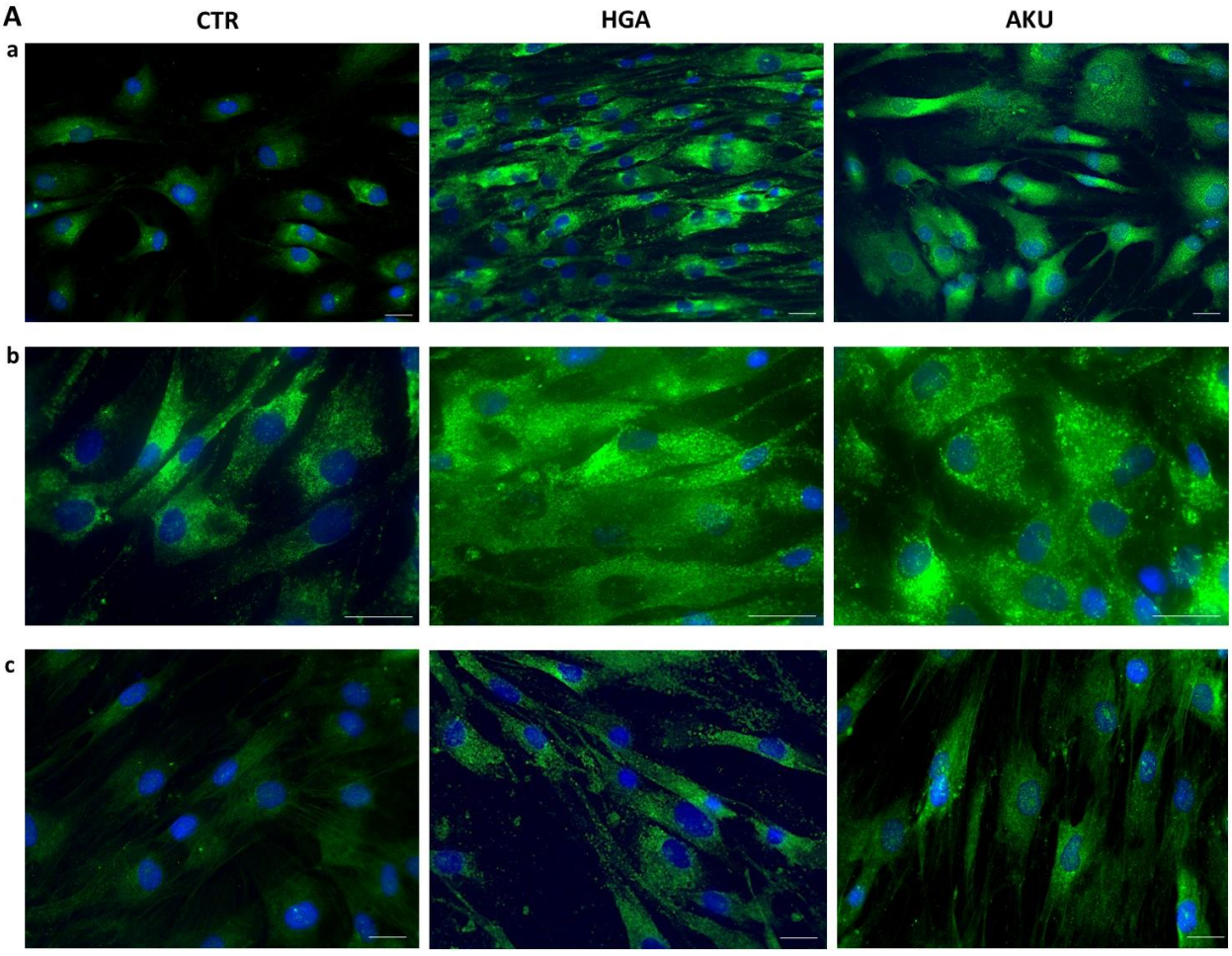


Figure 2: Detection of LAMP1 levels, through Western blot, in control, HGA-treated and AKU chondrocytes (A) and osteoblasts (B). The LAMP1 signals were normalized against the signal of the housekeeping protein GAPDH and expressed in the histogram graph as a percentage of the negative control.

The position of lysosomes in cells was detected through immunofluorescence against LAMP1. Both in AKU cell model, in which cells were treated with HGA for 21 days, and in AKU cells, lysosomes

were distributed in all the intracellular space, in particular concentrated in the peripheral region. On the opposite, control lysosomes had the characteristic physiological perinuclear localization. This peculiar lysosomal rearrangement had been showed in both AKU chondrocytes and osteoblast (Fig. 3A). Moreover, the data obtained from the quantification of LAMP1 fluorescence confirmed the Western blot results, since the substantial increment of lysosomes in AKU conditions was clear (Fig. 3B).



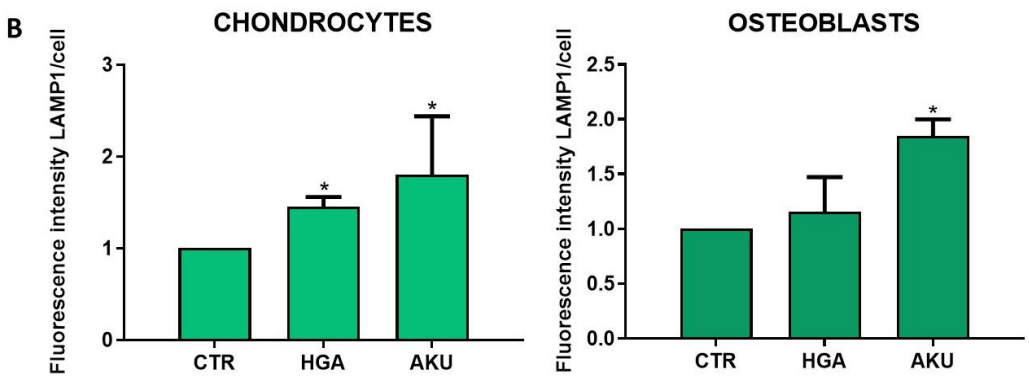


Figure 3: A) Immunofluorescence against LAMP1 in control, HGA-treated and AKU chondrocytes (a,b) and osteoblasts (c). Scale bar = 30 µm. B) Quantification of the LAMP1 fluorescence intensity for each cell. Results were expressed as a fold change relative to the control.

4.3.3 The ochronotic pigment was stored in lysosomes

The destiny of the pigment in cells was still unknown, despite it could be fundamental information in the attempt of prevent the ochronosis accumulation. Considering the role of lysosomes in absorbing debris, toxic molecules and pathogens, our hypothesis was that lysosomes could internalize the pigment. This would be a strategy for isolating the pigment in a separate compartment, to reduce the toxicity given by its strong oxidant power. In addition, cells in this way could try to degrade the pigment. To prove our hypothesis, the lysosomal fraction was isolated from HGA-treated cells and untreated control cells, following a protocol that allowed to separate this subcellular component through a density gradient. To test the validity of protocol, we performed Dot blot on all the fractions obtained after ultracentrifugation (data no showed), and the technique confirmed that in the visible opaque layer created after centrifugation were concentrated all the lysosomes. Therefore, we collected that lane from control and HGA-treated cells. The lysosomal fractions were concentrated and tested again with Dot blot (Fig. 4A). To analyze if the ochronotic pigment was present in lysosomal fraction, we exploited its peculiar capacity to produce a fluorescent signal after stimulation with UV light (Braconi et al. 2010). So lysosomal fractions were uploaded in a polyacrylamide gel, and, after the run, were exposed to wavelength in the UV range. After a brief UV illumination, a fluorescent melanin-like band appeared only in the HGA treated sample (Fig. 4B). The result confirmed that at least part of the ochronotic pigment was accumulated in lysosomes.

Moreover, we analyzed merged microscope images in order to evaluate the co-localization of lysosomes and the pigment. Control and HGA-treated chondrocytes were stained with Fontana Masson and then incubated with antibody against LAMP1, following immunofluorescence detection. The images obtained revealed a co-localization of the black spots in HGA treated cells, corresponding to the ochronotic pigment, and the lysosomal vesicles (Fig. 4C).

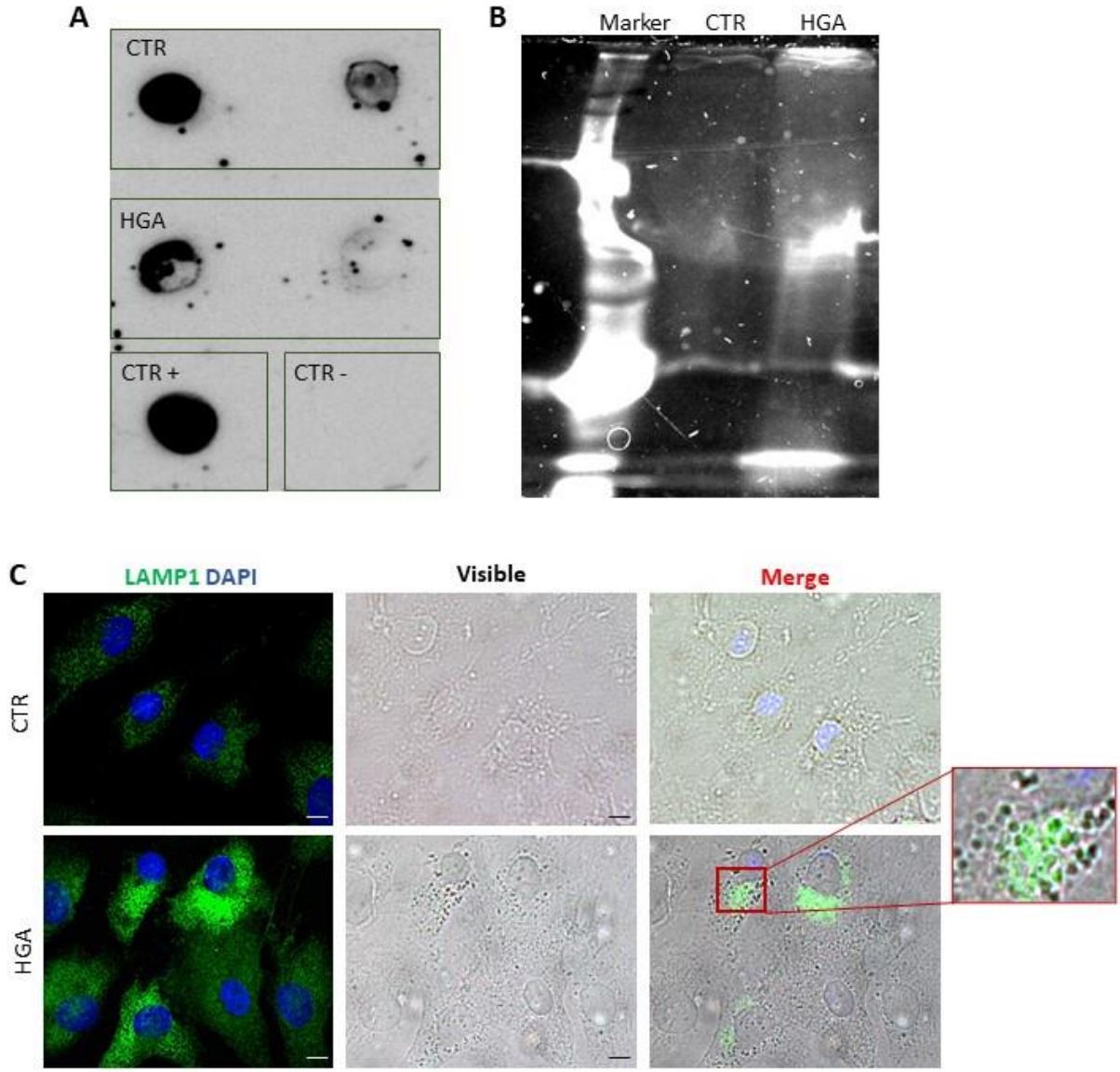


Figure 4: A) Dot blot with anti LAMP1 antibody on the lysosomal fractions extract from control and HGA-treated cells. Samples from the opalescence layer (on the right), and the layer above (left) were spotted. Positive control was obtained with a total protein extract, while negative control with a BSA solution 2 mg/ml. B) Lysosomal fraction tested with Dot blot were separated in a polyacrylamide gel, and the ochronotic pigment signal was revealed after UV light exposition. C) Control and HGA treated chondrocytes were incubated with anti LAMP1 antibody and stained with Fontana Masson. Merge images were created with ImageJ software. Scale bar = 10 µm.

Summarizing, in our hypothesis HGA and its oxidative products were naturally uptake from cells. The process continued, until their high concentration in the cytosol led the formation of ochronotic pigment subunits. This triggered the development of oxidative stress, with increase of ROS, that had several damaging effects on cellular subunits. The sufferance of the cells due to the presence of toxic

compounds allowed the increase of lysosomes number and the activation of autophagic pathway. However, as a defense mechanism, cells stored the pigment subunits in lysosomes. The failure in pigment degradation determined the accumulation of lysosomes with undigested substrates in their inner. So, organelles became inactive and stalled in the peripheral zone of the cytosol (Fig. 5).

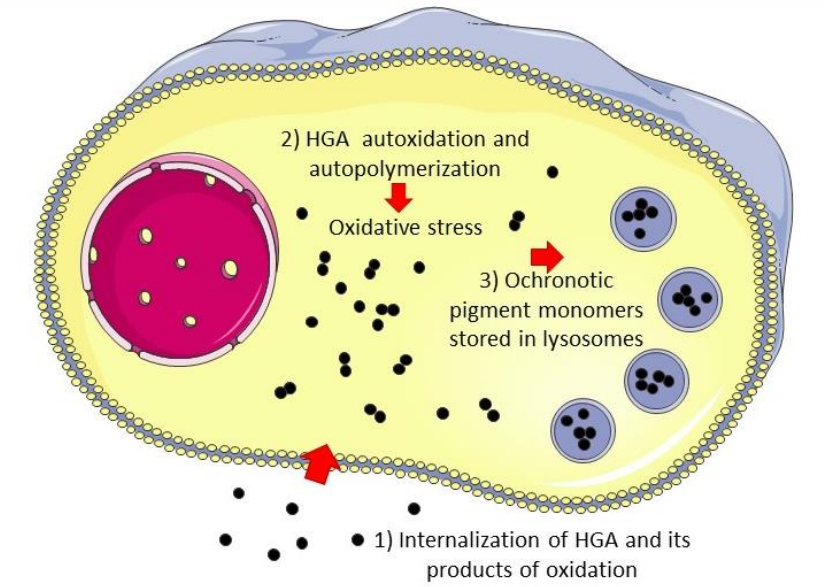


Figure 5: Schematic representation of the cellular uptake of HGA. The molecule is subjected to auto-oxidation and auto-polymerization reactions, resulting in oxidative stress and cellular sufferance. For this reason, pigment subunits generated in cells are collected in lysosomes, that increased in number and assumed a peripheral localization.

4.3.4 HGA accumulation caused an increase in intracellular vesicles number

Considering the demonstrated role of lysosomes in the attempt to limit the pigment toxicity, also the vesicular and endocytic/exocytose pathway could be involved. In order to demonstrate this, chondrocytes treated or not with HGA for 1, 2 and 3 weeks, were incubated with dextran conjugated with fluorescein. The dye was passively internalized in all the vesicles and could be revealed with fluorescent microscope. The results proved that, in AKU cell model, vesicles grew in number and size, occupying increasingly larger areas in cytoplasm (Fig. 6). Particularly, this was more evident after the longer incubation with HGA. Therefore, the intracellular HGA not merely determined the lysosomes increase but, as expected, also affected the endo/exocytose pathway. Autophagy and endosomal system are strictly connected, and the degradative and secretory function are intersected upon stressing stimuli (Buratta et al., 2020). Considering that vesicles contained dextran, their

formation was linked with the culture medium uptake. This means that HGA and pigment stimulated the new formation of lysosomes and vesicles as a protective reaction, with an increasing of intracellular trafficking. Thereby, there was, over time, a constant increase and accumulation of vesicles, with simultaneously HGA uptake and pigment formation, until the level of oxidative stress became intolerable, causing cells death (Millucci et al., 2015).

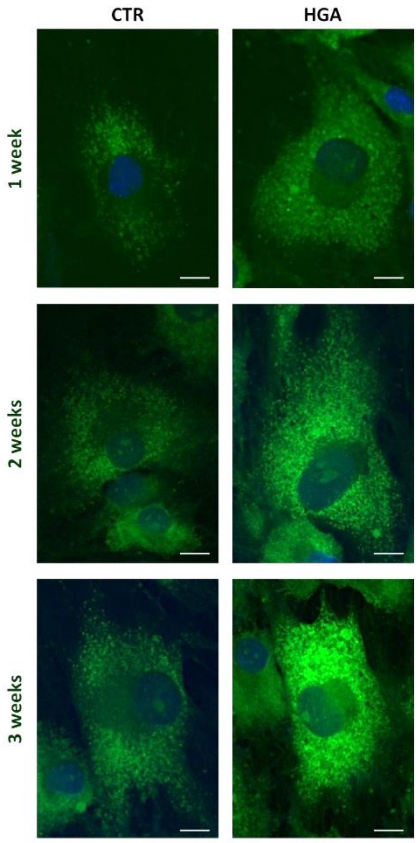


Figure 6: Chondrocytes treaded with HGA for 1, 2 and 3 weeks, compared with the untreated control. Intracellular vesicles were detected trough internalization of dextran conjugated with fluorescein. Scale bar= 10 μm.

4.4 Discussion

Despite several discoveries done about AKU in the last years, also thanks to the development of valid in vitro models, many molecular processes are still poorly understood. In particular, the destiny of the ochronotic pigment in the cells was never clarified. The aim of this work was to investigate, for the first time, the lysosomal and vesicular phenotype in AKU, and explore the hypothesis that the pigment was taken up by lysosomes. Our results showed that in AKU cells and cell models the number

of lysosomes was significantly higher compared to the control, with a substantial increase proportional to the time of treatment. This represents a cellular response to the oxidative stress accumulation, caused by HGA and his oxidative products. Indeed, it was already known that ROS promote the nuclear translocation of the transcriptional factor TFEB, the master regulator of autophagy induction and lysosomal biogenesis (Zhang et al., 2016).

Lysosomal biogenesis is a regulated process (Sardiello et al., 2009), and is activated as a consequence of intracellular accumulation of materials that need to be stored and degraded. For example, after sucrose incubation in cells, LAMP1 level increased, reaching a pick after 21 days treatment (Karageorgos et al., 1997). In AKU, because of the block in the conversion of HGA to maleylacetoacetic acid, HGA produced mainly in the liver circulates in the blood and accumulates in different districts, as the cartilage. Here, the molecules are absorbed from the cells, and the auto-polymerisation reactions start. This process was well represented in the in vitro model, where HGA was added in medium, and cells uptake and accumulate it in their inner. After 3 weeks, in the cells could be observed the formation of small pigment units. The oxidative stress and inflammation derived determined in cells the occurrence of suffering signal, since AKU cells showed typical damage marks (Spreafico et al., 2013), as the vacuolization, until to the activation of the apoptotic pathway (Millucci et al., 2015). In patients' cartilage, the pigment is located at the beginning in chondrocytes and in their territorial matrix, but with the progression of the disease, it proliferates in all the cartilage, leading the ochronotic arthropathy (Gallagher et al., 2016). More specifically, probably the pigment subunits generated in cells, after HGA uptake, are again excreted in the extracellular matrix, where polymerize and deposit on collagen fibrils. Pigment development and accumulation is an irreversible process, that progresses in patients over several years, to the catastrophic consequences of joins and tissue destruction.

With the progressive pigment formation, cells attempt to isolate and degrade it through lysosomal vesicles. The same condition took place in cells exposed to melanin, thus it had been reported that lysosomal enzymes decreased the amount of melanin and colour (Park et al., 2016), or in Parkinson cells, in which lysosomes up-took and cleared α-synuclein aggregates (Lee et al., 2004). Only Weidenfeld et al showed that, in macrophages treated with HGA, ochronotic pigment accumulated in lysosomes (Weidenfeld et al. 2019). In the present work, we corroborated this observation, detecting the characteristic pigment emission after UV lamp irradiation in a lysosomal fraction isolated from chondrocytes treated with HGA. Moreover, merge images showed the localization of the dark pigment in the LAMP1 positive vesicles. This evidence opens new interesting perspectives in research, with the purpose of block the ochronosis progression in AKU patients.

Vesicles and lysosomes are extremely dynamics and can move in retrograde or anterograde direction, through dynein or kinesin motors connected to the microtubules. Their position in the cytosol is not random, but strictly regulated and mediated by several pathways (Ba et al., 2018). Different stimulus, as pathogens infection or nutrients starvation (Korolchuk et al., 2011; Neefjes et al., 2017), activate the organelles transport. Lysosome positioning is a regulated by a complex interaction of signals and is strictly connected with their functions. In AKU cells it was observed an altered disposition of lysosomes: the organelles were localized in the peripheral region of the cytosol, whereas in the negative control cells lysosomes were concentrated in the perinuclear region. The acidification of cytosolic pH induces the translocation of lysosomes to the periphery (Heuser, 1989), and this condition could be caused by the ROS accumulation and consequent oxidative stress condition. The same condition has been detected in cancer, where lysosomes shift from the centre to the peripheral cytoplasm (Pu et al., 2016). Peripheral lysosomes interact easier with the plasma membrane and the early compartment of the endocytic pathway. Therefore, these organelles, in AKU cells, internalize the pigment, and because of their incapacity of degrade it, the pigment is accumulated and released again in the extracellular matrix. Is know that the internal pH of peripheral lysosomes is more alkaline than those in the perinuclear region, and this results in the reduction of their proteolytic capacity (Johnson et al., 2016). The loss of lysosomal activity impairs mitochondrial functionality, leading alterations in organelles function and reduction in cellular lifespan (Carmona-Gutierrez et al., 2016). Actually, the inefficiency in degradation triggers deleterious downstream events such as hyperactivation of inflammasome and enhanced apoptosis (Sergin et al., 2015). For this reason, lysosomes proteins and their pathways components could be considered as a potential therapeutic target, in order to reduce intralysosomal pH, thereby enhancing their function and degradative power (Bonam et al., 2019). The impairment of organelles was also proved by the less levels of Cathepsin D in AKU chondrocytes, compared to the control (Braconi et al., 2012).

Given that lysosomes play the role in responding quickly to the various metabolic conditions, to prevent damage or death, it is interesting to study their physiology in disease conditions. Indeed, lysosomes play a role in the inflammatory regulation, with a pro- or anti-inflammatory action, depending on the stage of the inflammatory response (Ge & Li, 2014).

CHAPTER 5:

HGA induces DNA damage: new light on molecular aspects of Alkaptonuria

Manuscript in preparation

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Abstract

Alkaptonuria (AKU) is an ultrarare genetic disease, caused by accumulation of homogentisic acid (HGA), which leads to the occurrence of a widespread chronic inflammation and oxidative stress. In this work, it was studied the effects of HGA on the DNA, using *in vitro* AKU model based on human primary chondrocytes. Our results showed that HGA incubation caused in cells significant DNA damage and nucleolar stress. The amount of damage decreased in time, suggesting the activation of DNA repair systems. Additionally, our findings revealed that HGA was not directly involved in genome breaks, which would be caused by HGA indirect oxidative effects.

5.1 Introduction

Prolonged condition of oxidative stress, with high concentration of ROS, as happens mostly in inflammatory diseases, causes in cells deoxyribonucleic acid (DNA) damages (Ames et al. 1993), such as strand breakage, base modification and base adducts formation (Halliwell 1996). This situation is extremely dangerous, because could generate mutations, genetic instability and cell death. Furthermore, chronic inflammation, through the generation of reactive nitrogen species (RNS), leads to nitrative damages on nucleic acids, and is related to the risk of developing human cancer at various sites (Sawa and Ohshima 2006). Therefore, oxidative DNA damages had been detected in several pathological conditions, characterized by oxidative stress and chronic inflammation, since it had been hypothesized that the damage plays a key role in the pathogenesis of disorders (Cooke, Olinski, and Evans 2006).

Cells have different mechanisms responsible for the recognition of DNA damage: the generation of double-strand breaks (DSBs) triggers the re-localization of many DNA damage response (DDR)

proteins. In particular, in response to DSBs, the histone variant H2AX is phosphorylated by the DNA damage-activated kinases ATM, ATR, and DNA-PK, to form γH2AX foci (Stiff et al. 2004), that specifically attract repair factors. Together with DDR recruitment by γH2AX, genome damages induce in cells the expression of several factors and proteins coordinating the defense response. Among these, a key factor, considered the genome guardian, is the tumor suppressor protein p53. P53 has a central role in cellular response against endogenous or exogenous damages, by orchestrating DDR. Moreover, the fundamental contribution of p53 depends on its capability to stop the cell cycle, giving time to the repair machineries for restore genome stability (Williams and Schumacher 2016). In this way, the replication of cells carrying mutations is avoided, and organism is protected by cancer development.

Furthermore, cellular oxidative stress has effects on the nucleolar morphology and functionality, resulting in the nucleolar stress manifestation (Yang, Yang, and Yi 2018). The mammalian nucleolus is comprised of three distinct regions: the fibrillar center, the dense fibrillar component and the granular component, composed of proteins with specific locations, such as fibrillarin and nucleolin. In physiological condition, these two nucleolar proteins are co-localized, while hallmark of oxidative stress is the nucleolin dispersion. Specifically, stressed nucleolus can assume a "rings" phenotype, characterized by perinucleolar nucleolin rings, with fibrillarin in the center, or "dispersed" phenotype, which lead to the dispersion of the nucleolin into the entire nucleus (He et al. 2018).

AKU is an autosomal recessive disease, characterized by a constant oxidative stress condition, that generates chronic inflammation and secondary A amyloidosis (Millucci et al. 2012; 2014; 2015). The disorder is caused by the deficiency of the enzyme homogentisate 1,2-dioxygenase (HGD) (Ascher et al. 2019), that acts in the metabolism of aromatic amino acids phenylalanine and tyrosine. HGD loss of function leads the accumulation of homogentisic acid (HGA) (La Du et al. 1958). HGA undergoes polymerization reactions, generating a typical black pigment called ochronotic, that results highly toxic for the body. Moreover, HGA, in presence of oxygen, is spontaneously autoxidized (Bernini et al. 2021), with the consequent formation of high reactive HGA-derived products and reactive oxygen species (ROS). These molecules attack cellular components, causing, among others, protein carbonylation, lipid peroxidation and unbalance of antioxidant defenses (Braconi et al. 2013; 2015; 2016).

Despite several progresses on knowledge of AKU molecular mechanisms, the effect of HGA accumulation on genome integrity was never analyzed. However, considering the inflammatory nature of the disorder, it could be a fundamental information for patients' care. In the present study, it was investigated whether HGA was directly involved in DNA damage, and its effects on the

genome integrity. In particular, γH2AX and p53 were used as sensors to quantify DNA breaks and cellular response. Moreover, it was revealed nucleolar morphology after HGA treatments using nucleolin and fibrillarin staining. Results showed that HGA affected indirectly DNA, causing strand breaks and nucleolar stress. These findings provided a more comprehensive understanding of the disease, at cellular and molecular level, which can be useful for the interpretation of some aspects of AKU and for the research of new therapeutic approaches.

5.2 Materials and methods

5.2.1 Analysis of the direct effect of HGA on genetic material

In order to analyze if HGA directly affected DNA, different concentrations (0.046 mM, 0.1 mM and 0.33 mM) of the molecule were incubated with 1 µg of a 300 bp plasmid, at 37°C for 30 min. Plasmid incubated in water was used as a negative control, while plasmid incubated with $FeSO_4$ 0.66 mM, Na_2-EDTA 1.25 mM and $FeSO_4$ 0.66 mM, Na_2-EDTA 1.25 mM, H_2O_2 5 mM, molecules known to be genotoxic, were used as positive controls. Following incubation, loading dye was added to the mixture, and 15 µL of each sample were loaded onto a 1% (w/v) agarose gel, stained with ethidium bromide 0.5 µg/ml. Electrophoresis was conducted at 100 volts in Tris-Borate-EDTA buffer.

5.2.2 Cells culture

Human articular cartilage fragments were obtained with informed consent from patients undergoing surgery for total hip or knee replacement. The study received approval from the Local Ethics Committee. Immediately after surgery, chondrocytes were isolated from articular cartilage, as previously described (Grigolo et al. 2002). Human primary chondrocytes were maintained in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS (Fetal Bovine Serum) and 1% penicillin/streptomycin at 37°C, in 5% CO_2 atmosphere.

5.2.3 Comet assay

The technique of single-cell electrophoresis Comet Assay was performed for detect DNA strand breaks on individual cells. 2x 10^5 cells/well were seeded in 6-wells plates, starved with FBS 1% medium ON and treated for 3 h with different concentration of HGA (0.046 mM, 0.066 M and 1 mM), with H_2O_2 0.1 mM to set up the positive control and with fresh medium without supplements

in the negative control condition. It was done the alkaline comet assay, following the protocol of Lu et al. (Lu, Liu, and Yang 2017). The experiment was performed in triplicate.

5.2.4 Immunofluorescence

Cells were seeded at 4 x 10^4 cells/well density on sterilized 13 mm glass coverslips in 24-well plates. Cells were grown until reached 80% confluence, then were exposed to serum-starvation, with FBS 1% ON. The day after, cells were incubated with medium containing 0.046 mM HGA for 1h, 3 h and 6 h, with medium without any supplementation as a negative control and with H_2O_2 0.1 mM to obtain the positive control. After treatments, chondrocytes were washed twice with PBS and fixed with 4% formaldehyde for 15 min at RT. Then, samples were permeabilized for 1 h with a solution of PBS/ BSA (Bovine Serum Albumin) 1%/ TritonX100 0,02%, incubated with primary antibodies at 4°C ON, washed with PBS three times, incubated with secondary antibody for 1 h, and washed again. Coverslips were mounted on glass slides using Mowiol Mounting Medium and photographed with Zeiss Axio Lab.A1 microscope, at 40x and 60x magnification. The detection of the phosphorylate form of histone H2AX was performed using anti phospho-histone H2A.X (Ser139) antibody (clone JBW301, Sigma) diluted 1:100, followed by incubation with anti-rabbit IgG Alexa Fluor 488 diluted 1:100 (A-11008, Thermo fisher). To analyze nucleolar stress, samples were immunolabeled with rabbit anti NCL antibody (HPA023981,Sigma) and mouse monoclonal anti FBL antibody (WH0002091M1,Sigma) together, and then with anti-rabbit IgG Alexa Fluor 488 (A-11008) and anti-mouse IgG Alexa Fluor 546 (A-11003,Thermo fisher). Each experiment was performed in triplicate.

5.2.5 Western Blots

Primary human chondrocytes were seeded at 1,5 x 10^5 cells/well density into 6-well plates. The day after, serum starvation was induced through cells incubation with DMEM + FBS 1% ON. Then, cells were incubated with complete medium supplemented with 0.046 mM HGA, H_2O_2 0.1 mM for the positive control or with any addition for the negative control. Total protein extracts were obtained by lysing cells in RIPA buffer containing 1% of protease and phosphatase inhibitors. 30 μg of proteins were denatured, separated in 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membrane was cut in 3 parts, blocked with 5% BSA in TBST 0,01% and incubated overnight at 4°C with anti phospho-histone H2A.X (Ser139) antibody (clone JBW301,Sigma) diluted 1:500, polyclonal anti TP53 antibody (Sigma) diluted 1:1000 and anti GAPDH Peroxidase Conjugated, (clone GAPDH-71.1,Sigma) diluted 1:50000. After 3 washes with TBST, membranes

were incubated with anti-mouse IgG Peroxidase antibody (Sigma) for the detection of phosphorylated H2AX and anti-rabbit IgG Peroxidase antibody (Sigma) for the detection of p53. Both the antibodies were diluted 1:80000 and incubated for 1 h at RT. The signal was recorded with Immobilon Crescendo HRP Substrate (Merk) and visualized by ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc.). Analysis of band intensities was performed by Image-Quant TM TL analysis software. Values were normalized against GAPDH intensity, and expressed as a percentage of the negative control.

5.3 Results

5.3.1 HGA did not directly affect the genetic material

Plasmids have a running speed in electrophoretic gel based on their shape, that could be supercoiled, open circular and linear. The shape assumed depends on the presence of breaks in the strand, indeed the open circular form has a nick in the structure, while the linear form a double break. Thus, through the detection of the plasmid conformation, it could be evaluated the genotoxicity of a substances. Plasmids incubated with $FeSO_4$, Na_2-EDTA and H_2O_2 assumed the linear and the open circular forms, because the substances caused DNA cleavages. On the other hand, the negative control preserved the supercoiled uncut form. Plasmids incubated with all the HGA doses preserved their native conformation, indicating that HGA did not directly induce DNA damage and strand breaks (Fig.1).

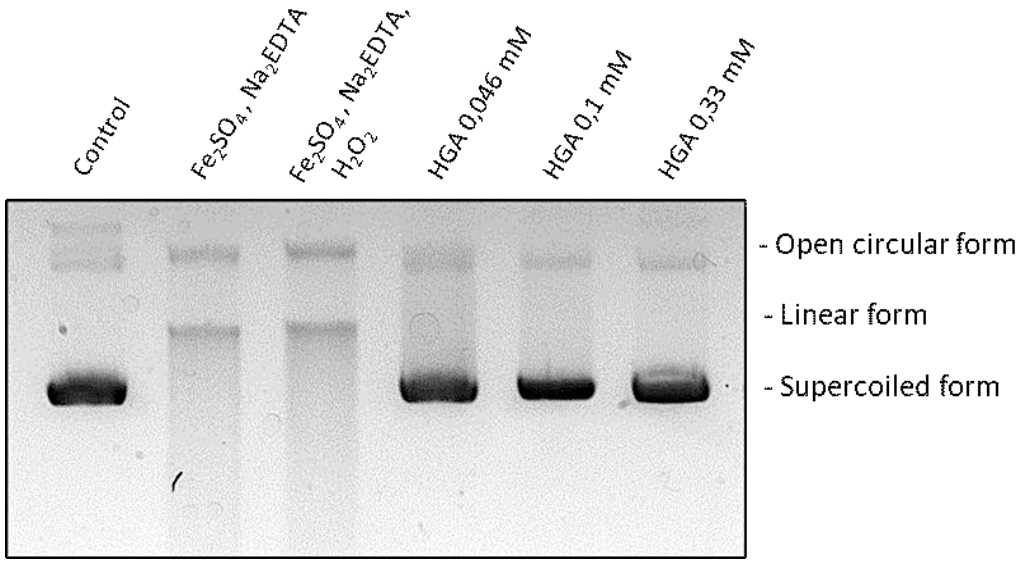

Figure 1: Plasmid incubated with different compounds and run through electrophoresis gel. The running speed depended on their conformation, that revealed if they were subject to DNA breaks.

5.3.2 HGA induced DSBs and foci formation

The ability of HGA to induce DSBs in the genome had been evaluated with Comet assay. This technique, consisting in a single cell electrophoresis, allows to estimate the DNA damage through the nuclei tails detection. Indeed, the tail length, consisting of DNA fragments, is proportion to the number of DNA breaks. Results showed that control nucleus preserved its physiological spherical shape, while in the positive control it was evident the comet tail. Chondrocytes treated with HGA, in all the tested concentrations, were subjected to genome breaks, noticeable from the tail formed. The amount of damage seemed to be similar in the different conditions. This demonstrated that HGA had a cytotoxic effect and induced the worst type of DNA damage, represented by DSBs.

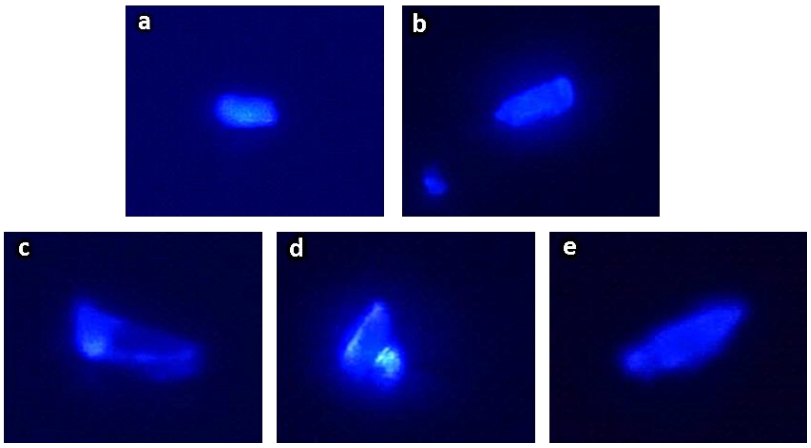

Figure 2: Comet assay: nuclei were stained with DAPI, and subjected to electrophoresis. The tail detected was proportional to the amount of DNA DSBs. The condition analyzed were negative control (a), positive control set up treating cells with H_2O_2 (b), and chondrocytes incubated with HGA 0.046 mM (c), 0.066 mM (d) and 0.1 mM (e).

DNA ruptures were evaluated also through detection of H2AX phosphorylation. Indeed, phosphorylation of histone H2AX in its C-terminal Ser139 residue, with the formation of γ-H2AX, represents an alarm signal that cells implement following DSBs. The phosphorylation induces the formation of foci, that increase DNA accessibility, leading to the recruitment and accumulation of specific DDR proteins (Mah, El-Osta, and Karagiannis 2010). Immunofluorescence against γ-H2AX allowed to reveal the quantity of foci in primary human chondrocytes treated with HGA at different time points. Results showed that cells incubated with HGA significantly exhibited DSBs, as could be deduced by the high amount of signal. Similarly, also nuclei of H_2O_2 treated cells showed high signal levels, while negative control cells had not fluorescent points (Fig. 3). Interestingly, the number of

foci revealed decreased with the time of incubation.

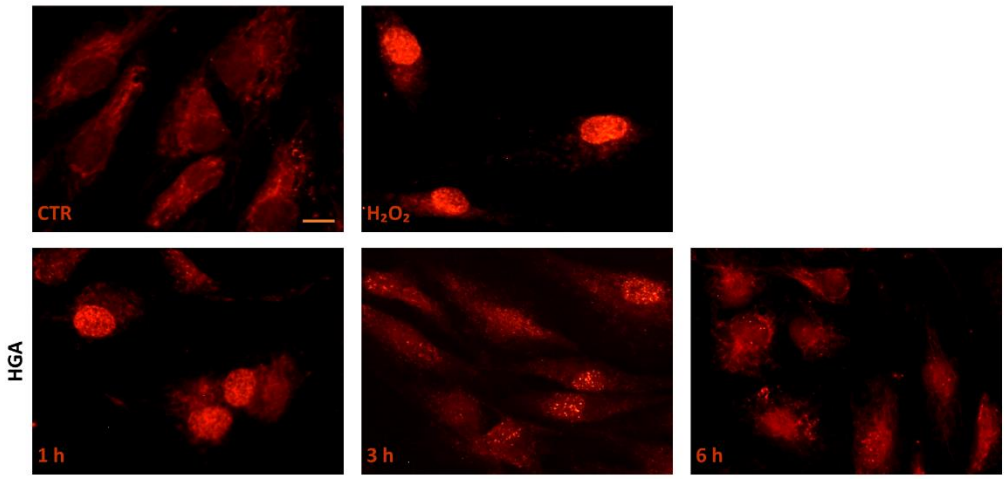

Figure 3: Immunofluorescence against γH2AX in cells treated with HGA, compared to positive and negative controls. Scale bar = 10 µm

5.3.3 HGA induced H2AX phosphorylation and p53 expression

Phosphorylation of histone H2AX following by HGA cells incubation was confirmed by Western Blot analysis. Therefore, the molecule led damages accumulation on DNA, with the consequent activation of cellular responses. Moreover, Western blot showed that HGA-induced DNA damage activated the expression of p53 protein, the principal factor that regulates the cellular response and actives signaling pathways (Fig. 4). Indeed, p53 mediates the transcriptional activation of genes involved in cell-cycle arrest, apoptosis, or DNA repair (Lakin and Jackson 1999). Therefore, p53 overexpression, compared to the negative control, demonstrated that HGA-exposed cells activated the complex of response mechanisms, that is fundamental for cells destiny. Indeed, depending by the gravity of damage and repair response, cells could undergo to premature senescence or apoptosis.

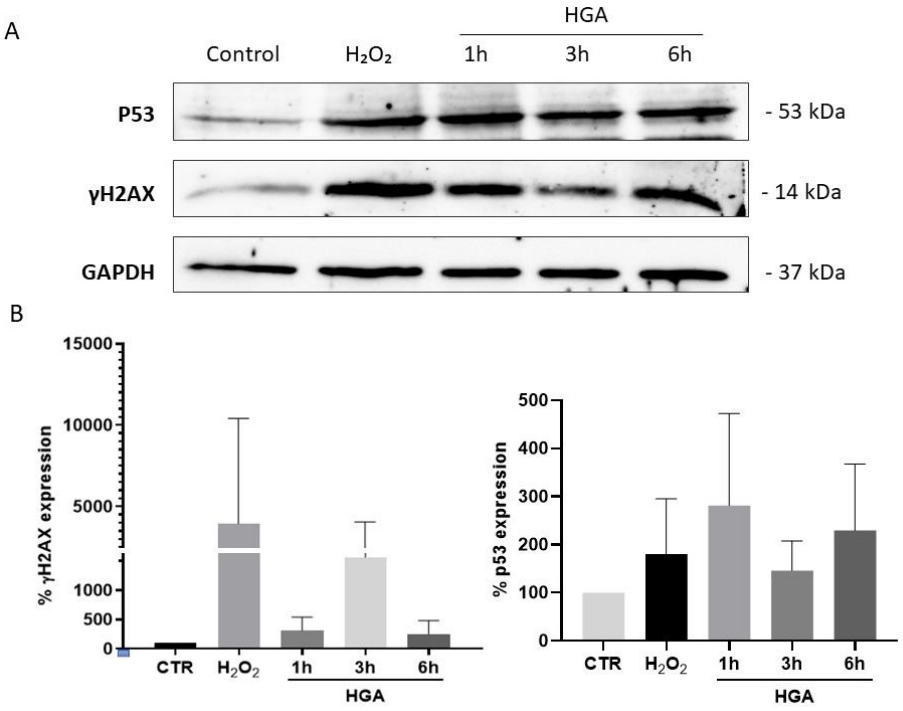

Figure 4: Western blot for detect H2AX phosphorylation and p53 expression (A). Signals were normalized against the housekeeping protein GAPDH and values were expressed as a percentage of the negative control (B).

5.3.4 HGA induced nucleolar stress

Several lines of evidence have revealed the role of the nucleolus as a sensor for various cellular stresses, during which some nucleolar proteins translocate from the nucleolus to the nucleoplasm. Thus, these translocations and redistributions are considered indicators of nucleolar stress (Yang, Yang, and Yi 2018). It had been evaluated the nucleolar morphology in chondrocytes after HGA exposure for different times. As expected, in the untreated condition, nucleolin and fibrillarin were concentrated in the nucleolus fibrillar region, while in the positive control, the nucleolin assumed the perinucleolar ring disposition (Fig. 5). This occurred also following the incubation with HGA, so the nucleolar ring phenotype proved that nucleoli were subjected to stress. Moreover, increasing the HGA incubation time, it could be observed a decrease of nucleolar rings number, suggesting that nucleoli restored their physiological morphology.

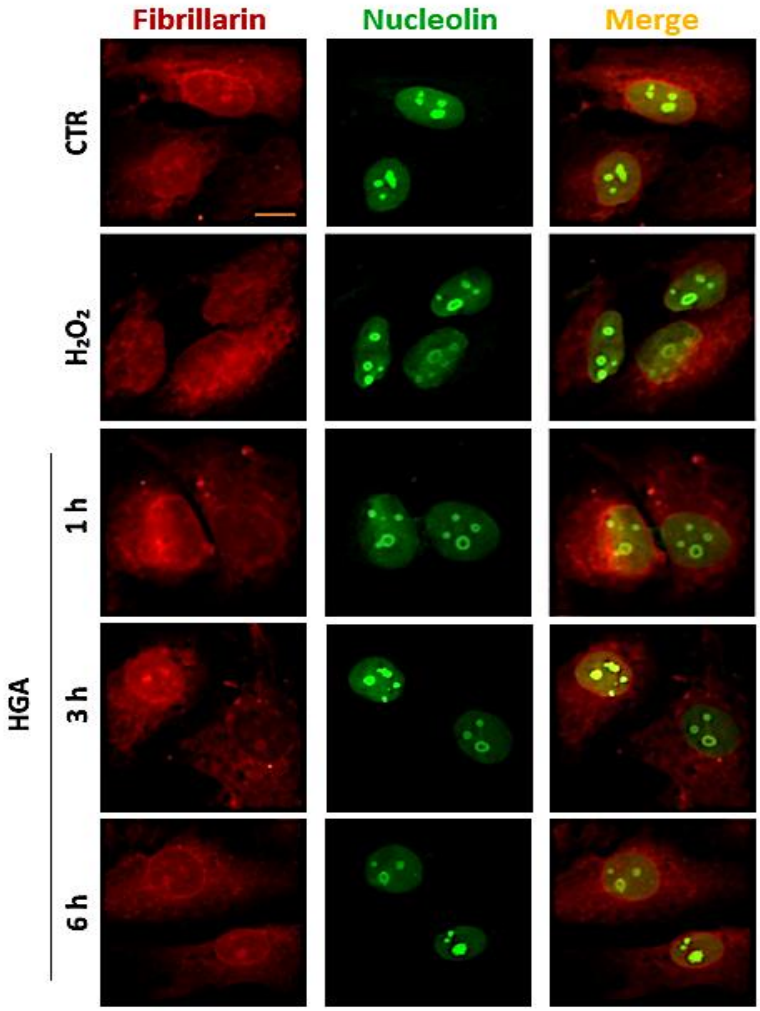

Figure 5: Immunofluorescence of fibrillarin, nucleolin and their merge, in cells incubated with medium (CTR), H_2O_2 and HGA for 3 time points. Scale bar = 10 µm

5.4 Discussion

In AKU patients, HGA accumulation, and the consequent oxidative stress, cause several damages, that especially affect joints and cartilage. In this work the molecular effects of HGA on chondrocytes had been deeply studied, with particularly interest on the integrity of genetic material. It had been shown that genome of cells incubated with HGA underwent to several strand breaks, as demonstrated by the γ-H2AX foci accumulation and the comet tail formation. Furthermore, it was proved that HGA caused nucleolar stress, resulting in the nucleoli typical ring phenotype. Previous works showed that in chronic diseases, characterized by high levels of cellular oxidative stress, ROS overproduction induced oxidative DNA damage, which, if unrepaired, could cause mutations and cell toxicity (Peluso et al., 2020). This occurred in different kinds of cancer, but also in rheumatoid arthritis (Altindag et

al. 2007) and in metabolic syndrome (Demirbag et al. 2006).

The genome stability is strongly preserved through a complex network of signaling and the activation of many different pathways, collectively known as DDR, in response to damage. The rapidity and redundancy of DDR are necessary to prevent the accumulation of mutations and errors in the genome, that could cause dramatic consequences as cellular death, or the occurrence of neurodegenerative disorders and tumors (Jackson and Bartek, 2009). The activation of cellular damage response signals it had been showed in AKU model by the overexpression of p53 protein. This step is crucial for the repair of DNA ruptures, before cells replication. Moreover, in case of high damages accumulation, cells remain in senescence status, or undergo to "programmed cellular suicide". It could be speculated that this situation happens in AKU, where patients' cells are continuously exposed to HGA and ROS, since it was demonstrated the activation of chondroptosis process AKU cells (Millucci, Giorgetti, et al. 2015).

The estimated half time for the DNA repair is in the range from 1.2 to 4 h, depending on the nature and intensity of the harmful stimulus (Collins and Squires 1986). From our results, it could be observed that the genome was mostly damaged between 1 and 3 h of HGA incubation. Contrarily, after 6 h of treatment, the DNA damage dramatically decreased, highlighting that the DNA repair proteins were activated, and cells were able to correct the defects accumulated. However, it must be considered that AKU patients' cells are continuously exposed to HGA and consequentially to the oxidative DNA damage.

Hiraku et al. (Hiraku, Yamasaki, and Kawanishi 1998) analysed the mechanism of HGA activity on the genetic material. Their study demonstrated that HGA plus Cu(II) caused site-specific DNA cleavage, at the cytosine residue in the 5P-ACG-3P, while HGA and Cu(II) alone didn't cause damage. This happened because the Cu(II)-catalyzed HGA autoxidation generate H_2O_2, that reacts with Cu(I) to form the Cu(I)-peroxide complex, capable of causing oxidative DNA damage. This is in line with the present results, in which it was showed that HGA alone, when incubated with a plasmid, was not able to cleave DNA. So, HGA itself is not genotoxic, but, when administrated to cells, its extremely reactivity lead oxidative chain reactions and ROS production, that affect cellular genome. In conclusion, it could be affirmed that HGA-induced DNA damages are the result of its indirect oxidative effect.

To date, no evidences reported the association of AKU with the occurrence of cancer (Avadhanula et al. 2020). Nevertheless, it could be hypothesized that the AKU/cancer relationship could be affected by the low number of patients analyzed, because of the rarity of the disease.

CHAPTER 6:

The combined administration of methotrexate and antioxidants as therapeutic strategy in the chronic inflammatory diseases' treatment

6.1 Introduction

Currently, more than 50% of all deaths in the world are caused by inflammatory related diseases, such as ischemic heart disease, cancer, diabetes mellitus, chronic kidney pathologies, and autoimmune and neurodegenerative disorders. Thus, chronic inflammatory conditions are considered the most significant global cause of death (Furman et al. 2019), and the necessity of keep investing in research is clear. Chronic disorders are characterized by the presence, in patients, of unbalanced level of reactive oxygen species (ROS), by the overexpression of proteins involved in inflammatory pathways and by the recruitment of inflammatory cells. In particular, oxidative stress and inflammation are strictly connected. Generally, inflammation starts when a dangerous stimulus actives immune system. After its activation, immune cells release proinflammatory cytokines and chemokines, that induce ROS production, including superoxide, nitric oxide, hydrogen peroxide, hydroxyl radical, peroxynitrite and hydrochlorous acid, triggering oxidative stress (Khansari, Shakiba, and Mahmoudi 2009). In chronic condition, the recurrent production of ROS leads oxidative damages accumulations, with detrimental effects on different cellular components. On the other hand, ROS induce inflammation. For these reasons, is fundamental nowadays be able to detect levels of inflammatory and oxidative stress markers, such as chemical components, cells, or specific proteins, in order to follow the progression and severity of the disease, but also to analyze if therapies are having effects. Moreover, markers could be used themselves as therapeutic targets in research (Ansar and Ghosh 2016). Considering that the measure of ROS is hampered by their very short half-life, commonly oxidative stress is quantified through the amount of oxidative modifications incurred by proteins and lipids. Among these, lipids peroxidation is an extremely frequent oxidative stress consequence, due to lipids' abundance of reactive double bonds. Peroxidation generate malondialdehyde (MDA), that could interact with proteins or cause itself potentially problems, as atherogenesis (Ho et al. 2013). Others targets of radicals and oxidants are proteins, with the consequent formation of multiple oxidative products. The most frequent reaction is the addition of carbonyl to proteins (Davies et al. 1999), that occurs in the early phases of oxidative stress. The reaction is extremely stable and irreversible, leading proteins to loss of function or degradation. Generally, the carbonylation is the result of ROS direct attack, but it could also derived from the reaction with 4-hydroxynonenal (HNE),

a modified aldehydes generated from lipid peroxidation (Castro et al. 2017). Despite cells are able to metabolize HNE, 4-HNE adducts in proteins could be followed by rearrangement and then determinate protein degradation. The most common and easy way to detect inflammatory development is through pro-inflammatory cytokines quantification, although other pathways could be investigated. Different inflammatory stimuli lead the expression of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) inducible enzymes, that play a fundamental role in the inflammatory signaling. Indeed, COX-2 is responsible to inflammatory prostaglandins production, while iNOS generates high levels of nitric oxide, that determinates cellular cytotoxicity and tissue damage. Pathways activated by these enzymes contribute to the occurrence of pain, swelling and joint destruction in arthritis (Needleman and Manning 1999). Furthermore, during early stages of inflammation, is systemically expressed the serum amyloid protein (SAA), under interleukins activation signal. SAA concentration dramatically increases following the inflammatory signal expansion, reaching levels 1000-fold higher than normal. Consequently, SAA, along with C-reactive protein (CRP), is a common serum biomarker for assessing disease severity of inflammatory conditions (Sorić Hosman, Kos, and Lamot 2021). In some situation, when the inflammation persists, the sustained high concentration of SAA and its abnormal processing cause protein aggregation, forming insoluble A-amyloid fibrils. Amyloid deposits stick to major organs, carrying the risk of organ failure and death. This phenomenon, called secondary A-amyloidosis, is a late inflammatory complication that occurs predominantly in rheumatoid arthritis and AKU patients (Millucci, Ghezzi, Paccagnini, et al. 2014; Obici et al. 2009; Targońska-Stępniak and Majdan 2014).

In vitro cellular models are extensively used for study the molecular common characteristics of the inflammatory signal and the efficiency of pharmacological approaches. A common osteoarthritis model, that is simple, easy to manipulate and cheap, was set up treating osteoarticular cells with IL-1β interleukin. This cytokine is expressed during early phases of chronic inflammation, inducing the activation of all the following signals, and triggering the amplification of inflammation. Therefore, through cells exposure to IL-1β is possible to induce all the biological changes that affect cells during inflammation and chronic conditions, and at the same time obtain a simple and expandable model for testing drugs (Johnson, Argyle, and Clements 2016).

Chronic inflammatory conditions, such as rheumatoid arthritis, juvenile idiopathic arthritis, juvenile dermatomyositis, systemic lupus erythematosus, vasculitis and severe psoriasis, are treated from many years with methotrexate (MTX), a drug with anti-inflammatory activity at low doses. Indeed, MTX acts on the inflammatory cells and cytokines expression (Dolhain et al. 1998), by inhibiting the pro inflammatory cytokines productions (Barrera et al. 1994; Gerards et al. 2003), reducing pro-

inflammatory cells (Dolhain et al. 1998) and suppressing NF-kB activation (Majumdar and Aggarwal 2001). Although MTX is effective for the treatment of these pathologies, it could cause several adverse effects, including lung and liver damages, skin reactions and decrease in the activity of immune system. Wherefore, the research is still highly active, with the purpose of find new therapeutic approaches still effective, but with a concurrent reduction of collateral effects. A popular approach, already tested with different compounds, involves the use of drugs co-administration in lower concentrations. The strategy allows taking advantage from drugs combinations, increasing their property, or keeping the same effect with lower doses, with a consequent reduction of side effects. For this reason, appear evident that the combination of MTX with antioxidants molecules could be extremely convenient for the treatment of the chronic inflammation disorders. Specifically, the use of N-acetylcysteine (NAC) and ascorbic acid (ASC) together as antioxidants compounds is attractive, considering that are both safe, widely use in medicine, and cheap. It had been demonstrated that both NAC (Eshraghi et al. 2020) and ASC (Da Silva et al. 2018), summed with their antioxidant power, have proinflammatory property and allow the decrease of cytokines production. Moreover, it had been showed that their use together is convenient for inhibit the oxidant and aggregative behavior of ASC (D'Agostini et al. 2000; Tinti et al. 2010).

Combined therapies were already tested in treatments involving MTX (Barrera et al. 1995), NAC (Eshraghi et al. 2020) and ASC (Shivavedi et al. 2017).

6.2 Aim of the study

The aim of the present study was to determine whether MTX and antioxidants NAC + ASC could be used in combination for treat disorders characterized by chronic inflammation. Antioxidants co-administration with MTX could allow to decrease the MTX dosage, with a consequent reduction of its side effects, without impairing the efficiency of the treatment. To set up an inflammatory disease model, Saos-2 cell line was induced with IL-1β, while oxidative stress was modeled treating cells with H_2O_2. The effect of the drugs, alone and in combination, was analyzed measuring different markers of oxidative stress and inflammation. Moreover, results obtained from treatments with two different doses of MTX were compared.

6.3 Material and methods

6.3.1 Cells culture and treatments

Human osteosarcoma cell line Saos-2 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) high glucose with 1% glutamine, supplied with 10% of Fetal Bovine Serum (FBS) and 100mg/ml of penicillin/streptomycin (P/S), and maintained at 37°C in humidified atmosphere of CO_2 5%.

Stock solution of 10 mM MTX was obtained dissolving the powder in NaOH, and was stored at -80°C. Antioxidants NAC and ASC were prepared fresh, dissolving the powder in PBS to obtain 10 mM stock solutions. IL-1β 10 mM stock was sterilely prepared in PBS-BSA 1% and stored at -20°C. H_2O_2 working solutions were obtained from H_2O_2 30% w/v (Sigma). All the compounds were filtered with 0,22 μm filter. Immediately before use, all stock solutions were serial diluted in complete medium to obtain desired final concentrations.

To perform experiments, Saos-2 were plated in multi-well and, when sub-confluence was reached (80-85%), cells were serum starved (FBS 0.1%) for 24 h. Then, cells were pre-treated for 24 h with two different concentrations of MTX (1 nM and 0.1 nM), Antioxidants (NAC 1 μM + ASC 1 μM) or their combination at the same doses. After pre-treatment, in all the conditions, except negative control that was grown only with culture medium, were added, depending on the experiment, IL-1β with a final concentration of 10 ng/ml, or H_2O_2, reaching a final concentration of 100 μM or 150 μM. All the treatments were made in starvation medium.

6.3.2 Western blots

Saos-2 in 6-well plates were pre-treated with MXT, Antioxidants or both, and inflammation was induced with IL-1β 10 ng/ml for 24 h. To prepare samples, cells were lysed with RIPA buffer supplemented with 1% of phosphate and protease inhibitors, sonicated for 7 min and centrifuged. The protein concentration was quantified by bicinchoninic acid (BCA) protein assay, and samples were prepared with 30 μg of protein, denatured with Laemmli Buffer and boiled for 5 min. For the detection of carbonylated proteins, samples were incubated for 30 min in the dark with a derivatized solution, to label carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). Differently from others, in Western blot against SAA, samples consisted of cells culture supernatants, concentrated with Amicon filters (Merk-Millipore). In this case, it was loaded the same volume of supernatants and the corresponding protein lysates, for GAPDH quantification.

Proteins were separated in sodium dodecyl sulphate-polyacrylamide gels (SDS–PAGE) and then transferred on nitrocellulose membranes. For protein carbonylation detection, samples were loaded in duplicate, and one gel was stained with Coomassie Blue method, to quantify the total amount of proteins.

The primary antibodies used included: rabbit anti-COX-2 monoclonal antibody (Cell Signalling, 1:1000), rabbit anti-iNOS polyclonal antibody (Invitrogen, 1:1000), mouse anti-α-DNP polyclonal antibody (Sigma-Aldrich, 1:10000), mouse anti-SAA polyclonal antibody (Santa Cruz, 1:200), anti-GAPDH HRP-conjugated (Sigma-Aldrich, 1:50.000). Secondary antibody used were: goat anti-rabbit HRP-conjugated antibody (Sigma-Aldrich, 1:80000) and goat anti-mouse HRP-conjugated antibody (Sigma-Aldrich, 1:50000). The immunoreactive bands were detected using ECL substrate (Luminata Crescendo, Merck Millipore) and images were acquired with ImageQuant LAS4000 machine (GE Healthcare). The signals were measured with the software ImageQuant TL. Protein levels were normalized against the protein loading control GAPDH, or in the case of carbonylated protein, against the total protein amount. The means of normalized values were expressed as a percentage of the negative control (100%).

6.3.3 Quantitative detection of Interleukin IL-6

The amount of IL-6 released into the culture medium of cells treated with MTX, Antioxidants or both for 24 h and then stimulated with IL-1β for other 24 h, was measured through IL-6 ELISA kit (Thermo Fisher) following the manufacturer's instructions. Optical density was read at 450 nm wavelength. The concentration of IL-6 for each condition was calculated using the IL-6 standard curve.

6.3.4 ROS quantification through flow cytometry

The level of ROS in cells was quantified through flow cytometry, with the instrument Muse Cell Analyzer (EMD Millipore) and the specific Muse Oxidative Stress kit. Firstly, two different H_2O_2 concentrations (150 µm and 100 µm) and three times of treatment (30 min, 2 h and 4 h) were tested, to select the best condition that simulate oxidative stress in cells. Based on this result, the treatment with H_2O_2 150 µm for 4 hours was chosen for the following experiments. Then, Saos-2 were pre-treated with drugs for 24 h, and oxidative stress was induced. At the end of the stimulation, cells were detached with trypsin, centrifuged, and dissolved in the Assay buffer to achieve the final concentration of 10^7 cells/ml. Each sample was incubated in the dark with Muse Oxidative Stress

Reagent working solution (1:800 from the stock solution) for 30 min at 37°C. After that, samples were analyzed with Muse instrument, that provided a graph with two distinct peaks, one representing normal homeostasis cells (ROS -) and the other including cells with outliers levels of oxidative stress (ROS +). The values of ROS+ and ROS- were given as a percentage of the cells analyzed by the instrument.

6.3.5 Lipid oxidation analysis with TBARS method

TBARS (Thiobarbituric Acid Reactive Substances) technique allows to rapidly measure levels of lipid peroxidation, an index of oxidative stress. The assay quantifies the amount of malondialdehyde (MDA), which is a product of lipid oxidation, through its reaction with thiobarbituric acid (TBA). The TBA-MDA adduct is a pink chromogen, that can be spectrophotometric measured.

To perform experiments, Saos-2 were pre-treated as described and treated with H_2O_2 100 μM for 24 h. At the end of treatments, cells were homogenate in ice with Triton X100 0.5% and sonicated for 7 min. Then samples were incubated for 1 h at 100°C in a solution of 0.4% TBA, 15% trichloroacetic acid and 0.1% butylated hydroxytoluene in 0.25 M HCl, and the absorbance was read at 534 nm. The mean of each condition was calculated, and values obtained were expressed as a fold change of negative control and represented with a bar plot.

6.3.6 Immunofluorescence against SAA

Saos-2 were seeded in 24-well plates, on sterile glass coverslips, at density of $3x10^4$ cells/well. After 24 h of pre-treatment with drugs, IL-1β was added to all the wells, with the exception of the negative control. After 6 days, slides were fixed with 4% paraformaldehyde, washed with PBS and permeabilized with 0.02% Triton X-100 in PBS/BSA 1%. Then, coverslips were incubated with mouse anti-SAA antibody (Santa Cruz) diluted 1:100 in PBS/BSA 1% ON at 4°C. The day after samples were washed with PBS and exposed for 1 h in the dark to the secondary antibody anti-mouse Alexa Fluor 546 conjugated (Thermo Fisher), diluted 1:100 in PBS/BSA 1%. Coverslips were washed again, mounted and visualized with fluorescent microscope (Zeiss AxioLabA1). Fluorescence intensity quantification was carried out with ImageJ software, by measuring the intensity signal of each cell. Means of each condition were expressed as a fold change of the negative control.

6.3.7 Congo red staining

Congo red staining allows to detect amyloid fibrils through its binding, that is visible with a green-yellow birefringence signal. A 0.6 % Congo Red solution was dissolved in 0.3 % NaCl 80 % EtOH, filtered with glass fiber filter and added to coverslip of fixed cells. After 3 h incubation, slides were washed with milliQ water, dehydrated with increasing concentrations of EtOH, clarified with xylene and mounted with Eukitt mounting medium (Sigma-Aldrich) on the slide support. Coverslips were observed using a polarized filter, and the birefringence was measured by the function Measure of ImageJ software. Means of birefringence intensity were expressed as fold change of the negative control.

6.3.8 Statistical analysis

All the experiments were performed 3 or more times. The results were expressed as their means + standard deviation. Statistical significance was calculated with ANOVA one way test, and expressed as *$p<0,05$ and **$p<0,01$.

6.4 Results and discussion

6.4.1 Effect of the co-administration of MTX + Antiox in the reduction of inflammation

The aim of the present study was to propose a combination of MTX at low doses and antioxidants compounds NAC + ASC as a remedy for diseases characterized by chronic inflammation. An *in vitro* model of inflammation had been set up inducing Saos-2 cells with IL-1β, and different inflammatory markers had been evaluated, to compare the efficacy of drugs alone to their combination. Specifically, it was analyzed if the well-known anti-inflammatory effect of MTX could be improved by the co-administration with NAC + ASC (indicated as Antiox). For this purpose, it was analyzed the expression of COX-2 (Fig. 1A) and iNOS (Fig. 1B), enzymes largely responsible of the occurrence of inflammation (Simon 1999; Zamora, Vodovotz, and Billiar 2000), since are commonly used targets of anti-inflammatory drugs (Hämäläinen et al. 2008; Zarghi and Arfaei 2011). Moreover, it had been measured treatments' effect on the amount of protein carbonylation (Fig. 1C), a post-translational modification that occurs in chronic diseases (Colombo et al. 2020). Results showed that all the tested conditions were effective in the reduction of inflammation. Interestingly, co-treatment with MTX and Antiox was more powerful compared to single treatments. In particular, the use of MTX 0.1nM + Antiox had a stronger effect compared to MTX 0.1nM or MTX 1nM alone. This suggested that the

use of the proposed combination in cure of chronic diseases could lead to the reduction of MTX dose, with consequently fewer negative effects for the body.

The potency of the co-treatment had been highlighted also through the detection of IL-6 expression in cells supernatant (Fig. 2). IL-6 takes part of the acute inflammatory response, and plays a pivotal role in the transition from acute to chronic (Gabay 2006), in the immune response and hematopoiesis. Therefore, IL-6 is extremely important in the pathogenesis of inflammatory chronic diseases, and also commonly used as therapeutic target. For instance, IL-6 is a central mediator of anemia in chronic disorders, as renal disease and rheumatoid arthritis (Raj 2009). MTX exhibited a not dose-response action on levels of the marker, as was previously detected (Noack and Miossec 2019). However, the co-administration with Antiox allowed to obtain enhanced effects in the MTX 1nM + Antiox condition.

The increasing positive effect of drugs combination could be a consequence of their different mechanism of action, added up together. MTX acts on adenosine signaling, causing an increase in extracellular adenosine concentration, that induces the downregulation of TNF-α and NF-kB, and inhibits T cells proliferation (Brown, Pratt, and Isaacs 2016). Moreover, growing findings had revealed that NAC and ASC acts on inflammatory pathways, together with their oxidant scavenging effect. In particular, NAC exerts its anti-inflammatory action decreasing the production of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α (Uraz et al. 2013). This is added to its antioxidant effects, given by NAC ability to reduce free radical damages. Also ASC has a role in inflammatory response, by influencing neutrophil chemotaxis, supporting phagocytosis and enhancing differentiation and proliferation of B- and T-cells (Carr and Maggini 2017). Thus, NAC and ACS, as demonstrated from the results, could be used as adjuvant to implement MTX action in the inflammatory suppression. This could lead to obtain a pharmaceutical solution strongest and more efficacious, with few adverse effects. This aspect results particularly attractive in the cure of chronic inflammation, considering the requirements of life-long treatments.

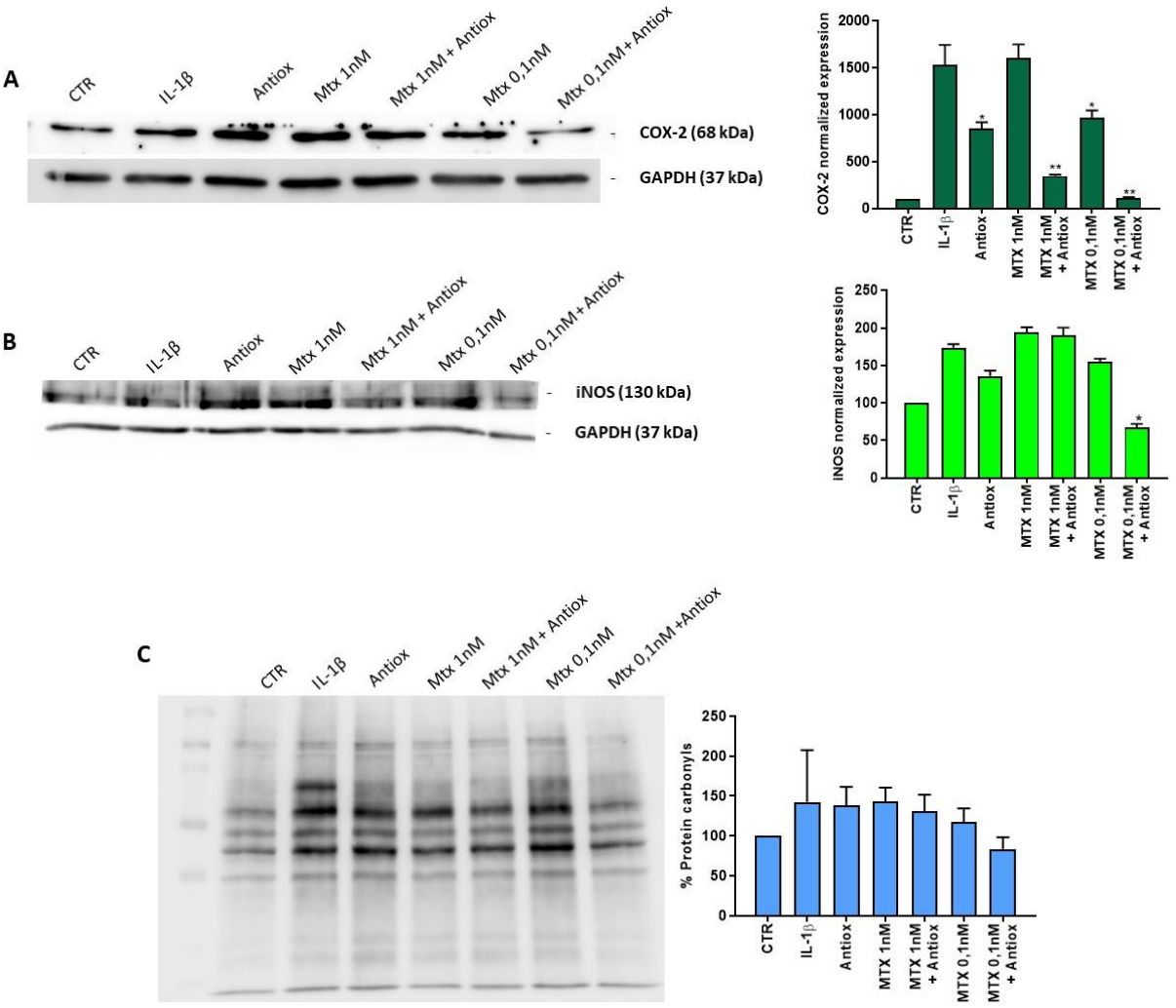

Figure 1: Western blots of COX-2 (A), iNOS (B) and carbonylated proteins (C). In the bar graphs were reported the values of band intensity signals normalized against housekeeping protein GAPDH (or total proteins) and expressed as a percentage of the control.

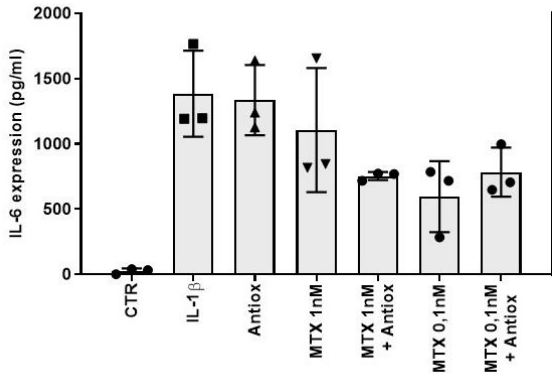

Figure 2: IL-6 concentrations (pg/ml) detected in cells supernatants with ELISA assay.

6.4.2 Effect of the co-administration of MTX + Antiox in the reduction of oxidative stress

Considering that in many chronic diseases, as occur in AKU, the inflammation is triggered by oxidative stress, is fundamental to propose a therapy that could act on inflammation but concurrently also has antioxidants effects (Hussain et al. 2016). This could reduce oxidative damages accumulated in chronic disorders, in which the natural antioxidant defenses are generally overwhelmed (Liu et al. 2018).

For this reason, it had been analyzed the effect of the studied compounds on markers of oxidative stress, that was induced in cell with H_2O_2. The antioxidant effect of NAC and ASC was already deeply studied and demonstrated. On the contrary, the effect of MTX is ambiguous. Some study reported its antioxidant property, due to its capability of scavenging free radicals and ROS, probably thanks to the structural phenolic ring (Clemens et al. 2020). On the other hand, some study supported the pro oxidant effect of MTX, that increased the amount of hydrogen peroxide released (Gressier et al. 1994). Here, we would understand if the co-administration of Antiox with MTX allows molecules to keep their antioxidant property, or could even improve that, as happened with the inflammation.

The amount of cells affected by oxidative stress was measured with Muse Cell Analyser instrument, and expressed as the ROS + cells percentage of the total. Firstly, it was identified the condition that better allowed to detect oxidative stress (Fig. 3A), corresponding to cell treatment with H_2O_2 150 µM for 4 h. Then, all the conditions were analyzed. Results showed that, in both the MTX tested concentrations, the co-administration of MTX with Antioxidants allowed to reduce ROS+ levels, compared to the MTX alone treatments (Fig. B-C). This result supported the convenience of compounds co-administration for counteract inflammatory pathologies.

It had been also quantified, with TBARS method, the lipid peroxidation, as a direct effect of ROS on cells. In this case, the co-administration had an impact comparable to the effect of MTX alone. Moreover, MTX + Antiox conditions were more effective than the Antioxidants alone. It was not clear if the positive effect in oxidative stress inhibition of MTX + Antiox was due to a summed antioxidant action of the compounds, or, on the opposite, could derive by the decrease of pro-oxidant MTX effect implemented by NAC + ASC. Anyway, data confirmed the convenience in the use of a combined treatment of MTX and Antioxidant, that, in addition to the beneficial impact in the inhibition of inflammation, were able also to counteract oxidative stress. It was further confirmed the evidence of the advantage in the use of the minor MTX dose supplemented with antioxidants as a pharmacological therapy. Indeed, it allowed to obtain the similar or improved beneficial effects,

employing less quantity of MTX.

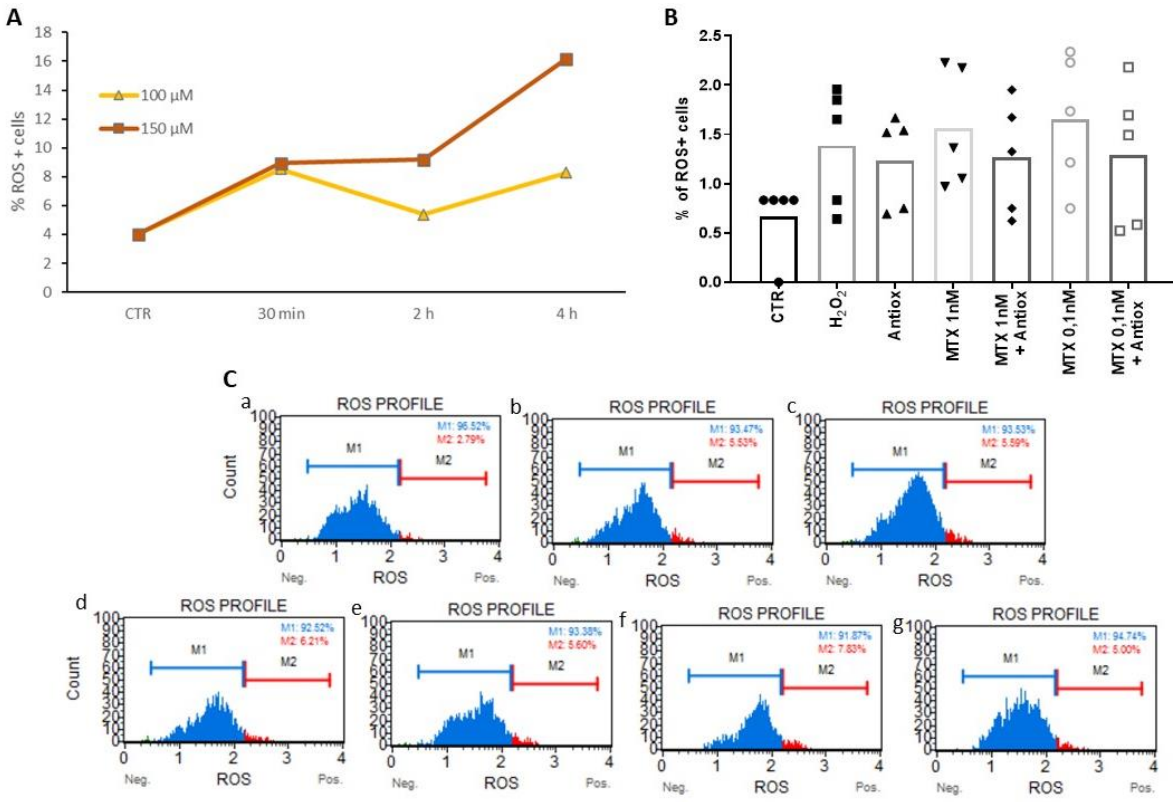

Figure 3: Quantification of cells affected by oxidative stress (ROS+), expressed as % of total cells, through Muse Cells Analyser. A) ROS+ cells were detected after H_2O_2 treatment (100 µM and 150 µM) for 30 min, 2 h and 4 h. B-C) ROS+ levels in cells pre-treated with the compounds for 24 h and induced with H_2O_2 150 µM for 4 h. Data from all the experiments were reported in the bar graph (B) and plots obtained from the instrument were shown (C). The blue pick represented the ROS- amount of cells, while the red one was constituted by ROS+ cells (a: CTR, b: H_2O_2, c: Antiox, d: MTX 1 nM, e: MTX 1 nM + Antiox, f: MTX 0.1 nM, g: MTX 0.1 nM + Antiox)

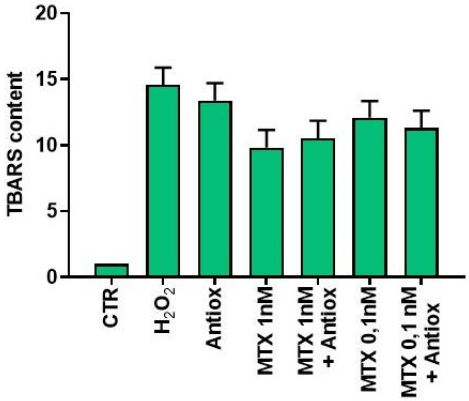

Figure 4: Spectrometric quantification of TBARS content in cells treated with 2 doses of MTX, Antiox or their combination. Oxidative stress was induced with H_2O_2 for 24 h. Data were expressed as a fold change of the negative control.

6.4.3 Effect of the co-administration of MTX + Antiox in the reduction of amyloidosis

Serum amyloid A 1 (SAA1) is one of the most expressed protein during the acute phase of inflammation, in which it increases up to 1000-fold (Sorić Hosman et al. 2021). When SAA1 is highly concentrated, the protein is prone to lose its folding, exposing its hydrophobic fraction. This leads SAA1 aggregation, that starts in cells, until amyloid deposits disturb the vesicular integrity and cells undergo to apoptotic death. Then, amyloid accumulates outside the cell, and occurs the fibrillation process (Claus et al. 2017). Amyloid fibrils stick to different organs, as heart, liver and kidney, causing cells death, tissues destruction and the global failure of organs. Diseases characterized by chronic infection or chronic inflammation, idiopathic and haematologic disease, tumor, are strongly associated with the development of AA amyloidosis (Brunger et al. 2020).

Accordingly, the necessity to analyze also this aspect during the study of drugs efficacy is evident. In particular, it had been evaluated the effect of MTX, Antiox and their combination on the cellular production of SAA and amyloid fibrils depositions. The amount of SAA produced by cells, and secreted in the culture medium, had been detected through Western blot (Fig. 5A). Results showed that the drugs co-treatment inhibited SAA expression more powerfully than treatments with the corresponding dose of MTX or Antiox alone. Again, the condition of MTX 0.1 nM + Antiox seemed to be the most effective. The SAA intracellular deposits and amyloid fibrils development had been revealed respectively with immunofluorescence against SAA (Fig. 5B) and Congo red staining (Fig. 5C). In this case, the difference between treatments with MTX alone or with Antiox addition was not visible, considering that MTX alone achieved completely the amyloid deposition, with a signal comparable to the negative control condition. This is not surprising, given that in the *in vitro* cellular model the amount of amyloid deposits detectable is extremely scarce. The deeply known anti-inflammatory activity of MTX explain its positive effect in the counteraction of amyloidosis. We can anyway speculate that in vivo, where the amyloidosis in more substantial, the stronger effects of MTX + Antiox, that had been highlighted in the reduction of inflammation and oxidative stress, as well as in the inhibition of SAA expression, could result also in the reduction of amyloid fibrils accumulation.

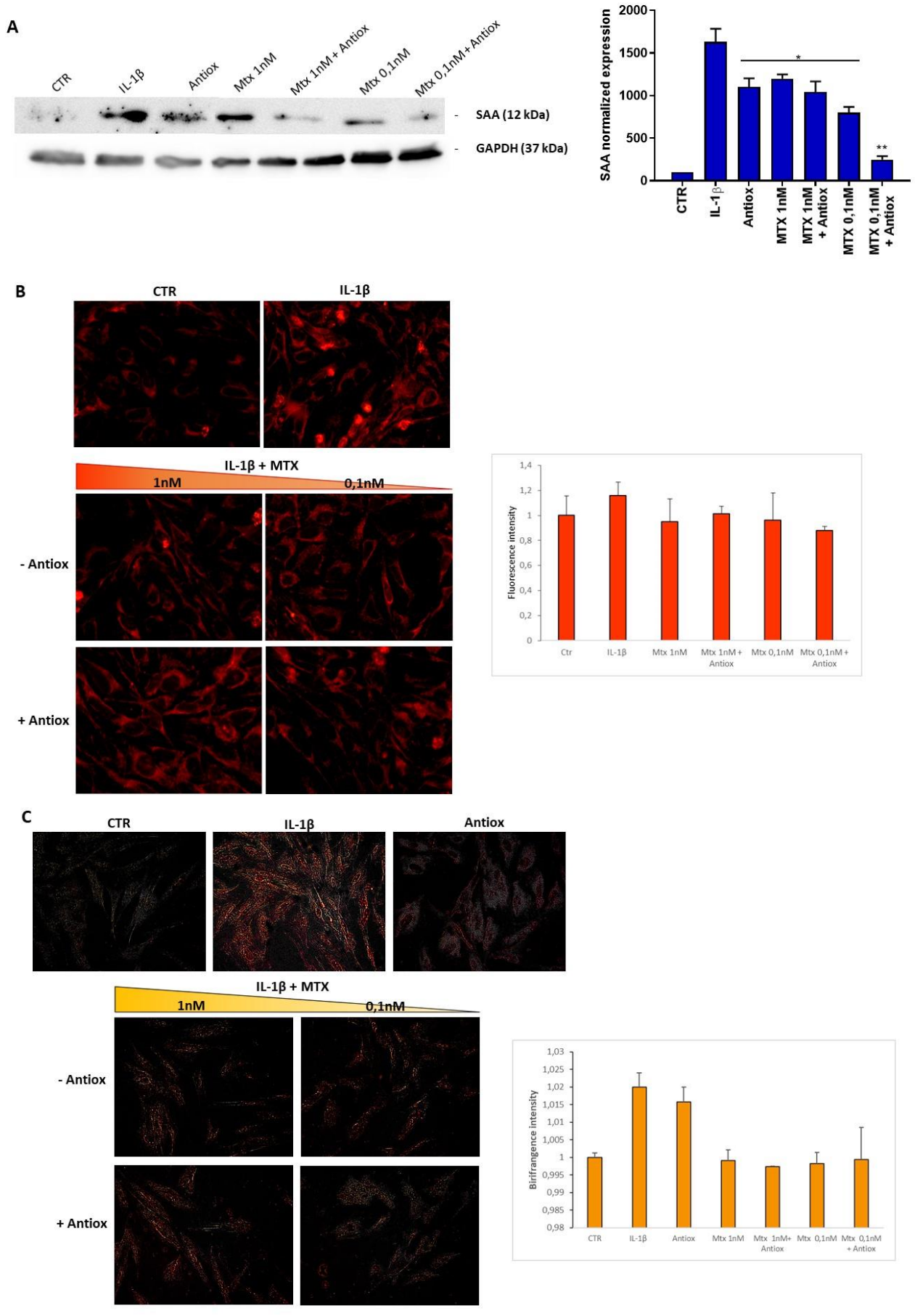

Figure 5: Study of amyloidosis markers in Saos-2 cells exposed to different treatments. A) SAA expression

detected in cellular medium through Western blot. B) SAA intracellular deposits revealed with Immunofluorescence and quantified with ImageJ software. C) Amyloid fibrils showed with Congo red staining, and quantified with ImageJ software.

6.5 Conclusion

The aim of the present work was to study the efficacy of combined therapy of MTX and antioxidant compounds NAC and ASC, in the treatment of chronic inflammatory diseases. It had been demonstrated that the co-administration of MTX and Antiox inhibited the expression of inflammatory markers and SAA, and reduced the oxidative stress. In particular, co-treatment had better effects in the inhibition of markers compared to the single treatments. This had been shown with both the MTX concentrations tested. So, the use of MTX 0.1 nM + Antiox for the treatment of inflammation was more effectiveness of the use of MTX 1 nM. This finding could allow several advantages in the treatment of chronic diseases, due to the reduction of MTX dose and its consequent adverse effects. Moreover, the formulation proposed appeared to be extremely convenient. MTX not merely preserved its anti-inflammatory property, but it was emphasized by antioxidants. In addition, the combination was more efficient also against oxidative stress. Therefore, antioxidants blocked the oxidative effect of MTX, and on the contrary increased their anti-oxidants properties. These results appeared particularly interesting, in a framework in which chronic disorders are the major cause of patients disability and premature death.

CHAPTER 7:

Effect of combined treatment of methotrexate and antioxidants on in vitro models of alkaptonuric ochronosis

7.1 Introduction

In Alkaptonuria (AKU) disease, homogentisate 1,2-dioxygenase (HGD) deficiency causes the block of tyrosine catabolic pathway, with consequent accumulation of homogentisic acid (HGA). Chemical characteristics and high reactivity of HGA and its derived molecules cause oxidation of cellular macromolecules and production of ROS, with the occurrence of oxidative stress (Braconi et al. 2013). Moreover, HGA is subjected to auto polymerization reactions, leading to the production of a peculiar melanin-like dark pigment (Roberts et al. 2015) called ochronotic pigment. In AKU patients, part of HGA is daily excreted in urine, giving them a typical black coloration, while the remaining part accumulates and circulates in the body. The pigment generated from HGA is accumulated in the body, and settles mostly in the connective tissue, but also in skin, glands, kidneys, osteoarticular cells and cardiovascular system (La Du et al. 1962; Helliwell, Gallagher, and Ranganath 2008; Laschi et al. 2012). It had been reported that in cartilage the ochronosis starts with the deposition of pigments in chondrocytes. Then, pigment spreads to the calcified matrix and to the surrounding tissues (Ranganath et al. 2019).

The pigment deposition on tissues is the basis of the morbidity in AKU. This condition is extremely harmful, and causes tissues impairment proportional to ochronosis progression. One of the mostly affected compartment is connective tissue of joints (Vigorita, Marino, and Lucas 2016), where the pigment leads bone fragility, tendon rupture and degeneration of cartilage, affected by the detachment of small fragments. These damages determinate in patients the occurrence of osteoarthritis (Al-Ajlouni et al. 2020; Selvi et al. 2000), scoliosis and motion limitations. AKU clinical manifestations appeared after the third decade of life, getting worse with time, following the advancement of pigmentation. For these reasons, the counteraction of ochronotic pigment spreading could be fundamental for the prevention of AKU injury.

In correspondence of AKU ochronotic pigment it had been shown the presence of amyloidogenic fibrils (Millucci et al. 2012; Millucci, Braconi, et al. 2015). Indeed, AKU belongs to the secondary amyloidogenic diseases, and amyloid deposits, composed by serum amyloid A (SAA) and serum amyloid P (SAP) proteins, had been found in several AKU tissues, such as salivary glands, cartilage,

synovia, aortic valve (Millucci, Ghezzi, Bernardini, et al. 2014). This condition exacerbates the tissues degradation and articulation failures, and need to be addressed during patients' pharmaceutical treatments.

Considering the oxidative nature of the damages in AKU, patients were generally treated with antioxidants. The beneficial effects of antioxidants had been already proven experimentally. In particular, the addition of antioxidants in AKU serum model led the decrease of protein carbonylation and melanin-like pigment formation (Braconi, Laschi, Amato, et al. 2010), while *in vitro* cell model treatment inhibited SAA production, pro-inflammatory cytokine release, membrane lipid peroxidation (Spreafico et al. 2013) and ochronotic pigment development (Tinti et al. 2010). Despite this, antioxidants are not enough to counteract the inflammatory characteristics of AKU. Moreover, the fact that AKU is an A-amyloidosis type II disease (Millucci et al. 2012) needs to be considered for its therapy. Methotrexate (MTX) is one of the mainstays for treatment of inflammatory disorders. At low doses, it exert an anti-inflammatory effect, and is already worldwide used for the treatment of several chronic pathology, including rheumatoid arthritis (Weinblatt 2013). In particular, arthritic patients treated with MTX had a reduction of pain, joint damage and a slowing of the disease progression. In addition, MTX counteracted the secondary amyloidosis plaques formation, as was shown also in AKU model (Millucci et al. 2012). For these reasons, MTX is a suitable candidate for the treatment of AKU. On 29 August 2016, European Medicines Agency (EMA), with European Commission, designed MTX orphan drug (EU/3/16/1723) for the treatment of AKU.

7.2 Aim of the study

In this work it had been evaluate the effects of MTX administration, in presence or absence of different antioxidant compounds, on the development of ochronotic pigment and amyloid deposition in AKU models. The disorder had been modeled using human primary chondrocytes and osteoblasts, derived from articular joints, the most affected compartment in the disease, in order to preserve physiological characteristics and closely simulate the patients' tissues conditions. The purpose of the study was to determine if the MTX co-administration with antioxidants led a stronger reduction of ochronosis and amyloidosis compared to the MTX alone. For this purpose, 3 MTX concentrations had been used, alone or with antioxidants, for long-time treatments, that allowed the pigment and amyloid development in cellular model. The idea was to propose a new therapeutic approach for AKU, that combines and amplifies the benefits derived from the single compounds. This strategy is not new in the scenario of the cure against rheumatoid arthritis (De et al. 2018; Rahman et al. 2018), but could be extremely innovative and advantageous for AKU treatment. Indeed, the efficacy due to

the co-administration could allow the use of lower MTX doses, with a consequent reduction of its side effects. The antioxidants used in this study were Taurine (TAU) and N-acetilcysteine (NAC) + ascorbic acid (ASC), that already showed their positive anti-oxidant effect in AKU cellular models (Spreafico et al. 2013).

7.3 Materials and Methods

7.3.1 Isolation and culture of primary human chondrocytes and osteoblasts

Primary human chondrocytes and osteoblasts were isolated from samples of articular cartilage and spongious bone, immediately obtained after surgery following the procedure of extraction (see paragraph 3.3.1). Cells were maintained in culture in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) or FBS Ultra Low for osteoblasts, and 1% of Penicillin/Streptomycin (P/S). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

7.3.2 Cells treatments

Stock solutions of MTX, NAC and ASC were prepared as described in the paragraph 6.3.1. TAU stock solution (10 mM) was obtained dissolving the powder in PBS. MTX was freshly diluted in culture medium to obtain final concentrations of 1 nM, 0.1 nM and 0.01 mM, while NAC, ASC and TAU stock solutions were diluted in culture medium to reach the final concentration of 10 µM. HGA 10 mM stock solution was prepared in mQ water and filtered. HGA was freshly diluted to 0.046 mM concentration in culture medium, and administrated to cells for setting AKU models.

Chondrocytes or osteoblasts were seeded in 24-well plates, above 13-mm diameter coverslips, previously sterilized with ethanol and UV light. Cells were plated in a concentration of 30.000 cell/well, to start the treatments with a cellular confluence of 70%-80%. Experiments consisted of a 24 h pre-treatment with MTX, alone or combined with antioxidants, followed by the treatment with HGA. The medium was changed every 3 days, adding fresh compounds. The control condition was grown with complete medium without any inducer. Within each experiment, all comparisons were carried out between cells from the same donor. All conditions were prepared in triplicate.

7.3.3 Fontana Masson staining and image analysis

After 3 weeks of treatment, cells were fixed with paraformaldehyde and stained with Fontana-

Masson, that allowed to reveal the presence of ochronotic pigment thanks to its affinity to Ag^+ ions. Fontana Masson staining was performed following the protocol descripted in paragraph 3.3.3. The images were acquired with Zeiss AxioLabA1 microscope, at 10X magnification.

Ochronotic pigment was quantified with ImageJ software, from NIH. In order to standardized images, their brightness levels were corrected, bringing to the same in all the conditions. Images were transformed in logarithmic images, using function Process>Math>Log, and inverted (Edit>Invert). Then, individual cells were selected drawing their outlines, and, for each cell, the value of color intensity was measured (Analyse>Histogram/Measure) (Yamamoto et al. 2008). For each condition, the mean value of colour intensity was calculated. Values were normalized to the control, set as 100%, and expressed as its percentage.

Data were expressed as the means ± standard deviation between experiments and represented through histogram graphs. To evaluate statistical differences between conditions, multiple-measurement ANOVA analysis was performed. Statistically significant differences between AKU model and treatments were indicated as *$p<0.05$ and **$p<0.001$, while statistically difference between negative control and AKU model was marked with §$p<0.001$.

7.3.4 Congo Red staining

In order to detect amyloid fibrils, samples were stained with Congo Red method (Romhányi 1971). After 4 weeks of treatments, slides were washed briefly in PBS, fixed in 4% paraformaldehyde for 15 min at RT, washed again and stained with Congo Red stain. Coverslips were incubated for 1-2 h with a solution of 1% Congo Red (see paragraph 6.3.7). After the staining, samples were rinsed in distilled water 3 times, dehydrated with increasing concentrations of ethanol and xylene, and mounted on coverslips. The images were acquired using a polarized light microscope (Zeiss AxioLabA1), at 40X magnification.

7.4 Results and discussion

7.4.1 Effect of MTX and antioxidants on ochronotic pigmentation

In order to evaluate the effect of MTX and its combination with antioxidants in the reduction of ochronotic pigment, cells were pre-treated with drugs, and then co-treated with HGA. After 3 weeks, pigment depositions were revealed through Fontana Masson staining and quantified with ImageJ software. It was already shown that the pre-treatment for 24 h with drugs, before HGA addition, was

more effective than the simultaneously treatment (Tinti et al. 2010). We could hypothesize that pre-treatment makes cells more prone to respond to HGA effects, through the previously internalization of drugs. Antioxidants efficient dosages were already found (Spreafico et al. 2013), while the range of MTX doses used was calculated starting by the quantity of molecules present in blood of rheumatoid arthritis patients subjected to MTX treatment (generally 7 mg/week). From this value, it had been extrapolated the amount of MTX that effectively reached the cartilage and chondrocytes. The concentration of HGA used for the AKU model creation was already established (see paragraph 3.4.1).

Results showed that MTX administration to chondrocytes, in all tested concentrations, decreased the presence of pigment caused by the HGA treatment. Considering that MTX could exhibit antioxidant property (Zimmerman et al. 2017), probably it could allow to counteract the autoxidation of HGA and the formation of oxidized derived molecules. This resulted in the slowing of ochronotic pigment development. Interestingly, MTX acted on the ochronosis reduction in a not dose-dependent manner. This kind of activity was already highlighted in the inhibition of cytokines production induced by MTX in cellular model of arthritis (Noack and Miossec 2019). Moreover, when MTX was administrated with antioxidants, its effect on the pigment reduction was stronger compared to the treatment with MTX alone. This happened with all the tested concentrations, supporting that the improved co-administration effect occurred in all the conditions. Specifically, the combined effect that led the pigment decrease was demonstrated through MTX co-administration with either TAU (Fig. 1) and NAC + ASC (Fig. 2). Taurine is an amino acid with antioxidants activity (Jong, Azuma, and Schaffer 2012), that is already used as supplementary treatment against mitochondrial diseases, metabolic syndrome, cancer, cardiovascular diseases and neurological disorders (Jong, Sandal, and Schaffer 2021). NAC is a drug deeply pharmacological exploited for its antioxidants and cytoprotective capabilities (Ezeriņa et al. 2018), along with its application as a mucolytic agent in respiratory conditions. Its safety is supported by years of clinical use. The use of ASC was recommended, along with diet restriction, to AKU patients, from the first occurrence of symptoms. Thanks to its antioxidant effects, it allowed a decrease of HGA levels and joint pain (Morava et al. 2003). However, ASC has a dual nature: despite it belongs to antioxidants scavenging free radicals, it could act also has a pro-oxidative factor (Kaźmierczak-Barańska et al. 2020), and is associated with a 2-fold increasing risk of kidney stones development (Thomas et al. 2013). These adverse effects of ASC are prevented by NAC (D'Agostini et al. 2000). Indeed, ASC and NAC co-administration to AKU model was strongly convenient, and effective in inhibition of HGA-induced chondrocyte apoptosis, pigment deposition and decrease of proteoglycans release (Tinti et al. 2010).

The pigment reduction induced by drugs treatment was demonstrated not only in human primary chondrocytes, but also in human primary osteoblasts (Fig. 3). Also in this case, the co-treatments with MTX and antioxidants led a better result in term of ochronosis inhibition, compared to the treatments with only MTX alone.

Thereby, the enhancement of drugs positive effects when administrated together had been kept in different conditions. MTX and antioxidants acted in the counteraction of HGA oxidative reactions by different ways, but is still no clear if their impact together was the result of a summary or synergic effect. It had been demonstrated that the combinatorial therapy of MTX and allylpyrocatechol, a molecule with antioxidants activity, attenuated the progression of arthritis, revealed by their effectiveness in the inhibition of periostitis, bony erosion along with cellular infiltration, synovial hyperplasia, and cartilage degeneration. Moreover, the co-treatment allowed to prevent the rise in levels of pro-inflammatory cytokines and oxidative stress (De et al. 2018).

The principal problem in the use of MTX for long-period treatments comes from its toxicity, and the numerous adverse effects that induces in the body. Thereby, the increase of efficacy due to its administration together with antioxidants could allow the use of lower doses, that results less dangerous. Moreover, it had been shown that antioxidants as promegranate and carvacrol had a protective action against oxidative bone marrow damages induced by MTX. So, the use of antioxidants together with MTX, may help to reduce some adverse effects of the drug (Şen et al. 2014).

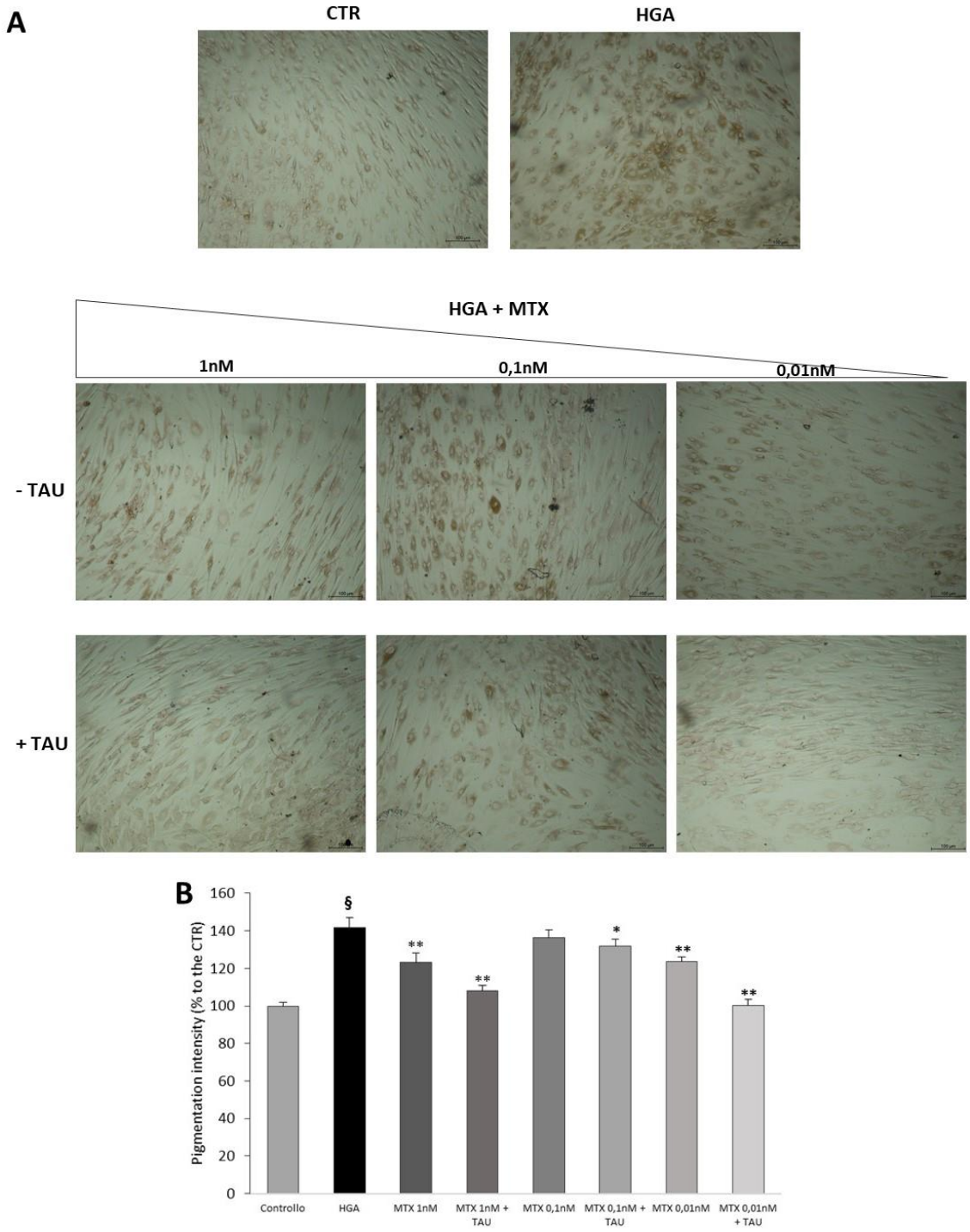

Figure 1: Human articular chondrocytes pre-treated for 24 h with different concentrations of MTX (1 nM, 0.1 nM, 0.01 nM), in absence or presence of TAU, and co-treated with HGA for 3 weeks. The negative control (CTR) was cultured with complete medium only. Cells were stained with Fontana Masson stain (A) and ochronotic pigment was quantified with ImageJ software (B). Scale bar = 100 µm.

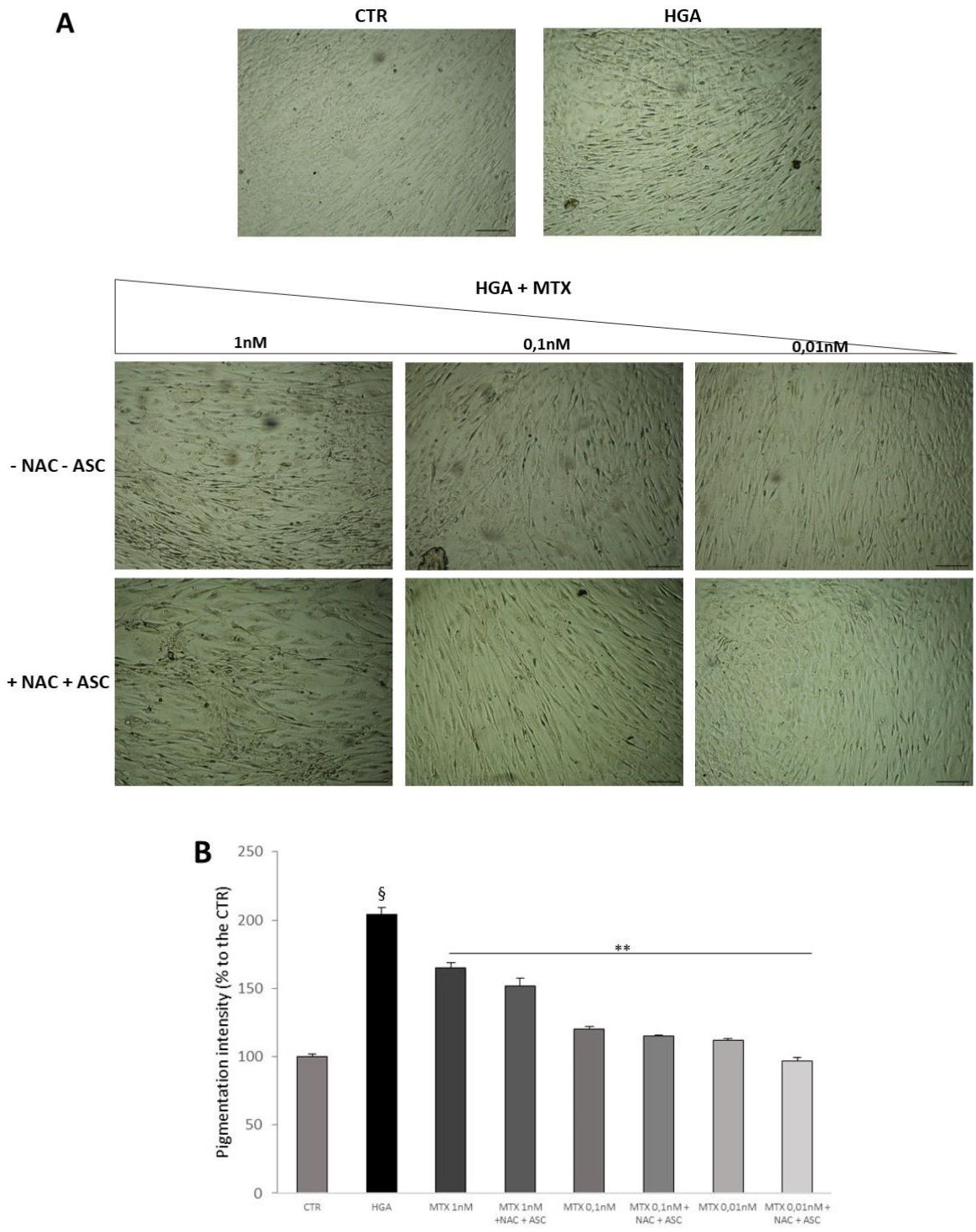

Figure 2: Human articular chondrocytes pre-treated for 24 h with different concentrations of MTX (1 nM, 0.1 nM, 0.01 nM), in absence or presence of NAC+ASC, and co-treated with HGA for 3 weeks. The negative control (CTR) was cultured with complete medium only. Cells were stained with Fontana Masson stain (A) and ochronotic pigment was quantified with ImageJ software (B). Scale bar = 100 µm.

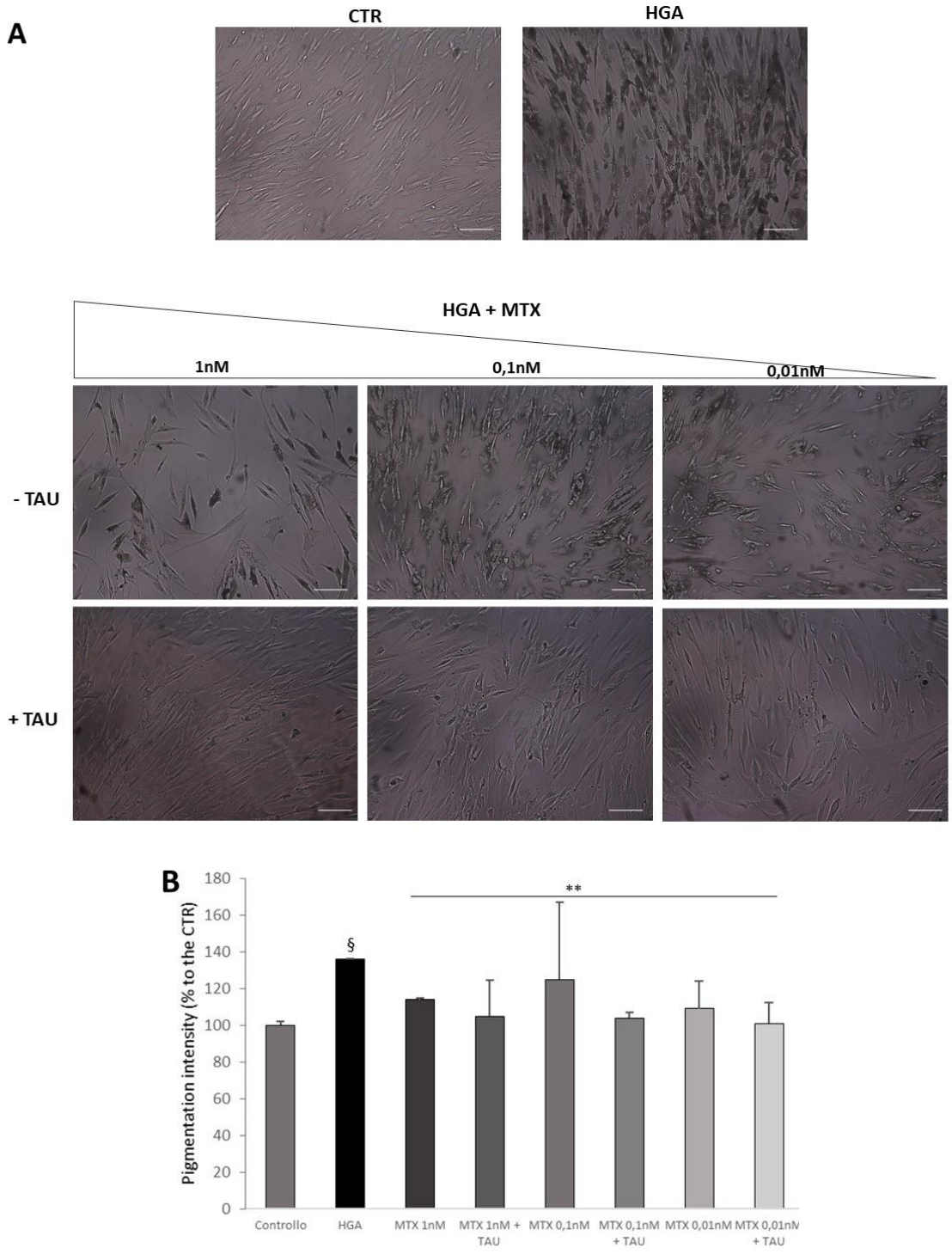

Figure 3: Human articular osteoblasts pre-treated for 24 h with different concentrations of MTX (1 nM, 0.1 nM, 0.01 nM), in absence or presence of TAU, and co-treated with HGA for 3 weeks. The negative control (CTR) was cultured with complete medium only. Cells were stained with Fontana Masson stain (A) and ochronotic pigment was quantified with ImageJ software (B). Scale bar = 100 µm.

7.4.2 Effect of MTX and antioxidants on amyloid production

MTX at low doses is widely recognized to have anti-inflammatory action (Chan and Cronstein 2002), since is one of the most effective medications to treat rheumatoid arthritis. In particular, MTX administration results in a reduction of amyloidosis fibrils and SAA levels in patients (Nakamura 2008). The most reliable mechanism of action that explain the anti-inflammatory property of low doses of MTX concerns its ability to block the activity of the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC). The inhibition of ATIC, involved in purine metabolism, causes an increase in extracellular release of adenosine, a powerful anti-inflammatory mediator (Brown et al. 2016). Indeed, adenosine acts as an inhibitor of T cells activation and proliferation and neutrophils recruitment.

It was already shown that MTX reduced in vitro HGA-induced A-amyloid aggregates (Millucci et al. 2012). In the present study, it had been explored the effect of MTX in different doses, alone or co-administrated with antioxidants, on the amyloidosis formation induced by HGA. The amyloid production in human chondrocytes was evaluated through Congo Red staining, in samples pre-treated with MTX, alone or with TAU, and then co-incubated with HGA. Images were acquired with a polarized light microscope, that allowed to detect the amyloid deposits through their birefringence. Results showed that HGA-induced amyloid formation was dramatically reduced in cells treated with compounds, compared to the AKU model, in which the birefringence was strongly visible (Fig. 4). In particular, all conditions tested were able to drastically inhibit amyloid formation, since the amyloidosis birefringent fibrils were not detectable anymore. Thus proved the strong efficacy of MTX in amyloidosis inhibition, that was evident after treatment with all the concentration tested. The model did not allow to evaluate if the co-administration with antioxidants improved the anti-amyloidogenic effect, considering that it was totally exerted by MTX. Despite this, we could speculate that in patients, were the amyloidosis is more diffuse and fibrils are bigger and stronger, the combined effect of drugs could deliver an additive and so improved effect.

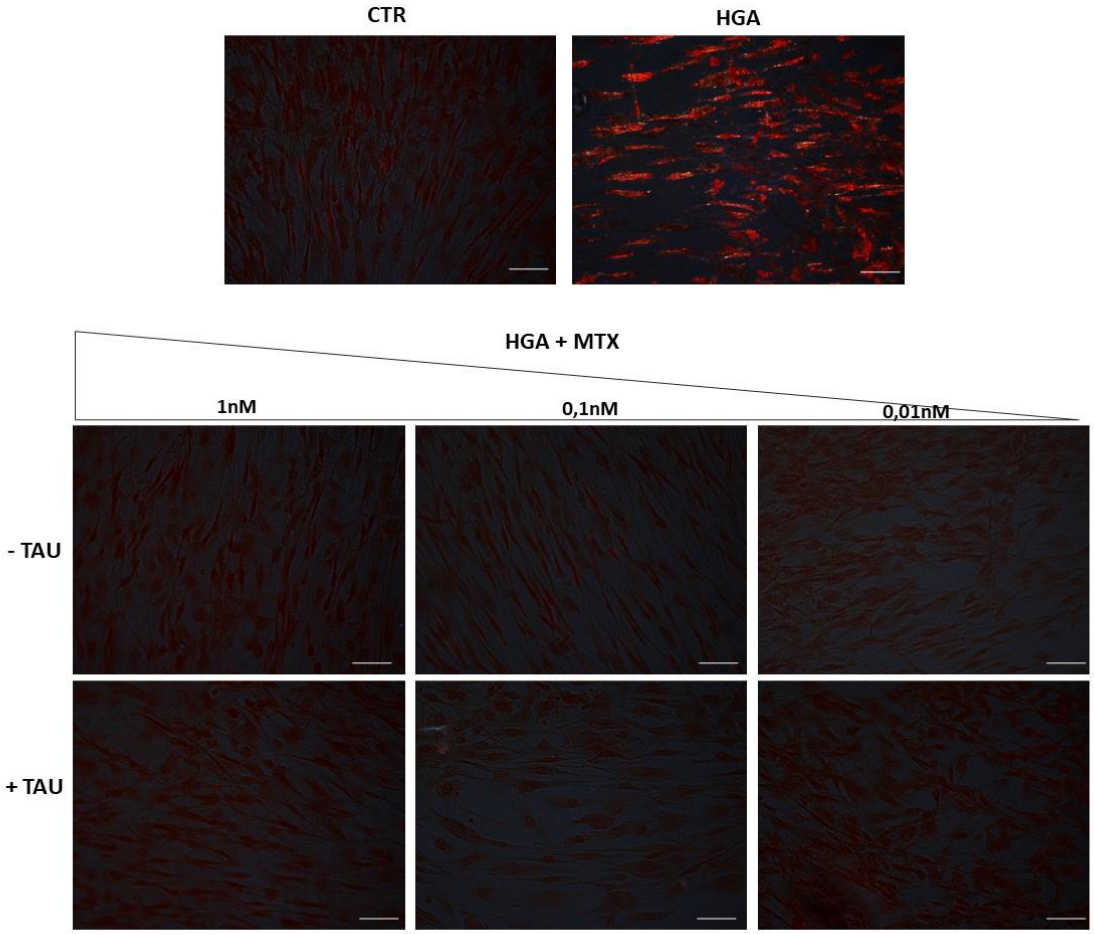

Figure 4: Human articular chondrocytes pre-treated for 24 h with different concentrations of MTX (1 nM, 0.1 nM, 0.01 nM), in absence or presence of TAU, and co-treated with HGA for 4 weeks. The negative control (CTR) was cultured with complete medium only. Cells were stained with Congo red stain and amyloid deposits were revealed exposing samples to polarized light. Scale bar = 100 µm.

7.5 Conclusion

In the present study, it had been proposed the association of MTX with antioxidants for the treatment of AKU. It had been studied the effect of combined drugs on AKU *in vitro* models, set up with human primary chondrocytes and osteoblasts, considering that the osteoarticular compartment is the most affected in the disease. Results showed that MTX reduced the development of ochronotic pigment and blocked the amyloidosis formation. The impact on ochronotic pigment was significantly incremented when the drug was co-administrated with antioxidants, both TAU and NAC+ASC. Probably antioxidant property of the compounds exerted an additive effect. Indeed, we could assume that molecules counteracted the autoxidation of HGA, that underwent oxidative reactions causing the final ochronotic pigment formation.

Ochronotic pigment and amyloid fibrils deposition in body leads in AKU patients the degeneration of tissues, with rupture of tendons, cartilage and bone fragmentations, and joints loss of function. Thus, slowing these processes could bring enormous benefits to patients' quality of life and amount of pain. Moreover, thanks to the combination of their antioxidative and anti-inflammatory property, the drugs combination counteracted both oxidative stress and chronic inflammation, two principal molecular aspect of the disease. Additionally, considering the several side effects that MTX causes to patients, the increment in its therapeutic power implemented by antioxidants, allowed to use lower doses of the drug, leading numerous advantages for patients.

Therefore, the co-administration of MTX and antioxidant compounds could be extremely effective for the pharmacological treatment of AKU.

CHAPTER 8:

Preliminary study of the inflammatory signal activation in HGA-treated blood cells

8.1 Introduction

Alkaptonuria (AKU) belongs to chronic inflammatory disorders. In particular, the inflammatory stimulus is caused by the permanence of oxidative stress condition, resulting from the homogentisic acid (HGA) accumulation. Oxidative stress factors stimulate the expression of pro-inflammatory cytokines, detected both in AKU chondrocytes (Braconi et al. 2012) and in HGA-treated AKU chondrocytes model (Spreafico et al. 2013). The body uptake of tyrosine and phenylalanine with diet is constant, so the HGD catabolic pathway is active, leading an unstopped HGA production. HGA is principally synthesized in the liver, but is carrying to all the body through the blood, where the inflammatory signal spread. Despite part of the molecules are excreted through urine or polymerized, forming the ochronotic pigment, AKU patients' blood has constantly high levels of HGA. This leads the expression of inflammatory markers and amplification of signal, with the occurrence of chronic inflammatory condition. Indeed, AKU is characterized by pathological features classically associated to collateral effects of inflammation, such as the secondary A amyloidosis (Millucci et al. 2012) and angiogenesis (Millucci et al. 2016).

Cytokines are small proteins secreted by cells in response to a specific stimulus, with a role in the cells interactions and communications. In particular, cytokines are involved in the initiation and persistence of inflammatory response, due to pathogen invasion or injury, and could be divided in two macro groups: pro-inflammatory and anti-inflammatory cytokines (Zhang and An 2007). Pro-inflammatory cytokines are principally expressed by immune cells after a potentially dangerous stimulus, triggering the propagation and amplification of inflammatory signal. In particular, pro-inflammatory cytokines act on the cell immune response, by regulating their growth, activation, differentiation, and migration (Turner et al. 2014). Moreover, cytokines induce production and release of further cytokines, that could drive, in case of infection or some pathology, to the "cytokine storm" phenomenon. Thus, the relevance of monitor cytokines level to follow diseases' progression is clear, considering that their concentration is strictly connected and proportional to the gravity of inflammation (Koelman et al. 2019). Actually, cytokine are already widely used both as biomarkers and therapeutic targets (Kany, Tilmann Vollrath, and Relja 2019).

8.2 Aim of the study

The impact of HGA in AKU on blood cells is still unexplored, despite this information could be fundamental to counteract the chronic inflammation and its detrimental effects on the organism. In this preliminary study, it had been created for the first time an AKU model exposing immune cells to HGA, in order to study its effects on the cytokines production and activation of the inflammatory signal. In this way, it had been reproduced the condition suffered by AKU immune cells, constantly in contact with HGA and its derived molecules.

In particular, the inflammatory occurrence in AKU was modeled by exposing different types of blood cells to increasing doses of HGA. Then, it was analysed if HGA directly stimulated the cytokines expression, following their production in time.

8.3 Material and methods

8.3.1 Cells isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood or from 1-day-old buffy coats, obtained from healthy donors, through centrifugation in density gradient of Ficoll-sodium diatrizoate. To obtained PBMC cells, the white layer between plasma and Ficoll was collected, centrifuged at 250 g for 15 min, and washed 3 times with PBS. Then, cells were suspended in PBS and counted. Also the pellet formed after Ficoll gradient centrifugation, consisting of red blood cells and granulocytes, was collected, and used for purify neutrophils. Specifically, cells were incubated at 37°C for 30 min with dextran 6% in PBS, centrifuged at 250g for 10 min, washed with PBS and exposed to a hypotonic shock. After a centrifugation and other 2 washing steps, the neutrophils obtained were suspended in PBS and counted.

Human monocytic cell line THP-1 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 0,12% sodium bicarbonate, and splitted 1:3 twice per week.

8.3.2 Induction experiments

HGA stock solution was prepared dissolving the powder in milliQ water to a final concentration of 10 mM and filtering. To be sure that the inflammatory response detected was stimulated by HGA, instead of possible contaminants, the HGA stock solution was tested with a specific kit that measured endotoxins concentration, following the manufacturer's instruction.

To perform experiments, cells freshly isolated were plated in 48-well plates at 1.5 *10^6 cells/ml density in RPMI 1640 medium with the addition of 2% FBS for PBMC cells and 10% for neutrophils. Then, cells were immediately treated, for 24, 48 or 72 h, with different concentrations of HGA, ranging from 1 mM to 0.05 mM or with Lipopolysaccharide (LPS), ranging from 500 ng/ml to 10 ng/ml, as positive control.

Monocytic cells were obtained from PBMCs plated and let grow ON. The day after, the culture medium with non-adherent cells was discarded, and monocytic cells, attached to the plate, were treated with inducers.

To measure cytokines in THP-1 supernatant, cells were seeded in 48-well plates at 1.5 *10^6 cells/ml density, in enriched inducers medium. Moreover, to stimulate the THP-1 cells differentiation to macrophages, cells were treated for 48 h with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA), and then exposed to inducers.

8.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

At the end of the treatments, cell supernatants were collected and used to quantify cytokines through ELISA method. The human CXCL8 and MCP-1 ELISA were performed following protocols developed in S. Struyf lab (Abouelasrar Salama et al. 2020), using antibodies purchased from R&D Systems. Human IL-1β and TNFα were measured with Duoset ELISA kits, following the manufacturer's instructions.

8.4 Results and discussion

8.4.1 Stimulation of CXCL8 induced by HGA

The HGA stock was analysed with a specific kit and resulted not contaminated by any endotoxin. This confirmed that all the following results describing the inflammatory signal activation derived exclusively by HGA effects.

As a first step, it was analysed if HGA could directly stimulate in immune cells the cytokines production, or if the inflammation in AKU is only due to secondary signals triggered by oxidative stress. For this purpose, it was measured the amount of CXCL8 in the supernatant of PBMC cells and neutrophils after 24 h of treatment with HGA, using concentrations in the range of HGA plasma levels found in patients (Tinti et al. 2010). CXCL8 is a chemotactic factor that mediates neutrophils

recruitment, leukocyte trafficking and phagocytosis stimulation. It belongs to signals behind the inflammatory cascade, and has a fundamental role in tumour progression (Bie et al. 2019). Therefore, its upregulation clearly indicates the onset of inflammation. Results proved that HGA had a direct effect on the CXCL8 expression and consequently on inflammation induction (Fig. 1A). Indeed, cells treated with 0.5 and 0.3 mM HGA expressed the cytokines up to 2 times more compared to the negative control. Nonetheless, the levels of the cytokine were low compared to the positive controls, consisting in induction with LPS. By the way, it need to be considered that LPS is a bacterial endotoxin able to immediately activate a strong immune response (Sampath 2018). Diversely, it could be hypnotized that, in AKU disease, the chronic inflammation is generated from a combination of direct HGA effect on immune cells and indirect stimuli, derived from high concentrated ROS and oxidant HGA derived molecules. In particular, patients' body is constantly exposed to HGA and oxidative stress. This could explain the lower signal obtained *in vitro,* compared to patients' situation. As expected (Porreca et al. 1999), PBMCs cells produced higher levels of CXCL8, compared to neutrophils. For this reason, PBMCs cells had been used for the following experiments. Moreover, the level of cytokine expression was HGA-dose dependent, and the concentrations of 0.1 and 0.05 mM seemed too low for stimulate inflammation.

The lower signal obtained with HGA induction, compared to LPS, could also depend on which pathway was activated by HGA. It could be possible that HGA needed more passages, and consequently more time, to exert its effect on CXCL8 expression. To evaluate this hypothesis, CXCL8 expression was detected after 24 and 72 h of PBMCs induction (Fig. 1B). Again, it was confirmed that HGA was able to stimulate the inflammatory response in PBMCs cells, inducing CXCL8 production that reach a concentration 7 time more than negative control, when cells were treated with HGA 1 and 0.5 mM for 24 h. However, the levels of cytokine after longer HGA induction were similar or less compared to the 24 h treatment. It can be concluded that HGA acts directly on pathways responsible for inflammation arising.

Discussed results allowed to identify the best HGA-treatment condition that induced inflammation. In particular, the optimal was represented by the stimulation of PBMC cells for 24 h with 1 – 0.3 mM HGA. This could constitute the basis for the set up, for the first time, of AKU *in vitro* model based on immunity cells system, leading huge advantages in the study of the pathology, considering its inflammatory character.

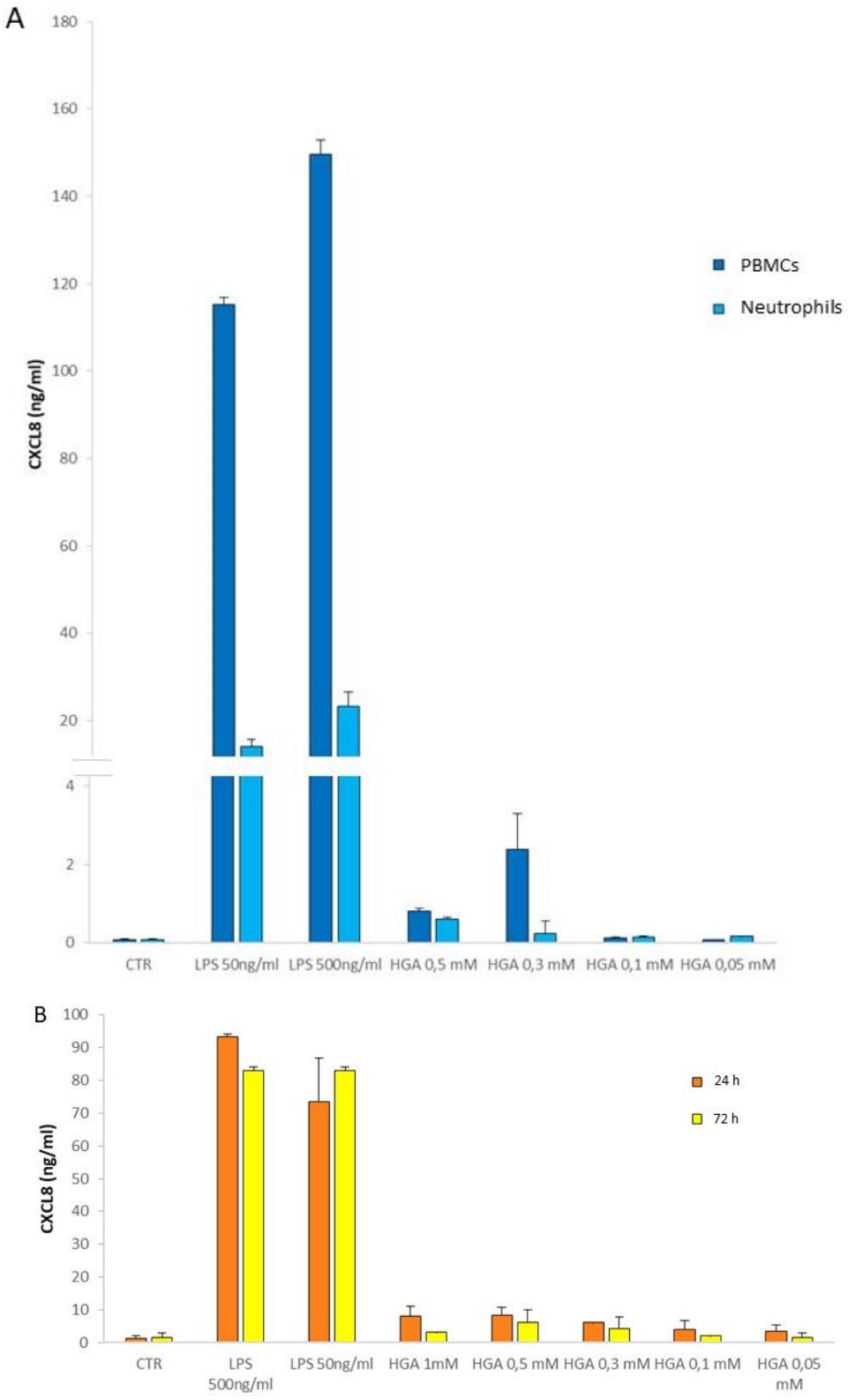

Figure 1: CXCL8 concentrations measured through ELISA in supernatant of cells. It was analysed the expression in PBMC cells and neutrophils after 24 h of treatment (A), and the expression in PBMC cells after 2 time points induction (B).

8.4.2 Time points of CXCL8 expression

Found that HGA stimulated CXCL8 expression, it was analysed the progression of CXCL8 secretion over time. PBMC cells were induced for 24 h with HGA 0.3 mM. This concentration was chosen because it was already selected as the optimal dose for cell line model set up (Tinti, Taylor, et al. 2011). Results showed that, in all time points, CXCL8 concentration was higher in LPS and HGA treated cells, compared to the not treated cells (Tab. 1). In PBMCs treated with HGA, the CXCL8 expression was very low after 2 h, then increased with the time of stimulation, reaching a peak at 24 h time point, when cytokine concentration was 5 time more than the negative control. After that, amount of CXCL8 decreased lightly, confirming that 24 h time point was appropriate for cytokines detection. This reflected the cytokines kinetics of mRNA formation in response to immune-cell stimuli (Fan et al. 1998).

	2h	6h	16h	24h	48h	72h	96h
CTR	0,125	0,075	10,19	0,16	0,197	0,243	1,022
LPS 50 ng/ml	11,058	62,1	>66.27	>66.27	>66.27	>66.27	>66.27
HGA 0.3 mM	0,04	0,452	13,764	0,808	0,413	1,15	1,393

Table 1: CXCL8 concentrations (ng/ml) detected through ELISA in supernatants of PBMC cells at different time points.

8.4.3 Detection of other cytokines in HGA-treated PBMCs

Following CXCL8 detection, the analysis was extended to other crucial components of the inflammatory response. The concentrations of MCP-1, TNFα and IL-1β were measured in PBMCs supernatants, after 24 h of stimulation with different concentrations of HGA, with LPS to obtain the positive control and without inducers for the negative control. Results revealed that cell stimulated with HGA produced not traceable quantity of TNFα and IL-1β (Tab. 2). We could hypothesize that cytokine levels were subjected to a small increase after HGA induction, but too low for be detectable with standard ELISA method. The goodness of experiments was proved by the detection of cytokines levels in positive controls. Differently, MCP-1 levels were detectable, and cells stimulated with HGA 1 mM produced higher amount of MCP-1 compared to the negative control. Despite this result confirmed the direct effect of HGA of cytokines production, surely it need deeply investigation, changing stimulation conditions or using more sensitive methods.

	TNFα (ng/ml)	IL-1β (ng/ml)	MCP-1 (ng/ml)
CTR	und	und	0,048125
LPS 500ng/ml	2,611	40,6255	5,2665
LPS 50ng/ml	2,92975	35,922375	5,996
HGA 1mM	und	und	0,1315
HGA 0.5 mM	und	und	0,03375
HGA 0.3 mM	und	und	0,02475
HGA 0.1 mM	und	und	0,018
HGA 0.05 mM	und	und	0,01475

*und= value under detection threshold

Table 2: Concentrations of MCP-1, TNFα and IL-1β detected through ELISA in supernatants of PBMC cells after 24 h of treatments.

8.4.4 HGA treatment of different immune cells

To obtain a detailed analysis of the inflammatory response in AKU, and set up the best *in vitro* model, resulted convenient study the HGA effect on different immune cells. For this reason, the investigation had been extended to other cell types. Specifically, it was analysed the difference in response between the monocytic cellular line THP-1, and THP-1 stimulated to differentiate into macrophages. THP-1 cells had been used in several studies, to investigate the immunomodulatory effects of small molecules (Jakopin and Corsini 2019), with the advantages brought by immortalized cells. In all the time points, the HGA-treated THP-1 cells produced higher concentration of CXCL8 compared to the negative control, in a dose-dependent way (Fig. 2). Macrophages did not result more responsive to HGA induction than THP-1. Moreover, also in this model, the best inflammatory response was achieved after 24 h of induction. Resulted showed that THP-1 HGA-stimulated cells could be used, among with PBMC cells, for *in vitro* investigations. These cells have several advantages over primary PBMCs, as their homogenous genetic background, which abolishes donor variability. Further, they

are cheap, easily accessible, and don't carry blood contaminants (Chanput, Mes, and Wichers 2014).

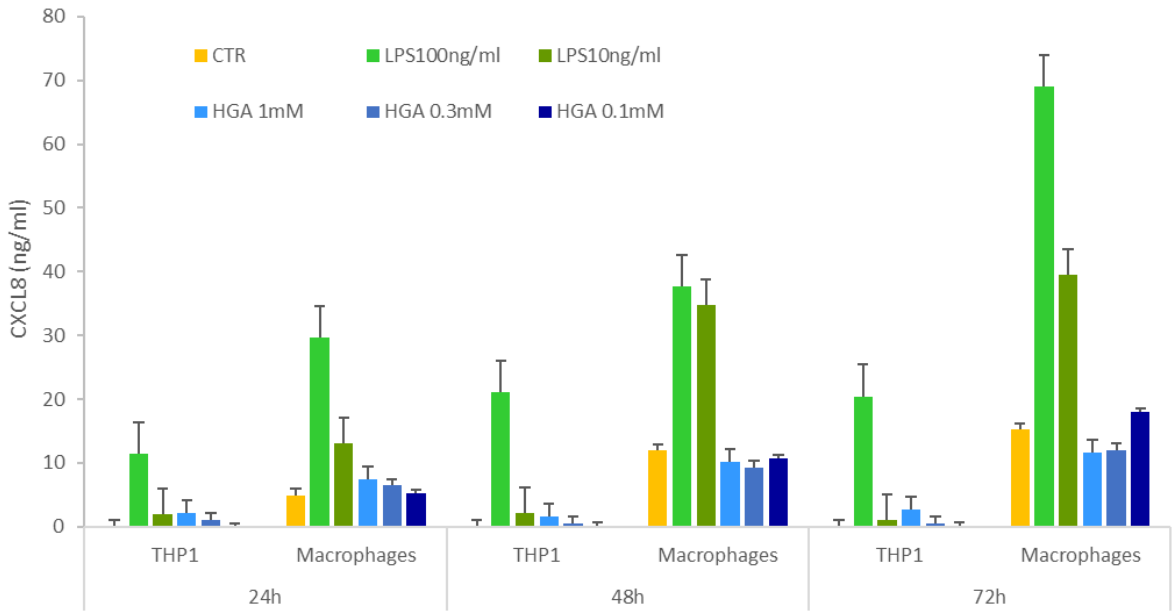

Figure 2: CXCL8 concentrations (ng/ml) detected through ELISA in supernatant of THP-1 cells and THP-1 cells differentiated in macrophages, after stimulation with different doses of HGA, LPS (positive control) or without any stimulation (negative control, CTR). The supernatant was taken in 3 time points.

The CXCL8 concentration had been also measured, after 24 h and 48 h of treatment, in supernatant of monocytes, to study if these cells were more responsive compared to PBMC cells. For instance, is known that LPS induced cytokine release varies between THP-1, PBMCs, monocytes, or whole blood. This depend by the differences between cells phenotypes (Schildberger et al. 2013). Monocytes were obtained from freshly isolated PBMCs, growing cells ON in plate and removing floating cells the day after. Also in monocytes, HGA stimulated the inflammation, visible through CXCL8 expression, in a dose-dependent way, with the higher level detected after treatment with HGA 1 mM (Fig. 3). The trend showed after 24 h of induction was maintained in 48 h treated samples. CXCL8 produced by monocytes did not differ by the values of concentration measured in PBMCs samples, proving that monocytes were not more responsive that PBMCs, as already observed in the comparison between THP-1 and THP-1 derived macrophages.

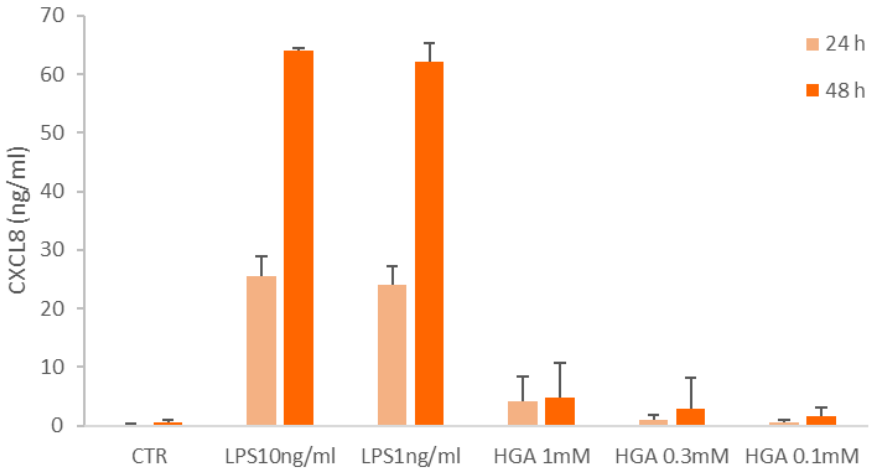

Figure 3: CXCL8 concentrations (ng/ml) detected through ELISA in supernatant of monocytes after 24 h and 48 h of treatments.

8.5 Conclusion

In the present study, it was analysed for the first time the effect of the administration of HGA to immune cells. It was demonstrated that the molecule acted as pro-inflammatory stimulus with a direct effect on cells, leading the CXCL8 expression. The effect of HGA on inflammation in AKU patients is propagated and amplified by the oxidative stress and ochronotic pigment accumulation (Braconi et al. 2015). Several types of immune cells were stimulated with a range of HGA concentrations, for different time points. It was established that the higher inflammatory response could be detected after 24 h of treatment with HGA ranging from 1 mM to 0.3 mM. Indeed, after 1 day of HGA administration there was reached the pick of cytokine expression. Moreover, the most responsive cells were PBMCs, THP-1 and monocytes. Also MCP-1 expression was positively stimulated by HGA, in contrast to TNFα and IL-1β that were not detectable. Further experiments will be required to better understand the pro-inflammatory pathway activated by HGA. All these preliminary observations open interesting prospective for the deeply insight of the ultra-rare disorder, mostly characterized by chronic inflammation and manifestations connected, as amyloidosis. For the first time, AKU was modeled using blood cells, and this could be extremely useful for the comprehension of immune cells involvement and inflammatory pathways activated. Indeed, this information could be used to identify new potential therapeutic pharmacological targets.

CHAPTER 9:

Appendix

- **PBS (Phosphate buffered saline):**

8 g/L NaCl

0.2 g/L KCl

1.15g/L Na_2HPO_4

0.2g/L $KH_2 PO_4$

pH= 7.4

- **RIPA buffer:**

50 mM TRIS

150 mM NaCl

0,5% sodium deoxycholate

0,1% SDS

1% Tergitol

- **Laemmli Sample Buffer (2X):**

4% SDS

20% glycerol

0.004% bromphenol blue

0.125M TRIS-HCl, pH 6.8

10% 2-mercaptoethanol

- **Running buffer (TGS) 10 X for Western blot:**

30 g/L TRIS

144 g/L Glycine (1920 nM)

1% SDS

- **Transfer buffer for Western blot:**

3 g/L TRIS

14,4 g/L Glycine

20% EtOH

- **SDS-PAGE gel:**

8-12% (separating gel) or 4% (stacking gel) of bis acrylamide (30% solution)
25% TRIS-HCl 1,5 M pH 8.8 (separating gel) or TRIS-HCl 1 M pH 6.8 (staking gel)
1% SDS
0,5% APS (1g/ml)
10 µL/10 ml TEMED

- **TBS (10X):**

24 g/L TRIS

88 g/L NaCl

pH= 7.6

CHAPTER 10:

Final conclusions and future prospects

In the present thesis, some unexplored molecular aspects of Alkaptonuria (AKU) were investigated, and it was studied the efficiency of a formulate, based on the combination of methotrexate (MTX) and antioxidants, for AKU and chronic inflammatory disorders treatment. The discussed results open intriguingly prospective for dealing with the disease and improve the patients' quality of life.

The finding of lysosomes implication in AKU and the discovery that ochronotic pigment was intracellularly stored in these organelles allow to focus the attention on the role of these organelles in the disease. Indeed, drugs that specifically target lysosomal function and activity could be used in the future to block the extracellular pigment deposit or to stimulate its endogenous degradation. Another outcome of HGA exposition to cells, showed during this project, is incurred by genome. Indeed, it was demonstrated that HGA had indirectly deleterious effects on DNA, mostly consisting of strand breaks. Moreover, it was highlighted that HGA treated cells exhibited nucleolar stress. These observations underline the requirements to frequently monitor patients, to be able to rapidly intervene in case of cancer masses development. Moreover, is stressed the necessity to combine antioxidants drugs to AKU treatments. Additionally, during this thesis project, it was set up a new model based on the incubation of immune cells with HGA, aiming to study inflammatory pathways. In particular, it was demonstrated the ability of HGA to directly stimulate pro-inflammatory cytokines expression. Inflammation development is a crucial aspect for the deep understanding of AKU, considering that the disease is characterized by a severe chronic inflammatory condition. All these findings add new knowledge about this condition, that is worldwide poorly studied for its rarity. The deeply comprehension of what happens in patients' body, constantly exposed to high concentration of HGA, could also inspire new possible treatments.

In this contest, it was proposed a new therapeutic approach, based on the combination of MTX, a widely used anti-inflammatory drug, and antioxidants molecules. The co-administration of compounds it had been proved to be more efficient in the counteraction of oxidative stress, inflammation and amyloidosis compared to the single treatments. Moreover, the efficiency of co-administration was kept also using a low dose of MTX, leading the advantage of side effects limitation. This opens extremely interesting prospective for the treatment of AKU, but appears also relevant for cure all the disorders characterized by chronic inflammation. The encouraging results obtained during this project motivate us to continue in this direction, expanding the *in vitro* study to *in vivo* models and finally clinical trials.

Actually, one of the limits of research on AKU is the absence of a valid *in vivo* model, considering

that mouse with *hgd* deletion doesn't exhibit chronic pigment accumulation and joints dysfunctionality. Therefore, in this thesis work it had been showed preliminary results obtained from the set up of an *in vivo* Zebrafish model. Considering that fishes grown with HGA developed ochronosis, this could represent the basis for the development of a new suitable and simple *in vivo* model for AKU research.

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