Proteins involved in oxidative stress in leiomyoma tissues treated with ulipristal acetate

BLENDI URA[1], LORENZO MONASTA[1], YERALDIN CHIQUINQUIRA CASTILLO DE SPELORZI[2], GIORGIO ARRIGONI[3,4], CINZIA FRANCHIN[3,4], STEFANIA BIFFI[1], MICHELANGELO ALOISIO[5], BARTOLOMEA GAITA[2], DANILO LICASTRO[6], EMMANOUIL ATHANASAKIS[1], FEDERICA SCRIMIN[1], GUGLIELMO STABILE[1], FEDERICO ROMANO[1], GIOVANNI DI LORENZO[1] and GIUSEPPE RICCI[1,7]

[1]Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo', I-34137 Trieste; [2]Life Sciences Department, University of Trieste, I-34128 Trieste; [3]Department of Biomedical Sciences, University of Padova, I-35121 Padova; [4]Proteomics Center, University of Padova and Azienda Ospedaliera di Padova, I-35129 Padova; [5]Central RNA Laboratory, Istituto Italiano di Tecnologia, I-16163 Genova; [6]ARGO Open Lab Platform for Genome Sequencing, AREA Science Park, I-34149 Trieste; [7]Department of Medical, Surgery and Health Sciences, University of Trieste, I-34148 Trieste, Italy

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Abstract. Uterine leiomyoma presents the highest incidence among benign tumors of the female reproductive tract. The present study compared the proteome of leiomyoma treated with ulipristal acetate with that of untreated leiomyoma to investigate protein expression patterns in relation to oxidative stress. Paired tissue samples from seven treated and untreated leiomyomas were collected and the proteome was analyzed by two-dimensional gel electrophoresis (2-DE). Western blotting was used to validate the results of 2-DE, and mass spectrometry was used to identify proteins. The tissue expression of 30 proteins was markedly affected by treatment with ulipristal acetate. Bioinformatics analysis revealed that several of the differentially expressed proteins were involved in the degradation of hydrogen peroxide and the synthesis of reactive oxygen species. The present study suggested the involvement of oxidative stress as a novel mechanism of action of ulipristal acetate. These findings require further investigations to understand the role of ulipristal acetate in the treatment of the leiomyoma.

Introduction

Uterine leiomyoma is a benign tumor that affects 70-80% of women, which is the highest incidence amongst the benign tumors of the female reproductive tract (1,2). This neoplasm is responsible for serious health problems (3) and is characterized by abnormal extracellular matrix (4), altered phosphoproteins (5), dysregulated chaperones (6), and alterations in the proteins involved in cell migration (7).

The management of leiomyoma is an emerging clinical issue as the symptoms, which include poliabortivity, pain, and heavy uterine bleeding (3), can severely affect women and female fertility.

For the management of symptomatic leiomyoma, surgical techniques, such as myomectomy and hysterectomy, are traditionally used (8). However, due to the high morbidity of uterine leiomyoma, there is urgent need for a more effective therapeutic approach, capable of improving the quality of life of these patients (9).

For the treatment of symptomatic leiomyoma patients there are, in addition to surgery, pharmacological therapies based on gonadotropin-releasing hormone agonists and selective progesterone receptor modulators (10), that can effectively reduce the volume of the leiomyoma (11). Among them, ulipristal acetate (UPA) is a selective progesterone receptor modulator (SPRM) used preoperatively to control leiomyoma growth (12). It controls progesterone receptor activity and leads to apoptosis of leiomyoma cells. However, the exact mechanism of action of this drug is still unknown (13). Interestingly, Maruo *et al* (14) reported that treating leiomyoma cell cultures with UPA induced the expression of matrix metalloproteinase MMP1 and MMP8 and decreased the expression of TIMP, while treatment of myometrium cells with UPA did not lead to protein dysregulation.

The objective of our study was to identify dysregulated proteins in leiomyoma tissue treated with UPA and in untreated leiomyoma, to gain an insight into the mechanism of action of this drug.

Materials and methods

Uterine leiomyoma samples. Seven premenopausal patients treated with ulipristal acetate and seven untreated patients who

Correspondence to: Dr Blendi Ura, Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo', 65/1 Via dell'Istria, I-34137 Trieste, Italy
E-mail: blendi.ura2006@burlo.trieste.it

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underwent hysterectomy for symptomatic uterine leiomyomas were enrolled. To be eligible for this study, all patients signed a written informed consent form. The median age of the patients was 42 years, with a minimum of 35 and a maximum of 48 years.

All study procedures complied with the Declaration of Helsinki and were approved by the Ethical Review Board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy).

Oncologic patients, human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) seropositive patients, and subjects with adenomyosis were excluded from the study. The patients were treated with UPA six months before surgery.

Tissue samples. All samples were collected from the central area of the leiomyomas, which were all benign and without atypical features. They were subserosal/intramural, with dimensions ranging from 4 to 6 cm. The samples were stored at -80˚C until proteomic analysis was performed.

2-DE and image analysis. 2-DE was performed as previously described (15). Briefly, clean samples of treated and untreated leiomyoma and myometrium (200 mg each) were homogenized in 1.2 ml of dissolution TUC buffer [7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 65 mM DTT and 0.24% Bio-Lyte (3-10)] with a protease inhibitor mix (2 mM PMSF, 1 mM benzamidine, 1 mM EDTA, 1 mM NaF). After vortex, the solutions were centrifuged at 10,000 x g at 4˚C for 30 min, and the protein content of the supernatant was determined using the Bradford assay. For 2-DE analysis, 400 μg of proteins from each sample (leiomyomas treated with UPA and untreated leiomyomas) were used. ReadyStrip™ pH 4.0-7.0 and 3-10 NL 18-cm immobilized pH gradient (IPG) strips were rehydrated in a dissolution buffer at 50 V for 12 h at 20˚C, and isoelectric focusing (IEF) was performed in a PROTEAN IEF Cell (Bio-Rad Laboratories). After IEF, serial incubations were performed: First, the IPG strips were equilibrated for 20 min in an equilibration buffer [6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 30% glycerol and 1% DTT] and then in equilibration buffer containing 4% iodoacetamide instead of DTT. For the second dimension, the equilibrated IPG strips were transferred to a 12% polyacrylamide gel. After electrophoresis, the gels were fixed in 40% methanol and 10% acetic acid for 1 h, and then stained for 16 h with SYPRO Ruby. After SYPRO Ruby destaining, the gels were stained for 48 h with colloidal Coomassie Brilliant Blue. The 2-DE gels were scanned with a Molecular Imager PharosFX System. Two experimental replicates were performed for each sample. For all gels, molecular weights were determined by comparison with Precision Plus Protein Prestained Standards (Bio-Rad Laboratories, Inc.) covering a range from 10 to 250 kDa, and analyzed using the Proteomweaver 4.0 software (both from Bio-Rad Laboratories, Inc.).

Quantification of spot levels. 2-DE image analysis was performed using the Proteomweaver 4.0 software, matching all gels from seven treated and seven untreated leiomyomas. to identify quantitative differences. Fold change was calculated as the ratio between the mean %V of the treated and untreated leiomyomas. Differences were considered significant when the ratio of the mean percentage relative volume (%V) (%V = V(single spot)/V(total spot)) was ±1.5-fold and satisfied the non-parametric Wilcoxon test ($P<0.05$).

Trypsin digestion and MS analysis. After excision from 2-DE gels, the spots were washed four times with 50 mM NH_4HCO_3 and acetonitrile (ACN; Sigma-Aldrich; Merck KGaA) and dried under vacuum in a SpeedVac system. Three microliters of 12.5 ng/μl sequencing grade modified trypsin (Promega Corporation) in 50 mM NH_4HCO_3 were added for gel spot digestion; samples were digested overnight at 37˚C. Peptide extraction was performed with three changes of extraction solution of 50% ACN/0.1% formic acid (FA; Fluka), peptide mixtures were dried under vacuum and stored at -20˚C until mass spectrometry (MS) analysis.

For MS analysis, samples were dissolved in 12 μl of 3% ACN/0.1% FA, and 4 μl of each sample were analyzed by LC-MS/MS on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Inc.) coupled with a nano-HPLC Ultimate 3000 (Dionex; Thermo Fisher Scientific, Inc.). Peptides were separated in a 10 cm pico-frit column (75 μm ID, 15 μm Tip; New Objective) packed in-house with C18 material (Aeris Peptide 3.6 μm XB-C18, Phenomenex). H_2O/FA 0.1% and ACN/FA 0.1% were employed as eluents A and B, respectively, and peptides were analyzed at a flow rate of 0.25 μl/min using a linear gradient of eluent B from 3 to 40% in 20 min. A Data Dependent Acquisition (DDA) was used: A full scan between 300 and 1,700 Da was performed at high resolution (60,000) on the Orbitrap. The ten most intense ions were selected for CID fragmentation and acquisition of MS/MS data in low resolution in the linear ion trap.

Raw data files were analyzed with the Proteome Discoverer 1.4 software package (Dionex-Thermo Fisher Scientific, Inc.) and searched with the Mascot Search Engine (version 2.2.4, Matrix Science). Spectra were searched against the human section of the Uniprot database (version July 2018) using the following parameters: Enzyme specificity was set to trypsin with 1 missed cleavage allowed, precursor and fragment ions tolerance were 10 ppm and 0.6 Da, respectively. Carbamidomethylcysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. Proteins were considered as positive hits if at least 3 unique peptides for each protein were identified with high confidence (FDR <0.01%).

Western blotting. Protein extracts (50 μg) used for 2-DE were separated by 12% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked by treatment with 5% dry milk in TBS-Tween 20. After milk saturation, the membrane was incubated overnight at 4˚C with 1:300 diluted primary rabbit polyclonal antibody against PDIA3, 1:300 diluted primary rabbit polyclonal antibody against HSPB1, 1:5,000 diluted primary rabbit polyclonal antibody against ACTG2. The membrane was washed three times in TBST for 10 min and then incubated for 90 min at 4˚C with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Sigma-Aldrich; Merck KGaA) at 1:3,000 dilution. Protein expression was visualized by chemiluminescence

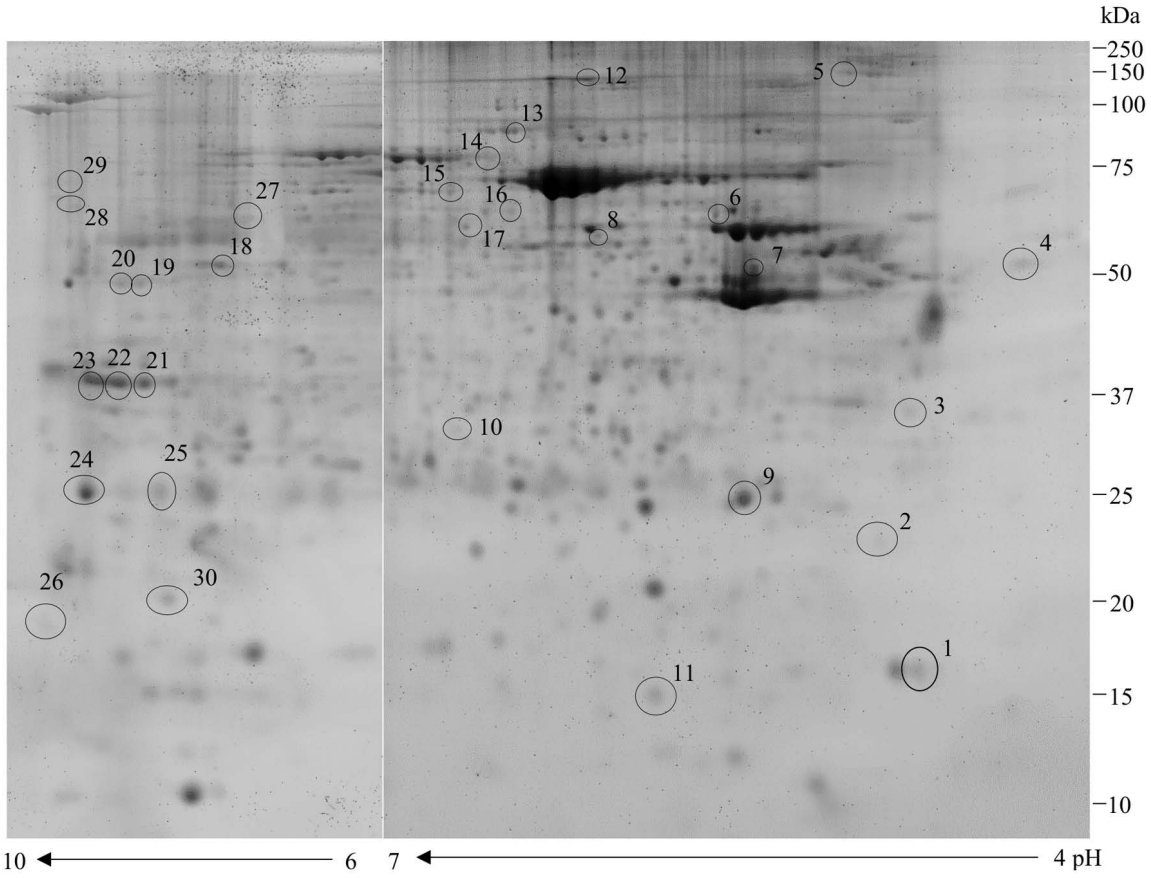

Figure 1. Master gel with the differentially abundant spots among treated and untreated leiomyoma with both pH range 4-7 and 6-10.

(SuperSignal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific, Inc.), and the intensity of the signals was quantified by VersaDoc Imaging System (Bio-Rad Laboratories, Inc.). No technical replicates were performed for each sample (5 cases and 5 controls were analyzed). The intensities of the immunostained bands were normalized with the protein intensities measured by Red Ponceau (Sigma-Aldrich; Merck KGaA) from the same blot.

Ingenuity pathway (IPA) analysis. Dysregulated proteins identified by MS in the treated and untreated leiomyomas, were analyzed by IPA (Qiagen GmbH) with a P-value <0.05 indicating a statistically significant value. Selected genes were used to generate bio-functions. For the filter summary, only associations with high (predicted) confidence or that had been experimentally observed, were considered.

Statistical analysis. Statistical analyses were carried out with the non-parametric Wilcoxon signed-rank test for matched samples for both 2-DE and western blotting data. P<0.05 was considered to indicate a statistically significant difference. All the analyses were conducted with Stata/IC 14.1 for Windows (StataCorp LP).

Results

Proteomic studies. In this study, we performed comparative proteomic analysis of leiomyoma tissues treated and untreated with ulipristal acetate with the aim of generating 2-DE reference maps and identifying dysregulated proteins. Gel-pairs correlation analysis highlighted an average matching efficiency of ~80%. Fig. 1 shows the 5,000 protein spots detected on the gel with ReadyStrip pH 4.0-7.0 and 6.0-10.0, for both types of proteomes. As reported in Table I, 13 protein spots were significantly upregulated (>1.5-fold), and 17 were significantly downregulated (<0.6-fold) in treated and untreated leiomyomas. Mass spectrometry analysis was applied to identify the 30 proteins modulated by the treatment, searching the MS/MS data against the human section of the UniProt database.

Western blotting study of altered proteins. In this study, we validated the downregulation of PDIA3, HSPB1, and ACTG2. Following bioinformatic analysis, we selected HSPB1, an optimal candidate due to its involvement in the inhibition of the synthesis of reactive oxygen species, and PDIA3 and ACTG2, for which antibodies are commercially available. Fig. 2 shows a quantitative comparative western blot analysis of PDIA3, HSPB1, ACTG2 expression in a cohort of five treated and five untreated leiomyoma tissue samples (previously analyzed through 2-DE). To normalize the results of our WB analysis, we determined the total protein content of each sample by Red Ponceau. The reason for this was that, according to our previous results (16) the proteins that are usually selected as encoded by housekeeping genes (i.e., β-actin, tubulin or GAPDH) are upregulated in leiomyoma and, thus, they are

Table I. Dysregulated proteins identified by mass spectrometry in treated compared with untreated leiomyoma.

Accession number	Spot number	Protein description	Gene symbol	Molecular weight, kDa	Protein score	Fold change[a]	P-value
P38919	17	Eukaryotic initiation factor 4A-III	EIF4A3	46.8	117.11	6.60	0.0277
P17661	8	Desmin	DES	53.5	740.50	5.50	0.0180
P49903-2	14	Selenide, water dikinase 1	SEPPHS1	42.9	85.70	3.12	0.0180
P50990-3	11	T-complex protein 1 subunit theta	CCT8	59.6	289.66	2.80	0.0180
P06733	18	Alpha-enolase	ENO1	47.1	564.98	2.62	0.0180
A0A0C4DGN4	9	Zymogen granule protein 16 homolog B	ZG16B	22.7	395.54	2.56	0.0180
A6NL76	29	Actin, alpha skeletal muscle	ACTA1	42	37.86	2.40	0.0273
P01024	16	Complement C3	C3	187.1	1,179.72	2.39	0.0310
A0A087WWT3	12	Serum albumin	ALB	69.3	319.93	2.00	0.0180
Q86YZ3	21	Hornerin	HRNR	282.3	145.20	1.96	0.0277
P51911-2	28	Isoform-2 of Calponin-1	CNN1	33.1	140.28	1.74	0.0273
P30041	6	Peroxiredoxin-6	PRDX6	25	729.76	1.60	0.0277
P23284	26	Peptidyl-prolyl cis-trans isomerase B	PPIB	23.7	124.27	1.56	0.0178
Q01995	22	Transgelin	TAGLN	22.6	213.46	0.64	0.0180
O60814	2	Histone H2B type 1-K	HIST1H2BK	13.8	302.83	0.62	0.0180
P00441	3	Superoxide dismutase [Cu-Zn]	SOD1	15.9	288.96	0.60	0.0178
P51911	23	Calponin-1	CNN1	33.1	140.28	0.48	0.0180
Q01518-2	27	Adenylyl cyclase-associated protein 1	CAP1	51.9	109.18	0.47	0.0180
P04792	5	Heat shock protein beta-1	HSPB1	22.7	758.06	0.44	0.0178
P30101	10	Protein disulfide-isomerase A3	PDIA3	56.7	425.61	0.41	0.0180
P63267	13	Actin, gamma-enteric smooth muscle	ACTG2	41.8	233.17	0.39	0.0178
P02647	30	Apolipoprotein A-I	APOA1	30.7	1,181.10	0.38	0.0273
B0YJC4	4	Vimentin	VIM	53.6	339.62	0.36	0.0180
G3V4W0	7	Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	33.6	387.32	0.30	0.0180
Q01995	19	Transgelin	TAGLN	22.6	343.38	0.30	0.0277
Q01995	20	Transgelin	TAGLN	22.6	608.19	0.26	0.0180
Q6P452	15	Annexin A4	ANXA4	35.8	299.01	0.26	0.0277
P69905	24	Hemoglobin subunit alpha	HBA1	15.2	90.15	0.23	0.0277
Q5D862	25	Filaggrin-2	FLG2	248	26.81	0.23	0.0277
F8VPF3	1	Myosin light polypeptide 6	MYL6	16.9	205.98	0.03	0.0178

[a]Fold change was defined as the ratio of the mean %V according to the formula $\%V = V_{single\ spot}/V_{total\ spot}$ of treated leiomyoma vs. untreated leiomyoma.

not adequate to be used as controls for normalization. Because we could not establish which proteins should be considered as housekeeping in our samples, we decided to apply a total protein content normalization method, as reported in several other publications (17,18).

Functional analysis. The core analysis with IPA was performed on the proteins identified by mass spectrometry, comparing treated and untreated leiomyomas. Fig. 3 shows the networks in which these proteins are most involved, i.e. the degradation of hydrogen peroxide and the synthesis of reactive oxygen species. The regulatory network that controls the degradation of hydrogen peroxide includes three of the proteins identified: HSBA1, PRDX6, SOD1. In particular, PRDX6 and SOD1 are associated with the activation of the degradation of hydrogen peroxide, while eight other proteins participate in the network responsible for the synthesis of reactive oxygen species. Among these, TAGLN, C3, ALB, PDRX6 are involved in the

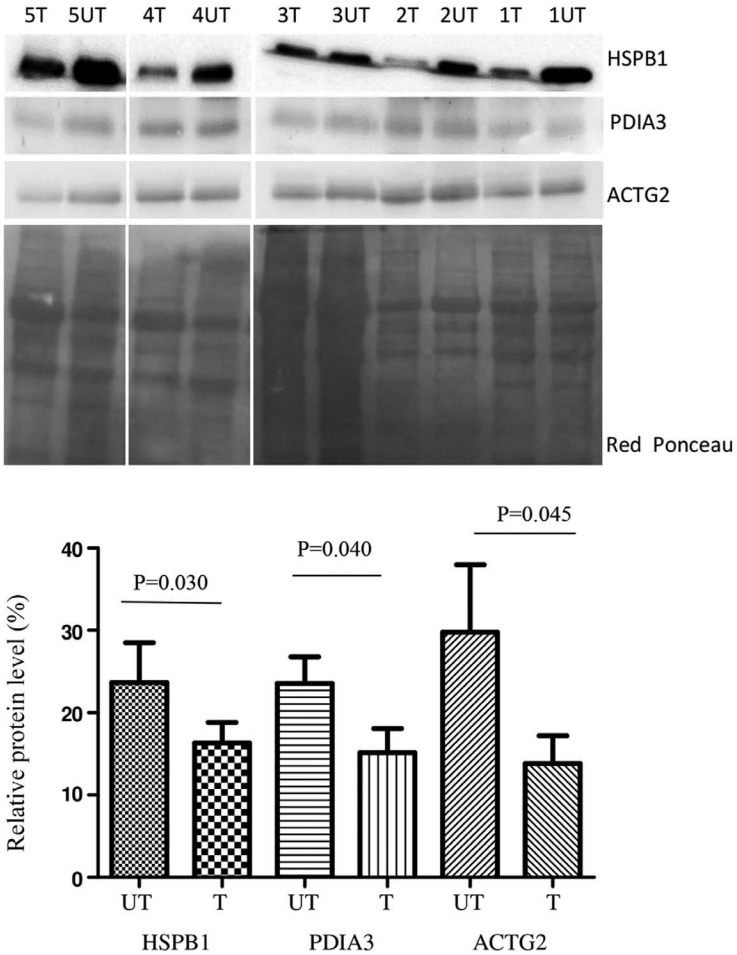

Figure 2. Western blot analysis of HSPB1, PDIA3 and ACTG2 in T and UT leiomyoma samples. The intensity of the immunostained bands was normalized against the total protein intensities measured from the same blot stained with Red Ponceau. Numbers 1-5 indicate the patients. The bar graph shows the relative expression (band density) of HSPB1, PDIA3 and ACTG2. The results are shown as a histogram ($P<0.05$) and each bar represents the mean ± standard deviation. T, treated; UT, untreated; HSPB1, heat shock protein family B (small) member 1; PDIA3, protein disulfide isomerase family A member 3; ACTG2, actin γ2, smooth muscle.

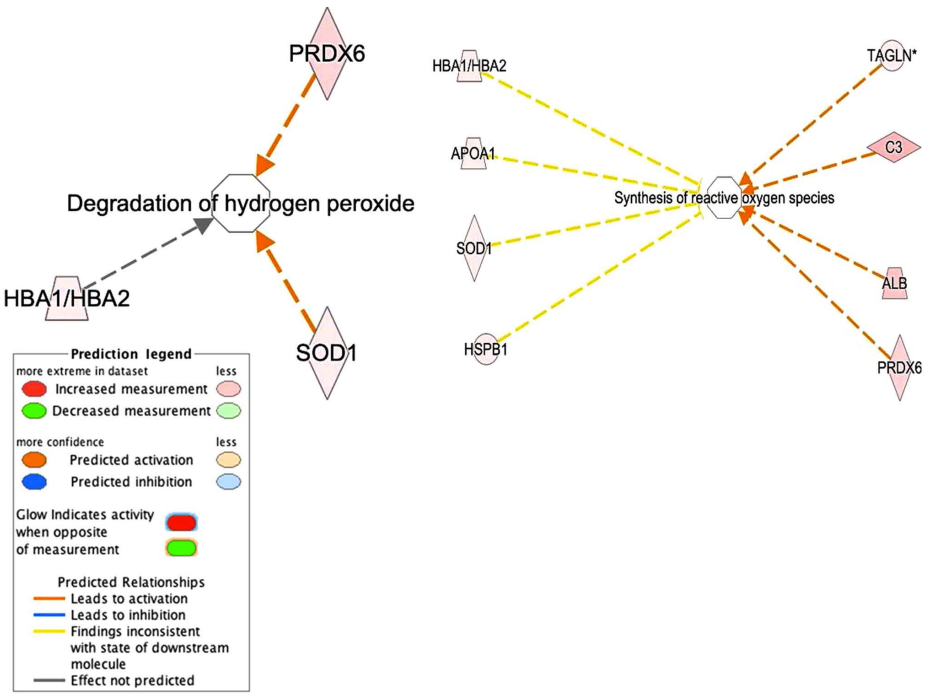

Figure 3. Network of one of the most significant bio-functions. Degradation of hydrogen peroxide and synthesis of reactive oxygen species. The network was constructed using ingenuity pathway analysis.

activation of the synthesis of reactive oxygen species, while HBA1, APOA1, SOD1, HSPB1 are implicated in the inhibition of synthesis of reactive oxygen species. PRDX6 is linked both to the degradation of hydrogen peroxide and to the synthesis of reactive oxygen species.

Discussion

In response to the urgent need for an efficient therapeutic approach for leiomyomas, selective progesterone receptor modulators (SPRMs) might offer a valid option (19,20). This study represents a first attempt to characterize the proteomic profile of leiomyomas after treatment with UPA, in order to identify dysregulated proteins correlated with the drug's mechanisms of action.

Through a combination of 2-DE and mass spectrometry, we identified 30 proteins dysregulated by the pharmacological treatment. Subsequent western blot analysis confirmed the altered expression of three of these proteins: HSPB1, PDIA3, and ACTG2.

Peroxiredoxin 6 (PDRX6), an enzyme involved in the reduction of water and alcohol, exerts its enzymatic activity on H2O2, fatty acids and phospholipid hydroperoxides (21,22).

In our study, PDRX6 was up-regulated (in treated leiomyoma samples) suggesting that its detoxifying function in cancer cells might bear some relation with the mechanisms of action of the drug.

SOD1, which contributes to the destruction of toxic free radicals (23,24) activating the degradation of hydrogen peroxide, also appeared to be up-regulated in treated leiomyoma. Considering that SOD1, as well as PDRX6, are involved in the destruction of free radicals protecting the cell from oxidative stress.

APOA1 is involved in the mechanism of reverse transport of cholesterol from peripheral tissues back to the liver for excretion in the bile (25). Interestingly, it has been shown that APOA1 overexpression can improve abnormal lipid metabolism in non-alcoholic steatohepatitis (NASH). This protective effect is due to its ability to decrease the levels of reactive oxygen species and suppress COX-2-induced inflammation in hepatocytes (26,27). In our study, this protein was down-regulated and this effect could bear some connection with the activity of the drug on leiomyoma cell.

HSPB1 is a molecular chaperone capable of preventing the aggregation of misfolded proteins, an event which is easily triggered/induced in a variety of different types of cancer cells, following treatment with Erastin, a ferroptosis-inducing agent (28,29).

The phosphorylation of HSPB1 reduces the iron-mediated production of lipid reactive oxygen species, thus conferring cancer cells protection against ferroptosis (30,31).

The disruption of HSPB1 expression following UPA treatment, suggests that there may be a correlation between production of lipid reactive oxygen species, ferroptosis, and drug treatment.

TAGLN is a cytoskeletal protein of the calponin family that has been shown/suggested to contribute to replicative senescence (32,33). Downregulation of TAGLN in leiomyomas treated with UPA, induces the synthesis of reactive oxygen species, causing an increase of these reactive species inside the cell.

To our knowledge, this is the first study that applies proteomics to investigate the mechanisms underlying the effectiveness of the treatment of leiomyoma with ulipristal acetate. Using bioinformatic tools, we identified several proteins associated with oxidative stress, suggesting that the mechanisms of action of the drug may be related to its effect on oxidative stress The main limitation of the study is that it doesn't address the functional aspects of the mechanisms of action of UPA. Another limitation of the study is the lack of technical replicates of western blotting.

However, in terms of clinical application, our study offers the advantage of addressing directly the mechanisms involved not only in hormonal metabolisms but also in protein expression, with the aim of identifying new and more effective molecules.

We need to clarify that the aim of our study was to investigate the implication, in terms of oxidative stress, of using ulipristal acetate in leiomyoma from a biochemical point of view. We believe that our findings could direct further studies toward the research for new molecules (in addition to the ulipristal acetate, which has been currently removed from the market) with a specific target to reduce oxidative stress. The ulipristal acetate has been removed from market under EMA (European Medicines Agency) recommendation due to rare side effects to the liver.

In conclusion, by analyzing the leiomyoma tissue of patients treated with UPA and comparing their proteomic profiles with samples obtained from patients receiving no treatment, we found some markers of interest. Several proteins involved in oxidative stress were down- or up-regulated following UPA treatment. Since oxidative stress is critical in a variety of conditions, including cancer, the ability of UPA to modulate this event is highly promising. Therefore, we believe that additional studies would help better understand the role of UPA in the treatment of leiomyoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BU, FS, FR, SB and GR conceived and designed the experiments. YCCDS, MA, BG, CF and EA performed the

experiments. BU, DL, GDL, GS, GA and LM analyzed the data. BU, LM, FR, SB, FS and GR wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All study procedures complied with the Declaration of Helsinki and were approved by the Institutional Review Board of the Institute for Maternal and Child Health-IRCCS 'Burlo Garofolo' (Trieste, Italy) (RC 27/2017). All patients signed a written informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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