EXPERT REVIEWS

Redox proteomics gives insights into the role of oxidative stress in alkaptonuria

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Daniela Braconi, Lia Millucci, Lorenzo Ghezzi and Annalisa Santucci*

Dipartimento di Biotecnologie, Chimica e Farmacia, via Fiorentina 1, Università degli Studi di Siena, 53100 Siena, Italy
**Author for correspondence:*
Tel.: +39 057 723 4958
Fax: +39 057 723 4903
annalisa.santucci@unisi.it

Alkaptonuria (AKU) is an ultra-rare metabolic disorder of the catabolic pathway of tyrosine and phenylalanine that has been poorly characterized at molecular level. As a genetic disease, AKU is present at birth, but its most severe manifestations are delayed due to the deposition of a dark-brown pigment (ochronosis) in connective tissues. The reasons for such a delayed manifestation have not been clarified yet, though several lines of evidence suggest that the metabolite accumulated in AKU sufferers (homogentisic acid) is prone to auto-oxidation and induction of oxidative stress. The clarification of the pathophysiological molecular mechanisms of AKU would allow a better understanding of the disease, help find a cure for AKU and provide a model for more common rheumatic diseases. With this aim, we have shown how proteomics and redox proteomics might successfully overcome the difficulties of studying a rare disease such as AKU and the limitations of the hitherto adopted approaches.

KEYWORDS: antioxidants • cartilage • joints • homogentisic acid • ochronosis • protein oxidation • rare diseases

Alkaptonuria (AKU, OMIM: 203500) is a serious, autosomal recessive, multisystem degenerative disorder of great historical and medical interest. AKU is ultra-rare, being characterized by an extremely low incidence, which is estimated to be one in 250,000–1,000,000 in most ethnic groups [1,2], though countries exist where the disease is much more common, such as Slovakia where the incidence rises to 1:19,000 [3], or Dominican Republic [4]. More than a century ago, AKU was one of the first conditions for which Mendelian recessive inheritance was proposed [5]. Nevertheless, it took almost one century before the morbidity was associated to a deficiency of activity of homogentisate 1,2-dioxygenase (HGD, E.C.1.13.11.5), enzyme converting homogentisic acid (HGA) to maleylacetoacetic acid in the degradation pathway of tyrosine and phenylalanine (FIGURE 1) [6]. HGD has been reported to be expressed in liver, kidney, prostate, small intestine, colon and recent evidence of its expression in human osteoarticular cells was provided [7].

The molecular characterization of AKU progressed very slowly despite a manifest historical interest, and since its identification, there have been a number of descriptions of the clinical features of the disease, reporting the effects of excess HGA, which is not fully excreted with urines (turning black due to spontaneous oxidation of HGA under aerobic/alkaline conditions, allowing a preliminary diagnosis) but instead partly accumulated in the body during life. Such an accumulation is prominent in connective tissues, to which a dark brown-black discoloration is imparted. This phenomenon is known as 'ochronosis' based on the color of the pigment, and it represents the hallmark of AKU. As a genetic disease, AKU is present at birth, but observable ochronotic manifestations and symptoms are delayed, typically beginning in the third decade of life. The reasons for such a delayed manifestation of the disease have not been clarified yet. Ochronosis appears as blue/black pigmentation of the eye and ear, whereas the most severe manifestations are at the articular level and include premature severe disabling

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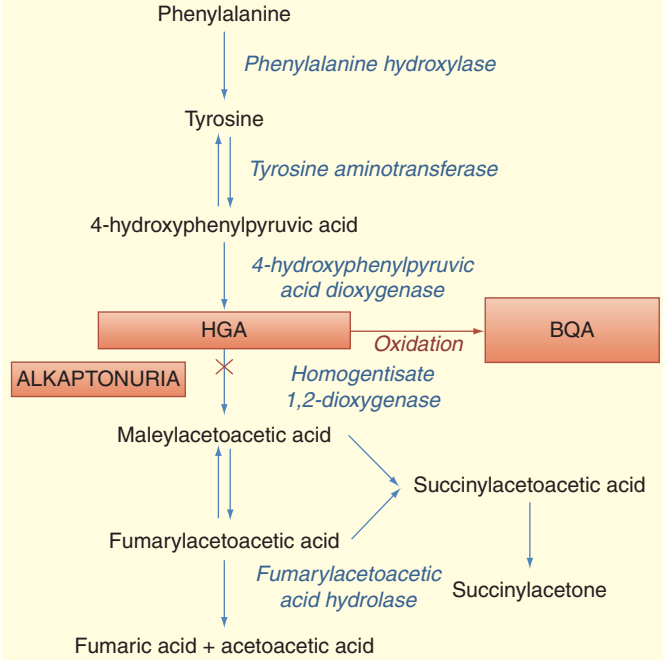

Figure 1. Phenylalanine and tyrosine degradation pathway in human. The enzyme deficiency in AKU (highlighted) leads to accumulation of HGA which undergoes spontaneous oxidation into BQA. This is considered to trig oxidative stress and polymerization ultimately leading to the production of ochronotic pigments in AKU.
AKU: Alkaptonuria; BQA: Benzoquinone acetic acid; HGA: Homogentisic acid.

osteoarthritis-like joint damages, which significantly affect and reduce patient's quality of life [8]. AKU sufferers are also more prone to fractures, ruptures of ligaments and tendons. Stones (salivary, gall bladder, prostate and renal) and aortic valve diseases [1,9] can be observed as well.

Manifestations of AKU at cardiovascular level are due to deposition of ochronotic pigment within connective tissue, a phenomenon noted intraoperatively which is considered to trig dystrophic calcification and which makes AKU sufferers at risk to develop aortic valve disease and coronary artery disease more frequently than the general population, with a theoretical concern of abnormalities in atrioventricular conduction. This often leads to aortic valve replacement, with or without coronary artery bypass grafting [10]. Mitral and tricuspid valve involvement in AKU have been described as well [11,12].

Scribonio was the first who described in 1584, in a young male patient, the phenomenon of urine darkening accelerated by alkalinization [13]. Based on this observation, Boedeker defined this clinical condition as 'alkaptonuria' combining the Arabic words for 'alkaline' and 'to take', but it was only at the end of the 19th century that the compound responsible for the discoloration, already known as 'alcapton', was identified to be HGA [1]. The very first clinical manifestations of ochronosis were observed in *Hawra*, an Egyptian mummy dating from 1500 BC, during radiological and biochemical examinations which confirmed the ochronotic pigment as chemically identical to oxidized-HGA [14,15]. Nevertheless, despite such an evident historical interest in analyzing and characterizing the ochronotic pigment, its exact molecular composition and the mechanisms of its production are still obscure.

Diagnosis of alkaptonuria

Diagnostic confirmation of AKU following evidence of the homogentisic aciduria (urine blackening as the pathognomonic sign) can be made by measuring HGA levels, which in healthy individuals should be null, in blood or urine by capillary electrophoresis [16], NMR [17], chromatography and mass spectrometry (MS) [18–21]. Definitive proof can be obtained by identification of AKU-causing mutations (see below).

Arthritis is the most common clinical feature of ochronosis, which can lead to chronic pain, crippling and disability, eventually leading to postural deformities [22], but ochronotic arthropathy can be misdiagnosed because it might resemble osteoarthritis, ankylosing spondylitis or Paget [23,24]. Recently, the importance of nuclear magnetic techniques in helping the diagnosis of ochronotic arthropathy [25] and the possible use of spine radiograph in the diagnosis and staging of ochronotic spondyloarthropathy [26] have been pointed out.

Therapy of alkaptonuria

Today, no pharmacological treatments exist to alter the natural history of AKU, and the disease is consequently characterized by a poor prognosis. Current treatments are only palliative and do not tackle the intrinsic causes of AKU. Symptomatic therapy includes:

- A low-protein diet, which however is extremely difficult to maintain and whose efficacy is controversial [27];
- Physiotherapy and palliative treatment of the pain associated with alkaptonuric ochronotic arthropathy (only during the early stages) with FANS and analgesics;
- At later stages, surgery for total joint and heart valves replacement is usually required.

A first attempt to treat AKU was made by administering ascorbic acid (ASC) [28], though, the efficacy of this treatment has not be proven undoubtedly and, on the contrary, it has been associated with an enhanced oxidative stress under certain circumstances [29,30]. Growing experimental evidence documented the ability of ASC to promote harmful effects in cells exposed to reactive oxygen species (ROS)/reactive nitrogen species (NOS), in obvious contrast with the antioxidant function of the vitamin [31,32]. Nevertheless, recent experimental evidence was provided *in vitro* for a positive action of ASC when combined with N-acetyl cysteine (NAC), a drug combination that successfully reduced the formation of ochronotic pigment [33–35] and allowed to hypothesize its possible therapeutic use in AKU.

A clinical trial was carried out in USA to evaluate the possible use of nitisinone in AKU [36]. Nitisinone is already in compassionate use for the treatment of hereditary tyrosinemia type 1 because it is a potent inhibitor of the enzyme p-hydroxy phenyl pyruvate dioxygenase in the catabolism of tyrosine, thus

blocking the production of HGA (Figure 1) [1,37]. In this first trial, the drug proved to dramatically reduce urinary HGA but failed to demonstrate benefits in primary and secondary clinical parameters in AKU patients suffering from ochronotic arthritis [36]. More recently, also on the basis of what was evaluated in an AKU mouse model [38,39], two new clinical trials, one in the USA and another in Europe, have been undertaken to evaluate the use of nitisinone in treating AKU and ochronotic-osteo arthropathy. Although care should be taken about the right dose of drug to be administered to AKU patients and the most proper age to start the treatment [40], and also considering the fact that obviously the drug is not devoid of adverse effects [37], at the moment nitisinone is the most promising drug for the treatment of AKU.

Genetics of alkaptonuria

AKU patients are homozygous or compound heterozygous for loss-of-function mutations in *HGD* gene [2], which in humans is located on chromosome 3q21–23 [41]. Fernandez-Canon *et al.* cloned the human *HGD* gene and identified the first loss of function mutations, providing proof of the enzyme defect as a result of defect in this gene [2]. So far, several different mutations have been identified in the *HGD* gene in patients from various populations [2,42–46]. Extensive genetic screenings have been carried out in Slovak AKU patients [3,47,48], allowing ultimately the establishment of a dedicated database [49].

The determination of the crystal structure of the enzyme HGD was a spur for the study of the pathogenic effects of AKU mutations. Rodriguez *et al.* provided structural and functional analysis of *HGD* mutations by means of His-tagged mutant HGD proteins in *Escherichia coli* [50]. Apparently, no correlation between genotype and disease severity (in terms of excreted HGA) exists. The lack of such a clear correlation between genotype and phenotype may not be only explained by the variability in residual HGD enzymatic activity but also by the impact of patients' life-style. Thus so far genetic analysis, though informative, failed in clarifying the disease mechanisms. At this moment, for instance, when there is a need to assess AKU severity, only a questionnaire-based evaluation can be used [51,52]. This strongly highlights the need to fill such a lack of knowledge by adopting approaches, different from those hitherto applied to study AKU, paying a particular emphasis to post-genomics.

In vitro & *ex vivo* human models to study alkaptonuria

A better understanding of the molecular biology of human diseases allowed gaining knowledge about the pathophysiology of many conditions, which implied an enormous progress in modern medicine. Such progress has been limited for AKU. As mentioned previously, very little is known about the molecular mechanisms by which the metabolic disturbance in AKU leads to ochronosis and arthropathy. This was also one of the major obstacles to progress in developing specific therapeutic interventions for AKU and ochronotic arthropathy.

The clarification of AKU pathophysiological molecular mechanisms would allow a better understanding of the disease and provide a double advantage. On the one hand, this would help in finding a dedicated cure for AKU, ochronotic arthropathy and other AKU-related severe organ complications; on the other hand, since AKU can be considered as a model for more common rheumatic diseases such as osteoarthritis and rheumatoid arthritis [53], the social and economic relevance of AKU study would definitely be much wider. In this light, developing *in vitro* and *ex vivo* models reproducing the disease condition becomes fundamental.

It was initially thought that HGA may act as a chemical irritant causing inflammation and tissue degeneration, but more recent and convincing lines of evidence suggested that it is rather benzoquinone acetate (BQA), a by-product of its spontaneous oxidation, that induces and propagates a state of oxidative stress while concurring to the production of an ochronotic melanin-like pigment [54,55]. Overall, in the last years numerous unequivocal indications of the HGA-induced oxidative stress in AKU models were collected. The link between oxidative stress and tyrosine metabolism disorders is not new, as in the case of phenylketonuria [56] or tyrosinemia [57,58]. Compelling evidence also indicates the presence of oxidative stress in rheumatic diseases, including osteoarthritis and rheumatoid arthritis [59], pathologies that share common features with AKU. Interestingly, apart from similar symptomatology, rheumatoid arthritis and osteoarthritis may present a yellowish/brownish/grey pigmentation in cartilage, suggesting the presence of a kind of melanin-like pigment [55].

A range of human serum-, cell- and tissue-based human models have been established in the last years (schematically depicted in Figure 2) [30,33–35,53,60–63]. These human AKU models are based on exogenous addition of HGA range concentrations analogous to those found in AKU patients' plasma. Thanks to such AKU models, the *in vitro* conversion of HGA into BQA, the induction of oxidative stress and the production of the ochronotic pigment were demonstrated (Figure 3B–D) similarly to what observed in *ex vivo* AKU cells (Figure 3A). More specifically, a plethora of HGA-induced effects were described including, among the others:

- Reduction of viability and proliferation, induction of apoptosis, reduction of proteoglycan release and protein oxidation in human articular chondrocytes; those effects were partially restored by administering a combination of ASC and NAC as antioxidants [33];
- Production of a fluorescent melanin-like ochronotic pigment in human serum from healthy donors challenged with HGA (Figure 3B); the addition of several antioxidant compounds was beneficial in reducing/delaying the production of such a fluorescent ochronotic pigment [34];
- Enhanced lipid peroxidation, decreased activity of the enzyme glutathione reductase and a massive depletion of thiols (Figure 4B) in human serum challenged *in vitro* with HGA [61];

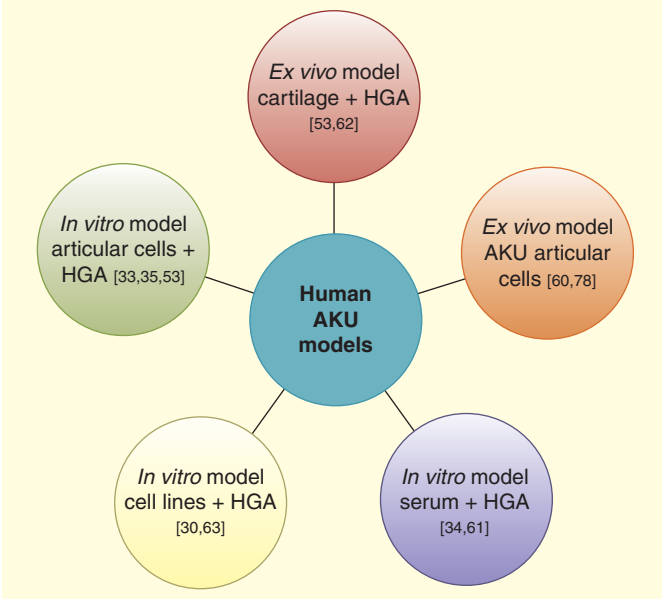

Figure 2. Human models of alkaptonuria. A range of *in vitro* and *ex vivo* serum-, cell- and tissue-based models were developed in the last years thanks to the exogenous addition of HGA and its spontaneous oxidation into BQA, helping to depict the pathophysiological mechanisms of the disease.
AKU: Alkaptonuria; BQA: Benzoquinone acetic acid; HGA: Homogentisic acid.

- Increased apoptosis, nitric oxide (NO) release and levels of pro-inflammatory cytokines in chondrocytes from AKU patients [60].

Proteomics & redox-proteomics of alkaptonuria

Biomarkers are objective molecular indicators of a condition/process that possess diagnostic, prognostic and predictive value. Several areas of medicine lack biomarkers to follow disease progression and response to therapy. This is not only true for AKU but also for more common human diseases such as inflammatory arthritis [64,65]. In the biomarker discovery process, genomics and transcriptomics are powerful methods; nevertheless, they cannot match the power of proteomics and MS, allowing for the simultaneous identification and quantification of proteins in complex biological mixtures, which provided the researchers with unprecedented and invaluable tools for the discovery of novel biomarkers which may help a predictive and personalized medicine in the future [66]. Moreover, the possibility to identify the post-translational modifications (PTMs) of proteins which are biologically and pathophysiologically relevant, is another key factor for the success of proteomic studies: since PTMs are of paramount importance in driving protein structure and function, their study is potentially the most informative among 'omic' approaches. For instance, it has already been shown that protein redox-changes may have a pivotal role in a range of arthritic disorders; similarly, a wide range of protein PTMs are emerging as unique, disease-specific biochemical markers for extracellular matrix remodeling in several connective tissue diseases [67].

Redox reactions within cells are tightly regulated and drive physiological functions, but when there is a perturbed balance between pro-oxidant and antioxidants systems, then oxidative stress occurs and possibly leads to the damage of macromolecules, especially proteins, often altering their structures and disrupting their biological functions [68]. Oxidized proteins are also prone to form aggregates, and the accumulation of oxidized/aggregated proteins is likely to impair fundamental cellular functions. Oxidation induced modifications of proteins, having relapses not only for structure but also for activity, unfolding and degradation, are gaining interest in the scientific community, as witnessed by the increasing number of redox proteomic studies. Due to their rapid reaction with ROS and their abundance within cells, proteins are of major interests for biochemists in search for oxidative stress biomarkers such as: protein carbonyls, thiol-oxidized proteins, HNE-modified proteins, nitrated proteins, glycated proteins and many others.

Cysteines are one of the most reactive groups among all amino acid side chains. They are exquisitely sensitive redox-reactive groups that can be reversibly/irreversibly oxidized with impacts for catalytic activity, metal binding or conformational alterations of proteins impacting on cell signaling, metabolism, gene regulation, proliferation, differentiation and apoptosis [69–72]. Furthermore, redox-regulated cysteine modifications can be transient, switching between physiological roles in redox signaling and functional alterations upon overoxidation. Nevertheless, due to their high reactivity, the analysis of redox-state of cysteines and their role in biological processes challenge the investigators with technical difficulties [73].

Carbonylation is the most common oxidative PTM of proteins after modification of thiol residues; being one of the most harmful reactions, it is often considered as a major hallmark of oxidative stress [74]. It can occur following metal-catalyzed oxidation, lipid peroxidation or glycoxidation. Since carbonylated proteins cannot be repaired, they accumulate and aggregate due to increased hydrophobicity eventually leading, if not degraded or eliminated, to cell death. Several biochemical and analytical methods are available to identify and quantify protein carbonyls [74] such as: biochemical and immunological techniques (merely providing a global information on modified proteins and oxidation levels) spectrophotometric and chromatographic assays (for quantification purposes), and mass spectrometry (for the exact identification of modified proteins and site of modification).

In AKU, a major obstacle in tackling the pathological features of the disease is the rarity of samples, which is not only due to the low incidence but also due to the difficulties in obtaining human tissues and cells from ochronotic patients (requiring very invasive sampling techniques). Moreover, ochronosis often causes severe damage to the tissues, so collection of suitable samples from biopsies may be problematic. These problems may be partially overcome by the adoption of AKU serum, cell and cartilage models. These models were characterized under different points of view, including proteomic and redox-proteomic analyses.

Human serum AKU model

Since blood plays a critical role in maintaining a correct redox-balance, a human serum-based AKU model was developed, where HGA-induced protein oxidation and *in vitro* production of ochronotic pigment were investigated by means of SDS-PAGE analysis [34]. Such a model was also used to evaluate the efficacy of anti-oxidant compounds in counteracting the HGA-generated oxidative stress. Since it is known that BQA con easily react with thiol groups, both protein carbonylation and oxidized protein thiols were analyzed. Human AKU serum model highlighted how HGA could promptly induce oxidation of serum proteins and allowed to identify which antioxidants could better counteract this phenomenon, protecting proteins from oxidation both in terms of carbonylation and protein thiol-oxidation. Interestingly, some of the most effective compounds (taurine, lipoic acid, phytic acid and a combined administration of ASC and N-acetylcysteine) among those tested could have a role in protecting against quinone toxicity, in counteracting lipid peroxidation, in restoring thiol pool or could act a s metal chelators. Some of them are already adopted for musco-skeletal diseases [34]. When the HGA-treated human serum was characterized by proteomics and redox-proteomics, the molecular targets of the *in vitro* HGA-induced damage were identified and indirect evidence of the auto-oxidation of HGA into BQA was provided, together with the ability of BQA to quickly bind serum proteins [61]. Overall, in HGA-treated serum altered levels of proteins involved in metal ion homeostasis, protein aggregation and with carrier functions were found. The same proteins were also found to be oxidized both in terms of carbonylation and thiol-oxidation (Figure 4A & Table 1), confirming the high susceptibility of thiol groups to oxidation in AKU and suggesting that increased pro-oxidant conditions occur in the blood of AKU sufferers, for whom a protein thiolation index was recently proposed [75]. These findings could let speculate about possible mechanisms allowing the development of alkaptonuric ochronosis, in other words, an imbalance of redox homeostasis of metals that can enhance protein oxidation in turn promoting protein aggregation (and also supporting the finding of AKU as an amyloidogenic disease), and transport of the generated BQA by serum carrier proteins, with propagation of oxidative damage within the entire body. At the articular level, where particular oxygen/nutrient conditions exist, and where a perfectly tuned redox homeostasis is required for the production of quality cartilage, BQA can start a cascade of events ultimately leading to its binding to macromolecules, polymerization and generation of the ochronotic pigment [61].

Human cell AKU models

Human articular chondrocytes were used to test the effects of HGA with or without ASC. The chosen HGA concentration was shown to induce intra- and extra-cellular deposition of ochronotic pigments. Such HGA concentration fell in the range of circulating HGA levels in AKU patients' serum [76]. The supplementation with ASC had a double reason: it is required *in vitro* for production and quality of cartilage [77]; and it is generally considered as an antioxidant. The evaluation of human chondrocytic protein repertoires revealed that ASC, when administered alone to cells, was associated with a general underexpression of proteins with structural functions as well as playing a role in the oxidative stress response (such as catalase, mitochondrial SOD and peroxiredoxin 1). HGA, on the contrary, induced an altered expression of proteins playing a role in determining protein fate and folding together and a reduction in levels of structural proteins, similar to protein expression profiles of osteoarthritic cartilage. The results obtained

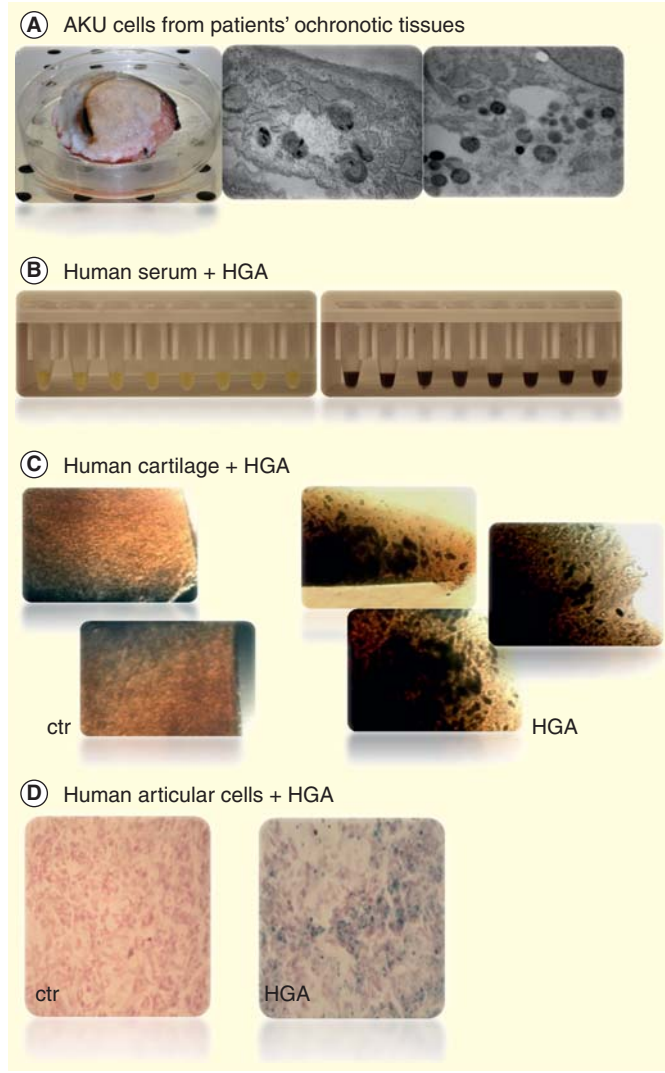

Figure 3. *Ex vivo* and *in vitro* ochronotic pigmentation. **(A)** The ochronotic pigmentation observable in cells isolated from AKU patients' tissues and propagated in laboratory is shown. **(B)** *In vitro*, the exogenous addition of HGA makes the human serum incubated at 37˚C turn brown due to the development of a melanin-like fluorescent ochronotic pigment. **(C)** Similarly, human cartilage grown in presence of HGA develops a visible ochronotic pigmentation. **(D)** The ochronotic pigment is also present intracellularly in human articular cells grown in the presence of HGA. AKU: Alkaptonuria; BQA: Benzoquinone acetic acid; HGA: Homogentisic acid.

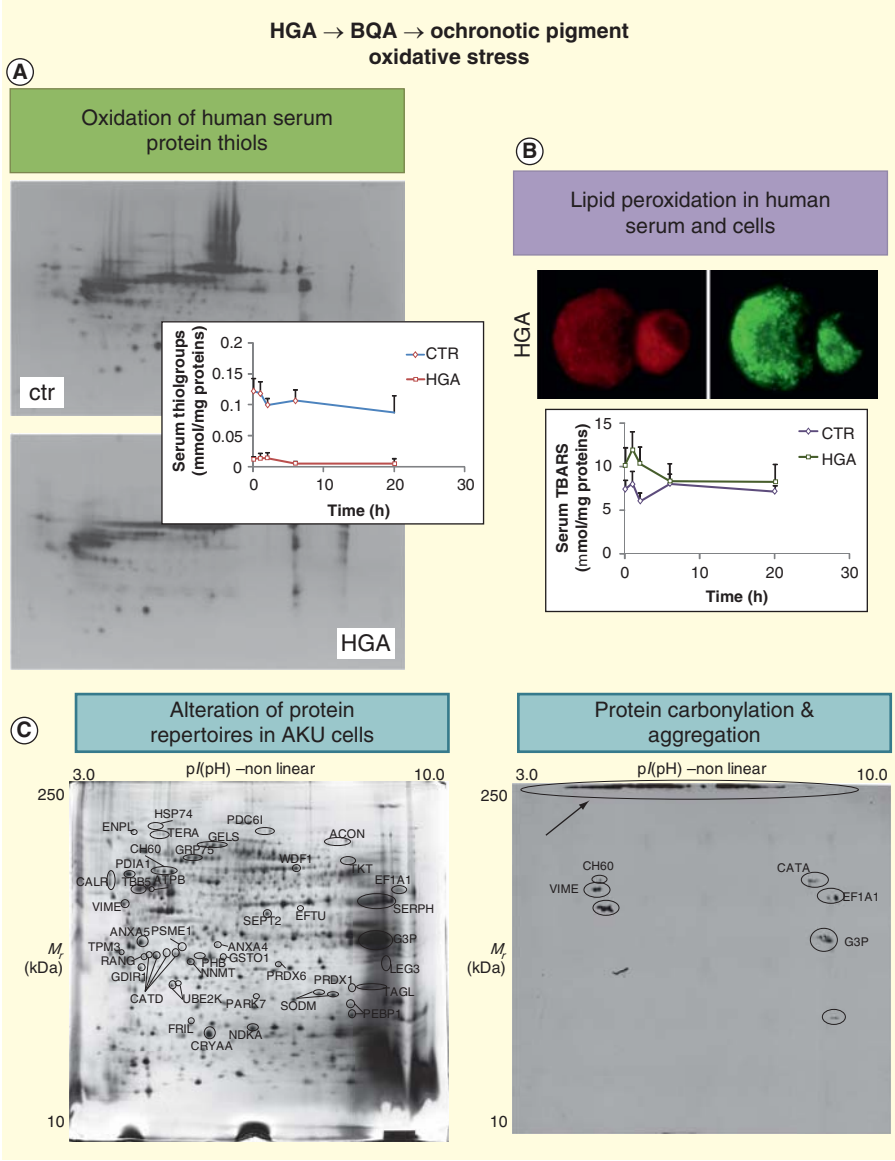

Figure 4. Oxidative stress in alkaptonuria. Several lines of evidence suggest that excess HGA can induce oxidative stress, which might be mediated by thiol depletion and oxidation of protein thiols, lipid peroxidation, and alterations at the protein level in terms of protein carbonylation and aggregation. BQA, the oxidative metabolite of HGA, and the ochronotic pigments can both promote and further propagate such an oxidative stress. AKU: Alkaptonuria; BQA: Benzoquinone acetic acid; HGA: Homogentisic acid.

when ASC and HGA were administered together confirmed the existence of an HGA-induced oxidative imbalance and an altered protein folding, which may help explaining the degeneration of articular tissues in AKU patients. In HGA/ASC-treated chondrocytes, the sub-repertoires of protein irreversibly oxidized to carbonyls (Table 1) confirmed a pro-oxidant action of ASC and HGA, their shared 'oxidative signature' and their ability to induce aggregation of oxidized proteins [30,33].

AKU cells

A biochemical, proteomic and redox-proteomic characterization of chondrocytes [60], osteoblasts [78] and synoviocytes (authors' unpublished data) isolated from biopsies of AKU patients was also undertaken. Proteomics and redox-proteomics of such *ex vivo* AKU cells validated the results previously observed in *in vitro* cell-based models, showing that AKU cells, besides an enhanced inflammation, experience dramatic alterations of proteins responsible of cell organization, protein folding and cell defense, especially those involved in adequate response to oxidative insults (Figure 4C). Several of these proteins were also found to be oxidized to carbonyls (Table 1). Noticeably, also in AKU cells, highly oxidized protein aggregates were found (Figure 4C), suggesting that this phenomenon could pose the basis, *in vivo*, for the production and deposition of the ochronotic pigment. Altogether, the picture seems to indicate profound alterations of chondrocytes structure and functions that might ultimately lead to 'a low quality' cartilage with an impaired ability to withstand loading forces.

Amyloidosis & oxidative stress in alkaptonuria

It has been suggested that HGA cannot be the sole causal factor for both intra- and extra-cellular ochronotic pigment deposition, and the potential role/presence of other unidentified proteins has been hypothesized [79]. Recent evidence has been provided on the presence of serum amyloid A (SAA) and serum amyloid P (SAP) in AKU *in vitro* and *ex vivo* models, which allowed to highlight the amyloid nature of the ochronotic pigment and to classify AKU as a secondary amyloidosis [53]. Amyloidoses are progressive diseases characterized by a lag phase, similarly to what happens in AKU whose nature is also progressive and whose symptoms are similar to those of other joint diseases where secondary amyloidosis is ascertained (rheumatoid arthritis, ankylosing spondylitis, familial Mediterranean fever) [80]. In AKU AA-amyloidosis, the clinical features might be secondary to the deposition of ochronotic pigment in connective tissues. In AKU patients presenting high SAA plasma levels, massive amyloid deposition may be related to the HGA-induced oxidative stress, suggesting that HGA itself may be involved in amyloid deposition. Remarkably, amyloid in AKU specimens co-localized with the ochronotic pigment and histological and biochemical analyses proved the intimate connection between oxidative stress and amyloid deposition in AKU, paving the way for a vicious cycle promoting chronic

inflammation (Figure 5). It has been shown that HGA produces melanin [29] and that melanin enhances inflammation. Consequently, in AKU the chronic accumulation of HGA and its oxidative derivative BQA, probably cause a variety of reactions promoting inflammation and mediating tissue damage. If, on the one hand, the production of the ochronotic pigment may represent a defense mechanism to counteract oxidative stress, on the other, it can further promote inflammation and eventually aberrant production of proteins involved in amyloidogenesis (Figure 5). This may in turn induce the production of melanin as a reaction of cells to counteract oxidative stress. The chronic presence of melanin may cause a further inflammatory stimulus resulting in overproduction of SAA and SAP finally causing the formation of amyloid (Figure 5).

The coexistence of chronic inflammation and oxidative stress in AKU is also supported by the positive correlation found between ochronotic pigmentation and staining for 4-hydroxynonenal (4-HNE), a known marker of lipoperoxidation, in AKU specimens from different tissues [Authors' Unpublished Data]. The link between oxidative stress, lipoperoxidation and amyloidosis is clear [81] and 4-HNE has been acknowledged as one of the most reactive lipid-derived molecules [82]. In addition, 4-HNE has been already associated to amyloid deposits in primary and secondary amyloidosis [83] as well as in Alzheimer's disease [84]. At the molecular level, furthermore, it has been observed that AKU cells have low levels of cathepsin D [53,60], a protein with major roles in completing SAA catabolism, preventing SAA from accumulating and serving as a precursor of AA amyloid fibrils [85].

Expert commentary

According to the 'oxidative stress theory' by Sies [73,86] aging is associated to decreased antioxidant defenses and increased ROS production, allowing oxidatively damaged macromolecules to accumulate. Age-related increase in protein carbonylation as well as in 4-HNE-protein adducts following lipid peroxidation have been well documented [87]. In recent years, due to the acknowledgments of the physiological functions exerted by ROS, their role in aging has been reviewed as well, and now there is mounting evidence that oxidative stress should be rather defined on the basis of a pro-oxidizing shift in the thiol-redox state and the consequent dysfunction of redox-sensitive proteins, explained with the 'redox stress hypothesis' [88].

The age-related decline in plasma glutathione (GSH) and low molecular weight thiols, concomitant with an increase in their oxidized forms, is well documented [89] and mounting evidence has indicated how perturbations in the thiol/disulfide homeostasis and an oxidative shift in the thiol/disulfide redox potential in plasma are associated not only with aging but also with a range of diseases [75]. Moreover, at the intracellular level, where GSH represents the most abundant non-protein thiol compound with a variety of direct and indirect functions in protecting from oxidative stress, it has been recently demonstrated that even mild redox imbalance of the GSH/GSSG ratio, harmless for most cell types, may be toxic for others [90].

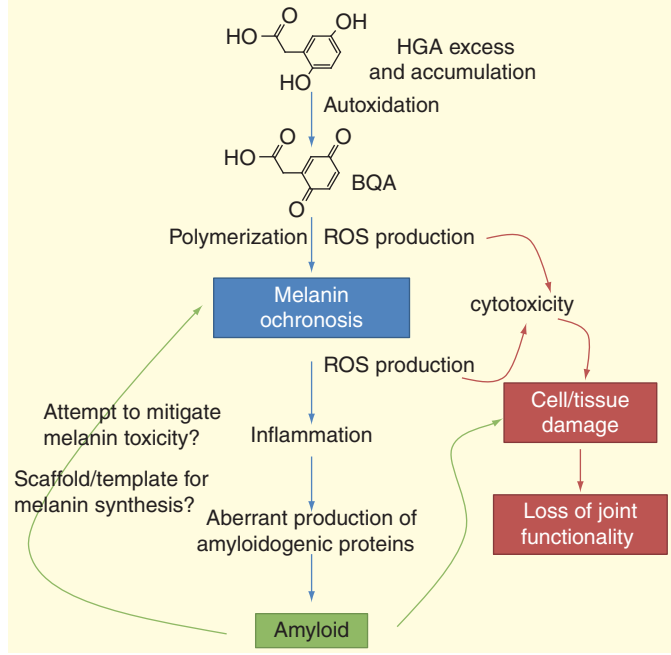

Figure 5. Schematic representation of ochronotic pigment and amyloid formation in alkaptonuria. *HGD* gene mutations cause HGA chronic accumulation and its auto-oxidation produces BQA, thus inducing melanin production. ROS are generated both during and after the occurrence of ochronosis. Such a repetitive oxidative insult is cytotoxic and inflammatory and causes cartilage degeneration and joint functions impairment. On the other hand, a chronic inflammatory status, in the absence of adequate defense responses against oxidative stress, induces aberrant production of amyloidogenic proteins finally resulting in secondary amyloid deposition. Amyloids and their precursors are also cytotoxic. The production of amyloid may also be an endeavor to mitigate melanin's cytotoxicity and/or function as a scaffold or template for its synthesis, analogously to what occurs physiologically in melanogenesis.
AKU: Alkaptonuria; BQA: Benzoquinone acetic acid; HGA: Homogentisic acid; ROS: Reactive oxygen species.

Thanks to the human serum AKU model, it has been shown that HGA addition is associated to a quick and massive depletion of plasma free thiols; thus, such an imbalance should be hypothesized to occur throughout whole AKU patients' life. Although AKU ochronosis manifests around the third-fourth decade of life, it should be imagined indeed as a repeated and constant oxidative damage sustained by circulating excess HGA spontaneously converting to BQA with ROS production, reacting with proteins and further propagating the oxidative insult. Such an insult might have more serious consequences with aging of patients, when diminished antioxidants are conceivably no more sufficient to counteract the HGA-induced stress, but it might also be mediated by the ochronotic pigment itself, similarly to what happens with melanin pigments [55]. More importantly, it has been shown that HGA might induce protein carbonylation and aggregation of carbonylated proteins, but especially how HGA can massively oxidize protein thiols, which is likely to negatively alter the redox balance. This

Table 1. Oxidatively modified proteins in human alkaptonuria models.

Entry	Protein name	Oxidation (carbonyls)	Oxidation (thiols)	Involved in redox-balance	Involved in amyloidogenesis
P10809	60 kDa heat shock protein, mitochondrial	Yes [60]		Accumulates in response to oxidative stress [97]	
P60709	Actin	Yes [30]			
P01009	α-1-antitrypsin	Yes [61]			
P04217	α-1B-glycoprotein	Yes [61]			
P08697	α-2-antiplasmin	Yes [61]			
P02765	α-2-HS-glycoprotein		Yes [61]		
P01023	α-2-macroglobulin		Yes [61]		
P07355	Annexin 2	Yes [30]			
P02647	Apolipoprotein A-I	Yes [61]	Yes [61]	May provide potent protection inhibiting the generation of pro-inflammatory oxidized lipids due to oxidative damage by free radicals [98]	Amyloidosis 8 (AMYL8) [MIM:105200] [99–101]
P27797	Calreticulin	Yes [30]			
P04040	Catalase	Yes [30,60]		Protects cells from the toxic effects of H_2O_2	
P07339	Cathepsin D				Altered levels in AKU chondrocytes [53,60] Acid protease active in intracellular protein breakdown. Involved in the pathogenesis of several diseases such as Alzheimer disease and AA amyloidoses
P00450	Ceruloplasmin	Yes [61]	Yes [61]	Copper-binding plasma glycoprotein thought to be involved in oxidative modification of lipoproteins, it plays a role in iron homeostasis having ferroxidase activity without releasing radical oxygen species. May also play a role in pulmonary antioxidant defense. The role of ceruloplasmin in regulating oxidative stress and iron homoeostasis makes it a strong candidate for regulation by alteration of cellular redox balance [102]	
P10909	Clusterin	Yes [61]	Yes [61]		Inhibits formation of amyloid fibrils (*in vitro*) [103–105]
P09871	Complement C1s subcomponent		Yes [61]		
P68104	Elongation factor 1-α 1	Yes [30,60]		May mediate accelerated synthesis of free thiol-containing proteins in response to oxidative stress [106]	

The presence of protein carbonyls and/or oxidized protein thiols is indicated together with the role of proteins in redox balance or their involvement in amyloidogenic processes or amyloid diseases.
AKU: Alkaptonuria; Hb: Hemoglobin; PARK7: Parkinson disease 7.

Table 1. Oxidatively modified proteins in human alkaptonuria models (cont.).

Entry	Protein name	Oxidation (carbonyls)	Oxidation (thiols)	Involved in redox-balance	Involved in amyloidogenesis
P14625	Endoplasmin (or 94 kDa glucose-regulated protein or Heat shock protein 90 kDa β member 1)				Altered levels in AKU chondrocytes [53,60]
P02679	Fibrinogen-γ chain		Yes [61]		
P06396	Gelsolin				Altered levels in AKU chondrocytes [53,60] Amyloidosis 5 (AMYL5) [MIM:105120]
P04406	Glyceraldehyde-3-phosphate dehydrogenase	Yes [30,60]		Redox-sensitive protein, undergoes oxidative stress-induced aggregation [107] and translocation into the nucleus [108]; it functions as NO sensor [109]	Oxidative stresses induce amyloid-like aggregation of glyceraldehyde-3-phosphate dehydrogenase *via* aberrant disulfide bonds of the active site cysteine, and the formation of such abnormal aggregates promotes cell death [107]
P00738	Haptoglobin	Yes [61]		Has antioxidant activity; a major function is to bind Hb to form a stable complex and thereby prevent Hb-induced oxidative tissue damage [110]	
P34932	Heat shock 70 kDa protein 4				Altered levels in AKU chondrocytes [53,60] Chaperone that attenuates protein aggregation and toxicity, has anti-apoptotic effects [111]
P11142	Heat shock cognate 71 kDa protein	Yes [30]		Contains redox-sensitive thiols [112]	
P02790	Hemopexin	Yes [61]		It is the major vehicle for the transportation of heme in the plasma, thus preventing heme-mediated oxidative stress and heme-bound iron loss [113]	
P99002	Immunoglobulin heavy chain α	Yes [61]			
P02750	Leucine-rich α-2-glycoprotein		Yes [61]		
P02760	Protein AMBP		Yes [61]		
P07237	Protein disulfide-isomerase	Yes [30]		Multifunctional protein catalyzing the formation, breakage and rearrangement of disulfide bonds; it regulates cell redox homeostasis [114]. Contains two thioredoxin domains	Altered levels in AKU chondrocytes [53,60] Prevents the neurotoxicity associated with ER stress and misfolding, protects against protein aggregation [115]

The presence of protein carbonyls and/or oxidized protein thiols is indicated together with the role of proteins in redox balance or their involvement in amyloidogenic processes or amyloid diseases.
AKU: Alkaptonuria; Hb: Hemoglobin; PARK7: Parkinson disease 7.

Table 1. Oxidatively modified proteins in human alkaptonuria models (cont.).

Entry	Protein name	Oxidation (carbonyls)	Oxidation (thiols)	Involved in redox-balance	Involved in amyloidogenesis
Q99497	Protein DJ-1	Yes [30]		Redox-sensitive chaperone and sensor for oxidative stress; eliminates H_2O_2 and protects cells against H_2O_2-induced cell death. May act as an atypical peroxiredoxin-like peroxidase that scavenges H_2O_2. Following removal of a C-terminal peptide, displays enhanced cytoprotective action against oxidative stress-induced apoptosis [116–119]	Altered levels in AKU chondrocytes [53,60] Parkinson disease 7 PARK7 [MIM:606324] Prevents aggregation of α-synuclein
P02787	Serotransferrin	Yes [61]		Exported iron is scavenged by transferrin, which maintains Fe^{3+} in a redox-inert state and delivers it into tissues preventing oxidative damage [120]	
P50454	Serpin H1	Yes [30]			
P02768	Serum albumin	yes [61]		Has antioxidant functions [121]	
P38646	Stress-70 protein, mitochondrial (75 kDa glucose-regulated protein or Heat shock 70 kDa protein 9 or Mortalin)	Yes [30]			Altered levels in AKU chondrocytes [53,60] Chaperone with anti-apoptotic functions, protects from amyloid-induced toxicity [122]
Q01995	Transgelin				Altered levels in AKU chondrocytes [53,60,123]
P02766	Transthyretin		Yes [61]		Amyloidosis, transthyretin-related (AMYL-TTR) [MIM:105210]
P08670	Vimentin	Yes [60]			

The presence of protein carbonyls and/or oxidized protein thiols is indicated together with the role of proteins in redox balance or their involvement in amyloidogenic processes or amyloid diseases.
AKU: Alkaptonuria; Hb: Hemoglobin; PARK7: Parkinson disease 7.

reasoning implies that with aging, when antioxidant defenses decrease, AKU patients will likely be no more able to properly counteract the HGA-induced oxidative stress, so that more severe clinical symptoms will appear. Similarly, an enhanced accumulation of lipid peroxidation products able to exert their damaging actions on proteins can be hypothesized. Furthermore, it should always borne in mind that individuals are continuously exposed to a range of oxidant stressors that can have additive or even synergistic effects [91]; in such a scenario, the HGA-induced oxidative stress in AKU sufferers could be further emphasized. The central inflammatory feature of AKU ochronotic arthritis, together with the role of inflammatory cytokines in cartilage and bone metabolism, suggests the following conceptual framework: inflammation, oxidative stress and amyloid deposition share common signaling-pathway components that meet at cross-roads in articular tissue microenvironment. Overlapping clinical factors associated with pigment deposition, amyloid formation and tissue degeneration in AKU provide further support for a shared disease process.

Several molecular targets of the oxidative insults generated by HGA and possibly BQA were identified (Table 1), and molecular mechanisms for the *in vivo* generation of ochronotic pigment can be hypothesized. Of clear relevance for alkaptonuric patients, experiencing oxidative stress throughout their whole life, were also the findings of ASC as an enhancer of HGA-induced oxidative stress [29,30], that underlined the need to explore new antioxidant therapies [92]. The efficacy of human AKU models in the preliminary evaluation of the efficacy of antioxidant compounds in counteracting the oxidative effects, induced by excess HGA, were successfully highlighted, thus establishing new basis to set up adequate pharmacological strategies in AKU.

Five-year view

Basic science is fundamental in piecing together the puzzle of rare diseases such as AKU and in assessing a drug for their treatment. As a spur to such a daunting task, we should always bear in mind that research on rare diseases has often yielded a deal of information in many related and unrelated areas to an extent that is completely out of proportion considering the number of cases [93]. Rare diseases are indeed gaining credibility as contributors to common diseases (in the case of AKU, osteoarthritis and rheumatoid arthritis can be suggested), adding knowledge and in parallel increasing the number of beneficiaries of the research [94].

It has already been postulated that sometimes changing levels of a modified protein might represent better biomarkers than changes in the protein's expression itself [65]. Here we have shown how proteomics and redox proteomics, highlighting which proteins/pathways are negatively affected by oxidative stress in AKU, can help the identification of protein biomarkers and the choice of proper pharmacological interventions for AKU and eventually pave the way for the design of new drugs, as recently demonstrated for a range of human diseases [95].

The identification of measurable biomarkers in rare diseases is obviously hindered by the low number of cases (and consequently samples to be analyzed), so negatively affecting the evaluation of their sensitivity, specificity and predicting values. Nevertheless, the identification of pharmacological treatments and an adequate follow-up of patients would absolutely need prognostic and diagnostic biomarkers also in this field. This was affirmed recently within an international effort formed jointly by the US NIH and the European Commission (EC), known as 'International Rare Disease Research Consortium' (IRDiRC), whose ambitious aim was to develop new tools for the diagnosis and pharmacological treatments of all the known rare diseases by the year 2020 [96]. With this aim, we have shown how proteomics and redox-proteomics might successfully overcome the difficulties of studying a rare disease such as AKU and the limitations of the hitherto adopted approaches.

Financial & competing interests disclosure

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Key issues

- Physio-pathology of alkaptonuria is still obscure.
- No genotype-phenotype relationship apparently exists in alkaptonuria, making post-genomics mandatory.
- Oxidative stress plays a fundamental role in physiopathology of ochronosis and alkaptonuria-related amyloidosis.
- Human alkaptonuria serum-, cell- and tissue-based models have been set up.
- Proteomics and redox-proteomics of alkaptonuria cells and serum models revealed strong homogentisic acid (HGA)-induced protein oxidation.
- Carbonylation, thiol oxidation and benzoquinone acetate-binding are the main HGA-induced protein modifications observed.
- HGA-induced structural/functional modifications are mainly directed toward proteins with a role in folding, metal homeostasis, response to stress (mainly oxidative) or functioning as carriers; some of them are involved in amyloidogenic processes.
- These proteins may be considered molecular hallmarks of alkaptonuria and may provide the basis of an 'oxidative-stress signature' of the disease.

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