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ABSTRACT

Osteoarthritis (OA) and osteochondrosis (OC) are two of the main challenges in orthopaedics, whose definitive diagnosis is usually based on radiographic/arthroscopic evidences. Their early diagnosis should allow preventive or timely therapeutic actions, which are generally precluded from the poor relationships occurring between symptomatology and radiographic evidences. These limitations should be overcome by improving the knowledge on articular tissue metabolism and on molecular factors regulating its normal homeostasis, also identifying novel OA and OC biomarkers suitable for their earlier diagnoses, whenever clinical/pathological inflammatory scenarios between these joint diseases seem somewhat related. To identify proteins involved in their aetiology and progression, we undertook a differential proteomic analysis of equine synovial fluid (SF), which compared the protein pattern of OA or OC patients with that of healthy individuals. Deregulated proteins in OA and OC included components related to inflammatory state, coagulation pathways, oxidative stress and matrix damage, which were suggestive of pathological alterations in articular homeostasis, plasma-SF exchange, joint nutritional status and vessel permeability. Some proteins seemed commonly deregulated in both pathologies indicating that, regardless of the stimulus, common pathways are affected and/or the animal joint uses the same molecular mechanisms to restore its homeostasis. On the other hand, the increased number of deregulated proteins observed in OA with respect to OC, together with their nature, confirmed the high inflammatory character of this disease. Some deregulated proteins in OA found a verification by analysing the SF of injured arthritic joints following autologous conditioned serum treatment, an emergent therapy that provides positive results for both human and equine OA. Being the horse involved in occupational/sporting activities and considered as an excellent animal model for human joint diseases, our data provide suggestive information for tentative biomedical extrapolations, allowing to overcome the limitations in joint size and workload that are typical of other small animal models.This article is part of a Special Issue entitled: Farm animal proteomics.

Keywords: Osteoarthritis Osteochondrosis HorseSynovial fluid Proteomics

1. Introduction

Osteoarthritis (OA) and osteochondrosis (OC) represent two of the main challenges in equine orthopaedics; these diseases are different in terms aethiopathogenesis, synovial joint pathology, clinical presentation and diagnostic/prognostic challenges but, being directed on the same articular-subchondral bone unit, can similarly compromise horse performances, causing career-limiting or career-ending lameness [1–3]. Equine OA is a slow, progressive disease that is characterized by cartilage degeneration, subchondral bone sclerosis, osteophyte formation, and varying degrees of periarticular tissue fibrosis [3,4]. Although the definitive diagnosis is usually based on radiographic and arthroscopic findings, lesions in cartilage and soft tissues often precede bone destruction and joint space narrowing/ deformity [3,4]. A general presence of synovial inflammation in OA has been observed, although its role is a matter of controversy. Equine OA exact aetiology has not been clarified yet and no effective treatments have been discovered [5], most available information were extrapolated from other species, most notably from humans, dogs, and laboratory animals. On the other hand, equine OC has been defined as a primary disease of growth cartilage, which causes disturbance in the process of endochondral ossification [6–8]. It occurs on both articular surface and metaphyseal growth plates; because of joint

incongruity, articular OC may lead to OA [9]. Although some equine OC lesions that cause clinical lameness have been well documented, few early lesions have been studied and the exact sequence of events leading to this disease is yet to be elucidated [10,11]. The causes of OC remain uncertain, but various factors have been implicated, such as skeletal growth rate, nutrition, genetics and physical activity [8].

According to what reported above, there is a limitation in equine OA and OC early diagnosis, which results from the poor relationships occurring between clinical signs and diagnostic imaging findings [12,13]; delayed diagnosis precludes preventive or timely therapeutic actions. These limitations should be overcome by improved knowledge on articular tissue metabolism and molecular factors regulating its normal homeostasis. Proposed diagnostic markers were suggestive of an inflammatory and cartilage turnover condition [14–17] and proved partially effective in early disease diagnosis. In OA, for example, deregulated levels of IL-1a, IL-6 and TNFa, have been reported, although the functional role of these pro- and anti- inflammatory mediators has still to be completely defined [18]. A similar condition was verified for collagen type I and II fragments, prostaglandin E2, osteocalcin, glycosaminoglycans, hyaluronic acid (HA) and proteins related to cartilage turnover, i.e. cartilage oligomeric matrix protein (COMP), matrix metallo-proteinases (MMPs) and disintegrin-metalloproteinases with thrombospondin motifs (ADAMTs), [14,19–26]. Some of these molecules have also been proposed as OC biomarkers [10, 14,27–29], but with the limitations mentioned above [14].

Proteomic approaches applied to human rheumatology investigations have recently proved that studying the expression pattern of synovial fluid (SF) proteins, and its fluctuation during pathological states, may be helpful in clarifying the biology of joint diseases [30–37]. Thus, a number of deregulated proteins have been identified in various human articular pathologies, such as OA [32,37,38], rheumatoid arthritis (RA) [30,37] and juvenile idiopathic arthritis (JA) [36,39], also clarifying many aspects of the SF-plasma exchange [40]. SF was verified as an ideal fluid for biomarker analysis because its composition reflects the joint tissue metabolism and provides information on locally active immunological defence and tissue healing mechanisms [35,41–44]. It was verified that quantitative protein composition of SF is often different with respect to that of plasma and better descriptive of the joint pathophysiological condition [31,35,39,41,43–45]. Thus, SF analysis may represent a complement to diagnostic imaging techniques for detection of early signs of OA and OC. Moreover, synoviocentesis is not such invasive as arthroscopy and other sensitive imaging modalities, sometimes not suitable for all equine joints, where general anaesthesia is often required and may need the use of intra-articular contrast media to identify early cartilage surface alterations [12].

In this study, we undertook a proteomic analysis of equine SF in order to identify proteins involved in the aetiology and progression of these joint diseases. In particular, we compared the pattern of SF proteins from OA or OC equine joints with that of healthy counterparts. This analysis was performed to increase the knowledge on the molecular events associated with both articular diseases as well as to identify deregulated proteins for their diagnosis, to be further validated in dedicated studies. Identified deregulated protein in OA were further assayed by analysing SF of injured arthritic joints following autologous conditioned serum (ACS) treatment, an emergent therapy providing positive results for both human and equine OA [46–48], whose pharmacological mechanism and treatment modalities need further investigations [49]. ACS is a serum enriched in anti-inflammatory cytokines and several growth factors, including IL-1Ra, IGF1-like, PDGF, and TGF-a. ACS treatment is based on the effect of these molecules on the inflammatory response and cartilage erosion [46,47,50,51]. Being the horse involved in occupational/sporting activities and considered as an excellent animal model for human joint diseases [8,52], our data can also provide suggestive information for tentative biomedical extrapolations, allowing to overcome the limitations in joint size and workload that are typical of other small animal models.

2. Materials and methods

2.1. Sample collection

SF samples were obtained by arthrocentesis of different models of high-motion joints, namely metacarpophalangeal and medial femorotibial joints from 10 patients with OA, metacarpo/metatarsophalangeal

and femoropatellar joints from 10 patients with OC, and metacarpo/metatarsophalangeal and femoropatellar joints from 10 healthy animals (CTR). All animals did not have pharmacological treatments before SF sample collection. The details of the samples used in this study are provided in Table 1.

Pathological SF samples were obtained either during diagnostic procedures or prior to arthroscopy from clinical cases referred to the University of Perugia Veterinary Teaching Hospital, following guidelines of the Animal Care and Use Committee of the University of Perugia. Owners of clinical patients signed waivers for their samples to be used in this study. OA diagnosis was based on clinical signs, response to intra-articular analgesia and positive radiographic (periarticular marginal osteophytes, narrowed joint space, subchondral bone sclerosis or lysis) and arthroscopic (cartilage fibrillation, thinning of the cartilage and/or presence of wear lines) findings. OC diagnosis was obtained from radiographic and arthroscopic findings by revealing the presence of osteochondral fragments of metacarpo/metatarsophalangeal (sagittal ridge of the third metacarpus/ metatarsus) and femoropatellar (lateral trochlear ridge of femur) joints. Representative examples of patients with OA and OC are reported in Fig. 1.

Control SF samples were obtained from the joints described above, which had no macroscopic and radiographic abnormalities at post-mortem examination, from animals euthanized for reasons other than orthopaedic diseases. After SF collection, metacarpo/metatarsophalangeal, femoropatellar and medial femorotibial joints of healthy horses were dissected to verify the absence of macroscopic abnormalities; if macroscopic changes consisting of OC/OA lesions were present, the corresponding SF samples were excluded from this study.

2.2. ACS preparation and treatment

ACS was prepared according to kit manufacturer's instructions (Orthokine System ®, Orthogen, Dusseldorf, Germany). Briefly, a 50 ml blood sample was collected from each horse in a syringe with glass beads, under aseptic conditions. After incubation for 24 h, at 37 °C, blood was centrifuged at 2100 x g for 10 min; the serum was removed under sterile conditions, filtered through 0.2 μm membrane, divided into individual doses and stored at -80 °C until used. ACS-treated horses received four ACS joint injections performed under aseptic conditions; each injection was performed every 7–10 days. Injected volume was 3–5 ml depending on the joint size. SF for proteomic analysis was aspirated before the first ACS (-ACS) and the last ACS (+ACS) injection. Indeed, veterinary examination performed after a 3 months follow-up demonstrated that seven out of ten treated horses returned to their athletic activity with workloads similar to those performed before the OA onset.

2.3. Sample processing and 2D-gel electrophoresis

SF samples were placed on ice immediately after collection, mixed with protease inhibitor cocktail P27124 (Sigma) according to manufacturer's instructions, centrifuged at 3000 x g for 20 min, at 4 °C, to remove cells and debris, and stored at -80 °C. Sample supernatants were precipitated with a cold 1:12:1 v:v methanol/ acetone/tributylphosphate mixture for 90 min, at -20 °C, and then centrifuged at 14000 x g, for 35 min, at 4 °C. Pellets were solubilised in 8 M urea, 2 M thiourea, 2% CHAPS, 30 mM Tris, 100 mM 1,4-dithiothreitol and 0.8% ampholytes (Bio-Rad, Hercules, CA, USA). Four hundreds μg of SF proteins were focused on IPG strips, 18 cm long, pH 4–7 and 3–10, by using a Protean IEF Cell (Bio-Rad) at 20 °C; low initial voltage was followed by a voltage gradient from 10,000 to 95,000 Vh, with a limiting current of 50 mA/strip. After focusing, proteins were reduced by incubating the IPG strips with 5 mM tributylphosphine in 10 mL of equilibration buffer (375 mM Tris–HCl pH 8.8, 6 M urea, 20% w/v glycerol, 2% w/v SDS) for 14 min, and then alkylated with 2.5% w/v iodoacetamide in 10 mL of equilibration buffer containing trace of bromophenol blue for 14 min.

Electrophoresis in second dimension was carried out in 9–16%T gradient slab poly- acrylamide gels (180 × 240 × 1 mm) with a Protean apparatus (Bio-Rad), using electrophoresis buffer (25 mM Tris HCl pH 8.3, 1.92 M glycine and 1% w/v SDS), at 40 mA/gels, until the dye front reached the bottom of the gel. 2-DE gels were stained with colloidal Coomassie G250; resulting images were acquired by using a GS-800 imaging system

(Bio-Rad). Each biological sample was analysed in triplicate. pI and molecular mass values were estimated by running a sample containing SF proteins together with a mixture of protein standards (Bio-Rad). Protein concentration was estimated by using the Bradford assay (Bio-Rad).

2.4. Gel image and statistical analysis

Digitized images of the stained gels were analyzed by using the PDQuest (ver 7.4) 2-D analysis software (Bio-Rad), which allowed spot detection, landmark identification, aligning/ matching of spots within gels, quantification of matched spots and their analysis, according to manufacturer's instructions. Manual inspection of the spots was performed to verify the accuracy of automatic gel matching; any errors in the automatic procedure were manually corrected prior to the final data analysis. The spot volume was used as the analysis parameter for quantifying protein expression. The protein spot volume was normalized by using the program "total quantity in valid spots" normalization mode. 2-DE maps were grouped together by using the "replicate group" function. Fold-changes in protein spot levels were calculated between spot volumes in the pathological (OA or OC) group, relative to that in the control gels. Spots whose staining intensity was significantly different in the experimental groups and exhibiting a fold change >2 or <0.5 were further considered for MS identification; fold change value was calculated as ratio of average of spot value in OC or OA vs those of CTR.

2.5. Statistical analysis

To assess overall statistical significance of differential expression among pathological (OA or OC) and control samples, data from PDQuest-normalized volume spots were analysed by using the R program, version 2.13.0. At first, the normality distribution of the mean normalized volume of each spot (n=3) was evaluated by using the Shapiro-Wilk test ($P<0.05$). Next, as the results highlighted a parametric/ nonparametric distribution of spot intensity values, analysis of variance one-way ANOVA and Wilcoxon test were performed. In both cases, a P value<0.05 was considered as significant. Statistical significance of the data before (-ACS) and after (+ACS) was evaluated by using a general linear model analysis of variance, with the horse serving as a random effect and time considered as the fixed effect. Also in this case, a P value<0.05 was considered as significant.

2.6. Protein digestion and MS analysis

Spots from 2-DE were manually excised from gels, minced and washed with water. Proteins were in-gel reduced, S- alkylated and digested with trypsin, as previously reported [53]. Protein digests were subjected to a desalting/concentration step on μZipTipC18 pipette tips (Millipore Corp., Bedford, MA, USA) before MALDI-TOF-MS and/or nanoLC-ESI-LIT-MS/ MS analysis. During MALDI-TOF peptide mass fingerprinting (PMF) experiments, peptide mixtures were loaded on the instrument target together with CHCA as matrix, using the dried droplet technique. Samples were analysed with a Voyager-DE PRO mass spectrometer (Applied Biosystems, USA). Peptide mass spectra were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applied Biosystems).

Peptide mixtures were analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (ThermoFinnigan, USA) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Denmark) [53,54]. Peptide mixtures were separated on an Easy C_{18} column (10 × 0.075 mm, 3 μm) (Proxeon) using a gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile ramped from 5% to 35% over 15 min and from 35% to 95% over 2 min, at a flow rate of 300 nL/min. Spectra were acquired in the range m/z 400–2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 1 min). The mass isolation window and collision energy were set to m/z 3 and 35%, respectively.

2.7. Protein identification

MASCOT software package version 2.2.06 (Matrix Science, UK) [55] was used to identify spots unambiguously from an updated Equus caballus non-redundant sequence database (NCBI nr 2011/02/03). MALDI-TOF PMF data were searched using a mass tolerance value of 50 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. NanoLC-ESI-LIT-MS/MS data were searched by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. MALDI-TOF PMF candidates with a cumulative MASCOT score > 83 or nanoLC-ESI-LIT-MS/MS candidates with more than 3 assigned peptides with an individual MASCOT score > 25, both corresponding to $P < 0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

3. Results

Equine SF samples were analysed by using a proteomic approach based on 2-DE and integrated MS protein identification procedures. Pilot 2-DE experiments indicated that, as with other biological fluids, most of the synovial proteins were well represented within the pH range 4–7. IEF analysis within this pH range allowed improving spot resolution/detection, when compared with that performed in the range 3–10 (data not show). A typical 2D proteomic map, which was obtained for a healthy animal individual, is shown in Fig. 2. In order to evaluate the changes in equine SF protein pattern occurring in OA or OC with respect to control samples, comparative 2-DE gel analyses were then performed. Resulting 2D proteomic maps for synovial fluid showed 263 ± 38, 272 ± 27 and 242 ± 45 spots for healthy, OA and OC joints, respectively. Quantitative variations in the spot volumes between each pathological (OA or OC) and healthy (CTR) condition were calculated from the ratios of average spot density (10 samples in triplicate) in the OA group vs control and in OC group vs control, and are reported with the corresponding fold change values (Table 2).

3.1. Synovial fluid deregulated proteins in osteoarthritis

Comparative 2-DE analysis of SF from OA patients and healthy individuals revealed 66 protein spots exhibiting a significant quantitative variation ($P < 0.05$). Representative details of the gel images showing protein spots differentially represented are reported in Fig. 3. These spots were excised from the gel, digested with trypsin and subjected to MALDI-TOF PMF and/or nLC-ESI-LIT-MS/MS analysis. Table 2 shows the details of the protein identification results, together with the corresponding quantitative spot variation and statistical significance. The occurrence of multiple closely spaced spots, identified as the same protein species, occurring with the horizontal train aspect typical of glycosylated/phosphorylated plasma proteins, led to the final recognition of 17 deregulated protein entries in equine OA.

In particular, we observed a volume increase in OA for horizontal spot patterns corresponding to a-2-macroglobulin (a2- MG), complement component C4A (C4A), carboxylesterase D1 (CE-D1) and ceruloplasmin (CP). A similar trend was also observed for specific spots identified as serotransferrin (ST), anti-thrombin III (AT), vitamin D binding protein (VDBP), inter-a-trypsin inhibitor heavy chain H1 (ITI-H1) and apolipoprotein A-I (APOA1) isoforms. Finally, an apparent proteolysis of serum albumin (SA) was highlighted by the augmented levels of some spots apparently associated with corresponding protein fragments. In contrast, whole spot patterns corresponding to afamin (AF) and plasminogen (PL), or specific spots further assigned to immuno-globulin gamma 1 heavy chain constant region (IG -Hc1), immunoglobulin heavy constant gamma 5 (IgG -Hc5), transthyretin (TTR), haptoglobin (HP) and a-1-B glycoprotein (a1B-G) isoforms generally showed a significant volume decrease in OA.

Gene ontology analysis by DAVID software of deregulated proteins in OA indicated a common grouping of 5, 5, 7, 5, 9, 7, 4 and 3 entries in regulation of response to stimulus, response to wounding, response to stress,

homeostatic process, response to stimulus, transport, defence response, and acute inflammatory response, respectively (Supporting Information Table S1). Regarding functional categories, 16, 17, 12 and 17 entries were classified in signal, secreted, plasma and glycoprotein group, respectively (Supporting Information Table S2). IPA analysis clearly identified main canonical pathways associated with deregulated proteins in OA as acute phase response signalling (ALB, a2-MG, APOA1, C4A, CP, HP, PL and TTR) (P=2.85E-15) and coagulation system (a2-MG, PL and AT) (P=3.36E-06) (Supporting Information Figure S1A). Selected results from IPA network analysis are reported in Fig. 4A; this figure shows the system obtained by merging the two major networks that contain the largest number of interactions for deregulated proteins in OA and their direct/indirect relationships. It also includes some putative biomarkers of OA (HA, MMPs, IL-1a, and others), already identified by using non-proteomic methodologies.

3.2. ACS treatment effects

A comparison of the SF proteomic profile obtained before and after ACS treatment revealed an individual response of the animals to this therapy. Only variations with unambiguous fold changes or deriving from a number of animals major than 4 were further taken into consideration (Table 3). Among the 17 deregulated proteins associated with OA (see previous section), only 6 showed quantitative spot trends suggestive for a general improvement of joint conditions, as also evaluated by resolution of clinical signs recognized at veterinary inspection (see the dedicated experimental section for details). In particular, C4A, CE-D1, a2MG, CP, ST and APO-A1, which were up-regulated during OA, showed decreased levels after ACS treatment ($P<0.05$) (Table 3). This condition was suggestive for their deregulation in OA, although further experiments based on different biochemical approaches are needed to confirm their involvement in this disease.

3.3. Synovial fluid deregulated proteins in osteochondrosis

Comparative 2-DE analysis of SF samples from OC patients and healthy individuals revealed 28 protein spots showing a significant quantitative difference ($P<0.05$). Cropped gel images reporting differentially represented protein spots are shown in Fig. 5. These spots were submitted to analysis for protein identification, which led to the final recognition of 9 deregulated protein entries in equine OC. Corresponding results, together with quantitative spot variations and statistical significance, are reported in Table 2. In particular, a very high volume increase was observed for two spots further identified as fibrinogen regulated components corresponded to complement factor B (CB) and C4A spot patterns, and specific HP isoforms. On the contrary, spots assigned to a-actin (ACT), VDBP, CP, AF and SA were significantly down regulated (Fig. 5).

Gene ontology analysis of deregulated proteins in OC indicated a common grouping of 7, 3, 4, 3, 3 and 2 entries in response to stimulus, response to wounding, response to stress, defence response, response to nutrients and complement activation, alternative pathway, respectively (Supporting Information Table S3). Regarding functional categories, 8 entries (AF, CP, C4A, CB, FG, VDBP, HP and ALB) classified as being involved in plasma, signal and secreted, whereas others (CP, C4A, CB and HP) occurred in acute phase (Supporting Information Table S4). Also in the case of deregulated proteins in OC, IPA analysis clearly identified main canonical pathways as acute phase response signalling (ALB, C4A, CB, CP and HP) ($P = 1.04E-08$) and complement system (C4A and CB) ($P = 1.31E-04$) (Supporting Information Figure S1B). Selected results from IPA network analysis are reported in Fig. 4B, which illustrates the network system obtained by merging those that contained the largest number of interactions for deregulated proteins in OC, and their relationships. It includes as central nodes some pro- and anti-inflammatory mediators (IL-6 and TNFa) already identified by using non-proteomic approaches as putative biomarkers of OC.

4. Discussion

In this study, we analysed the protein profile of SF from healthy, osteoarthritic and osteochondritic equine joints

by a classical gel-based proteomic approach. Resulting 2-DE maps were generally equivalent to that obtained for well and diseased human counterparts, either for the number of spots and their pI/Mr values [38]. They demonstrated that SF proteomic analysis could reveal distinctive protein patterns in diseased equine joints. Observed protein variations in OA and OC, associated with components related to inflammatory state, coagulation pathways, oxidative stress and matrix damage, were suggestive of pathological alterations in articular homeostasis, plasma-SF exchange, joint nutritional status and vessel permeability. In particular, our proteomic data on equine OA were in good agreement with previous gel-based and gel-free studies on the human disease, as deduced for a2-MG, HP, CP, VDBP, APOA1, C4A, ITI-H1 and a1B-G isoforms [37,38]. Other deregulated proteins observed in these investigations were probably not detected here as result of the different proteomic technologies used [32,37,38]. Moreover, our data on equine OA partially resembled that obtained for human JA, as verified for ST, VDBP, APOA1, a2-MG, HP, CP, inter-a-trypsin inhibitor and Ig-chain isoforms [36,39,40], suggesting related pathological mechanisms between these diseases. Conversely, no comparison was possible between equine and human OC, since no proteomic data are available on the latter case. Moreover, no putative biomarkers of equine OA and OC [14], already proposed by non-proteomic approaches, were observed in this study. This finding was related to their low/high molecular mass value or their low relative abundance in SF. Some of these biomarkers are present in the networks resulting from IPA analysis of the deregulated proteins we detected in OA and OC, confirming the consistency of our data.

Some equine proteins (C4A, HP, and AF) seemed commonly deregulated in both animal pathologies indicating that, regardless of the stimulus, common pathways are affected and/or the animal joint uses the same molecular mechanisms to restore its homeostasis. On the other hand, the increased number of deregulated proteins observed in OA with respect to OC, together with their nature, was suggestive of the high inflammatory character of this disease, but also underlined different metabolic implications between OA and OC. In fact, although GO analysis generally classified OA deregulated proteins as acute phase components and/or involved in complement and coagulation cascades, and homeostatic processes, their quantitative changes have also to be considered in light of their specific role/function in joint tissue physiology. In particular, various deregulated proteins related to inflammatory status observed in OA have been reported also to play a direct/indirect effect on the enzymatic activity of matrix proteases and on extracellular matrix (ECM) turnover. For example, a2MG (up-regulated in OA–Table 2) modulates MMPs/ ADAMTs activity [56] and its macrophage production [57], plays a distinct role in ECM turnover [58] and is overexpressed in non-infectious equine arthritis [59], human OA, JA and RA [36,38,60]. Similarly, ITI-H1 (up-regulated in OA samples–Table 2) plays a prominent role in HA metabolism, affects the function of various matrix proteins and has been already associated with OA and RA [37,61]. Deregulated proteolytic activity described in OA joints [62,63], eventually attributable to MMPs or other proteases, found a positive confirmation in our proteomic data on OA (Table 2), which showed a decrease of intact SA and IgG components and a corresponding increase of SA fragments, as already observed for human RA [64,65].

Activation of the complement system (CS) has been implicated in the pathophysiology of various articular diseases [66]. In fact, some ECM proteins or their degradation products have been reported to activate complement pathways, which pro- mote joint inflammation [67], blood vessel permeability and cellular release of lysosomal enzymes, ultimately damaging articular cartilage [68]. Local production of CS components by cartilage chondrocytes and synoviocytes is an important issue in the pathophysiology of joint diseases [69–71]. Our proteomic data are in line with these observations and other proteomic studies on human OA and JA [36,38–40], and prove the activation of the CS either in OA and OC. However, different complement pathways seem triggered in these diseases, as indicated by the increase in C4A (OA) and that in C4A and CB (OC) (Table 2). This phenomenon has been already related to the differential expression of COMP or its fragments [67,72], a known biomarker of OA [19,25]. In particular, CB levels may suggest the occurrence of an alternative pathway related to defects of endochrondral ossification in OC. This alternative pathway has been already reported to putatively play a significant role in cartilage transformation to bone [66,73].

Differences between equine OA and OC were also observed for coagulation enzymes (Table 2). In particular, concomitant increase of AT and decrease of PL suggested a possible alteration of the coagulation system in OA (Supporting Information Figure S1A). AT augmented levels and reduced activity have been already reported in human RA [74]; AT inactivation was related to the high concentration of free HA [75], another known biomarker of equine OA. On the other hand, the over-expression of FG present in OC (Table 2) emphasized an altered coagulation also in this disease, with possible clot formation and inappropriate tissue calcification [76]. Its increased expression levels, together with that of AT, C4A and PL, found a positive confirm in previous studies on human OA, JA and OC [36,38,77].

In OA, a number of proteins involved in joint homeostasis appeared selectively deregulated, thus reflecting previous observations on differential joint and systemic circulation [78]. High levels of lipoproteins and lipids have also been reported to contribute to articular inflammation [79–81]; excessive lipids in OA cartilage have been described to determine matrix degradation [82]. In agreement with these observations, increased APOA1 and VDBP levels were observed in OA (Table 2). Toxicity of vitamin D overload with respect to bone resorption [83] and the negative effect of excess lipids on joints have been already documented [82]. In this context, the augmented CE-D1 levels present in OA (Table 2) should be also suggestive for a local attempt to catabolize noxious lipid esters, metabolic debris and toxicants [84]. Further inputs on joint homeostasis impairment in OA derived from the measured levels of ST, CP and AF (Table 2), which act as Fe, Cu and vitamin E carriers, respectively [85]. Imbalance in redox homeostasis and consequent ROS generation should contribute to cartilage degradation by different mechanisms acting on collagenase, proteoglycan synthesis and proteins involved in ECM turnover [86]. Oxidative damage, low vitamin E levels and Fe overload due to increased ST levels have already been reported in OA [87–89]. Other carrier proteins, such as TTR or SA, were found under-expressed in OA (Table 2), suggesting an alteration in the supply of important nutrients, vitamins and elements in pathological joints. Nutritional unbalances have been reported in the aethiopathogenesis of both OA and OC [3].

Contrarily to OA, decreased CP and VDBP levels were specifically observed in OC samples (Table 2). These findings are not surprising since OC is generally associated with a defect in cartilage-to-bone transformation. Down-regulated expression of these proteins can affect the expression of lysyl oxidase [90], a Cu-dependent enzyme involved in ECM remodeling and collagen cross-linking [91]; low Cu levels and decreased collagen cross-linking have been described in OC aethiopathogenesis [3,92]. On the other hand, reduced VDBP levels can induce alterations in cartilage and bone homeostasis requiring vitamin D for physiological turnover. VDBP role in joint diseases has been related to vitamin D binding protein-macrophage activating factor [93], a potent stimulator of osteoclastic activity also involved in bone remodeling and skeletal development [94]. A specific decrease of ACT was also observed in OC (Table 2), which was tentatively associated with a reduction of cartilage components and/or impairment in its cellular turnover [71,95,96].

The meaning of the proteomic profile changes observed in OA sample was partially confirmed by the results obtained from the comparison of SF proteomes from OA-affected patients before and after ACS treatment. This study was performed by comparing SF samples following a 30–40 days of ACS treatment (four injections), which were sampled from animals that in part (7/10) returned to previous levels of their athletic activities. Since ACS is enriched in IL-1Ra and several growth factors that diminish concentration of inflammation mediators and their effects [46,47,50,51] it was not surprising that positive acute phase proteins, such as C4A, a2MG and CP, were reduced following ACS treatment (Table 3).

5. Conclusions

This study describes the first application of classical proteomic technologies to the analysis of equine SF from patients with OA and OC. Distinctive protein patterns were observed, which confirmed previous observations on human arthritic joints [32,36–40] and unveiled functional networks with already known equine biomarkers [14]. Observed protein variations, associated with components related to inflammation, coagulation, oxidative stress and matrix damage pathways, were suggestive of pathological alterations in articular homeostasis, plasma-SF exchange, joint nutritional status and vessel permeability. Further holistic studies on deregulated metabolic

processes identified here for equine OA and OC are necessary; they could definitively benefit of the integrated "omic" platforms to unveil concomitant deregulation of proteins with different molecular mass and absolute concentration in SF. They will eventually disclose the role of some carrier proteins affecting nutrient supply in pathological joints, which may provide inadequate support to articular tissues to reverse the homeostatic challenges induced by OC and OA stimuli. Presence and possible effects of non-physiological deposits of fibrin, lipids and iron have also to be further explored.

Acknowledgements

This study was partially supported by grants from MIUR (PRIN2007_RKYAZS_001), Fondazione Cassa di Risparmio di Perugia (no. 2008.021.0376) and Regione Campania (Rete di Spettrometria di Massa, RESMAC).

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2012.02.008.

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Fig. 1– Representative radiographic images of equine joints. Panel A, dorsoproximal-palmarodistal view of a normal equine metacarpophalangeal joint. Panel B, dorsoproximal-palmarodistal view of an OA equine metacarpophalangeal joint; the arrows indicate osteophyte formation at the medial aspect of dorsomedial proximal phalanx an distomedial aspect of MCIII.

Panel C, plantarolateral-dorsomedial oblique view of a normal hock. Panel D, lantarolateral-dorsomedial oblique view of an OC equine hock with a large osteochondral fragment separated from the distal intermediate ridge of the tibia (as indicated by the arrow).

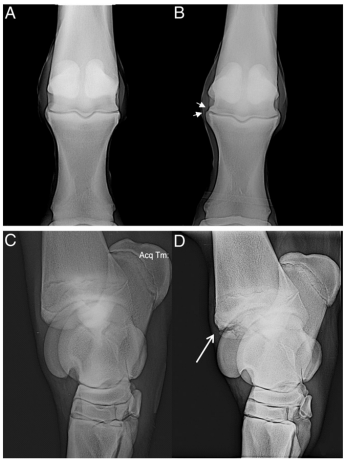

Fig. 2 – Representative 2-D gel (pH 4–7, 9–16%T) image for equine SF after staining with colloidal Coomassie. Differentially protein spots, exhibiting a statistically significant fold change in osteochondrosis and osteoarthritis are reported and numbered.

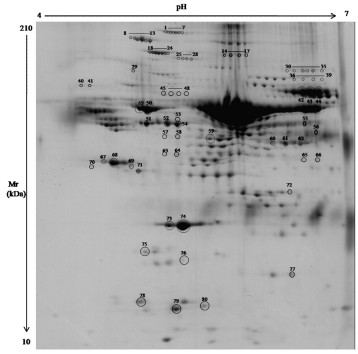

Fig. 3 – Differential 2-DE analysis of equine SF from healthy (CTR) and osteoarthritis (OA) affected animals. An expanded view of the gel portions including the deregulated proteins is shown. ITI-H1, inter-a-trypsin inhibitor heavy chain H1; APOA1, apolipoprotein A-I; a2-MG, a-2-macroglobulin; ST, serotransferrin; AT, antithrombin III; a1B-G, a-1-B glycoprotein; IG -Hc1,1 heavy chain constant region; IgG -Hc5, immunoglobulin heavy constant gamma 5; HP, haptoglobin; CP, ceruloplasmin; C4A, complement component C4A; AF, afamin; CE-D1, carboxylesterase D1; PL, plasminogen; VDBP, vitamin D binding protein; TTR, transthyretin; SA, serum albumin.

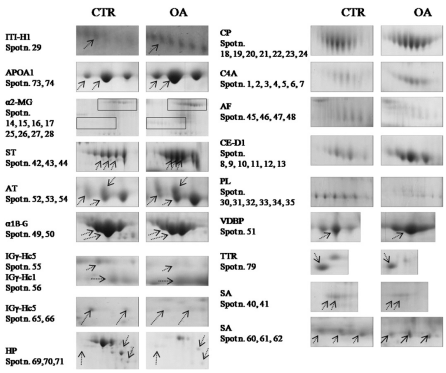

Fig. 4 – Networks of deregulated proteins in equine OA (panel A) and OC (panel B). Images were obtained by merging of networks found by the IPA software. Grey nodes represent MS-identified protein, whereas white nodes were inserted by the software. Various shapes represent different functional protein classes: concentric circles, groups or complexes; down-pointing triangles, kinases; diamonds, other enzymes; horizontal ovals, transcription regulators; vertical ovals, transmembrane receptors; vertical rectangles G-protein–coupled receptors; horizontal rectangles, ligand-dependent nuclear receptors; circles, other actors. Solid and dashed lines indicate direct or indirect interactions between nodes, respectively. Lines beginning and ending on the same node show self-regulation. Arrowhead shows directionality of the relationship.

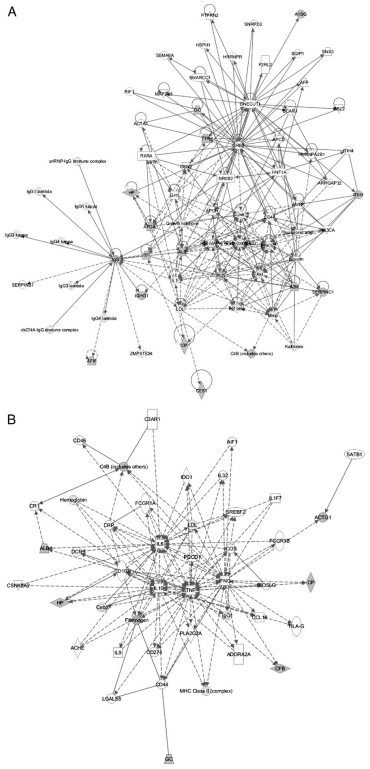

Fig. 5 – Differential 2-DE analysis of equine SF from healthy (CTR) and osteochondrosis (OC)-affected animals. An expanded view of the gel portions including the deregulated proteins is shown. FG, fibrinogen chain; CB, complement factor B; ACT, a-actin; CP, ceruloplasmin; VDBP, vitamin D binding protein; C4A, complement component C4A; AF, afamin; HP, haptoglobin; SA, serum albumin.

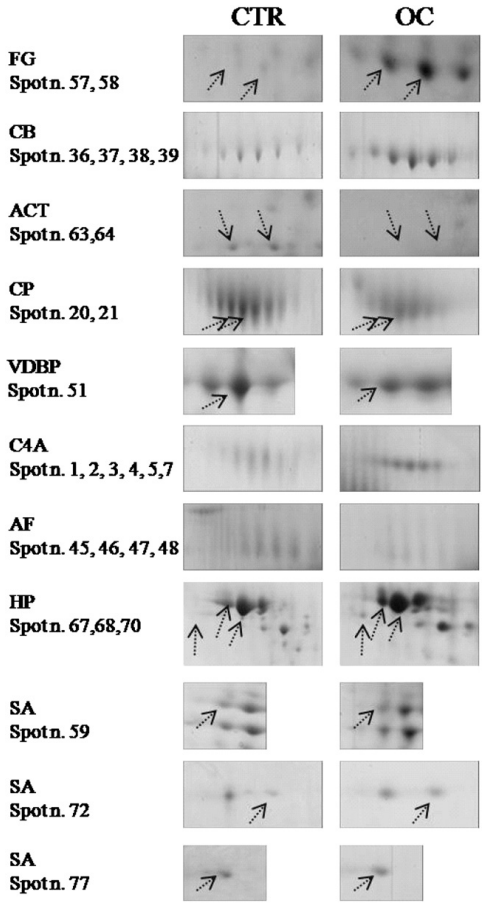

Table 1 – Horse and joint characteristics of synovial fluid samples. OA, osteoarthritis; OC, osteochondrosis; ACS, autologous conditioned serum.

	Control (n. 10)	OA (n. 10)	ACS (n. 10)	OC (n. 10)
Age (range)	1–14 years	4–10 years	4–12 years	1–1.5 years
Sex (n)				
Female	2	5	4	4
Male	2	1	1	6
Gelding	6	4	5	0
Joints (n)				
Metatarsophalangeal	4	0	0	6
Metacarpophalangeal	3	6	5	2
Femoropatellar	3	0	0	2
Medial femorotibial	0	4	5	0
Breed (n)				
Thoroughbred	2	6	3	8
Warmblood	8	4	2	0
Standardbred	0	0	2	2
Diagnosis/Group inclusion criteria	Absence of macroscopic and radiographic abnormalities of OA/OC at post-mortem. No history of orthopedic diseases or lameness at list 6 months before euthanasia	Lameness, positive diagnostic anaesthesia, radiographic and arthroscopic findings of OA	Lameness, positive diagnostic anaesthesia, radiographic and arthroscopic findings of OA	Radiographic and arthroscopic findings with or without joint effusion

Table 2 – Proteins with changed expression levels in the synovial fluid of horses suffering with osteoarthritis (OA) and osteochondrosis (OC). Spot number, protein name, gene number according to the NCBI database, theoretical and experimental pI and Mr values, peptide number, sequence coverage, Mascot score identification method, organism, fold change and relative P values are listed. EC, PMF and TMS indicate *Equus caballus*, MALDI-TOF peptide mass fingerprinting and nLC-ESI-LIT-MS/MS, respectively.

Spot	Protein name	NCBI Accession	Theoretical pI/Mr (kDa)	Experimental pI/Mw (kDa)	Peptides	Sequence coverage (%)	MASCOT Score	ID method	Matched/ observed peptides	Organism	Fold change (disease *vs* control)	P value
1	Complement component C4A	149732359	6.83/192	4.82/210	3	2	141	TMS		EC	3.4 OA	0.0007 OA
											5.5 OC	0.0002 OC
2	Complement component C4A	149732359	6.83/192	4.89/205	2	3	129	TMS		EC	2.2 OA	0.0019 OA
											4.9 OC	0.0013 OC
3	Complement component C4A	149732359	6.83/192	4.94/206	4	3	232	TMS		EC	2.9 OA	0.0299 OA
											4.5 OC	0.0006 OC
4	Complement component C4A	149732359	6.83/192	4.99/205	11	8	117	PMF	11/12	EC	3.1 OA	0.0015 OA
											6.3 OC	0.0013 OC
5	Complement component C4A	149732359	6.83/192	5.04/204	10	7	105	PMF	10/11	EC	2.6 OA	0.0023 OA
											3.7 OC	0.0017 OC
6	Complement component C4A	149732359	6.83/192	5.09/203	6	5	201	TMS		EC	3.7 OA	0.0019 OA
7	Complement component C4A	149732359	6.83/192	5.13/204	2	2	124	TMS		EC	9.7 OA	0.0001 OA
											10.1 OC	0.0009 OC
8	Carboxylesterase D1	149699076	5.51/61	4.58/158	4	10	188	TMS		EC	4.7 OA	0.0007 OA
9	Carboxylesterase D1	149699076	5.51/61	4.61/161	5	13	235	TMS		EC	4.6 OA	0.0015 OA
10	Carboxylesterase D1	149699076	5.51/61	4.62/160	10	22	115	PMF	10/15	EC	3.1 OA	0.0001 OA
11	Carboxylesterase D1	149699076	5.51/61	4.69/158	11	25	121	PMF	11/15	EC	2.1 OA	0.0127 OA
12	Carboxylesterase D1	149699076	5.51/61	4.64/160	6	16	258	TMS		EC	4.3 OA	0.0474 OA
13	Carboxylesterase D1	149699076	5.51/61	4.67/159	10	22	72	PMF	10/19	EC	2.1 OA	0.0304 OA
14	α-2-Macroglobulin	194211675	6.24/164	5.87/191	33	30	1298	TMS		EC	2.1 OA	0.0025 OA
15	α-2-Macroglobulin	194211675	6.24/164	5.93/191	7	6	300	TMS		EC	2.3 OA	0.0006 OA
16	α-2-Macroglobulin	194211675	6.24/164	6.04/192	21	20	820	TMS		EC	2.3 OA	0.0052 OA
17	α-2-Macroglobulin	194211675	6.24/164	5.97/191	32	21	1239	TMS		EC	2.4 OA	0.0053 OA
18	Ceruloplasmin	149729967	5.36/122	4.74/149	10	13	112	PMF	10/13	EC	2.5 OA	0.0057 OA
19	Ceruloplasmin	149729967	5.36/122	4.77/148	14	16	141	PMF	14/20	EC	2.0 OA	0.0266 OA
20	Ceruloplasmin	149729967	5.36/122	4.81/148	13	16	119	PMF	13/22	EC	2.1 OA	0.0055 OA
											0.4 OC	0.0115 OC
21	Ceruloplasmin	149729967	5.36/122	4.84/148	10	9	472	TMS		EC	2.1 OA	0.0056 OA
											0.5 OC	0.0422 OC
22	Ceruloplasmin	149729967	5.36/122	4.89/148	12	15	144	PMF	12/15	EC	2.4 OA	0.0075 OA
23	Ceruloplasmin	149729967	5.36/122	4.92/148	11	15	132	PMF	11/14	EC	4.1 OA	0.0172 OA
24	Ceruloplasmin	149729967	5.36/122	4.97/149	6	7	112	PMF	10/13	EC	3.1 OA	0.0061 OA
25	α-2-Macroglobulin	194211675	6.24/164	5.42/110	19	19	742	TMS		EC	2.5 OA	0.0300 OA
26	α-2-Macroglobulin	194211675	6.24/164	5.49/109	16	15	674	TMS		EC	4.3 OA	0.0449 OA
27	α-2-Macroglobulin	194211675	6.24/164	5.57/109	17	17	710	TMS		EC	3.4 OA	0.0145 OA
28	α-2-Macroglobulin	194211675	6.24/164	5.65/110	12	13	689	TMS		EC	3.5 OA	0.0384 OA
29	Inter-α-trypsin inhibitor heavy chain H1-like	149728587	6.55/101	4.69/102	3	5	165	TMS		EC	2.2 OA	0.0047 OA
30	Plasminogen	149743990	6.37/91	6.31/101	3	6	141	TMS		EC	0.2 OA	0.0013 OA
31	Plasminogen	149743990	6.37/91	6.41/102	4	7	160	TMS		EC	0.3 OA	0.0464 OA
32	Plasminogen	149743990	6.37/91	6.50/103	5	7	175	TMS		EC	0.1 OA	0.0001 OA

Table 2 (continued)

Spot	Protein name	NCBI Accession	Theoretical pI/Mr (kDa)	Experimental pI/Mw (kDa)	Peptides	Sequence coverage (%)	MASCOT Score	ID method	Matched/ observed peptides	Organism	Fold change (disease vs control)	P value
33	Plasminogen	149743990	6.37/91	6.60/102	4	4	98	TMS		EC	0.2 OA	0.0072 OA
34	Plasminogen	149743990	6.37/91	6.75/102	9	12	199	TMS		EC	0.5 OA	0.0119 OA
35	Plasminogen	149743990	6.37/91	6.88/101	7	10	210	TMS		EC	0.3 OA	0.0010 OA
36	Complement factor B	149732066	6.75/86	6.33/96	17	29	554	TMS		EC	2.2 OC	0.0324 OC
37	Complement factor B	149732066	6.75/86	6.42/97	17	30	536	TMS		EC	2. 4 OC	0.0138 OC
38	Complement factor B	149732066	6.75/86	6.49/98	14	19	489	TMS		EC	3.5 OC	0.0006 OC
39	Complement factor B	149732066	6.75/86	6.55/98	3	3	65	TMS		EC	3.8 OC	0.0001 OC
40	Serum albumin	126723507	5.95/69	4.18/134	6	12	191	TMS		EC	0.3 OA	0.0204 OA
41	Serum albumin	126723507	5.95/69	4.27/133	8	18	290	TMS		EC	0.3 OA	0.0421 OA
42	Serotransferrin	126352628	6.83/78	6.35/76	20	36	199	PMF	20/27	EC	2.3 OA	0.0005 OA
43	Serotransferrin	126352628	6.83/78	6.42/76	11	21	111	PMF	11/14	EC	3.3 OA	0.0011 OA
44	Serotransferrin	126352628	6.83/78	6.52/77	11	24	123	PMF	11/13	EC	2.2 OA	0.0028 OA
45	Afamin	149701611	5.49/69	4.96/77	3	7	99	TMS		EC	0.2 OA	0.0050 OA
											0.1 OC	0.0043 OC
46	Afamin	149701611	5.49/69	5.00/76	9	23	296	TMS		EC	0.1 OA	0.0012 OA
											0.1 OC	0.0035 OC
47	Afamin	149701611	5.49/69	5.11/78	5	10	165	TMS		EC	0.3OA	0.0291 OA
											0.2 OC	0.0142 OC
48	Afamin	149701611	5.49/69	5.26/76	3	10	130	TMS		EC	0.3 OA	0.0185 OA
											0.3 OC	0.0414 OC
49	α-1B-glycoprotein	194216172	8.74/68	4.68/65	8	25	68	PMF	8/27	EC	0.5 OA	0.0006 OA
50	α-1B-glycoprotein	194216172	8.74/68	4.73/66	9	26	90	PMF	9/22	EC	0.5 OA	0.0001 OA
51	Vitamin D binding protein	149701606	5.46/54	4.68/58	5	9	650	TMS		EC	2.1 OA	0.0036 OA
											0.5 OC	0.0513 OC
52	Antithrombin III	149708147	6.31/52	4.76/56	12	26	119	PMF	12/19	EC	2.2 OA	0.0054 OA
53	Antithrombin III	149708147	6.31/52	4.85/57	13	26	126	PMF	13/22	EC	2.1 OA	0.0188 OA
54	Antithrombin III	149708147	6.31/52	4.87/55	8	23	642	TMS		EC	2.3 OA	0.0005 OA
55	Ig γ 5 heavy chain constant region	18996195	5.95/35	6.70/55	6	30	73	PMF	6/12	EC	0.2 OA	0.0001 OA
56	Ig γ 1 heavy chain constant region	15020816	7.68/37	6.70/50	4	20	173	TMS		EC	0.3 OA	0.0004 OA
57	Fibrinogen γ chain	194208381	5.27/52	4.93/49	10	26	313	TMS	14	EC	7.1 OC	0.0027 OC
58	Fibrinogen γ chain	194208381	5.27/52	5.07/49	10	30	322	TMS	13	EC	7.5 OC	0.0010 OC
59	Serum albumin	126723507	5.95/69	5.49/47	36	54	466	PMF	36/40	EC	2.1 OC	0.0240 OC
60	Serum albumin	126723507	5.95/69	6.15/46	7	14	101	PMF	7/8	EC	3.1 OA	0.0354 OA
61	Serum albumin	126723507	5.95/69	6.30/46	12	22	162	PMF	12/15	EC	2.1 OA	0.0163 OA
62	Serum albumin	126723507	5.95/69	6.48/46	6	10	168	TMS		EC	5. 3 OA	0.0044 OA
63	Actin. cytoplasmic 1	46397332	5.29/42	4.93/46	13	38	599	TMS		EC	0.2 OC	0.0001 OC
64	Actin. cytoplasmic 1	46397332	5.29/42	5.06/46	9	37	289	TMS		EC	0.2 OC	0.0006 OC
65	Ig γ 5 heavy chain constant region	18996195	5.95/36	6.72/40	2	7	66	TMS		EC	0.2 OA	0.0019 OA
66	Ig γ 5 heavy chain constant region	18996195	5.95/36	6.76/40	3	15	76	TMS		EC	0.3 OA	0.0105 OA
67	Haptoglobin	149699777	5.59/38	4.29/41	4	15	164	TMS		EC	6.6 OC	0.0031 OC
68	Haptoglobin	149699777	5.59/38	4.37/40	8	28	256	TMS		EC	3. 3 OC	0.0112 OC
69	Haptoglobin	149699777	5.59/38	4.70/38	6	25	100	PMF	6/8	EC	0.4 OA	0.0004 OA
70	Haptoglobin	149699777	5.59/38	4.30/39	2	8	132	TMS		EC	2.5 OA	0.0194 OA
											3.7 OC	0.0003 OC
71	Haptoglobin	149699777	5.59/38	4.79/35	4	20	188	TMS		EC	0.4 OA	0.0526 OA
72	Serum albumin	126723507	5.95/69	6.26/31	28	39	326	PMF	28/35	EC	2.1 OC	0.0129 OC
73	Apolipoprotein A-I	149716548	5.65/30	4.85/24	12	41	622	TMS		EC	2.1 OA	0.0006 OA
74	Apolipoprotein A-I	149716548	5.65/30	5.05/24	13	38	167	PMF	13/22	EC	2.0 OA	0.0473 OA
75	Non-identified			4.62/20				TMS			3.8 OA	0.0004 OA
76	Non-identified			4.69/19				TMS			5.3 OA	0.0001 OA
77	Serum albumin	126723507	5.95/69	6.34/17	7	15	265	TMS		EC	3.8 OC	0.0370 OC
78	Non-identified			4.64/13				TMS			0.1 OA	0.0002 OA
79	Transthyretin	149720864	5.36/16	4.93/14	5	62	75	PMF	5/13	EC	0.5 OA	0.0212 OA
80	Serum albumin	126723507	5.95/69	5.43/14	4	9	163	TMS		EC	8.1 OC	0.0010 OC

Table 3 – Proteins with changed expression levels in synovial fluid of horses with OA after ACS treatment. Protein name, spot number, fold change, relative P value and number of horses out of all treated individuals with univocal response are listed. Clinical examination after a 3 months follow-up demonstrated that seven out of ten treated horses returned to their athletic activity with workloads similar to those performed before the OA onset. OA, osteoarthritis; ACS, autologous conditioned serum. C4A, complement component C4A; CE-D1, carboxylesterase D1; α2-MG, α-2-macroglobulin; CP, ceruloplasmin; ST, serotransferrin; APOA1, apolipoprotein A-I.

Protein	Spot	Fold Change (+ACS/-ACS)	P value	Proteomic observation in treated horses/total
C4A	1	0.38	0.0184	7/10
	2	0.31	0.0512	7/10
	3	0.43	0.0276	7/10
	4	0.40	0.0186	5/10
	5	0.39	0.0420	5/10
	6	0.47	0.0296	8/10
	7	0.33	0.0351	5/10
CE-D1	8	0.44	0.0359	4/10
	10	0.42	0.0410	7/10
	12	0.44	0.0096	9/10
	13	0.46	0.0118	7/10
α2MG	14	0.43	0.0332	4/10
	15	0.42	0.0481	4/10
	16	0.45	0.0466	4/10
	17	0.46	0.0485	4/10
	25	0.28	0.0293	5/10
	26	0.36	0.0304	5/10
	27	0.34	0.0326	6/10
	28	0.27	0.0363	7/10
CP	18	0.44	0.0146	6/10
	20	0.43	0.0058	6/10
	21	0.38	0.0180	6/10
	22	0.38	0.0229	5/10
	23	0.49	0.0476	4/10
ST	42	0.41	0.0257	5/10
	43	0.45	0.011	7/10
	44	0.33	0.0082	6/10
APOA1	74	0.39	0.0363	5/10