Biochemical and Proteomic Characterization of Alkaptonuric Chondrocytes

DANIELA BRACONI,1 GIULIA BERNARDINI,1 CLARETTA BIANCHINI,1 MARCELLA LASCHI,1 LIA MILLUCCI,1 LOREDANA AMATO,1 LAURA TINTI,2 TOMMASO SERCHI,1 FEDERICO CHELLINI,3 ADRIANO SPREAFICO,2 AND ANNALISA SANTUCCI1,2*

1Dipartimento di Biotecnologie, Università degli Studi di Siena, Siena, Italy
2Centro Interdipartimentale per lo Studio Biochimico delle Patologie Osteoarticolari, Università degli Studi di Siena, Siena, Italy
3Sezione di Reumatologia, Dipartimento di Medicina Clinica e Scienze Immunologiche, Università degli Studi di Siena, Policlinico Le Scotte, Siena, Italy

Alkaptonuria (AKU) is a rare genetic disease associated with the accumulation of homogentisic acid (HGA) and its oxidized/polymerized products which leads to the deposition of melanin-like pigments (ochronosis) in connective tissues. Although numerous case reports have described ochronosis in joints, little is known on the molecular mechanisms leading to such a phenomenon. For this reason, we characterized biochemically chondrocytes isolated from the ochronotic cartilage of AKU patients. Based on the macroscopic appearance of the ochronotic cartilage, two sub-populations were identified: cells coming from the black portion of the cartilage were referred to as “black” AKU chondrocytes, while those coming from the white portion were referred to as “white” AKU chondrocytes. Notably, both AKU chondrocytic types were characterized by increased apoptosis, NO release, and levels of pro-inflammatory cytokines. Transmission electron microscopy also revealed that intracellular ochronotic pigment deposition was common to both “white” and “black” AKU cells.

We then undertook a proteomic and redox-proteomic analysis of AKU chondrocytes which revealed profound alterations in the levels of proteins involved in cell defence, protein folding, and cell organization. An increased post-translational oxidation of proteins, which also involved high molecular weight protein aggregates, was found to be particularly relevant in “black” AKU chondrocytes.

J. Cell. Physiol. 227: 3333–3343, 2012. © 2011 Wiley Periodicals, Inc.

Abbreviations: AKU, alkaptonuria; BQA, benzoquinone acetic acid; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DNPH, 2,4-dinitrophenylhydrazine; DTE, 1,4-dithioerythritol; HGA, homogentisic acid; IAA, iodoacetamide; IEF, isoelectric focusing; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: Fondazione Monte dei Paschi di Siena; Contract grant number: GGP10058.
Contract grant sponsor: Telethon; Contract grant number: GGP10058.
Tommaso Serchi’s present address is Centre de Recherche Public - Gabriel Lippmann 41, rue du Brill L-4422 BELVAUX - GD Luxembourg.

Correspondence to: Annalisa Santucci, Università degli Studi di Siena, Dipartimento di Biotecnologie, via Fiorentina 1, 53100 Siena (SI), Italy. E-mail: santucci@unisi.it
Manuscript Received: 27 September 2011
Manuscript Accepted: 9 December 2011
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 29 December 2011
DOI: 10.1002/jcp.24033
Materials and Methods

Reagents

Unless otherwise indicated, all high quality reagents and antibodies were from Sigma–Aldrich (St. Louis, MO). All water used was Milli-Q (Millipore, Bedford, MA).

Isolation and culture of human chondrocytes from ochronotic AKU patients

AKU chondrocytes were obtained, after informed consent in accordance with the Declaration of Helsinki, from hip cartilage fragments of three alkaptonuric patients suffering from ochronotic arthropathy and undergone surgery for hip replacement. The study received approval from the Local Ethics Committee. The characteristics of patients enrolled for the study are schematically reported below:

- Patient #1: female, age 62, 4/4 backbone impairment, 4/4 articular joints impairment, two orthopaedic surgical interventions, urinary HGA level (24 h): 300 mg/dl.
- Patient #2: male, age 60, 4/4 backbone impairment, 4/4 articular joints impairment, five orthopaedic surgical interventions, urinary HGA level (24 h): 475 mg/dl.
- Patient #3: female, age 69, 4/4 backbone impairment, 4/4 articular joints impairment, two orthopaedic surgical interventions, urinary HGA level (24 h): 475 mg/dl.

At visual inspection, donors’ cartilages presented both a white and dark coloration and we obtained, from separate digestion of these different fragments, two cell populations that we named “white” and “black” chondrocytes. Immediately after surgery, cartilage was cut aseptically and minced in small pieces. Fragments were washed in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% penicillin/streptomycin solution and 0.2% amphotericin B. Chondrocytes were isolated by sequential enzymatic digestion: 30 min with 0.1% hyaluronidase, 1 h with 0.5% pronase, and 1 h with 0.2% collagenase at 37 °C in wash solution (DMEM þ penicillin/streptomycin solution þ amphotericin B). The cells suspension was then filtered twice using 70 μm nylon meshes, washed and centrifuged for 10 min at 700 g.

Cells were washed in Dulbecco’s modified Eagle’s medium (DMEM) containing 2% penicillin/streptomycin solution and 0.2% amphotericin B. Chondrocytes were isolated by sequential enzymatic digestion: 30 min with 0.1% hyaluronidase, 1 h with 0.5% pronase, and 1 h with 0.2% collagenase at 37 °C in wash solution (DMEM þ penicillin/streptomycin solution þ amphotericin B). The cells suspension was then filtered twice using 70 μm nylon meshes, washed and centrifuged for 10 min at 700 g.

It was linearly increased from 300 to 3,500 V during the first 3 h and then stabilized at 5,000 V for 22 h. (total 110 kV h). Prior to silver ammoniacal staining was carried out according to Switzer (Switzer et al., 1979) whereas gels to be transferred onto nitrocellulose (NC) membranes or 50 μg (2D gels to be silver stained) of proteins were adsorbed onto Immobiline Dry Strips (IPG 18 cm, nonlinear 3–10 pH range, Bio-Rad) for 10 h. Isoelectric focusing (IEF) was carried out with a Protean IEF cell (Bio-Rad). The voltage was linearly increased from 300 to 3,500 V during the first 3 h and then stabilized at 5,000 V for 22 h (total 110 kV h). Prior to silver ammoniacal staining was carried out according to Switzer (Switzer et al., 1979) whereas gels to be transferred onto NC were washed and equilibrated in a transfer buffer [50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20%, v/v methanol] and protein transfer was carried out using a semidy setting Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm² for 75 min. Protein transfer was checked by staining with 0.2% Ponceau S in 3% (v/v) TFA for 3 min and de-staining with water.

For the Western blot analysis of carbonylated proteins, after the IEF IPG strips were briefly rinsed with water and incubated at room temperature for 20 min with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 5% (v/v) TFA to allow derivatization of protein carbonyls (Reinecke et al., 2000), Strips were washed twice with a solution containing 8 M urea, 20% (v/v) glycerol, 9 M SDS, and 150 mM Tris–HCl pH 6.8. For the immunorevelation of protein carbonyls, NC sheets were incubated with rabbit anti-dinitrophenyl antibodies 1:10,000 (overnight at 4 °C), followed by peroxidase-conjugated anti-rabbit antibodies 1:7,000 (2 h at room temperature) and revelation was achieved through chemiluminescence (Immu-Star HRP, Bio-Rad). Protein spots of interest were identified by MALDI-TOF mass spectrometry (MS) or by gel matching with proteomic reference

Cytokine assay

The release of IL-1β, IL-6, IL-8, IL-10, and TNFα from AKU chondrocytes was evaluated in cell culture supernatant by means of multiplex assay for cytokine quantification (Bioplex, Bio-Rad, Hercules, CA). Cytokine concentrations were calculated using a standard curve established from serial dilutions of each cytokine standard as described in the manufacturer’s protocol and expressed as picograms per milliliter of culture medium.

Two-dimensional electrophoresis (2D-PAGE) and Western blot

Cells were washed twice with sterile PBS and resuspended in 50 μl of a buffer containing 65 mM DTE, 65 mM CHAPS, 9 M urea, and 35 mM Tris-base. Cell disruption was achieved by sonication briefly in an ice bath. Protein content in cell lysates was assessed according to Bradford (Bradford, 1976).

Cell lysates were first mixed with a buffer containing 8 M urea, 35 mM CHAPS, 10 mM DTE, and a trace of bromophenol blue. One hundred micrograms (2D gels to be transferred onto nitrocellulose (NC) membranes) or 50 μg (2D gels to be silver stained) of proteins were adsorbed onto Immobiline Dry Strips (IPG 18 cm, nonlinear 3–10 pH range, Bio-Rad) for 10 h. Isoelectric focusing (IEF) was carried out with a Protean IEF cell (Bio-Rad). The voltage was linearly increased from 300 to 3,500 V during the first 3 h and then stabilized at 5,000 V for 22 h. (total 110 kV h). Prior to silver ammoniacal staining was carried out according to Switzer (Switzer et al., 1979) whereas gels to be transferred onto NC were washed and equilibrated in a transfer buffer [50 mM Tris, 40 mM glycine, 1.3 mM SDS, 20%, v/v methanol] and protein transfer was carried out using a semidy setting Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm² for 75 min. Protein transfer was checked by staining with 0.2% Ponceau S in 3% (v/v) TFA for 3 min and de-staining with water.

For the Western blot analysis of carbonylated proteins, after the IEF IPG strips were briefly rinsed with water and incubated at room temperature for 20 min with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 5% (v/v) TFA to allow derivatization of protein carbonyls (Reinecke et al., 2000), Strips were washed twice with a solution containing 8 M urea, 20% (v/v) glycerol, 9 M SDS, and 150 mM Tris–HCl pH 6.8. For the immunorevelation of protein carbonyls, NC sheets were incubated with rabbit anti-dinitrophenyl antibodies 1:10,000 (overnight at 4 °C), followed by peroxidase-conjugated anti-rabbit antibodies 1:7,000 (2 h at room temperature) and revelation was achieved through chemiluminescence (Immu-Star HRP, Bio-Rad). Protein spots of interest were identified by MALDI-TOF mass spectrometry (MS) or by gel matching with proteomic reference.
maps already produced, calibrated, and characterized in our laboratories (Spreafico et al., 2006, 2009).

Image analysis

Images of gels and films were acquired (Image Scanner, Amersham Biosciences, Uppsala, Sweden) and analyzed with Image Master™ Platinum (Amersham Biosciences). For comparative proteomic analysis, spot% relative abundance was adopted; for multiple spots identified as different molecular species of a same protein, the average% relative abundance was calculated. For the quantitative analysis of immunorevealed protein carbonyls, the intensity of bands, which is automatically normalized by Image Master™ Platinum against the surrounding background, was chosen as the reference parameter.

Statistical analysis

All of the experiments were carried out in triplicate; data are presented as average values with standard deviation. Student’s t-test and multiple-measurement ANOVA analysis followed by the Bonferroni-type multiple comparison were used when necessary. Differences with at least a P-value ≤0.05 were considered significant. For comparative proteomic and redox proteomic analyses, only representative gels and films are shown.

Results

In this work, we characterized two distinct types of primary chondrocytes (namely “white” and “black” AKU chondrocytes) obtained from AKU patients suffering from ochronotic arthropathy who underwent surgery. TEM analysis revealed that both “white” and “black” AKU chondrocytes contained intracellular ochronotic pigments (Fig. 1) and showed a similar morphology. The finding of pigments in “white” chondrocytes is particularly noteworthy, because it demonstrated that deposition of ochronotic pigments occurred even in areas where no macroscopic evidence of pigmentation was observed (Kutty et al., 1973). Therefore, intracellular pigment deposition possibly precedes deposition in the extracellular matrix. Another noteworthy feature was the presence of ochronotic deposits of varying shapes and sizes also within some vacuoles (Fig. 1).

Both types of AKU cells showed a proliferation rate higher than the control (+30.8- and +8.9-fold-change for “white” and “black” chondrocytes, respectively) and “white” AKU chondrocytes had a proliferation rate higher than “black” ones (with a +6.1-fold change) (Fig. 2A). Since exposure to HGA may induce cytotoxicity (Kirkpatrick et al., 1984; Angeles et al., 1989), we investigated also cell apoptosis and found that “white” and “black” chondrocytic cultures were both characterized by a higher% of apoptotic cells when compared to the control (+1.5- and +2.0-fold-change, respectively) (Fig. 2B). Interestingly, the release of NO in AKU chondrocytes was significantly higher than in the control. Particularly, +33- and +126-fold-change values were recorded for NO levels in “white” and “black” chondrocytes, respectively (Fig. 2C).

Cytokine expression

To evaluate the inflammatory status of AKU ochronotic chondrocytes, we measured the levels of IL-1β, IL-6, IL-8, IL-10, and TNFα in cell culture supernatants. With the only exception of IL-1β in “black” AKU chondrocytes, which did not show any difference with respect to the control, for each assayed cytokine we found significantly higher levels in AKU cells, as shown in Figure 3. Interestingly, levels of both IL-1β and TNFα were significantly higher in “white” AKU chondrocytes, whereas “black” chondrocytes showed only a significant increase in TNFα levels. Upon injuries or diseases, cytokines such as IL-1β and TNFα can be found as major mediators of

Fig. 1. Deposition of ochronotic pigments, detected by TEM, in “white” and “black” AKU chondrocytes and their controls (non-AKU). Arrows indicate the presence of ochronotic pigments. Only representative images are shown.
inflammation within articular cartilages undergoing repair/ regeneration (Pelletier et al., 2001; Kobayashi et al., 2005; Wehling et al., 2009). Both cytokines induce an imbalance towards the catabolic pathway (Fernandes et al., 2002) through up-regulation of metalloproteinases (MMPs), aggrecanases, inducible NO synthase, and cyclooxygenase 2 (Umlauf et al., 2010). Their pro-inflammatory action is also mediated by an enhanced production of IL-6 and IL-8 (Umlauf et al., 2010) together with induction of apoptosis (Fischer et al., 2000; Aizawa et al., 2001; Aigner and Kim, 2002). Though players in a very complex scenario, IL-1β and TNFα are associated with cartilage degradation and synovial inflammation observed in OA (Fernandes et al., 2002).

IL-6 levels were significantly increased in AKU “white” and “black” chondrocytes with respect to the control (+2.9- and +3.7-fold-change, respectively), similarly to what we observed for IL-8 levels (+7.7-fold-change in “white” and +4.7-fold-change in “black” AKU chondrocytes). IL-6 is a co-factor of the catabolic effects of IL-1β (Flannery et al., 2000) enhancing the production of MMP-13 and, consequently, the IL-1β-induced degradation of proteoglycan in cartilage (Honorati et al., 2002). IL-8 belongs to the family of chemokines. Recently, a key role for the chondrocytic chemokine/chemokine receptor system in OA has been suggested (Facchini et al., 2005). Notably, IL-8 was used as a biomarker for rheumatoid arthritis on the basis of its elevated levels found in synovial fluid of affected patients (Kokebie et al., 2011).

IL-10 levels were 3.7 and 3.4 times higher in AKU “white” and “black” chondrocytes than in the control, respectively. In OA, cartilage and synovium show increased levels of IL-10 (Isozaki and Punnonen, 1997; Jorgensen et al., 1998; Moo et al., 2001) that are thought to inhibit the synthesis of other pro-inflammatory cytokines (Katsikis et al., 1994; Lechman et al., 1999). Nevertheless, the exact roles of IL-10 in inflammatory diseases, and especially in chondrocyte homeostasis, need to be

Fig. 2. Cell proliferation (A), apoptosis (B), and NO release (C) in “white” and “black” AKU chondrocytes and their control (non-AKU). Cell proliferation was assayed by measuring the DNA content of cell pellets; apoptosis was assayed by Annexin V-FITC/propidium iodide staining and flow cytometry; NO release in culture supernatants was assayed by Griess reagent, as detailed under Materials and Methods Section. Experiments were performed in triplicate; data are presented as average values with standard deviation. Statistical significance compared to non-AKU control (*P ≤ 0.05) and between “white” and “black” AKU chondrocytes ($)P ≤ 0.05) is indicated.

Fig. 3. Release of cytokines in “white” and “black” AKU chondrocytes and their control (non-AKU) by means of multiplex assay, as detailed under Materials and Methods Section. Experiments were performed in triplicate; data are presented as average values with standard deviation. Statistical significance compared to non-AKU control (*P ≤ 0.05) and between “white” and “black” AKU chondrocytes ($)P ≤ 0.05) is indicated. nd: non-detectable.
fully realized. In particular, the study of the interplay between IL-10 and TNF-α revealed that, if on the one hand IL-10 can strongly inhibit the TNF-α-induced down regulation of aggrecan expression, on the other it can only have minor effects in contrasting the TNF-α-induced collagen type II suppression (Muller et al., 2008).

**Comparative proteomics**

Silver staining allowed to reveal nearly 3,000 spots in “white” and “black” AKU chondrocyte proteomic maps (Fig. 4A, B). MALDI-ToF MS and gel matching with reference maps (Spreafico et al., 2006, 2009) allowed the identification of 78 gene products. Once set a statistically significant threshold ≥2.0 for fold-change values in protein relative abundance ratio, the quantitative analysis against the control proteomic map revealed that 34 and 41 proteins were differently expressed in “white” and “black” AKU chondrocytes, respectively. The description of differently expressed proteins and fold-change values are schematically reported in Table I (see also Supplementary Material). The functional classification of differently expressed proteins is reported in Figure 4C, D. The protein repertoires of “white” and “black” AKU chondrocytes were quite similar and the most significant alterations with respect to the control were found for proteins involved in:

(i) **Protein fate:** Twenty percent and 22% of proteins differently expressed in AKU “white” and “black” chondrocytes, respectively. We generally found an under expression of identified proteins involved in folding, maturation, and transport of proteins. It is noteworthy to mention the under-expression in “black” AKU chondrocytes of protein disulfide-isomerase (PDIA1), that is thought to contribute to the structural integrity of cartilage tissue, allowing a suitable response of load bearing joints to mechanical stress (Grimmer et al., 2006). We observed also an over-expression of programmed cell death 6-interacting protein (PDC6I) in both “white” and “black” AKU chondrocytes. Since PDC6I participates in the physiological sorting pathway for tyrosinase, a key enzyme in melanogenesis (Theos et al., 2005) an involvement of PDC6I in the production of ochronotic pigment in AKU at the cartilage level can be hypothesized.

(ii) **Cell structure and organization:** Fifteen percent and 24% of proteins differently expressed in AKU “white” and “black” chondrocytes, respectively. Changes in the organization and distribution of structural proteins characterize various pathologies, including OA (Fioravanti et al., 2003; Capin-Gutierrez et al., 2004; Holloway et al., 2004). Levels of several structural proteins were lower in AKU chondrocytes with respect to the control, such as vimentin (VIME), under-expressed in both “white” and “black” AKU chondrocytes, and gelsolin (GELS), under-expressed in “black” chondrocytes. VIME is the most sensitive sensor for mechanically induced stress, being necessary to maintain chondrocyte stiffness and allow a proper mechanotransduction (Li et al., 2010). GELS, regulating chondrocyte architecture and cell-matrix interactions, has

---

**Fig. 4.** Proteomic analysis. Silver stained 2D maps of “white” (A) and “black” (B) AKU chondrocytes. The comparative analysis carried out against a non-AKU chondrocytes control map (not shown) allowed the identification of differently expressed proteins (fold change at least 2 in protein% relative abundance) that are indicated with their abbreviated name. Functional classification of differently expressed proteins is indicated in (C) and (D) for “white” and “black” AKU chondrocytes, respectively. Representative images from a triplicate set are shown. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]
| Spot   | AN       | Gene   | Protein                                | Biological processes                           | loc. | CHW/CTR | CHB/CTR | CHB/CHW | ID |
|--------|----------|--------|----------------------------------------|-----------------------------------------------|------|----------|----------|----------|----|
|        |          |        |                                        | Metabolism                                    |      | C, Cs    | +2.1     | +2.3     | GM |
|        |          |        |                                        | Nucleotide, nucleoside, and nucleic acids      |      |          |          |          |     |
|        |          |        |                                        | Dihydropyrimidinase-related protein 2          |      |          |          |          |     |
|        |          |        |                                        | Nucleoside diphosphate kinase A                |      |          |          |          |     |
|        |          |        |                                        | Prohibitin                                    |      | Mt       | -11.9    | -3.2     | +3.7 GM |
|        |          |        |                                        | Inhibition of DNA synthesis, regulation of proliferation |      |          |          |          |     |
| Energy |          |        |                                        | Carbohydrate metabolism                       |      |          |          |          |     |
|        |          |        |                                        | Aconitase hydratase, mitochondrial             |      | Mt       | +3.7     | +2.8     | GM |
|        |          |        |                                        | Glyceraldehyde-3-phosphate dehydrogenase       |      | C, M     | -5.2     | -3.9     | MS |
|        |          |        |                                        | Tricarboxylic acid cycle; isocitrate from oxaloacetate: step 2/2 |      |          |          |          |     |
|        |          |        |                                        | Glycolysis (pyruvate from d-glyceraldehyde 3-phosphate: step 1/5). Independent of its glycolytic activity, it is also involved in membrane trafficking in the early secretory pathway and in oxidoreductase reactions |      |          |          |          |     |
|        |          |        |                                        | ATP synthesis, regulation of intracellular pH  | Mt   | -3.8     | -2.5     |          | GM |
|        |          |        |                                        | Fermentation; pyruvate fermentation to lactate; (S)-lactate from pyruvate: step 1/1 | C    | -2.5     |          |          |     |
|        |          |        |                                        | Transketolase                                  | C    | +2.2     | +2.0     |          | GM |
|        |          |        |                                        | Sedoheptulose 7-phosphate + d-glyceraldehyde 3-phosphate = d-ribose 5-phosphate + d-xylulose 5-phosphate; response to oxidative stress |      |          |          |          |     |
|        |          |        |                                        | Acid protease active in intracellular protein breakdown | Lys, Mel, S | -4.7     | -3.8     |          | MS |
|        |          |        |                                        | Protein biosynthesis                           | C    | -9.6     | -10.1    |          | GM |
|        |          |        |                                        | Protein biosynthesis                           | Mt   | -6.4     | -7.5     |          | GM |
|        |          |        |                                        | Molecular calcium-binding chaperone promoting folding, oligomeric assembly and quality control in the ER | C, ER, EM, S | -2.3     | -2.9     |          | GM |
|        |          |        |                                        | Control of cell proliferation and cellular aging, may also act as a chaperone; has anti-apoptotic functions | Mt   | -2.0     | -2.1     |          |     |
|        |          |        |                                        | Catalyzes the post-translational formation of 4-hydroxyproline in -Xaa-Pro-Gly- sequences in collagens |      | -2.2     |          |          | GM |
|        |          |        |                                        | and other proteins; it is involved in redox reactions |      |          |          |          |     |
|        |          |        |                                        | May function as a redox-sensitive chaperone and as a sensor for oxidative stress; prevents aggregation of alpha-synuclein | C, N, may be associated to Mt after oxidative stress | -3.3     | -3.4     |          | GM |
|        |          |        |                                        | Protein transport; may play a role in the regulation of both apoptosis and cell proliferation | C, Cs, Mel | +12.8    | +8.2     | -2.2     | GM |
|        |          |        |                                        | Catalyzes the formation, breakage, and rearrangement of disulfide bonds; at the cell surface, seems to act as a reductase that cleaves disulfide bonds of proteins attached to the cell. At high concentrations, functions as a chaperone that inhibits aggregation of misfolded proteins; at low concentrations, facilitates aggregation (anti-chaperone activity) | ER, Mel, M | -2.3     |          |          | GM |
|        |          |        |                                        | Phosphatidylethanolamine-binding protein 1      | C    | +16.4    | +27.2    |          | GM |
| Spot | AN   | Gene  | Protein                                      | Biological processes*                                                                 | loc.b | CHW/CTRc | CHB/CTRc | CHB/CHW | ID d  |
|------|------|-------|----------------------------------------------|--------------------------------------------------------------------------------------|-------|----------|----------|---------|-------|
| PSME1| Q06323| PSME1 | Proteasome activator complex subunit I       | Implicated in immunoproteasome assembly and required for efficient antigen processing | C, Pr | −3.2     | −2.4     | GM      |
| TERA | P55072| VCP   | Transitional endoplasmic reticulum ATPase    | ER-associated protein catabolic process, ER-unfolded protein response, protein ubiquitination, retrograde protein transport (ER to cytosol) | C, N  | −2.9     | GM       |
| UBE2K| P61086| UBE2K | Ubiquitin-conjugating enzyme E2 K            | Protein ubiquitination                                                                | C     | −2.6     | GM       |
|      |       |       |                                              |                                                        |       |          |          |         |       |
| Signal transduction |      |       |                                              |                                                        |       |          |          |         |       |
| GDIR1| P52565| ARHGDIA| Rho GDP-dissociation inhibitor 1               | Regulates the GDP/GTP exchange reaction of the Rho proteins                          | C     | −3.5     | −3.5     | GM      |
| RANG | P43487| RANBP1| Ran-specific GTPase-activating protein        | Inhibits GTP exchange on Ran; may act in an intracellular signaling pathway which may control the progression through the cell cycle by regulating the transport of protein and nucleic acids across the nuclear membrane | C, N  | −13.5    | −16.6    | GM      |
|      |       |       |                                              |                                                        |       |          |          |         |       |
| Cellular organization |      |       |                                              |                                                        |       |          |          |         |       |
| Cytoskeleton and microtubules |      |       |                                              |                                                        |       |          |          |         |       |
| GELS | P06396| GSN   | Gelsolin                                     | Binds to actin and to fibronectin preventing monomer exchange and promoting the assembly of monomers into filaments (nucleation) as well as sever filaments already formed. Defects in GSN are the cause of amyloidosis type 5 (AMYLS5) [MIM:105120]; also known as familial amyloidosis Finnish type. AMYLS5 is a hereditary generalized amyloidosis due to gelsolin amyloid deposition. It is typically characterized by cranial neuropathy and lattice corneal dystrophy. Most patients have modest involvement of internal organs, but severe systemic disease can develop in some individuals causing peripheral polyneuropathy, amyloid cardiomyopathy, and nephrotic syndrome leading to renal failure | C, Cs, S, amyloid | −2.0     | −2.2     | GM      |
| TAGL | Q01995| TAGLN | Transgelin                                   | Actin cross-linking/gelling protein. Involved in calcium interactions and contractile properties of the cell that may contribute to replicative senescence | C     | +9.2     | +17.0    | MS      |
| TBB5 | P07437| TUBB  | Tubulin beta chain                           | Major constituent of microtubules                                                     | C     | −2.9     | GM       |
| Cell cycle |      |       |                                              |                                                        |       |          |          |         |       |
| SEPT2 | Q15019| SEPT2 | Septin-2                                     | Cell division, mitosis                                                                | C     | +2.0     | GM       |
| Annexin family |      |       |                                              |                                                        |       |          |          |         |       |
| ANXA4| P09525| ANXA4 | Annexin A4                                   | Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis; anti-apoptosis | C     | −4.9     | −2.4     | +2.0    | GM    |
| ANXA5| P08758| ANXA5 | Annexin A5                                   | Calcium-regulated membrane-binding protein, anti-apoptosis                           | C, N, M | −2.3     | −2.6     | GM      |
| Intermediate filaments family |      |       |                                              |                                                        |       |          |          |         |       |
| VIME | P08670| VIM   | Vimentin                                     | Class-III intermediate filaments found in various non-epithelial cells                 | C     | −5.2     | −4.1     | GM      |
| Other functions |      |       |                                              |                                                        |       |          |          |         |       |
| LEG3 | P17931| LGALS3| Galectin-3                                   | Cell differentiation                                                                | N, C, M | −3.3     | −11.3    | −3.4    | GM    |
| TPM3 | P06753| TPM3  | Tropomyosin alpha-3 chain                    | Stabilization of cytoskeleton and actin filaments, cell motion                       | C, Cs  | −2.4     | GM       |
| WDFI | Q81WB7| WDFY1 | WD repeat and FYVE domain-containing protein 1| Phosphoryl transferase binding                                                        | C, N   | −2.6     | GM       |
| Spot          | AN   | Gene   | Protein                             | Biological processes                                                                 | loc. | CHW/CTR | CHB/CTR | CHB/CHW | ID  |
|--------------|------|--------|-------------------------------------|--------------------------------------------------------------------------------------|------|----------|----------|----------|-----|
| Cell rescue, defence, and stress                  |      |        |                                     |                                                                                      |      |          |          |          |     |
| CH60         | P10809| HSP60  | 60kDa heat shock protein, mitochondrial | Implicated in mitochondrial protein import and macromolecular assembly, may facilitate the correct folding of imported proteins, prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix | Mt   | -2.4     | -2.7     |          | GM   |
| CRYAA        | P02489| CRYAA  | Alpha-crystallin A chain (Heat shock protein beta-4) | Anti-apoptosis, protein folding, unfolded protein binding, response to heat | C    | 2.7      | 2.5      |          | GM   |
| CRYAB        | P02511| CRYAB  | Alpha-crystallin B chain            | Anti-apoptosis, protein folding, unfolded protein binding, response to heat | —    | 2.2      |          |          | MS   |
| ENPL         | P14625| HSP90B1| Endoplasmic                     | 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; response to hypoxia | C, ER, Mel | -3.6     | -4.8     |          | GM   |
| Other functions                       |      |        |                                     |                                                                                      |      |          |          |          |     |
| FRIL         | P02792| FTL    | Ferritin light chain               | Iron storage and homeostasis, involved in redox reactions                           | R    | +2.0     | +2.1     |          | GM   |
| NNMT         | P40261| NNMT   | Nicotinamide                      | Catalyzes the N-methylation of nicotinamide and other pyridines to form pyridinium ions (this activity is important for biotransformation of many drugs and xenobiotic compounds) | C    | -3.2     | -3.4     |          | GM   |

AN, accession number.
Proteins whose levels are altered in AKU "white" (CHW) and "black" (CHB) chondrocytes with respect to non-AKU control (CTR) chondrocytes.

aProtein biological processes.
bProtein sub-cellular localization: C (cytosol); Cs (cytoskeleton); CJ (cell junction); M (membrane); BM (basal membrane); EM (extracellular matrix); Mel (melanosome); Mt (mitochondrion); N (nucleus); Pe (peroxisome); Pr (proteasome); ER (endoplasmic reticulum); S (secreted); L (lysosome). Retrieved by UniProt knowledgebase (http://www.uniprot.org/).

cFold-change in protein% relative abundance (as average values in case of multiple spots): (+) over-expressed proteins, (-) under-expressed protein according to the ratio indicated. "A" and "P" indicate the absence or presence of proteins, respectively.
dIdentification method (GM: gel-matching; MS: MALDI-TOF mass spectrometry).
been indicated as a prognostic and diagnostic marker of OA (De Ceuninck et al., 2005). Together, VIME and GELS contribute together to synovial fluid functions (Gobezie et al., 2007).

(iii) Cell rescue, defence, and stress response: Thirty-two percent and 22% of proteins differently expressed in AKU “white” and “black” chondrocytes, respectively. AKU chondrocytes showed altered levels of proteins fundamental for the protection from oxidative stress, like previously indicated by our group in a chondrocytic line used as a model of AKU (Braconi et al., 2010b). Though alpha-crystallin A chain (CRYAA), peroxiredoxin-1 (PRDX1), peroxiredoxin-6 (PRDX6), and serpin H1 (SERPH) were over-expressed both in “white” and “black” AKU chondrocytes compared to the control, lower protein levels were found in “white” and “black” AKU chondrocytes for most of the identified proteins. This is the case of the mitochondrial 60 kDa HSP (CH60), alpha-crystallin B chain (CRYAB), endoplasmic (ENPL), glutathione S-transferase omega-1 (GSTO1), glutathione S-transferase P (GSTP1), heat shock 70 kDa protein 4 (HSP74), and mitochondrial superoxide dismutase (SODM). The simultaneous deficiency of these proteins reinforce the hypothesis that AKU cells cannot adequately control reactive oxygen species (ROS)- and quinone-mediated toxicity.

**Redox-proteomics**

Differently from what observed with the comparative proteomic analysis, when we investigated protein oxidation through immunorevelation of carbonylated proteins we found that “white” and “black” AKU chondrocytes showed quite distinct patterns (Fig. 5). Gel-matching with the corresponding silver stained replica gel allowed the identification of almost all the spots immunorevealed under the conditions tested (Table II).

In control chondrocytes, we could identify as carbonylated only the proteins G3P (energy) and VIME (cell organization). In AKU cells, whereas “white” chondrocytes had no resolved immunoreactive spots but only high molecular weight protein aggregates stacked on the top of the SDS–PAGE gel, “black” AKU chondrocytes showed, together with carbonylated high molecular weight protein aggregates, the following carbonylated proteins: G3P (energy), EF1A1 (transcription, synthesis, and turnover of proteins), VIME (cell organization), CATA and CH60 (cell rescue, defence, and stress).

**Discussion**

In this study we have provided, to the best of our knowledge, the first proteomic and redox proteomic characterization of ex vivo AKU chondrocytes as models of ochronotic arthropathy. Our main aim was to gain insights into those AKU-related pathophysiological processes that allow ochronosis to develop in connective tissues, especially joints. We were able to subculture two distinct populations, namely “white” and “black” AKU chondrocytes, on the basis of the macroscopic appearance of donors’ hip ochronotic cartilage from which the cells were obtained. Both cell types, maintained in culture conditions resembling the original pathological ones thanks to the addition of HGA to the culture medium, were characterized by increased apoptosis, NO release, and levels of pro-inflammatory cytokines similarly to what observed in OA, a pathology that share some features with alkaptonuric arthropathy. Interestingly, both the cell types contained intracellular ochronotic pigments, suggesting that ochronosis might involve joint cells before than the surrounding ECM. Profound alterations in the levels of proteins involved in cell defence, protein folding, and cell organization were found in AKU ochronotic chondrocytes together with an increased post-translational oxidation of proteins and oxidized high molecular weight protein aggregates.

One of the main functions of articular cartilage is to provide a structure dissipating excessive loading and withstanding tension, compression, and shearing forces (Juang et al., 2010).
Chondrocytes are crucial players in maintaining cartilage integrity by activating catabolic or anabolic processes in response to external stimuli. The catabolic program activated upon inflammation involves the production of proteases and the induction of apoptosis together with the suppression of matrix synthesis. Conversely, the anabolic program promotes the secretion of specific cytokines, protease inhibitors, the production of ECM and cell proliferation (Lotz et al., 1995). An imbalance towards an increase in catabolic activities is thought to be involved in the disruption of cartilage integrity leading to loss of joint functions (Blanco Garcia, 1999; Kim and Blanco, 2007). We have already demonstrated an inflammatory status in AKU (Selvi et al., 2000) and IL-6 and IL-8 over-expression in AKU chondrocytes (unpublished data). In this work, we confirmed our previous findings and also highlighted that levels of NO were increased in AKU ochronotic cells concomitantly with an enhanced release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNFx). Altogether, our results suggest that pro-inflammatory cytokines and NO might play important roles in cartilage and joint tissue disruption in AKU patients during the ochronotic progression.

NO plays pro-inflammatory and destructive effects in cartilages and it has been related to a variety of mechanisms promoting cartilage catabolism (Henrotin et al., 2005). Cytokines such as TNFx and IL-1β are important mediators of inflammation and cartilage degradation observed in OA (Cillero-Pastor et al., 2010). Both have been shown to modulate proteins regulating cytoskeleton, tissue organization, and cell morphology of human chondrocytes (Cillero-Pastor et al., 2010) and to induce the expression of PRDX1 (Cillero-Pastor et al., 2010), whose main function is to amend oxidative stress imposed by hydrogen peroxide. Similar results were confirmed in this work thanks to the proteomic characterization of AKU imposed by hydrogen peroxide. Similar results were confirmed et al., 2010), whose main function is to amend oxidative stress inhibition of metabolism-related proteins is a common feature of OA (Iliopoulos et al., 2010). Nevertheless, the over-expression of proteins with functions in energy and protein fate, such as G3P, PDIA1, HS47, and CALR, as a function of catabolic program was highlighted in a rat chondrocyte model exposed to pressure overload (Juang et al., 2010). Interestingly, in our work we found that G3P, PDIA1, and CALR were under-expressed in AKU ochronotic chondrocytes, probably pointing out to an impaired ability of cells to produce a cartilage structure optimally withstandig pressure forces. In this light, it is noteworthy to mention that load bearing joints are the first to show the deposition of ochronotic pigments (La Du, 1991).

IL-1β is a key mediator of inflammation and cartilage degradation in OA, but it also play a role in oxidative stress generation since it can induce an imbalance in the activity of fundamental anti-oxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, as demonstrated recently in chondrocytes (Mathy-Hartert et al., 2008). Though to a lesser extent, also IL-6 was found to induce such a disregulation of antioxidant enzymes. These changes can ultimately lead to a transient accumulation of ROS (Mathy-Hartert et al., 2008) which might induce, in turn, oxidation of cellular constituents. On this basis, and also taking into account the HGA-induced oxidative post-translational modifications of proteins that we highlighted in different AKU models (Braconi et al., 2010a,b; Tinti et al., 2010, 2011b), we performed a redox proteomic analysis to evaluate protein carboinylation in AKU ochronotic cells. Notably, in this work we found responses that were similar to what previously showed in a chondrocytic cell line treated with 0.33 mM HGA and used as an in vitro model of AKU ochronosis (Braconi et al., 2010b). We confirmed the identity of proteins most likely to undergo HGA-induced oxidation and also the production of high molecular weight aggregates of oxidized proteins, providing in turn indirect evidences of the auto-oxidation of HGA into BQA, which is known to promote protein aggregation mediated by oxidative phenomena. Protein oxidation and aggregation might help, in vivo, the production of ochronotic pigments.

In conclusion, the findings presented in this work support the deposition of ochronotic pigments (La Du, 1991).

### TABLE II. Redox proteomics of AKU chondrocytes

| Spot | AN | Gene | Protein | Biological processes | CTR | CHW | CHB |
|------|----|------|---------|---------------------|-----|-----|-----|
| G3P  | P04046 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Glycolysis, response to oxidative stress | + | + |
| Transcription, synthesis, and turnover of proteins | EEFIA1 | Elongation factor I-alpha 1 | Protein biosynthesis | + |
| Cellular organization | VIME | Vimentin | Structural constituent of cytoskeleton, cell motion | + |
| Cell rescue, defence, and stress | CATA | Catalase | Response to oxidative stress | + |
| | CH60 | HS60 | Protein folding and maturation, response to unfolded protein | + |

Identification of carbonylated (+) proteins in non-AKU control (CTR), AKU “white” (CHW) and “black” (CHB) chondrocytes.
Literature Cited

Aigner T, Kim HA. 2002. Apoptosis and cellular vitality: Issues in osteoarthritic cartilage degeneration. Arthritis Rheum 46:1986–1996.

Atawaz T, Kon T, Einhorn TA, Gersterfeld LC. 2001. Induction of apoptosis in chondrocytes by tumor necrosis factor alpha. J Orthop Res 19:785–796.

Augeles AP, Badger R, Gruber HE, Seegmiller JE. 1989. Chondrocyte growth inhibition induced by homocysteic acid and its partial prevention with ascorbic acid. J Rheumatol 16:512–517.

Blanco Garcia FJ. 1999. Catabolic events in osteoarthritic cartilage. Osteoarthritis Cartilage 7:308–309.

Bracconi D, Lisch H, Amato L, Bernardini G, Moro F, Marcolongo R, Spreafico A, Santucci A. 2001a. Oxidative stress and ascorbate intake in OA patients. J Rheumatol 28:2499–2503.

Bracconi D, Lisch H, Taylor AM, Bernardini G, Spreafico A, Tinti L, Gallagher JA, Santucci A. 2001b. Proteomic analysis of early-response to mechanical stress in neonatal rat mandibular condylar chondrocytes. J Cell Physiol 212:610–612.

Liu X, Li H, Wang JF, Wang H, Wu JS, Chen YX, Li X, Li S, Hua ZC. 2005. Proteomic analysis of early-response to mechanical stress in neonatal rat mandibular condylar chondrocytes. J Cell Physiol 212:610–612.

Manni M, Pappo V, Fabbri B, Benucci M, Janossy M, Chellini F, Galeazzi M, Marcolongo R. 2000. Chondroprotective effect of ascorbic acid on human articular cartilage. J Cell Biochem 81:119–122.

Moo V, Sieper J, Herzog V, Muller BM. 2001. Regulation of expression of cytokines and growth factors in osteoarthritic cartilage. J Cell Biochem 81:119–122.

Neville P, Scarpa P, Ferrari A, Giampietro A, Marcolongo R, Santucci A. 2011. Evaluation of antioxidant drugs for the treatment of osteoarthritic cartilage in an in vitro human cell model. J Cell Physiol 225:84–91

Tinti L, Spreafico A, Chellini F, Galeazzi M, Santucci A. 2010. Evaluation of antioxidant drugs for the treatment of osteoarthritic cartilage in an in vitro human cell model. J Cell Physiol 225:84–91.

Zannoni VG, Malwista SE, La Du BN. 1962. Studies on ochronosis. II. Studies on benzosuberic anhydride, a probable intermediate in the connective tissue pigment formation in alkaptonuria. Arthritis Rheum 5:547–556.