Alkaptonuria is a novel human secondary amyloidogenic disease

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Abstract

Alkaptonuria (AKU) is an ultra-rare disease developed from the lack of homogentisic acid oxidase activity, causing homogentisic acid (HGA) accumulation that produces a HGA-melanin ochronotic pigment, of unknown composition. There is no therapy for AKU. Our aim was to verify if AKU implied a secondary amyloidosis. Congo Red, Thioflavin-T staining and TEM were performed to assess amyloid presence in AKU specimens (cartilage, synovia, periumbilical fat, salivary gland) and in HGA-treated human chondrocytes and cartilage. SAA and SAP deposition was examined using immunofluorescence and their levels were evaluated in the patients’ plasma by ELISA. 2D electrophoresis was undertaken in AKU cells to evaluate the levels of proteins involved in amyloidogenesis. AKU osteoarticular tissues contained SAA-amyloid in 7/7 patients. Ochronotic pigment and amyloid co-localized in AKU osteoarticular tissues. SAA and SAP composition of the deposits assessed secondary type of amyloidosis. High levels of SAA and SAP were found in AKU patients’ plasma. Systemic amyloidosis was assessed by Congo Red staining of patients’ abdominal fat and salivary gland. AKU is the second pathology after Parkinson’s disease where amyloid is associated with a form of melanin. Aberrant expression of proteins involved in amyloidogenesis has been found in AKU cells. Our findings on alkaptonuria as a novel type II AA amyloidosis open new important perspectives for its therapy, since methotrexate treatment proved to significantly reduce in vitro HGA-induced A-amyloid aggregates.

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1 Introduction

Alkaptonuria (AKU; MIM no. 203500) is a rare disease (1:250,000–1,000,000 incidence) resulting from a deficiency of the enzyme homogentisate1,2-dioxygenase (HGO) that splits the aromatic ring of homogentisic acid (HGA, 2,5-dihydroxyphenylacetic acid), an intermediary product of tyrosine and phenylalanine catabolism in the liver [1]. This leads to the accumulation of HGA that cannot be further metabolized. A portion of HGA is excreted daily in the urine where it imparts a characteristic black discoloration upon oxidation. As in tissues, HGA oxidizes to benzoquinone acetic acid (BQA), which in turn forms HGA-melanin-based polymers [2], deposited in the connective tissue, most commonly the joints, cardiovascular system, kidney and skin [3], causing a pigmentation known as “ochronosis”. Polymer deposition in cartilage leads to degeneration, chronic inflammation and osteoarthritis. Musculoskeletal involvement is the most serious complication, leading to a severe and sometimes crippling form of arthropathy, which is the most common clinical presentation of AKU and often mimics ankylosing spondylitis [4]. AKU patients sometimes suffer from cardiovascular disease (frequent cause of death [5]) and kidney disease [6].

Although AKU pathological features are clinically described, its molecular basis has not been explored to any significant degree, because of the lack of suitable models to study the disease. We introduced novel human ochronotic cell, tissue and serum models and undertook pre-clinical testing of potential antioxidant therapies for AKU [1,7–11]. These models contributed to understanding HGA effects on cell viability [9,12], cell protein expression [9,10,12] and joint destruction in AKU [2]. Both intra- and extra-cellular pigmented deposition indicates that HGA cannot be the sole factor causing it and suggests the potential role/presence of other unidentified proteins [13].

There is no effective cure for AKU at the moment. Treatment is symptomatic, although this is recommended for early-stage of the disease while for the end-stage, total joint replacement is required.
Secondary amyloidosis (AA) is a serious complication of chronic inflammatory conditions such as rheumatoid arthritis (RA) and its amyloid deposition process involves a cleaved product of the acute-phase protein serum amyloid A (SAA) [14]. AA amyloidosis occurs in patients with poorly controlled chronic inflammatory disease, mainly RA, ankylosing spondylitis, and familial Mediterranean fever.

In the present paper, we provided experimental evidence that AKU osteoarticular tissue contains AA-amyloid deposits. This is the first report, to the best of our knowledge, of secondary amyloidosis associated with AKU. This opens new perspectives for AKU therapy and we also showed that methotrexate was able to significantly prevent in vitro HGA-induced A-amyloid aggregates.

2. Materials and methods

The whole study was conducted following the approval of the local University Hospital Ethics Committee. All patients gave a written informed consent prior to inclusion in the study.

All reagents were from Sigma-Aldrich (St. Louis, MO), if not differently specified.

2.1. AKU samples

Alkaptonuric specimens were obtained from seven AKU patients (Table 1). Healthy human articular cartilage was obtained from patients without any history of rheumatic diseases, who underwent surgical knee joint sampling. Tissue was removed only from healthy, glossy and completely intact articular cartilage surface.

2.2. AKU cell and tissue models

We previously developed original cell and organotypic ex vivo AKU models based on human chondrocytes or articular cartilage treated with 0.33 mM HGA up to the development of ochronosis, as described [10–12].

2.3. Congo Red (CR) staining


2.4. Thioflavin T (Th-T) staining

Samples incubated in 1% Th-T [17,18] were mounted and observed under a fluorescence microscope (excitation 450 nm, emission 482 nm).

2.5. Fluorescence microscopy

Synovia and cartilage samples in paraffin were cut in 3–5 μm slices and used for double immunofluorescence staining with anti-SAA and anti-serum amyloid P (SAP) antibodies (Santa Cruz Biotechnology, CA). Additional immunofluorescence assays were performed using anti-immunoglobulin light chain, anti-pre-albumin, anti-α-synuclein, anti-beta-2 microglobulin and anti-Pmel17 antibodies (all by Santa Cruz Biotechnology, CA). Intrinsic HGA-melanin fluorescence (excitation 633 nm and emission between 650 and 742 nm) was observed under a Rhodamine 123 filter.

2.6. Biochemical assays

Plasma SAA and SAP in AKU patients were measured by ELISA (Invitrogen-Life Technologies, Carlsbad, CA).

2.7. Statistical analysis

Student’s t-test was used when appropriate. Two-tailed analysis with P value lower than 0.05 was considered significant. Correlation analysis was performed using Pearson’s correlation.

2.8. Transmission electron microscopy (TEM)

AKU cartilage was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB) pH 7.2 for 3 h at 4 °C. After rinsing in CB, samples were post-fixed in 1% osmium tetroxide in CB for 2 h at 4 °C, dehydrated in a graded series of ethanol and embedded in a mixture of Epon–Araldite resins. Thin sections, obtained with a Reichert ultramicrotome, were stained with uranyl acetate and lead citrate and observed with a TEM (FeiTecnai G2 spirit at 80 K).

<table>
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<th>Knee</th>
<th>Asc</th>
<th>No</th>
<th>Asc</th>
<th>No</th>
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</thead>
<tbody>
<tr>
<td>Location of amyloid</td>
<td></td>
<td></td>
<td>Asc</td>
<td>N</td>
<td></td>
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| Table 1
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<td>F</td>
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<td>0/4</td>
<td>3/4</td>
<td>2/4</td>
<td>n.d.</td>
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<td>3/4</td>
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<td>n.d.</td>
<td>1/4</td>
</tr>
<tr>
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<td>3.43</td>
<td>134.99</td>
<td>99.36</td>
<td>117.70</td>
<td>87.14</td>
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<td>36.362</td>
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<td>46.615</td>
<td>47.966</td>
<td>25.434</td>
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<tr>
<td>SAE (mg/L)</td>
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<td>3.43</td>
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<td>3N,4N</td>
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</tr>
<tr>
<td>Location of amyloid</td>
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<td>Knee</td>
<td>Asc</td>
<td>No</td>
<td>Asc</td>
<td>No</td>
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</tr>
</tbody>
</table>

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2.9. Chondrocyte proteomic analysis

Cell cultures of AKU or healthy (control) chondrocytes were washed twice with sterile PBS and resuspended in a buffer containing 65 mM DTE, 65 mM CHAPS, 9 M urea, and 35 mM Tris-base. Cell disruption was achieved by sonication in an ice bath and protein content was assessed. A total of 50 μg of protein samples were submitted to 2D electrophoresis (2DE), as described [9]. Digitalized images were obtained by ImageScanner III (GE-Healthcare, Milan) and then qualitatively and quantitatively analyzed by the ImageMaster software (GE-Healthcare).

The increasing/decreasing index (fold change) was calculated as the ratio of spot relative volume between the different gel maps. Protein spot identification was obtained as described [9,19].

3. Results

3.1. Congo Red stained AKU cartilage, synovia and chondrocytes

CR staining under polarized light of AKU cartilage of elderly patients (58 to 69 years) showed green birefringence as well as ochronotic cartilage fragments (shards) while control healthy cartilage did not. The size and the prevalence of cartilaginous amyloid in AKU patients seemed to be related to disease progress. We observed interconnected amyloid deposits in AKU synovial tissues and ochronotic cartilage shards embedded in severely degraded synovium [Fig. 1A(H,L)]. Amyloid deposits appeared along the surface and more deeply [Fig. 1A(H,L)]. CR birefringence was superimposing the ochronotic shards [Fig. 1A, compare G with H and I with L]. CR-positive amyloid deposits were revealed in AKU chondrocytes isolated from patients [Fig. 1B].

3.2. Congo Red stained cell and cartilage AKU models

Using our AKU models [10–12] we confirmed CR staining of chondrocytic and cartilage pigmented areas [Fig. 1C] and at the same time we proved that the amyloid presence was due to HGA, suggesting its potential role in the formation of amyloid structures in vivo. Similar staining of deposits was visible in AKU patients’ cartilage [Fig. 1A(C–F)], perfectly reproducing the ex vivo situation, since CR birefringence of AKU cartilage model exactly overlapped the pigmented areas [Fig. 1C].

3.3. Thioflavin T stained AKU cartilage and AKU synovia and amyloid co-localized with melanin-like deposits

To confirm the presence of amyloid aggregates in cartilage and synovial tissue from AKU patients we performed the Th-T assay. Th-T fluorescence was evident and perfectly superimposing the ochronotic shards in AKU tissues [Fig. 2A]. Ochronotic deposits are defined as melanin-like pigments and we wanted to ascertain if such structures could potentially co-localize with amyloid deposits in AKU cartilage and synovia. Th-T fluorescence overlapped HGA-melanin fluorescence and double exposure of phase contrast and fluorescence allowed the simultaneous localization of amyloid and ochronotic shard [Fig. 2B].

3.4. AKU is a SAA- and SAP-mediated secondary amyloidosis

SAA and SAP deposition in AKU cartilage and synovial specimens was examined using immunofluorescence techniques. Co-localization of SAA with SAP staining was detected in all of the examined tissues [Fig. 3A]. No positivity for the presence of immunoglobulin light chains, pre-albumin, α-synuclein, beta-2 microglobulin and Pmel17 was observed (Table 1). The patterns of immunofluorescent staining did not appear to differ between SAP and SAA, although this latter was highly present in the cartilage from any AKU patient, suggesting a strong production and release of SAA by AKU chondrocytes and consequently high SAA and SAP circulating levels. Interestingly, SAA and SAP distribution in amyloid of AKU cartilage perfectly superimposed with HGA-melanin localization [Fig. 3B]. Indeed, high plasma levels of both SAA and SAP were found in all AKU patients [Fig. 4A,B]. AKU synovial sections showed particularly intensive SAP positivity in correspondence of ochronotic shards [Fig. 3A], especially in patients with high SAA and SAP plasma levels (Table 1, Fig. 4A,B).

High plasma levels of both SAA and SAP were found in all AKU patients (Fig. 4A,B). Four patients were receiving oral antioxidant therapy, for at least 6 months before the time of study. Patient 2 had received an anti-inflammatory treatment. Three cases were untreated. The SAA level of AKU patients (Fig. 4, middle panel) ranged 3.43–134.69 mg/L with a mean of 86.48 ± 13.04 mg/L, while those of the control group ranged 4.23–8.92 mg/L with a mean of 6.36 ± 2.82 mg/L; the difference was statistically significant (P = 0.001). SAA levels in patients who had not received any treatment (mean = 116.61 ± 20.44 mg/L) resulted significantly higher than controls (P = 0.043), Correlation between SAA level and some of the disease parameters revealed statistically significant positive correlation for the age (r = 0.362, P = 0.02) and disease duration (r = 0.698, P = 0.0001). This significant correlation indicated that age and disease severity in AKU may be associated with raised SAA levels, thus reflecting also the progressive nature of type AA amyloidosis. Mean serum SAP in AKU patients was 43.06 ± 4.40 mg/L, which was statistically different (P = 0.001) from the values in 4 healthy controls (10 ± 3.5 μg/L). All AKU patients showed high SAP plasma levels, apparently not influencing disease severity (Fig. 4B). In the control population serum SAP was not related to age.

3.5. Congo Red stained periumbilical fat and salivary gland AKU specimens

Confirmation of systemic amyloidosis in AKU patients was obtained by CR staining of AKU abdominal fat aspiration and labial salivary gland biopsies (Fig. 4C), that has been proven as highly sensitive and reliable method for diagnosis of secondary amyloidosis [20,21]. Minor (labial) salivary gland and subcutaneous abdominal fat tissues from AKU patients showed amyloid presence in all samples examined (Fig. 4C).

3.6. Methotrexate (MTX) was able to prevent amyloid and to decrease pro-inflammatory cytokine release in an in vitro AKU chondrocytic model

Our in vitro AKU model allowed a semi-quantitative analysis of the production of amyloid due to HGA addition and its reduction (−97.2%) due to a treatment with 10−9 M MTX (Fig. 5A), a concentration in the range of that administered to RA patients to keep low SAA plasma levels and thus control/reverse secondary amyloidosis [22]. HGA-treated chondrocytes released high levels of pro-inflammatory cytokines and MTX treatment proved to be able to decrease them or even restore control levels (Fig. 5B).

3.7. TEM observation of amyloid deposits in AKU cartilage

The darkness of AKU cartilage is the feature that differentiates ochronotic articular cartilage from other forms of arthritis. To better investigate the ultrastructure of amyloid deposits in AKU tissue, we performed an electron microscopical study of AKU cartilage samples. Using our AKU model allowed a semi-quantitative analysis of the production of amyloid due to HGA addition and its reduction (−97.2%) due to a treatment with 10−9 M MTX (Fig. 5A), a concentration in the range of that administered to RA patients to keep low SAA plasma levels and thus control/reverse secondary amyloidosis [22]. HGA-treated chondrocytes released high levels of pro-inflammatory cytokines and MTX treatment proved to be able to decrease them or even restore control levels (Fig. 5B).

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fibris that appeared wavy and sometimes fragmented with loss of periodicity is also visible when amyloid fibris merge with the collagen fibris that were always mixed with the dispersed pigment [Fig. 6(A,D)].

3.8. Proteomic analysis of AKU chondrocytes

Proteomic analysis of chondrocytes from AKU patients revealed the abnormal expression of proteins involved in amyloidogenic...
A relationship between proteolytic cleavage of gelsolin and increased important role in amyloidogenesis and inhibits amyloid-namics and mitochondrial function in neurons [28]. Gelsolin plays an Alzheimer’s disease and its overexpression signi-
is induced following processing of the amyloid precursor protein in high molecular weight protein aggregates [9]. Transgelin expression
and in cell and animal models [24,25], like also Protein DJ-I, known to prevent α-synuclein aggregation [26]. AlphaB-crystallin is also a novel mediator of chondrocyte matrix gene expression that may contribute to altered chondrocyte metabolism during OA development [27], but possibly also in AKU. AlphaB-crystallin had been previously found underexpressed in HGA-treated chondrocytes [9]. More generally, in our previous paper we found that HGA induced alteration of protein folding in human chondrocytes and caused production of high molecular weight protein aggregates [9]. Transgelin expression is induced following processing of the amyloid precursor protein in Alzheimer’s disease and its overexpression significantly alters actin dy-namics and mitochondrial function in neurons [28]. Gelsolin plays an important role in amyloidogenesis and inhibits amyloid-β fibrilization. A relationship between proteolytic cleavage of gelsolin and increased

### 4. Discussion

We present here original results showing that alkaptonuria is a novel secondary amyloidosis. All the conventionally adopted and uni-versally accepted methods (CR and Th-T staining, TEM) succeeded in unequivocally assessing the presence of amyloid in our tissue and cel-

![Fig. 2. Thioflavin T stained AKU cartilage and AKU synovia and amyloid co-localized with melanin-like deposits. A) Th-T fluorescence of AKU synovial and cartilage specimens shown by confocal microscopy. Melanin fluorescence was revealed under a Rhodamine 123 filter. Cartilage was from AKU Patient 7, synovia was from AKU Patient 3. Analogous results were obtained from specimens of other patients. Bar: 30 μm; B) co-localization of melanin and amyloid was revealed by merge of Th-T and melanin fluorescence. DIC: differential interference contrast. Bar: 22 μm. Representative images from a triplicate set are shown.](image)

![Fig. 3. SAA and SAP were present in amyloid deposits of AKU cartilage and synovia and both co-localized with melanin. A) SAA and SAP deposition in AKU cartilage and syno-

vial specimens was detected by dual immunofluorescence technique. AKU cartilage and synovia showed high levels of SAA deposit superimposing SAP deposits. Positive staining for SAA and SAP was particularly intense in correspondence of ochronotic shards. Bar: 75 μm; B) AKU cartilage sections were dual-stained using antibodies specific for SAA SAP and compared to melanin fluorescence, resulting in a perfect co-localization of amyloid deposits and pigmented areas. Cartilage specimen was from Patient 6 and synovia specimen was from Patient 1. DIC: differential interference contrast. Bar: 150 μm. Representative images from a triplicate set are shown.](image)
expressing HGO may be affected in this disease: joints [36], heart [5], kidney [37], liver [38], eyes [4], marrow [39], bladder [40], and lung [41]. It is necessary to correctly define three forms of melanin (two of them are natural): i) DOPA-melanin or eumelanin synthesized in melanosomes of the melanocytes of the skin and in melanosomes of retinal-pigment epithelium, and ii) neuromelanin (NM) or DOPAmine-melanin synthesized in DOPAminergic neurons during all life long (NM accumulates linearly in nervous system during normal aging). A third type is the abnormal melanin, HGA-melanin found only in AKU.

Fig. 4. A, B) High SAA and SAP plasma levels in AKU patients. SAA (A) and SAP (B) plasma levels related with age and disease severity, thus reflecting also the progressive nature of AA amyloidosis. Experiments were performed in triplicate; data are presented as average values ± standard deviation. C) Congo Red staining of AKU subcutaneous periumbical fat and AKU salivary gland tissues. Examples of CR fat smears and labial salivary gland biopsy of AKU Patients 3 and 5, under normal and in polarized light. CR staining confirmed the presence of amyloid deposition. Magnification 20×, a,b 10×. Representative images from a triplicate set are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Remarkably, we found that alkaptonuric amyloid co-localized with HGA-melanin ochronotic pigment. Our unprecedented findings are the first case, to our knowledge, in which ochronotic pigment is directly associated with amyloid. This evidence suggests that HGA polymer may be involved in amyloid deposition. The association of AKU and amyloidosis is in keeping with evidence that synoviocytes and chondrocytes may be important producers of amyloid in RA [14].

Reactive systemic AA amyloidosis is one of the most severe complications of several chronic rheumatic disorders [42]. Problems associated with these pathologies may present joint symptoms similar to AKU (stiffness, swelling, and movement limitation) due to the deposition of amyloid in the synovial membranes of the joint or of the tendon sheaths. In AKU AA-amyloidosis, the clinical features could be secondary to the deposition of ochronotic pigment in connective tissues. Amyloidoses are progressive diseases, with a lag period before the appearance of AA amyloidogenesis [42], congruent with the progressive nature of AKU whose symptoms are analogous to other joint diseases with ascertained secondary amyloidosis (RA, ankylosing spondylitis, familial Mediterranean fever). A case of acute anterior uveitis as the initial presentation of AKU mimicking ankylosing spondylitis has been recently reported [4]. Both uveitis and ankylosing spondylitis are SAA secondary amyloidoses.

Alkaptonuric arthritis resembles osteoarthritis (OA), but clinically is more like RA and in most patients with AKU there are frequent periods of acute inflammation as in RA. RA chondrocytes serve as a source of intra-articular SAA, suggesting an active role in RA pathogenesis [14]. Compared to RA, secondary amyloidosis is a new complication of AKU. We detected AA-amyloid in longstanding AKU patients, confirming amyloidosis to be a progressive disease.

Since SAA plasma levels do not correlate with age while SAA serum concentration provides prognostic information [43], the different SAA levels found in our AKU patients could help to grade AKU severity whose scoring system [44,45] is so far not established at the molecular level. The striking co-localization of HGA-melanin and amyloid suggests the participation of fluorescent oxidized HGA pigment in the formation of amyloid aggregates and a link between HGA oxidation and amyloid deposition. Melanin acts by trapping free radicals and its synthesis...
Table 2
Comparative proteomics of human AKU chondrocytes. Proteins whose synthesis was altered in AKU chondrocytes versus controls.

<table>
<thead>
<tr>
<th>Spot</th>
<th>AN*</th>
<th>Gene</th>
<th>Protein</th>
<th>Biological processes‡</th>
<th>AKU chondrocytes/ ctr*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP75</td>
<td>P38646</td>
<td>HSPA9</td>
<td>Stress-70 protein, heat shock 70 kDa protein</td>
<td>Implicated in the control of cell proliferation and cellular aging. May also act as a chaperone. Anti-apoptotic functions</td>
<td>−2.1</td>
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<tr>
<td>PARK7</td>
<td>Q99497</td>
<td>PARK7</td>
<td>Protein DJ-1</td>
<td>May function as a redox-sensitive chaperone and as a sensor for oxidative stress.</td>
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<tr>
<td>PDA1</td>
<td>P07237</td>
<td>P4HB</td>
<td>Protein disulfide-isomerase</td>
<td>Prevents aggregation of SNCA.</td>
<td>−2.3</td>
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<tr>
<td>GELS</td>
<td>P06396</td>
<td>GSN</td>
<td>Gelsolin</td>
<td>Catalyzes the formation, breakage and rearrangement of disulfide bonds. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, facilitates aggregation (anti-chaperone activity).</td>
<td>−2.0</td>
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<tr>
<td>TAGL</td>
<td>Q01995</td>
<td>TAGLN</td>
<td>Transgludin</td>
<td>Bind to actin and to fibronectin. Calmodulin-activated, actin-modulating protein that binds to the plus (or barbed) ends of actin monomers or filaments, preventing monomer exchange (end-blocking or capping). It can promote the assembly of monomers into filaments (nucleation) as well as sever filaments already formed. Defects in GSN are the cause of amyloidosis type 5 (AMYL5) [MIM: 105120], also known as familial amyloidosis Finnish type.</td>
<td>−17.0</td>
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<tr>
<td>ENPL</td>
<td>P14625</td>
<td>HSPI081</td>
<td>Endoplasmolin, 94 kDa glucose-regulated protein, GRP-94, Heat shock protein 90 kDa beta member 1</td>
<td>Molecular chaperone that functions in the processing and transport of secreted proteins. Functions in endoplasmic reticulum associated degradation (ERAD). Has ATPase activity. Plays a role in protein folding and transport, has anti-apoptotic functions.</td>
<td>−4.8</td>
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<tr>
<td>HSP74</td>
<td>P34932</td>
<td>HSPA4</td>
<td>Heat shock 70 kDa protein 4</td>
<td>Stress response, plays a role in the unfolded protein response.</td>
<td>−5.4</td>
</tr>
<tr>
<td>CATD</td>
<td>P07339</td>
<td>CTSD</td>
<td>Cathepsin D</td>
<td>Acid protease active in intracellular protein breakdown. Involved in the pathogenesis of several diseases, AA amyloidosis included.</td>
<td>−3.8</td>
</tr>
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* AN: accession number.
‡ Protein biological processes retrieved by UniProt knowledgebase (http://www.uniprot.org/).
— Fold-change in protein % relative abundance (as average values in case of multiple spots); (+) over-expressed proteins, (−) under-expressed protein according to the ratio calculated between AKU and control (ctr) cells.

In our knowledge, the first case of amyloid associated with melanin outside the melanosomal compartment under pathological conditions. AKU is the second pathology after Parkinson’s disease (PD) where amyloid is associated with a melanin-based pigmentation and also a parallel has been drawn between A-beta and DOPA-melanin with respect to the relation of these molecules and Alzheimer’s disease (AD) and PD [48,50]. AD and PD are neurodegenerative diseases traditionally associated with amyloid fibrils, produced by β-amyloid and α-synuclein aggregation-prone proteins, respectively. The destruction of connective tissue by HGA is reminiscent of the neurotoxicity of 6-hydroxy dopamine (6-OHDA) [21]. Indeed, an association of PD and AKU has been reported [50]. Amyloid and melanin have different structures but share several common features with respect to synthesis, accumulation in aging, affinity for metals and roles in cell protection or toxicity, this latter mediated by inflammation by both types of molecules, and they can enter into a physiological or pathological process depending on the cell context [32,35]. In PD the colocalization of α-synuclein, the protein whose aggregation induces the formation of amyloid, and DOPA-melanin may facilitate the precipitation of α-synuclein and the consequent neuronal damage [49,50]. Analogously to PD, it is not clear if HGA-melanin is part of the toxic events that underlie AKU or a protective response that may slow the disease.

We suggest that, analogously to rheumatoid arthritis, AA is a secondary complication of AKU, due in this case, to a chronic inflammatory status derived from HGA-benzoquinone acetic acid (BQA)-melanin-induced oxidative stress.

5. Conclusions

Our findings on AKU as a novel AA amyloidosis open new perspectives for its treatment. In fact, AA tissue amyloid resolves following the cessation of inflammatory stimuli, the impetus that maintains high SAA plasma levels. This principle is supported by the excellent outcome of liver transplantation in patients affected by some forms of amyloidosis. For AKU amyloidosis, our present findings are supported by the completely successful reversal of ochronotic arthropathy following liver transplantation [41]. The control of the underlying inflammatory disorder can result in regression of the disease, as
proven for some secondary amyloidogenic musculoskeletal disorders sharing clinical features with AKU. Suppression of SAA below 10 mg/L halts the progression of the disease and is associated with prolonged survival, with reversal of amyloid deposition and with recovery of organ function [26]. Low doses of MTX are safe and effective for the routine treatment of inflammatory arthritis and it has been successfully adopted to keep low SAA levels in RA in order to prevent and regress secondary amyloidosis [18]. MTX proved to have an excellent efficacy to inhibit the production of amyloid in our AKU model chondrocytes, suggesting the introduction of its use in AKU therapy. This treatment would be useful especially for those symptomatic AKU patients for whom the therapy with nitisine (the only orphan drug so far recognized for alkaptonuria) failed in a clinical trial [51].

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