Identification of possible new salivary biomarkers of stress in sheep using a high-resolution quantitative proteomic technique

Damián Escribano[a]*, Anita Horvatić[b], Maria Dolores Contreras-Aguilar[c], Nicolas Guillemin[b], Jose Joaquín Cerón[c], Marina Lopez-Arjona[c], Maria Luisa Hevia[d], Peter David Eckersall[b,e] PD, Xavier Manteca[b], Vladimir Mrljak[b]

[a] *Department of Animal and Food Science, School of Veterinary Science, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès (Barcelona), Spain.*

[b]*ERA Chair FP7, Internal Diseases Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Croatia.*

[C]*Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo s/n, 30100 Espinardo, Murcia, Spain.*

[d]*Department of Animal Production, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo s/n, 30100 Espinardo, Murcia, Spain.*

[e]*Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Bearsden Rd, Glasgow, G61 1QH, UK.*

* Corresponding author. Tel.: +34 93.518.1352.

E-mail address: Damian.Escribano@uab.cat (D. Escribano).

Abstract

The aim of this study was to identify biological pathways and proteins differentially expressed in the saliva proteome of sheep after the application of a model of stress, using high-resolution quantitative proteomics. In addition, one of the proteins differently expressed was verified and evaluated as a possible biomarker of stress in this species. Saliva paired samples from eight sheep before and after the application of a model of stress based on shearing were analysed using tandem mass tags (TMT). The TMT analysis allowed for the identification of new stress-related metabolic pathways and revealed 13 proteins differentially expressed between before and after the stress. Six of these proteins pertain to four major metabolic pathways affected, namely: canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. The rest of proteins were unmapped original proteins such as acyl-coenzyme-A-binding protein; complement C3; alpha-2-macroglobulin isoform-X1; type-II small proline-rich protein; lactoferrin; secretoglobin family-1D-member; and keratin, type-II cytoskeletal 6. Of these proteins, based on its biological significance and specific immunoassay availability, lactoferrin was selected for further validation. The immunoassay intra- and inter-assay coefficients of variation were lower than 13%. The method showed good linearity under dilution and recovery, and the detection limit was low enough to detect salivary lactoferrin levels. A significant decrease ($P < 0.01$) in salivary lactoferrin concentration in the sheep following the application of the model of stress was observed, suggesting that this protein could be a potential salivary biomarker of stress situations in sheep.

Keywords: Ovine, Lactoferrin, Saliva, Stress, TMT analysis

1. Introduction

In recent years, there has been growing interest for the identification of biomarkers in saliva to monitor animal welfare. Saliva sampling is a noninvasive and stress-free procedure and can represent an alternative to blood sampling, which has been demonstrated to be a confounding factor in stress studies of animals (Merlot et al., 2011; Escribano et al., 2013). In addition, the ability to perform multiple saliva procedures without an evident influence on the stress response of animals has been an advantage in trials in which the immediate response of an animal to a stressor is studied (Heintz et al., 2011). Furthermore, saliva collection requires only modest levels of personnel training, facilitating animal monitoring in remote locations (Fuentes et al., 2016).

Proteomic techniques have become widely employed as useful tool for the search of novel biomarkers in biofluids. The comparative quantitative analysis of complex protein samples can be achieved either with two-dimensional gel electrophoresis–based proteomics or gel-free mass spectrometry-based approaches using isobaric tagging options such as isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT). These novel technologies that employ isobaric tags have emerged in the last few years and are becoming widely employed, since they are highly reproducible and sensitive and allow for the relative simultaneous quantification of differentially labelled peptides (Baeumlisberger et al., 2010; Dayon et al., 2011; Giron et al., 2011). In veterinary science, recent investigations using these novel quantitative technologies to detect possible biomarkers of diagnostic and understand the prognosis of diseases in dogs (Martínez-Subiela et al., 2017; Franco-Martínez et al., 2018) have been reported. Also, studies performed in sheep using these techniques to evaluate the alterations in the intestine associated with under weaning stress using jejunum samples (Cui et al., 2018) or to consider age-related changes in the cerebrospinal fluid (Chen et al., 2018) have been published. However, to the best of our knowledge, no reports currently

exist on the use of isobaric tagging technology in saliva samples to detect possible biomarkers of stress in sheep.

Despite this lack of published research, in the last several years, there has been increasing interest in the measurement of saliva biomarkers to evaluate stress in sheep, and analytes such as cortisol, alpha-amylase, and lipase have been assayed to determine their increase in concentration after different stressful stimuli (Fuentes et al., 2016; Mesori et al., 2017; Contreras-Aguilar et al., 2018). The insight in various biological pathways in saliva induced by stress as well the identification of new proteins that can change in stressful situations can help researchers to gain knowledge about the status of salivary secretory proteome components and to identify new possible biomarkers that may be helpful to prevention of the stress situations.

The hypothesis of our study was that the application of TMT proteomic technology in sheep saliva samples could uncover novel pathways and identify new proteins capable of changing in situations of stress that could be considered as novel stress biomarkers. To test this hypothesis, the objectives of the present study were; (1) the identification of the metabolic pathways and proteins differentially represented in the saliva after stress by proteomic approach; (2) the selection of at least one protein, differentially expressed after stressors, and its validation by immunoassay and evaluation as a possible biomarker of stress in sheep.

2. Materials and methods

2.1 Animal and sampling procedures

Details describing sheep model, in which salivary biomarkers of stress were evaluated, was published in a recent paper (Contreras-Aguilar et al., 2018).

Saliva samples of eight Montesina sheep, sub-breed of Manchega, originally from southeast Spain, located at the Education Farm of the University of Murcia (Spain), were

used. The day of the experiment, the whole flock of 40 sheep was moved to a compound of 26 m^2 outside in the open air, and the sheep were acclimatized for 30 minutes. A basal saliva sample (baseline or the sample before the application of a stress stimulus) was taken from sheep selected prior to stress induction. The animals were removed from the flock one at a time and the stress stimulus implemented, which consisted of being sheared with a shearing machine for approximately five minutes. The samples collected just after shearing were used in the proteomic study, and the samples were selected on the basis of significant increases of salivary biomarkers (cortisol, alpha-amylase and lipase) showed by Contreras et al. (2018).

Saliva samples were obtained using placing a sponge in the mouth. When sponges were thoroughly moist, they were placed in plastic tubes (Salivette; Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany), centrifuged (3,000 g for 10 min, 4°C), and the supernatant was aliquoted and kept at −80°C until analysis. The results of salivary biomarkers from freshly obtained saliva samples were reported in the previous study (Contreras-Aguilar et al., 2018). The aliquots of samples were stored, approximately for a year, at -80°C until proteomic analysis (Internal Disease Clinic, Faculty of Veterinary Medicine, University of Zagreb in Zagreb, Croazia).

2.2 Proteomic study of saliva samples from sheep and Liquid Chromatography Mass Spectrometry (LC–MS/MS)

Two groups were formed in the proteomic study, one containing eight samples obtained prior to the stress induction and another containing eight samples of the same animals obtained after the stress. Saliva proteins were acetone-precipitated (six volumes of ice-cold acetone, overnight), dissolved in 100 mM of triethylammonium bicarbonate (TEAB) (pH 8.5) and protein concentration was obtained by bicinchoninic acid (BCA) assay. A pooled

sample, generated by mixing equal protein quantity of all sixteen samples was employed as an internal standard in all TMT six-plex experiments.

For each sample, proteins were submitted to reduction, alkylation and digestion and labelled using six-plex TMT reagents following manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA) with some modifications, as reported by Martínez-Subiela et al. (2017). In short, 35 µg of proteins was reduced with 200 mM of 1,4-Dithiothreitol (DTT) (Sigma-Aldrich, S t. Louis, MO, USA), alkylated with 375 mM of iodoacetamide (Sigma-Aldrich, S t. Louis, MO, USA) and precipitated with ice-cold acetone (VWR Corp., Radnor, PA, USA) overnight. Saliva samples were then centrifuged and the acetone was eliminated by decantation. Afterward, 50µL of 100 mM TEAB buffer were used to resuspend the pellets and digested with trypsin (Promega Corp., Madison, WI, USA) overnight at 37°C (trypsin-to-protein ratio 1:35, w/w). The reagents of TMT labelling were equilibrated and resuspended with anhydrous acetonitrile LC–MS grade (Thermo Fisher Scientific, Waltham, MA, USA) and added to each saliva sample. For one hour the labelling reaction was incubated (at room temperature) and then 5% hydroxylamine for 15 minutes (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample for its inactivation. Samples were mixed at same amounts (5 µg) and then were vacuum-dried and kept at -80°C before additional LC–MS/MS analysis. The LC−MS/MS analysis was performed using the Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberley, UK) and the Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as reported by Horvatić et al. (2018). For protein identification and relative quantification Proteome Discoverer (version 2.0., Thermo Fisher Scientific, Waltham, MA, USA) was used for the SEQUEST search against Ovis aries FASTA files downloaded from NCBI database (18/05/2018, 70016 sequences) according to parameters set as follows: precursor and fragment mass tolerances of 10 ppm and 0.02 Da, two trypsin missed cleavage sites, respectively; carbamidomethyl (C),

146 oxidation (M), fixed peptide modification, deamidation (N,Q) and TMT six-plex (K, peptide
147 N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification
148 was calculated using the Percolator algorithm. Proteins with at least two unique peptides and
149 5% FDR were considered successfully identified. Protein quantification was based on relative
150 intensities of reporter ions representing differentially labelled peptides selected for MS/MS
151 fragmentation. To compare relative quantification data, for each protein between the TMT
152 six-plex experiments, an internal standard was used.
153
154 *2.3 Validation of lactoferrin in sheep as possible salivary biomarker of stress*
155 *2.3.1 Lactoferrin assay*
156 Lactoferrin was measured using a commercial sheep enzyme-linked immunosorbent
157 assay (ELISA) kit specific to lactoferrin (sheep lactotransferrin ELISA kit; Cusabio Biotech.,
158 Ltd. Houston, TX, USA), according to manufacturer instructions.
159
160 *2.3.2 Analytical validation*
161 Sheep saliva samples taken before and after applying the model of stress described
162 above were employed for the validation study of the ELISA assay. The following parameters
163 were evaluated: intra- and inter-assays precision, linearity, recovery and the limit of detection.
164 The intra- and inter-assays precision expressed as coefficients of variation (CVs) were
165 calculated by analysing two pools of saliva samples containing, low and high concentrations
166 of lactoferrin. Each pool was prepared by mixing saliva samples with similar concentrations
167 of lactoferrin (previously quantified by the ELISA method used in our study). Inter-assays
168 CVs were obtained by measuring five time the same pools in different days. Each CV was
169 calculated as the percentage of the standard deviation (SD) of the replicates divided by the

mean. To avoid possible variations due to cycles of thawing and freezing, the saliva samples were aliquots and only was unfrozen the aliquot needed for each assay.

The linearity under dilution was used to evaluate the accuracy of the assay. For it, two sheep saliva samples with high lactoferrin concentrations were serially diluted in varying concentrations (e.g., 1:2, 1:4, 1:8, 1:16, 1:32) with an assay buffer. Afterwards, linear regression between the observed and expected results was performed and the slope, y-intercept, and coefficients of determination (R^2) were calculated.

The detection limit, defined as lowest concentration of lactoferrin that assay can distinguished from zero value, was calculated based on mean value of 10 replicate determination of the assay buffer (zero standard) plus three SDs.

2.3.3 Evaluation of lactoferrin changes after stress

The ability of the ELISA assays to distinguish between the stress levels shown by the sheep was investigated by comparing saliva samples from 14 sheep before and after the application of the stress model by shearing for five minutes as previously described for the proteomic study. The samples analysed were from the 8 sheep using the first part related to proteomic approach and from 6 additional sheep. 8 sheep were the same used in the first part related to proteomic approach and another 6 sheep more until completely a total of 14 used before by Contreras-Aguilar et al. (2018).

2.4 Gene ontology pathways

The proteomic results obtained in the study were employed for the gene ontology (GO) analysis. The proteins, encoding in ovine genes, differentially expressed were transformed to their human arthologs employing the Ensembl orthologs database and its tool for data mining BioMart (http://www.ensembl.org/index.html). Obtained genes were used to

add their best known interactors (maximum of 10 per genes) according to the databases STRING-EMBL, IntAct and Reactome by the utilization of the Cytoscape (v3.6.1) plug-in CluePedia (v1.5.2) (Shannon et al., 2003; Bindea et al., 2013). Original proteins (differentially expressed between the two grouping conditions of data, data obtained before vs. data obtained after stress) and enriched proteins (best interactors of the original proteins) were then used to determine the GO terms over-represented in this set of proteins by way of the utilization of the Cytoscape plug-in ClueGO (v2.5.0) (Bindea et al., 2009) on the Homo sapiens GO-biological process (14/08/2018) (GO level from 3 to 8, minimum number of genes = 3, minimum percentage = 4, Kappa score threshold = 0.4, two-sided hypergeometric test with Bonferroni correction). For the REVIGO analysis the GO terms over-represented were submitted to remove redundant GO terms, the similarity allowed was 0.7 SimRel, and their functional description defined the groups related GO terms (Supek et al., 2011). Finally, pathway interactomes were designed in Cytoscape using the radial layout incorporating the GO data generated by ClueGO and ReviGO (e.g., the number of input genes in GO terms, associated p-value, GO/proteins relationships, GO groups).

2.5 Statistical analysis

In order to compare the abundances of proteins identified in the proteomic analysis between two groups of samples (group of samples before versus group of samples after stress stimulus) data, by logarithmic transformation, were normalized and Student's t-test (two-tailed, paired) was used. A $P < 0.05$ value was considered to be significant. Fold changes (FC) have been obtained with the formula FC=log2 (Group before /Group after) as has been reported before (Franco-Martínez et al., 2018). RStudio (v1.0.143) (R Studio Team. RStudio "RStudio Team. RStudio: Integrated Development Environment for R [Internet]. Boston,

MA: RStudio, Inc.; 2015. Available from: http://www.rstudio.com/," 2015) was employed for statistics.

Intra- and interassay CVs of assay were calculated as SD/mean value of repeated measurements and expressed as percentage (multiplied by 100). Detection limits, linearity under dilution, linear regression analyses also were performed with Rstudio. Because of the small sample size, the changes of lactoferrin in sheep between groups for clinical analysis, before and after of stress stimulus, were assessed by a non-parametric Wilcoxon matched-pairs test using a commercial statistics package (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA). A $P < 0.05$ value was considered significant.

3. Results

3.1 Proteomic changes of saliva samples from sheep following the model application

A total of 445 proteins were identified, 35 of which showed significant changes in relation to baseline or to their levels prior to the model application. These proteins appear in Table 1. Five of them showed a decrease after model application, namely: lactoferrin (P = 0.015); alpha-2-macroglobulin isoform X1 (P = 0.034); keratin, type II cytoskeletal 6A (P = 0.039); complement C3 (CC3) (P = 0.042); and secretoglobin family 1D member (SCGB1D2) (P = 0.049). Conversely, eight showed an increase after model application, namely: triosephosphate isomerase isoform X2 (P = 0.015), 14-3-3 protein gamma (P = 0.039), phosphoglycerate mutase 1 isoform (P = 0.015), acyl-coenzyme-A (CoA)-binding protein (P = 0.015), hemoglobin subunit beta isoform X2 (HBB) (P = 0.023), 14-3-3 protein zeta/delta isoform X1 (P = 0.041), type II small proline-rich protein (P = 0.039), and calmodulin-alpha (P = 0.039).

3.2 Results of validation of lactoferrin in sheep as possible salivary biomarker of stress

Analytical validation: The intra-assay CVs were 7.1% for the pool with a high lactoferrin concentration and 8.5 % for the pool with a low lactoferrin concentration. Additionally, inter-assay CVs were 13 % for the pool with a high lactoferrin concentration and 11% for the pool with a low lactoferrin concentration, respectively. A linear regression coefficient of 0.96 was observed when saliva samples with high concentrations diluted 1:2-fold or greater were analyzed. The analytical limit of detection calculated was 0.15 µg/mL. Differences between lactoferrin levels obtained before and after of the application of the stress model are presented in Figure 1. Sheep showed a significant decrease ($P < 0.01$) of salivary lactoferrin levels after shearing (median: 0.24 µg/mL; range: 0.21–0.26 µg/mL; 25-75th percentiles) in comparison with the levels observed prior to shearing (median: 0.33 µg/mL; range: 0.27–0.37 µg/mL; 25-75th percentiles).

3.3 Bioinformatics

Although 13 proteins representing unique genes were identified as being differentially expressed between before and after the stress, 38 further proteins were added in the enrichment step in GO analysis. From the GO analysis, four GO groups were defined as follow: canonical glycolysis (11 genes, -log10 $P = 18.3$), oxygen transport (five genes, -log10 $P = 7.6$), regulation of actin cytoskeleton reorganization (five genes, -log10 $P = 5.7$), and neural nucleus development (four genes, -log10 $P = 3.2$) (Figure 2). Representation of GO terms shows that canonical glycolysis is central and the most relevant pathway in the study (Figure 3). Proteins belonging to the guanine nucleotide exchange factor (RAPGEF3/4), optineurin (OPTN) and enolase-3 (ENO3) were at the crossroads between the most important GO terms despite the fact that they were not identified as differentially expressed in the current proteomic study.

4. Discussion

In this study was used for the first time the TMT technology for protein quantification is saliva of sheep. In addition, the main proteins differentially represented in this type of sample and related major metabolic pathways were identified following the application of a model of acute stress. GO analysis showed that four major metabolic pathways were found to be significantly affected in sheep saliva after the stress of shearing, namely: canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. In addition, thanks to use of this high-resolution quantitative proteomic technique, 13 proteins never before described in the saliva of sheep were observed to change after the experimental stress condition. Six of the proteins identified by TMT technology were related with the metabolic pathways. Phosphoglycerate mutase 1 isoform X1 (PGAM1) and triosephosphate isomerase isoform X2 (TPI1) were found to be upregulated in the canonical glycolysis. PGAM1 catalyses the transfer of phosphate groups from 3-phosphoglycerate to 2-phosphoglycerate, while the TPI1 genes encodes two proteins or enzymes, which catalyses in glycolysis the isomerization of glyceraldehydes 3-phosphate (G3P) and, in the gluconeogenesis, the dihydroxy-acetone phosphate (DHAP). Both proteins, as a part of glycolytic processes, and catalyse the breakdown of a carbohydrate into pyruvate, with the concomitant production of energy (Hitosugi et al., 2012). Therefore, these significant increases in concentration of the above mentioned enzymes might lead to hyperglycaemia in sheep. The hyperglycaemia represents an immediate response to stress in any species, providing rapid energy source to meet the energy requirements of fear and the "fight-or-flight" induced response (Surwit et al., 1992).

Another of the metabolic pathways identified in GO analysis of our study was that for oxygen transport represented by HBB, which was significantly upregulated after the stress of shearing. This protein is involved in transporting oxygen from the lungs to various peripheral

294 tissues. Therefore, HBB is a protein that could be involved in the “fight-or-flight”-induced
295 response in sheep in a manner intended to improve oxygen delivery and could also be related
296 to the stress response. This increase of expression of hemoglobin genes has been reported
297 before in mice subjected to acute social stress stimulus (Stankiewicz et al., 2014).
298 Calmodulin-alpha (CaM), which is related with the metabolic pathway of neural nucleus
299 development, was also found to be upregulated in saliva after the stress. CaM is a protein to
300 Ca2+-sensing that is important in many cellular processes (Stevens, 1983) and which
301 regulates a myriad of target proteins, such as G-protein-coupled receptor, ion channels, and
302 kinases. As an example, in the Ca2+-signalling pathway, CaM regulates and activates the
303 calcineurin and myosin light chain kinase IV (Kishi et al., 2018). Therefore, CaM plays part
304 in regulating different biological processes including proliferation, learning and metabolism
305 (Westerlund and Delemotte, 2018). In addition, calcium has an important role in the effects of
306 stress hormones (Malinovská et al., 1991). Regarding actin cytoskeleton reorganization, two
307 upregulated proteins were identified, namely: the 14-3-3 protein gamma (YWHAG) and the
308 14-3-3 protein zeta/delta isoform X1 (YWHAZ). The 14-3-3 proteins mediate interactions
309 between proteins involved in cell-cycle regulation and signal transduction, being important
310 for protein–protein interactions (Dubois et al., 1997). Therefore, these are adapter proteins
311 implicated in the regulation of a large spectrum of both specialized and general signalling
312 pathways. Stress can be defined as a state of threat to homeostasis (Chrousos and Gold, 1992),
313 and the stress response requires an appropriate coordination of multiple signalling pathways
314 to return to homeostasis. Therefore, these proteins could be related to the stress response,
315 although further studies should be performed to clarify their role.
316 The rest of the proteins TMT quantified affect by the stress of shearing were
317 unmapped in GO terms. The acyl-CoA-binding protein (DBI) and the type II small proline-
318 rich protein (SPRR2E) were upregulated, whereas CC3; A2M; SCGB1D2; Keratin, Type II

Cytoskeletal 6 (KRTL1); and lactoferrin were downregulated in saliva after the stress response.

DBI can act as an intracellular carrier, binding with high affinity to long- and medium-chain acyl-CoA esters. Furthermore, DBI is alble to displace diazepam from its recognition site located on the acid gamma-aminobutyric (GABA) type A receptor. Therefore, is possible that DBI module the funtion of the GABA receptor, acting also as a neuropeptide. (van Aalten et al., 2001). Acute stressors change the release of different neurotransmitters, such as acetylcholine or GABA in limbic areas of the brain (e.g., hippocampus, prefrontal cortex, nucleus accumbens, and amygdala), which are functionally interconnected, leading to the modulation and promotion of behavioural processes to cope with the stressor (Mora et al., 2012). Notably, in the present investigation, the SPRR2E cross-linked envelope protein of keratinocytes. The encoded protein, along with other family members, is a component of the cornified cell envelope that forms beneath the plasma membrane in terminally differentiated stratified squamous epithelia. This envelope serves as a barrier against extracellular and environmental factors. In humans, it has been shown to have a relationship with problem in skin such as psoriasis (Kainu et al., 2008). In our study, this response could be related with the stress caused by shearing, which is also skin-related, although further studies should be made to clarify this fact.

The downregulated proteins quantified by TMT in our study were associated with different acute reactions. CC3 played a central role in the activation of the complement system in both alternative and classical complement pathways. Derived from the proteolytic degradation of CC3, CC3a anaphylatoxin is a mediator of local inflammatory processes and acts as a chemoattractant for neutrophils (Ricklin et al., 2016). In addition, it induces the contraction of smooth muscle and improves vascular permeability (Ricklin et al., 2016), increasing the cardiac frequency and vascular permeability needed for an acute stress

response. The A2M is a protein that is responsible for the neutralization of proteolytic enzymes and, in humans, it is considered to be a hyperoxia biomarker (Bhattacharya et al., 2014). The SCGB1D2 proteins are widely expressed in endocrine-responsive organs. This protein may bind androgens and other steroids and may be under transcriptional regulation of steroid hormones (Jackson et al., 2011). The gen KRTL1 encoded a protein that belongs of the keratin gene family. These proteins, the type II cytokeratins, interfere during differentiation of simple and stratified epithelial tissues. In general, further studies should be performed to clarify the role of these downregulated proteins in the stress reactions.

Finally, lactoferrin, this secretory glycoprotein is found in body fluids produced by exocrine glands such as the mammary, salivary, and lacrimal (Lodernnal and Iers, 1995; Hayashi et al., 2017) and also in neutrophil granules of mammals (Wakabayashi et al., 2006). Numerous physiological roles have been associated to this protein, including in the protection against microbial infection, regulation of iron metabolism, and management of immune functions but also has anti-viral, anti-cancer, antioxidant, and anti-inflammatory activities (Wakabayashi et al., 2006; Hao et al., 2019). In relation to role of the lactoferrin in the ovine physiology has been evaluated previously in several studies, fundamentally, evaluating the effects of the lactoferrin bovine milk administration. For example, Wong et al. (1996 and 1998) showed that bovine milk contains several proteins and peptides having immunomodulatory and/or therapeutic potential effect in sheep. In a recent article, El-Ashker et al. (2018) suggested that, supplemented with Lactobacillus sp., is a possible nutritional supplements to support the immune system in healthy lambs. Due to its role in the protection against microbial infection or its anti-viral activities, it also has been administrated as prebiotic to prevent colonization and excretion Escherichia Coli (Yekta et al., 2011) or against the ovine pulmonary adenomatosis (Sozmen and Beytut, 2012).

In relation to stress studies, its administration after the application of different stressors has been associated with an analgesic effect in rodents (Takeuchi et al., 2003; Zimecki et al., 2005; Tsuchiya et al., 2006). Notably, it seems to exert anxiolytic and analgesic effects that are accompanied by an increase in nitric oxide production or the activation of the μ-opioid system. Due to its possible attenuation effect in the impact against stress stimulus, it has also been used in studies as a prebiotic for stress resistance in rodents (Mika et al., 2016; Peña-Juárez et al., 2016). Maekawa et al. (2017) reported that the lactoferrin ameliorates corticosterone-related acute stress and hyperglycemia in rats, and its effect, which could be attributed to the activation of the HPA axis. In a recent report performed in human, Shinjo et al. (2018) indicated the possible application of lactoferrin in managing psychological stress being able to exert a suppression effect on the changed in parasympathetic and sympathetic activities evoked by the calculation task. Therefore, the pathway of actuation in stress response it could be at both levels (HPA and autonomic nervous system (SNA)). In any way, lactoferrin appears have an important role in stress response acting as an adaptogen during stress (Aleshina et al., 2016).

However, to our knowledge, no studies have been performed in this specie where has been evaluated lactoferrin as stress biomarker, furthermore, few studies about this fact there have being published in veterinary medicine. Kim et al. (2011) found a significant decrease of lactoferrin in the blood levels of calves after stress due to weaning (around of 30% of its levels 315 to 216 μg/mL after 1 day post-weaning). In addition, in the only study performed on saliva in veterinary species, Huang et al. (2017) showed a decrease in saliva lactoferrin expression of pigs after restraint stress stimulus, although the authors did not observe significant changes ($P = 0.06$ after 30 min of immobilization, measured as fold change in relation to baseline by western blotting). Due to the facts that lactoferrin is one of the most differently expressed proteins after stress, that it has demonstrated stress-related changes in

several other species, and that it can be measured by use of a commercially available immunoassay in sheep, this protein was selected for the validation study. Our analytical results showed that immunoassay used is suitable for the application in sheep saliva samples with an excellent precision, accuracy, and sensitivity. The immunoassay enabled the detection of a significant stress-induced decrease in lactoferrin levels, around of 27% (0.33 to 0.24 µg/mL post stress). In addition, all sheep showed a decrease in lactoferrin concentrations in relation to the levels before the stress was applied, showing hardly any overlap.

In conclusion, four new metabolic pathways and 13 proteins differentially represented in the saliva of sheep after an application of acute stress using TMT quantitative proteomic technique are reported in this study. The new metabolic pathways found include canonical glycolysis, oxygen transport, neural nucleus development, and regulation of actin cytoskeleton reorganization. The new stress-related proteins identified in our study with this high-resolution quantitative proteomic technique could have the potential to be novel biomarkers of diagnostic or prevention of stress situations, specially lactoferrin, which can be measured by use of a commercially available immunoassay and which decreased in the sheep saliva following the application of the stressful stimulus used in our experiment.

Acknowledgements

This work was supported by a help of mobility "Jose Castillejo" of the "Ministerio de Educación, Cultura y Deporte" within the framework of the State Program for the Promotion of Talent and its Employability in I+D+i, the State Subprogram of Mobility, of the State Plan (Spain). DE has a post-doctoral fellowshisp "Juan de la Cierva Formación" supported by the "Ministerio de Economía y Competitividad", Spain. M. D. C.-A. was granted by the predoctoral contract 'FPU' of University of Murcia (R-605/2016), Spain. AH, NG, PDE and

VM were supported by the European Commission FP7 ERA Chair "VetMedZg" Project (grant number 621394).

Declaration of interest

The authors have declared that no competing interests exist.

Ethics statement

In this experiment only were used samples collected before in article Contreras et al., (2018) in accordance with the ethical standards of the Bioethical Commission of Murcia University (CEEA 431/2018).

Software and data repository resources

Our data are not deposited in an official repository.

References

Aleshina, G.M., Yankelevich, I.A., Zhakharova, E.T., Kokryakov, V.N., 2016. [STRESS-PROTECTIVE EFFECT OF HUMAN LACTOFERRIN]. [Article in Russian]. Russian journal of physiology 102, 846-851.

Baeumlisberger, D., Arrey, T.N., Rietschel, B., Rohmer, M., Papasotiriou, D.G., Mueller, B., Beckhaus, T., Karas, M., 2010. Labeling elastase digests with TMT: Informational gain by identification of poorly detectable peptides with MALDI-TOF/TOF mass spectrometry. Proteomics 10, 3905–3909.

Bhattacharya, S., Zhou, Z., Yee, M., Chu, C.Y., Lopez, A.M., Lunger, V.A., Solleti, S.K., Resseguie, E., Buczynski, B., Mariani, T.J., et al., 2014. The genome-wide transcriptional response to neonatal hyperoxia identifies Ahr as a key regulator. American journal of physiology. Lung cellular and molecular physiology. 307, L516-523.

Bindea, G., Galon, J., Mlecnik, B., 2013. CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. Bioinformatics 29, 661–663.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman,

W.H., Pagès, F., Trajanoski, Z., Galon, J., 2009. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–1093.

Chen, C.P.C., Preston, J.E., Zhou, S., Fuller, H.R., Morgan, D.G.A., Chen, R., 2018. Proteomic analysis of age-related changes in ovine cerebrospinal fluid. Experimental Gerontology 108, 181-188.

Chrousos, G.P., Gold, P.W., 1992. The concepts of stress and stress system disorders. Overview of physical and behavioural homeostasis. Journal of the American Medical Association 267, 1244–1252.

Contreras-Aguilar, M.D., Escribano, D., Quiles, A., López-Arjona, M., Cerón, J.J., Martínez-Subiela, S., Hevia, M.L., Tecles, F., 2018. Evaluation of new biomarkers of stress in saliva of sheep. Animal, 1-9. Animal, doi: 10.1017/S1751731118002707, Published online by Cambridge University Press 26 October 2018.

Cui, K., Wang, B., Zhang, N., Tu, Y., Ma, T., Diao, Q., 2018. iTRAQ-based quantitative proteomic analysis of alterations in the intestine of Hu sheep under weaning stress. PLoS One 19, 13(7):e0200680.

Dayon, L., Turck, N., Garci-Berrocoso, T., Walter, N., Burkhard, P.R., Vilalta, A., Sahuquillo, J., Montaner, J., Sanchez, J.C., 2011. Brain extracellular fluid protein changes in acute stroke patients. Journal of Proteome Research. 10, 1043–1051.

Dubois, T., Rommel, C., Howell, S., Steinhussen, U., Soneji, Y., Morrice, N., Moelling, K., Aitken, A., 1997. 14-3-3 is phosphorylated by casein kinase I on residue 233. Phosphorylation at this site in vivo regulates Raf/14-3-3 interaction. The Journal of Biological Chemistry, 272 28882-8.

El-Ashker, M., Risha, E., Abdelhamid, F., Ateya, A., 2018. Potential immune modulating properties and antioxidant activity of supplementing commercially available lactoferrin and/or Lactobacillus sp. in healthy Ossimi lambs. Polish Journal Veterinary Science. 2018 21, 705-713.

Escribano, D., Soler, L., Gutiérrez, A.M., Martínez-Subiela, S., Cerón, J.J., 2013. Measurement of chromogranin A in porcine saliva: Validation of a time resolved immunofluorometric assay and evaluation of its application as a marker of acute stress. Animal 7, 640–647.

Franco-Martínez, L., Tvarijonaviciute, A., Horvatić, A., Guillemin, N., Cerón, J.J., Escribano, D., Eckersall, E., Kocatürke, M., Yilmaz, Z., Lamy, E., et al., 2018. Changes in salivary analytes in canine parvovirus: A high-resolution quantitative proteomic study. Comparative Immunology, Microbiology and Infectious Diseases 60, 1–10. Comparative immunology, microbiology and infectious diseases, doi 10.1016/j.cimid.2018.09.011. Published online by ELSEVIER 24 September 2018.

Fuentes-Rubio, M., Fuentes, F., Otal, J., Quiles, A., Hevia, M.L., 2016. Validation of an assay for quantification of alpha-amylase in saliva of sheep. Physiology and Behavior 102, 259–265.

Giron, P., Dayon, L., Turck, N., Hoogland, C., Sanchez, J.C., 2011. Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. Journal of Proteome Research. 10, 249–258.

Hao, L., Shan, Q., Wei, J., Ma, F., Sun, P., 2019. Lactoferrin: Major Physiological Functions and Applications. Current Protein & Peptide Science 20, 139-144.

Heintz, M.R., Santymire, R.M., Parr, L.A., Lonsdorf, E.V., 2011. Validation of a cortisol enzyme immunoassay and characterization of salivary cortisol circadian rhythm in chimpanzees (Pan troglodytes). American Journal of Primatology 73, 903–908.

Hitosugi, T., Zhou, L., Elf, S., Fan, J., Kang, H.B., Seo, J.H., Shan, C., Dai, Q., Zhang, L., Xie, J., et al., 2012. Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. Cancer Cell 22, 585-600.

Horvatić, A., Guillemin, N., Kaab, H., McKeegan, D., O'Reilly, E., Bain, M., Kuleš, J., Eckersall, P.D., 2018. Quantitative proteomics using tandem mass tags in relation to the acute phase protein response in chicken challenged with Escherichia coli lipopolysaccharide endotoxin. Journal of Proteomics, in press, DOI: 10.1016/j.jprot.2018.08.009. Published online by Journal of Proteomics 13 August 2018.

Huang, Y., Liu, Z., Liu, W., Yin, C., Ci, L., Zhao, R., Yang, X., 2017. Short communication: Salivary haptoglobin and chromogranin A as non-invasive markers during restraint stress in pigs. Research in Veterinary Science 114, 27-30.

Jackson, B.C., Thompson, D.C., Wright, M.W., McAndrews, M., Bernard, A., Nebert, D.W., Vasiliou, V., 2011. Update of the human secretoglobin (SCGB) gene superfamily and an example of 'evolutionary bloom' of androgen-binding protein genes within the mouse Scgb gene superfamily. Human Genomics 5, 691-702.

Kainu, K., Kivinen, K., Zucchelli, M., Suomela, S., Kere, J., Inerot, A., Baker, B.S., Powles, A.V., Fry, L., Samuelsson, et al., 2009. Association of psoriasis to PGLYRP and SPRR genes at PSORS4 locus on 1q shows heterogeneity between Finnish, Swedish and Irish families. Experimental Dermatology 18, 109-115.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Research. 45(D1):D353–D361.

Kim, M.H., Yang, J.Y., Upadhaya, S.D., Lee, H.J., Yun, C.H., Ha, J.K., 2011. Stress of weaning influences serum levels of acute-phase proteins, iron-binding proteins, inflammatory cytokines, cortisol, and leukocyte subsets in Holstein calves. Journal of Veterinary Science 12, 151-157.

Kishi, H., Ye, L.H., Nakamura, A., Okagaki, T., Iwata, A., Tanaka, T., Kohama, K., 1998. Structure and function of smooth muscle myosin light chain kinase. Advances in experimental medicine and biology 453, 229-234.

554 Lehrer, R.I., Nguyen, T., Zhao, C., Ha, C.X., Glasgow, B.J., 2000. Secretory lipophilins: A
555 tale of two species. Annals of the New York Academy of Sciences 923, 59–67.
556
557 Lonnerdal, B., Iyer, S., 1995. Lactoferrin: molecular structure and biological function. Annual
558 Review of Nutrition 15, 93–110.
559
560 Maekawa Y, Sugiyama A and Takeuchi T 2017. Lactoferrin ameliorates corticosterone-
561 related acute stress and hyperglycemia in rats. The Journal of Veterinary Medical
562 Science 79, 412-417.
563
564 Malinovská, V., Matonoha, P., D'Andrea, V., Malinovský, L., Zechmeister, A., 1991. [The
565 role of calcium in the effect of stress hormones]. [Article in Czech]. Casopís lékařů
566 českých 130, 631-640.
567
568 Martinez-Subiela, S., Horvatic, A., Escribano, D., Pardo-Marin, L., Kocaturk, M., Mrljak, V.,
569 Burchmore, R., Ceron, J.J., Yilmaz Z 2017. Identification of novel biomarkers for
570 treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic
571 analysis. Veterinary immunology and immunopathology 191, 60–67.
572
573 Merlot, E., Mounier, A.M., Prunier, A., 2011. Endocrine response of gilts to various common
574 stressors: A comparison of indicators and methods of analysis. Physiology and Behavior
575 102, 259-265.
576
577 Messori, S., Pedernera-Romano, C., Rodriguez, P., Barnard, S., Giansante, D., Magnani, D.,
578 Dalmau, A., Velarde, A., Dalla Villa, P., 2017. Effects of different rest-stop durations at
579 control posts during a long journey on the welfare of sheep. Veterinaria Italiana. 53,
580 121-129.
581
582 Mika, A., Day, H.E., Martinez, A., Rumian, N.L., Greenwood, B.N., Chichlowski, M., Berg,
583 B.M., Fleshner, M., 2017. Early life diets with prebiotics and bioactive milk fractions
584 attenuate the impact of stress on learned helplessness behaviours and alter gene
585 expression within neural circuits important for stress resistance. The European Journal
586 of Neuroscience 45, 342-357.
587
588 Mora, F., Segovia, G., Del Arco, A., de Blas, M., Garrido, P., 2012. Stress, neurotransmitters,
589 corticosterone and body-brain integration. Brain Research 1476, 71-85.
590
591 Peña-Juárez, M.C., Campos-Rodríguez, R., Godínez-Victoria, M., Cruz-Hernández, T.R.,
592 Reyna-Garfias, H., Barbosa-Cabrera, R.E., Drago-Serrano, M.E., 2016. Effect of
593 Bovine Lactoferrin Treatment Followed by Acute Stress on the IgA-Response in Small
594 Intestine of BALB/c Mice. Immunological Investigations 45, 652-667.
595
596 Ricklin, D., Reis, E.S., Mastellos, D.C., Gros, P., Lambris, J.D., 2016. Complement
597 component C3 - The "Swiss Army Knife" of innate immunity and host defense.
598 Inmunological Reviews 274, 33-58.
599
600 Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N.,
601 Schwikowski, B., Ideker, T., 2003. Cytoscape: a software Environment for integrated
602 models of biomolecular interaction networks. Genome Research 13, 2498–2504.
603

Shinjo, T., Sakuraba, K., Nakaniida, A., Ishibashi, T., Kobayashi, M., Aono, Y., Suzuki, Y., 2018. Oral lactoferrin influences psychological stress in humans: A single-dose administration crossover study. Biomedical Reports 8, 426-432.

Sozmen, M., Beytut, E., 2012. An investigation of growth factors and lactoferrin in naturally occurring ovine pulmonary adenomatosis. Journal of Comparative Pathology 147, 441-451.

Stankiewicz, A.M., Goscik, J., Swiergiel, A.H., Majewska, A., Wieczorek, M., Juszczak, G.R., Lisowski, P., 2014. Social stress increases expression of hemoglobin genes in mouse prefrontal cortex. BMC Neurosciences 4, 15:130.

Stevens, F.C., 1983. Calmodulin: an introduction. Canadian Journal of Biochemistry and Cell Biology 61, 906–910.

Supek, F., Bošnjak, M., Škunca, N., Šmuc, T., 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One 6, e21800.

Surwit, R.S., Schneider, M.S., Feinglos, M.N., 1992. Stress and diabetes mellitus. Diabetes Care 15, 1413-1422.

Takeuchi, T., Hayashida, K., Inagaki, H., Kuwahara, M., Tsubone, H., Harada, E., 2003. Opioid mediated suppressive effect of milk-derived lactoferrin on distress induced by maternal separation in rat pups. Brain Research 979, 216–224.

Tsuchiya, T., Takeuchi, T., Hayashida, K., Shimizu, H., Ando, K., Harada, E., 2006. Milk-derived lactoferrin may block tolerance to morphine analgesia. Brain Research 1068, 102-108.

van Aalten, D.M., Milne, K.G., Zou, J.Y., Kleywegt, G.J., Bergfors, T., Ferguson, M.A., Knudsen, J., Jones, T.A., 2001. Binding site differences revealed by crystal structures of Plasmodium falciparum and bovine acyl-CoA binding protein. Journal of Molecular Biology 309, 181-92.

Wakabayashi, H., Yamauchi, K., Takase, M., 2006. Lactoferrin research, technology and applications. International Dairy Journal 16, 1241-1251.

Westerlund, A.M., Delemotte, L., 2018. Effect of Ca2+ on the promiscuous target-protein binding of calmodulin. PLoS Computational Biology 14(4):e1006072.

Wong, C.W., Regester, G.O., Francis, G.L., Watson, D.L., 1996. Immunomodulatory activities of whey fractions in efferent prefemoral lymph of sheep. Journal of Dairy Research 63, 257-267.

Wong, C.W., Liu, A.H., Regester, G.O., Francis, G.L., Watson, D.L., 1997. Influence of whey and puri®ed whey proteins on neutrophil functions in sheep. Journal of Dairy Research 64, 281-288.

Wong, C.W., Watson, D.L., Regester, G.O., Smithers, G.W., 1998. Comparison of immunomodulatory properties of purifed lactoferrin, lactoperoxidase and b-casein in

654 sheep. Journal of Dairy Research 65 697-701.
655
656 Yekta, M.A., Cox, E., Goddeeris, B.M., Vanrompay, D., 2011. Reduction of Escherichia coli
657 O157:H7 excretion in sheep by oral lactoferrin administration.Veterinary Microbiology.
658 150, 373-378.
659
660 Zimecki, M., Artym, J., Chodaczek, G., Kocieba, M., Kruzel, M., 2005. Effects of lactoferrin
661 on the immune response modified by the immobilization stress. Pharmacological
662 Reports : PR 57, 811–817.
663
664

665 Statistically significant expression changes of proteins in saliva of sheep with after model of
666 sympathetic activation (differentially expressed between the two grouping conditions of data,
667 data obtained of before vs. data obtained after stress). Ordained based on P value of Paired t-
668 test.
669

Protein asscension ID	Protein name	Paired t-test (P value)	Mean Before	Mean After	Fold Change
451799070	lactoferrin precursor	0.015	1.465	1.117	-0.39
254656113	Lactoferrin	0.015	1.468	1.128	-0.38
803057661	keratin, type II cytoskeletal 6A	0.015	1.465	1.117	-0.38
803289817	keratin, type II cytoskeletal 6A isoform X2	0.015	1.468	1.128	-0.37
426221202	acyl-CoA-binding protein	0.015	0.625	0.985	0.65
803131482	phosphoglycerate mutase 1	0.015	0.666	1.023	0.61
426253331	phosphoglycerate mutase 1 isoform X1	0.015	0.666	1.023	0.61
803266107	phosphoglycerate mutase 1 isoform X2	0.015	0.666	1.023	0.61
478694	Ig mu chain – sheep	0.015	1.587	1.168	-0.44
803249757	triosephosphate isomerase isoform X2	0.015	0.757	0.917	0.27
965928682	triosephosphate isomerase isoform X1	0.022	0.743	0.892	0.26
803341154	hemoglobin subunit beta isoform X2, partial	0.023	1.145	1.827	0.67
803220086	carcinoma-associated protein 2B isoform X2	0.023	1.490	1.027	-0.53
803055708	alpha-2-macroglobulin isoform X1	0.034	1.062	0.862	-0.30
803055710	alpha-2-macroglobulin isoform X2	0.034	1.062	0.862	-0.30
803055712	alpha-2-macroglobulin isoform X3	0.034	1.062	0.862	-0.30
803055714	alpha-2-macroglobulin isoform X4	0.034	1.062	0.862	-0.30
426219697	major allergen Equ c 1	0.039	1.505	0.955	-0.65
803236637	14-3-3 protein gamma	0.039	0.637	0.871	0.45
803244735	allergen Bos d 2	0.039	1.311	0.985	-0.41
803290165	keratin, type II cytoskeletal 6A, partial	0.039	1.440	1.086	-0.40
803043154	keratin, type II cytoskeletal 6A isoform X1	0.039	1.440	1.086	-0.40
57619320	type II small proline-rich protein	0.039	0.691	0.905	0.38
803205645	small proline-rich protein 2I	0.039	0.691	0.905	0.38
426256638	allergen Bos d 2	0.039	1.275	0.982	-0.37
803055820	calmodulin-alpha	0.039	0.906	1.162	0.35
165945	immunoglobulin mu chain, partial	0.039	1.453	1.157	-0.32
561876	CDS translation of the C_region	0.039	1.453	1.157	-0.32

966012747	Ig mu chain C region secreted form isoform X1	0.039	1.453	1.157	-0.32
803103838	14-3-3 protein zeta/delta isoform X1	0.041	1.388	1.571	0.17
112696	Protein kinase C inhibitor protein 1	0.041	1.376	1.557	0.17
2582411	immunoglobulin alpha heavy chain, partial	0.042	1.972	1.418	-0.47
803205348	complement C3-like	0.042	1.027	0.927	-0.14
426251872	secretoglobin family 1D member	0.049	1.805	1.032	-0.80

670

671

672 **Figure legends**

673

674 Fig.1. Lactoferrin concentrations in sheep (n = 14) before and after the application of stress
675 model base on shearing. The plot show median (line within box), 25th and 75th percentiles
676 (box) and 10th and 90th percentiles (whiskers). Asterisks indicated significant differences
677 between groups: $^{**}P < 0.01$.

678

679 Fig.2. GO terms over-represented in the pool of significantly expressed saliva proteins before
680 and after stimulus. GO terms are ordered by the number of significant genes/proteins of the
681 study associated with them according to Gene Ontology database (first y-axis). -log10 of p-
682 value for each GO term is represented on the second y-axis. GO terms which define a group
683 of similar GO terms (determined by ReviGO) are in bold.

684

685 Fig.3. Network representation of GO terms over-represented in the pool of significant proteins
686 and associated proteins. GO terms are represented by octagon shape, proteins by diamond
687 shape. Proteins are in red with a black border for proteins identified in the experiments, or
688 with a white border for proteins added by the enrichment step. GO nodes are filled by colours
689 corresponding to their GO group (determined by ReviGO). Name of GO terms defining 1
690 group are in bold. When a protein belongs to 1 GO term, a link is figured between the nodes
691 of protein and GO term. The network representation has been realized under Cytoscape using
692 the radial layout. Acronyms gene and related full name protein of each protein that appears in
693 the figure (Datas obtained of UniProt data base; (http://www.uniprot.org): 1) Mapped original
694 proteins: CALM2 (Calmodulin-2); HBB (Hemoglobin subunit beta); PGAM1
695 (Phosphoglycerate mutase 1); TPI1 (Triosephosphate isomerase), YWHAG (14-3-3 protein
696 gamma) and YWHAZ (14-3-3 protein zeta/delta). 2) Mapped enriched proteins: ACTR1A

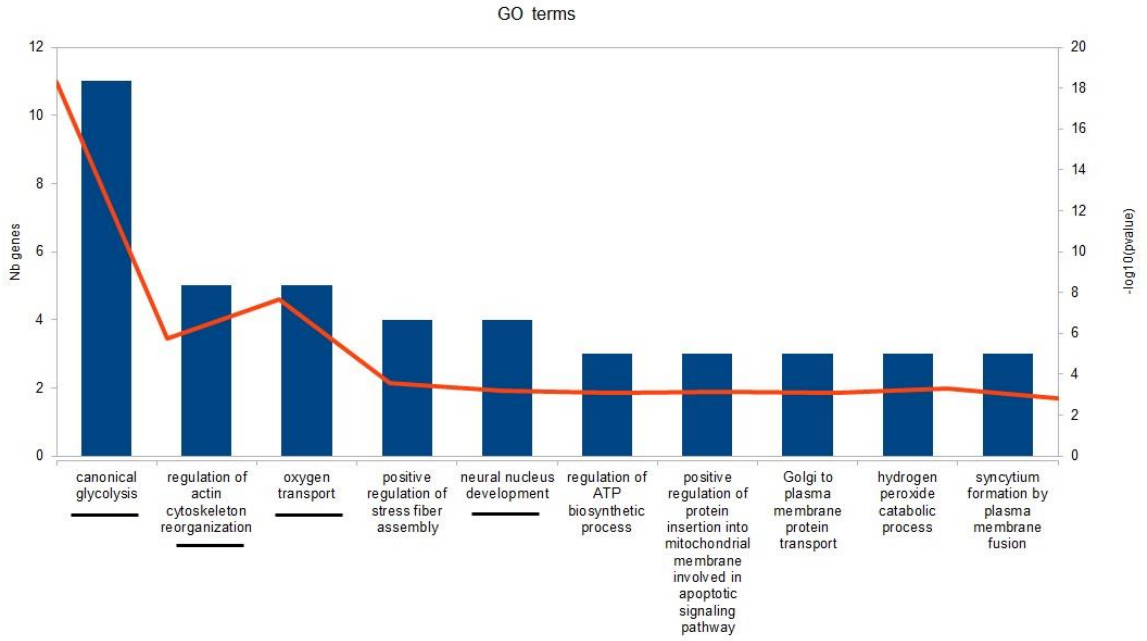
(Alpha-centractin); ALDOA (Fructose-bisphosphate aldolase A); ALDO B (Fructose-bisphosphate aldolase B); ALDO C (Fructose-bisphosphate aldolase C); CLU (Clusterin); ENO1 (Alpha-enolase); ENO2 (Gamma-enolase); ENO3 (Beta-enolase); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); GAPDHS (Glyceraldehyde-3-phosphate dehydrogenase, testis-specific); HBA1 (Hemoglobin subunit alpha-1); HBA2 (Hemoglobin subunit alpha-2); HBD (Hemoglobin subunit delta); HBE1 (Hemoglobin subunit epsilon); OPTN (Optineurin); PGK1 (PGK1); RAB10 (Ras-related protein Rab-10); RAPGEF3 (Rap guanine nucleotide exchange factor 3); RAPGEF4 (Rap guanine nucleotide exchange factor 4) and YWHAQ (14-3-3 protein theta). 3) Unmaped orinigal proteins: A2M (Alpha-2-macroglobulin); C3 (Complement C3); DBI (Acyl CoA-binding protein); KRT6B (Keratin, type II cytoskeletal 6B); LTF (Lactoferrin); SCGB1D2 (Secretoglobin family 1D member 2) and SPRR2E (Small proline rich protein 2E). 4) Ummapped enriched proteins: A1BG (Alpha-1B-glycoprotein); AKAP9 (A-kinase anchor protein 9); APCS (Serum amyloid P-component); APP (Amyloid precursor protein); CDKN1B (Cyclin-dependent kinase inhibitor 1B); CXCL13 (C-X-C motif chemokine 13); CXCR6 (C-X-C chemokine receptor type 6); FAM3C (Protein FAM3C); GNB5 (Guanine nucleotide-binding protein subunit beta-5); HSPG2 (Basement membrane-specific heparan sulfate proteoglycan core protein); INSL5 (Insulin-like peptide INSL5); MAFF (Transcription factor MafF); MAGED2 (Melanoma-associated antigen D2); NMUR1 (Neuromedin-U receptor 1); PGK2 (Phosphoglycerate kinase 2); TUBB4A (Tubulin beta-4A chain) and VTI1B (Vesicle transport through interaction with t-SNAREs homolog 1B).

719 Figure 1

720

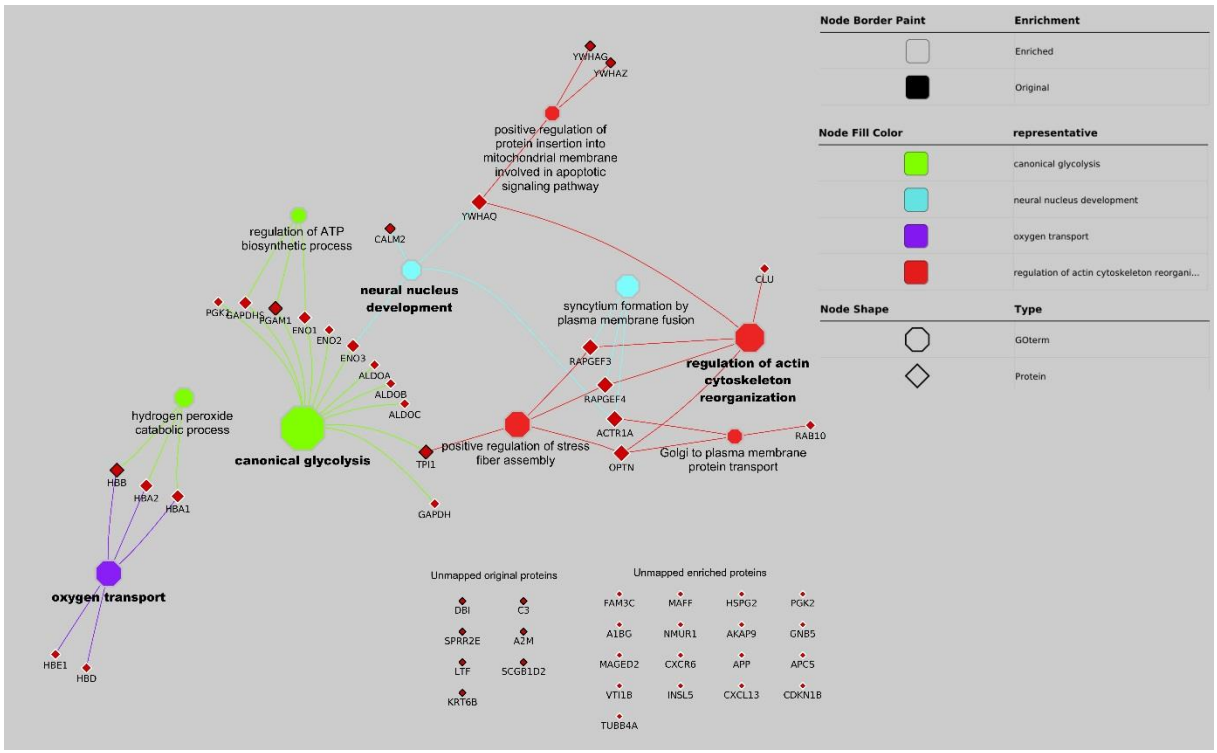
721

722 Figure 2



723

724


YWHAG
YWHAZ
positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway
YWHAQ
regulation of ATP biosynthetic process
CALM2
neural nucleus development
CLU
syncytium formation by plasma membrane fusion
RAPGEF3
RAPGEF4
regulation of actin cytoskeleton reorganization
PGK1
GAPDHS
PGAM1
ENO1
ENO2
ENO3
ALDOA
ALDOB
ALDOC
hydrogen peroxide catabolic process
canonical glycolysis
TPI1
GAPDH
positive regulation of stress fiber assembly
ACTR1A
OPTN
Golgi to plasma membrane protein transport
RAB10
HBB
HBA2
HBA1
oxygen transport
HBE1
HBD
Unmapped original proteins
DBI
C3
SPRR2E
A2M
LTF
SCGB1D2
KRT6B
Unmapped enriched proteins
FAM3C
MAFF
HSPG2
PGK2
A1BG
NMUR1
AKAP9
GNB5
MAGED2
CXCR6
APP
APC5
VTI1B
INSL5
CXCL13
CDKN1B
TUBB4A
Node Border Paint
Enrichment
Enriched
Original
Node Fill Color
representative
canonical glycolysis
neural nucleus development
oxygen transport
regulation of actin cytoskeleton reorgani...
Node Shape
Type
GOterm
Protein

725