Seminal plasma AnnexinA2 protein is a relevant biomarker for stallions which require removal of seminal plasma for sperm survival upon refrigeration

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Abstract

Some stallions yield ejaculates which do not tolerate conservation by refrigeration prior to artificial insemination (AI), showing improvement after removal of most of the seminal plasma (SP) by centrifugation. In this study, the SP-proteome of 10 different stallions was defined through HPLC/MS/MS and bioinformatic analysis in relation to the ability of the ejaculates to maintain semen quality when cooled and stored at 5ºC. Stallions were classified into three groups, depending on this ability: those maintaining good quality after direct extension in a commercial extender (*good*), stallions requiring removal of seminal plasma (RSP) to maintain seminal quality (*good-RSP*), and stallions, unable to maintain good semen quality even after RSP (*poor*). Pathway enrichment analysis of the proteins identified in whole equine seminal plasma using human orthologs was performed using g:profiler showing enriched Reactome and KEGG pathways related to hexose metabolism, vesicle mediated transport, post translational modification of proteins and immune response. Specific proteins overrepresented in stallions tolerating conservation by refrigeration included a peroxiredoxin-6 like protein, and transcobalamin-2, a primary vitamin B12-binding and transport protein. Also, the protein involved in protein glycosylation, ST3 beta-galactoside alpha-2,3-sialyltransferase 1 was present in *good* stallions. These proteins were nearly absent in *poor* stallions. Particularly, annexinA2 appeared as to be the most powerful discriminant variable for identification of stallions needing seminal plasma-removal prior to refrigeration, with a p =0.002 and a q value= 0.005. Overall this is the first detailed study of the equine SP-proteome, showing the potential value of specific proteins as discriminant bio-markers for clinical classification of stallions for AI.

Summary sentence

The seminal plasma protein Annexin A2 identifies ejaculates needing removal of seminal plasma prior to conservation in refrigeration.

Key words: seminal plasma, UHPLC/MS/MS, proteins, artificial insemination, horse.

Introduction

Despite artificial insemination (AI) being successfully used in equine breeding, several obstacles exclude valuable stallions from wider use in AI with refrigerated, extended semen. Semen from these stallions does not tolerate storage at 5ºC for a sufficient period of time to allow for shipping; this is manifested as a drop in motility below acceptable values for a commercial dose [1]. However, motility improves in the ejaculates from many of these stallions when most of the seminal plasma (SP) is removed by centrifugation [2, 3], suggesting a component of the SP plays a part in how tolerant the ejaculate is of being stored by refrigeration [4]. Seminal plasma in stallions is composed of secretions from the epididymis and the accessory glands of the male genital tract, particularly those from the prostate. Stallions produce ejaculates with large volumes and the SP is involved in different functions; from acting as a vehicle, nourishing and regulating spermatozoa viability [5-9] to interacting with the mare's endometrium [5, 10-12]. Although many proteins in equine SP have previously been characterized [6, 13-19], detailed proteomic studies of equine SP using shot gun proteomics are not yet available. The use of different omics is rapidly advancing our knowledge of sperm biology [20-24], and despite the importance of proteomics, it is a relatively recent field in spermatology and, in the particular case of equines, only two papers have been published to date using shot gun proteomics [25, 26]. However, there is a previous paper addressing the proteome of equine seminal plasma [18]. Nevertheless, proteomic studies are providing striking new findings in sperm biology. Owing to the enormous need to link novel findings in sperm biology, including the complexity and plasticity of metabolism and the presence of proteins involved in transcription and translation [20, 27, 28] suggesting a role in early embryo development; the present study aimed to: first provide an in depth description of the proteome of equine seminal plasma, and second to test the hypothesis that specific bio-markers which indicate the ability of ejaculates to withstand refrigeration for AI can be detected in equine SP.

MATERIAL AND METHODS

Reagents and media

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain), unless otherwise stated.

Semen collection and processing

Semen was collected from 10 stallions maintained as indicated under European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). All stallions were of proven fertility, with a median age of 11 years (range 7 to 20 years old), including 5 Andalusians, 1 Spanish Sport Horse, 1 Lusitano, 1 Arab, 1 Anglo-Arabian and 1 Spanish-Arabian horse. The study was supervised and approved by the ethical committee of the University (IB16030). Semen collection was carried out using a warmed Missouri model artificial vagina following standard protocols at our center, using a filter to separate the gel fraction. Immediately after collection, ejaculates were processed in the adjacent laboratory, and every ejaculate was processed using colloidal centrifugation [29, 30] to isolate SP. In brief, ejaculates were processed by single layer colloidal centrifugation using Equipure®(Nidacon Mölndal, Sweden) following the instructions provided by the manufacturer, and centrifuged for 20 min at 300 g at room temperature.

Experimental design

Three ejaculates from each of the 10 stallions with different abilities to withstand semen extension and refrigeration for AI were used in this study (30 biological replicates plus two technical replicates for each). Additional independent ejaculates from the same stallions were used for sperm functional analysis, where ejaculates were split into two aliquots and one was directly extended in INRA96® (IMV Technologies, L'Aigle, France), while the other had the seminal plasma removed by centrifugation before extension. Both aliquots were extended to 25 x10^6 sperm/ml in INRA 96® and kept refrigerated in volumes of 20 ml at 5ºC for 48 h. After 24 and 48 hours of incubation, total motility and velocities were measured in both groups (with and without seminal plasma) using computer assisted sperm analysis (CASA). Based on this analysis stallions were classified into three groups: *good* if total motility after 48h was >50% and curvilinear velocity (VCL) was >100μm/s , *good after removal of seminal plasma (good-RSP)*, if after centrifugation and removal of the bulk of seminal plasma motility after 48h was >50% and VCL was > 100μm/s and *poor*, for those ejaculates where even after removal of seminal plasma (RSP) total motility after 48h was < 50% and VCL was <100 μm/s

Sample preparation

Samples were processed immediately after collection. Aliquots of isolated SP were kept frozen at -80ºC until further analysis. Phase contrast microscopy was used to control the absence of spermatozoa, moreover SP was filtered (0.22μM) before snap freezing and further processing.

Protein solubilization

Aliquots of SP were solubilized in lysis buffer (C7:C7Bz0 [3-(4-heptyl) phenyl-(3-hydroxypropyl) dimethylammoniopropanesulfonate], 7M urea, 2M thiourea and 40 mM Tris (pH 10.4) and incubated under constant rotation at 4ºC for 1 hour.

Protein quantification

Protein quantification was performed using the 2-D Quant Kit (GE Healthcare, Sevilla Spain) following the manufacturer's instructions: https://www.gelifesciences.co.jp/tech_support/manual/pdf/80648622.pdf. All samples were normalized to obtain a final concentration of 100 μg of protein per sample.

In-solution trypsin digestion.

In total, 200 μL of solution obtained from the previous stage was mixed with 100 μl of 25 mM ammonium bicarbonate buffer pH 8.5 (100μg of protein in 300μL of solution). The addition of 30 μL of 10 mM Dithiothreitol (DTT) was used to reduce the proteins under incubation at 56 °C for 20 min. The proteins were then alkylated by adding 30 μL of 20 mM 3 indoleacetic acid (IAA) and incubated for 30 min at room temperature in the dark. Finally, digestion was performed by adding 1 μL of Trypsin Proteomics Grade (Sigma) (Trypsin solution: 1 μg/μL in 1mM HCl) at 37 °C for at least 3 h. The reaction was stopped by adding 10 μL of 0.1% formic acid and the solution filtered through 0.2 μm (hydrophilic PTFE) into a 2 mL dark glass vial. In the last step the samples were dried using a constant nitrogen current with the vial in a heating block at 35ºC. The dry samples were resuspended in 20 μl of buffer A, consisting of water/acetonitrile/formic acid (94.9:5:0.1)

UHPLC-MS/MS analysis.

The UHPLC/MS system used was an Agilent 1290 Infinity II Series UHPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High Speed Binary Pump coupled to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. The MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01) controlled the UHPLC and Q-TOF. Every sample was injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 μm, 150 × 2.1 mm, Agilent technologies), thermostatted at 55 °C, at a flow rate of 0.4 ml/min. The gradient program started with 2% of buffer B (water/acetonitrile/formic acid, 10:89.9:0.1) that remained in isocratic mode for 5 min and then increased linearly up to 45 % B over 40 min, further increasing up to 95 % B for 15 min and remaining constant for an additional 5 min. After this 70 min run, 5 min of post-time followed using the initial condition for conditioning of the column for the next run. The mass spectrometer was operated in positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 10 l/min at 250 °C. The sheath gas flow was set to 300 ºC. The peak to peak voltages of the capillary spray, fragmentor and octopole RF were 3500 V, 340 V and 750 V respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode, where the MS and MS/MS mass ranges were 50-1700 m/z and scan rates were 8 spectra/sec for MS and 3 spectra/sec for MS/MS. Auto MS/MS mode was used with precursor selection in abundance and a maximum of 20 precursors selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of -4.8. The same ion was rejected after two consecutive scans.

Data processing

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA). Briefly, raw data were extracted under default conditions as follows: non fixed or variable modifications were selected; [MH]+ 50–10000 m/z; maximum precursor charge +5; retention time and m/z tolerance ± 60 seconds; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals. The MS/MS search against the appropriate and updated protein database (https://www.uniprot.org/uniprot/?query=Equus+caballus&sort=score, accessed 4/07/20) was performed following these criteria: non fixed modifications were selected, and the following variable modifications were selected: carbamidomethylated cysteines, tryptic digestion

with a maximum of 5 missed cleavages, ESI-Q-TOF instrument, minimum matched peak intensity 50%, maximum ambiguous precursor charge +5, monoisotopic masses, peptide precursor mass tolerance 20 ppm, product ion mass tolerance 50 ppm, and calculation of reversed database scores. Validation of peptide and protein data was performed using auto thresholds with a %FR (false discovery rate) of 1.2%. The results for proteins were obtained as protein summarized using all validations; score >4 and % SPI (Scored Peak Intensity: the percentage of the extracted spectrum that is explained by the database search result) 60.

Seminal plasma proteome analysis

The proteins identified in SP were queried for gene ontology (GO) terms (http://geneontology.org) according to cellular component (CC), biological process (BP) and molecular function (MF) and classified using (PANTHER v.14.0) (http://www.pantherdb.org), and g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) following detailed published protocols [31-33]. The lists of proteins were queried against the equine proteome database (https://www.uniprot.org/taxonomy/9796) for significant enrichment using the Fisher´s exact test corrected with a False Discovery Rate (FDR) set at *P*<0.05.

Enrichment analysis of pathways in the seminal plasma proteome

PANTHER (http://www.pantherdb.org/pathway/pathwayList.jsp) and KEGG pathway (https://www.genome.jp/kegg/) [34-37] analysis were used to identify biological pathways likely to be active in seminal plasma. The significance of the presence of the protein list was queried against the equine proteome database using a FDR <0.05 and Fisher's exact test. g:Profiler was also used to perform an enrichment analysis [33]. Due to the increased depth of the human proteome in terms of annotation, the equine annotations were transformed to their human orthologs using g:Profiler (https://biit.cs.ut.ee/gprofiler/orth) and a pathway enrichment analysis and visualization was performed again using g:Profiler and Cytoscape analysis using Reactome (https://reactome.org).

Network analysis
Cytoscape (https://cytoscape.org) plug in ClueGo was used to identify functionally grouped gene ontology terms in equine seminal plasma as described in [38, 39].

STRING (https://version-10-5.string-db.org) was used to identify potential functional partners of specific proteins.

Identification of discriminant proteins in seminal plasma

Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins in the seminal plasma based on spectral counts among stallions classified in function of the period of time that their ejaculates can be stored under refrigeration. Variance filtering was used to reduce the noise and improve the detection of discriminant variables [40]. Identification of discriminant variables between subgroups of stallions (*good*, *good-RSP*, and *poor*) was performed by fitting a linear model for each variable with the condition of semen lifespan under refrigeration as predictor. The P-values were adjusted for multiple testing with the Benjamini-Hochberg method [41], variables with adjusted p values below 0.1 were considered significant. T-tests between two different conditions were also performed and differences were considered significant when $P < 0.05$. Data were Log_2 transformed and normalized, and comparisons were performed filtered by fold change >2.

Computer-assisted Sperm Analysis (CASA)

Sperm motility and velocities were assessed with a Computer-Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) [30, 42]. Samples were loaded into a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a warmed stage at 38 °C. Sixty consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41) and 500 spermatozoa per sample were then analyzed in random fields. Spermatozoa VAP > 35 µm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile. The following parameters were measured: percentages of linearly motile spermatozoa, and circular (VCL) straight line (VSL) and average (VAP) velocities in µm/s.

Statistical analysis

The normality of the motility and sperm velocity data was assessed using the Kolmogorov-Smirnoff test. Paired t-tests and One-way ANOVA followed by Dunnett's multiple comparisons test were

performed using GraphPad Prism version 7.00 for Mac, La Jolla California USA, (www.graphpad.com).

RESULTS

Three ejaculates from each of 10 stallions were used in this study, and 3,544 different proteins were identified and matched to the equine proteome database. The complete list of SP-proteins is provided as supplementary material (Suppl table 1).

Gene ontology analysis of seminal plasma proteins

Gene ontology (GO) analysis (biological processes BP) of SP-proteins returned enriched terms related to metabolism (Table 1), only terms with a fold enrichment > 2 are provided. Interestingly, terms related to redox regulation were also present including the terms *cellular response to hydrogen peroxide* (GO:0070301) and *response to hydrogen peroxide* (GO:0042542). Gene ontology terms for cellular components (CC) are provided in Table 2 and include terms related to *immunological synapse* (GO:0001772) responses and *extracellular space (GO:0005615)* and *extracellular region* (GO:0005576). The g:profiler analysis of orthologs revealed, in addition to terms related to metabolism, an enrichment in GO terms (cellular components) related to *extracellular vesicles* (GO:1903561), *extracellular exosome* (G0:0070062) *and vesicle mediated transport* (GO:0016192) (Fig 1.), suggesting a role for SP in promoting cell-to-cell communication. Notably, the term *animal organ development* (GO:0048513) was observed in the enrichment.

Pathway enrichment analysis

The results of the pathway enrichment analysis using g:profiler with human orthologs are given in the Manhattan plot in Fig 2 and accompanying table. Reactome and KEGG pathways related to *hexose metabolism*, *vesicle mediated transport, post translational modification of proteins*, and pathways related to the *immune response* were enriched.

Functional network analysis

The ClueGo analysis employed gene ontology and KEGG data resources to classify the proteome of the equine seminal plasma in a functional network. In order to reduce the complexity of the network the fusion option was applied. The network obtained is presented in Figure 3. The functional network obtained suggested important roles of seminal plasma proteins in the regulation of ovulation, of TGF-β and of mitochondrial membrane permeability.

Impact of the equine SP-proteome on the preservation of chilled semen

Stallion ejaculates were collected and evaluated in terms of life span after extension and refrigeration at 5ºC for 48h. Three different outcomes were evident (Fig 4); *good* were those stallions in which total motility and curvilinear velocity (VCL) were >50% and 100 μm/s respectively. Some stallions required SP-elimination to remain above these values, *good-RSP* and a third group classified as *poor* was unable to maintain these values despite RSP. The proteome was studied in these three groups, and differences observed between groups with significant differences in the number of proteins over or underrepresented in each group. In particular, numerous proteins were detected as being underrepresented in the *poor* group of stallions. In order to identify specific proteins in each of the groups that may explain the differences observed, an ANOVA was performed, and a heat map constructed (Fig 5), specific proteins overrepresented in both groups of *good* stallions included a *peroxiredoxin-6 like protein*, and *transcobalamin-2*, a primary vitamin B12-binding and transport protein. Also, the protein involved in protein glycosylation, *ST3 beta-galactoside alpha-2,3-sialyltransferase 1* was present in the *good* stallions (Fig 6). These proteins were nearly absent in *poor* stallions and the glucagon receptor was absent in samples from stallions in the *poor* group. Following this, volcano plots were used to explore potential candidates that may explain differences between stallions, and revealed that *poor* stallions were characterized by low levels of antioxidant proteins, particularly *catalase, superoxide dismutase* and *peroxiredoxin 6 like protein (Fig 7C)*, while *good* stallions presented high amounts of peroxiredoxin 6 like protein (Fig 7A) and *annexin A2* was enriched in stallions requiring SP-removal (Fig. 7B).

More specifically, and due to its practical importance, differences between stallions that need SP-removal to withstand refrigeration, from those that withstand refrigeration after extension in the presence of SP were explored, focusing on which proteins were differentially expressed by two groups of stallions classed as: *good* or *good-RSP*. In the volcano plots, two proteins (*annexinA2* and

spectrin beta chain) were underrepresented in *good* stallions while overrepresented in *good-RSP* stallions (Fig 8a-b). In particular, annexinA2 was revealed as a powerful discriminating variable for identification of *good-RSP* stallions (Fig 9), with a p =0.002 and a q value= 0.005. STRING was used to identify potential functional partners of this protein, and included *Protein S100-A10, Kalirin, Obscurin, Plasminogen, Plasminogen activator, Trio Rho guanine nucleotide exchange factor, Annexin A11, Caspase4 and Caspase 9* (Fig 8C).

Discussion

This study investigated the proteome of SP in horses using a shot gun proteomics approach and a bioinformatic enrichment analysis. Although previous studies have addressed equine SP [12, 16, 43-48], this is, to the authors' knowledge, the first study focused not only on the detailed description of the SP-proteome but also linking specific SP-proteins with the ability of the ejaculate to withstand storage by refrigeration at 5°C for 48h. Enrichment analysis suggested that equine SP proteins play important roles in the metabolism of hexoses, extracellular vesicles and in response to hydrogen peroxide, since these terms were significantly enriched. Hexose metabolism and extracellular vesicles are linked, acting as a support mechanism for spermatozoa. Prostasomes, a specific class of extracellular vesicles, express glycolytic enzymes with capacity for ATP production [49-51]. Interestingly, the abundance of SP-proteins involved in carbohydrate metabolism relates to stallion fertility [18]. The proteins involved in these metabolic pathways are present in equine prostasomes [50] which can be linked to the enrichment findings in our study. Another relevant function of SP, enriched in our analysis, was the response to stress and to hydrogen peroxide. Catalase has been reported as a key antioxidant present in equine SP that is derived primarily from prostatic secretions [47]. In addition, in relation to redox regulation, the GO term NADH regeneration (GO:0006735) was significantly enriched in equine SP. Finally, another group of enriched terms were those related to post-transcriptional modification of proteins, particularly SUMOylation, with the reactome pathways *SUMOylation and SUMO E3 ligases SUMOylate target proteins*. Since the spermatozoa is a translationally and transcriptionally silent cell, it relies heavily on post translational modifications for its regulation [52, 53]. This finding suggests a major role of SP in regulating sperm functionality; further supported by the study of functional networks. ClueGO analysis revealed a highly interconnected network of SP-proteins, with particularly relevant functions concerning the regulation of ovulation, TGF-β and mitochondrial membrane permeability. The existence of

ovulation inductor factors in equine seminal plasma have been suggested previously [54] and further research is warranted.

In addition to enrichment analysis, the proteome in 10 stallions routinely used in a commercial facility producing seminal doses was studied. They were classified into three groups according to the ability of their ejaculates to withstand extension and posterior conservation by refrigeration with or without removal of SP. Differences in the proteome among these groups were investigated, in an attempt to identify specific proteins or groups of proteins, that may explain the different ability of ejaculates to withstand conservation by refrigeration at 5º C . Numerous proteins were less abundant in samples with a reduced life span under refrigeration, many of these proteins were related to redox regulation, including catalase, peroxiredoxin 6 and superoxide dismutase. Differences among stallions in superoxide dismutase and glutathione peroxidases content have been previously reported [55]. In addition, another protein with antioxidant activity, HSP-90 [56] was found to be less abundant in *poor* samples. Interestingly, alteration of redox regulation inactivates HPS-90 due to the action of reactive electrophilic aldehydes generated during the loss of redox homeostasis [57]. All together, these findings support the hypothesis that ineffective redox regulation is behind the limited period of time that these ejaculates can be conserved by refrigeration. Other proteins related to metabolism, like oxoglutarate dehydrogenase, glyceraldehyde 3 phosphate dehydrogenase or proteins involved in cell viability like PI3K were also reduced in this group of stallions. The total absence of the glucagon receptor in *poor* samples found in our study is also noteworthy. This receptor has recently been described in human spermatozoa [58]. In this study glucagon fueled phosphatidylinositol-3-kinase (PI3K)/AKT signaling and was reversed by the protein kinase inhibitor H89, indicating a dependence of glucagon signaling on protein kinase A (PKA). A stimulatory action of glucagon on lactate dehydrogenase and glucose-6-phosphate dehydrogenase (G6PDH) activities was observed in the above-mentioned study. Peroxiredoxin 6 was found to be most abundant in *good* stallions and catalase was also abundant, but with no difference in levels from stallions that needed removal of seminal plasma to achieve a good period of conservation.

The protein *ST3 beta-galactoside alpha-2,3-sialyltransferase 1* (ST3GAL1) was highly enriched in *good* stallions. This protein plays a role in post-transcriptional modification of proteins, and interestingly the gene for ST3GAL1 has previously been associated with sperm storage duration in the oviduct in chickens [59]. This finding falls in line with the special dependence of post transcriptional modifications for the proper functioning of the spermatozoa. The comparison

between stallions that needed removal of most SP (*good-RSP*) and those which did not require this procedure (*good*) identified a number of proteins as better candidates for bio-markers, with annexin highly enriched in *good-RSP* stallions, while barely present in *good* stallions. Annexins have a wide variety of cellular functions, including cell proliferation, differentiation, apoptosis, migration, membrane repair and inflammatory response [60]. Although annexin can be considered a good potential marker to distinguish samples that will require SP-removal, the reasons behind this remain to be fully understood, but potential mechanisms involved may include an increase in inflammatory responses [61] or accelerated progression of apoptosis in these stallions [62, 63]. Equine annexins may stimulate the production of reactive oxygen species, a mechanism involved in these responses [61]. The network analysis also supported this hypothesis, with annexin interacting with proteins involved in cell survival and immune regulation.

In conclusion, this study provides the first detailed description of the proteome of equine seminal plasma. Bioinformatic analysis revealed major roles related to hexose metabolism, that may be mediated by extracellular vesicles, probably involving prostasomes. In addition, findings are suggestive of major roles of SP in redox regulation and immune response regulation. The finding that the varied presence of specific proteins from groups of SP-proteins have potential value as markers for the ability of ejaculates to withstand refrigeration, and whether or not they may need SP-removal to achieve this goal is of particular practical interest. Moreover, findings offer new potential lines of research, with applications both in sperm biotechnologies and reproductive medicine.

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Table 1. Panther overrepresentation test for proteins of interest identified by UHPLC/MS/MS GO analysis of equine (*Equus caballus*) seminal plasma.

GO biological process (BP)	Fold-enrichment	P value
Hemoglobin metabolic process (GO:0020027)	40.14	1.32E-04
ATP generation from ADP (GO:0006757)	21.15	1.28E-07
Glycolytic process (GO:0006096)	21.15	1.28E-07
ADP metabolic process (GO:0046031)	16.81	5.05E-07
Purine ribonucleoside diphosphate metabolic process (GO:0009179)	14.90	1.04E-06
Purine nucleoside diphosphate metabolic process (GO:0009135)	14.90	1.04E-06
Pyruvate metabolic process (GO:0006090)	14.57	1.20E-06
Ribonucleoside diphosphate metabolic process (GO:0009185)	14.25	1.37E-06
Nucleoside diphosphate phosphorylation (GO:0006165)	13.95	1.55E-06
Nucleotide phosphorylation (GO:0046939)	13.66	1.77E-06
Cellular response to hydrogen peroxide (GO:0070301)	12.32	8.74E-05
Response to hydrogen peroxide (GO:0042542)	11.02	3.01E-05
Carbohydrate catabolic process (GO:0016052)	10.86	1.54E-06
Nucleoside diphosphate metabolic process (GO:0009132)	10.57	8.28E-06
Cellular response to antibiotic (GO:0071236)	8.65	1.05E-04
ATP metabolic process (GO:0046034)	5.59	1.37E-04
Cellular response to organic cyclic compound (GO:0071407)	4.16	1.16E-05
Carbohydrate metabolic process (GO:0005975)	3.89	2.39E-05
Response to drug (GO:0042493)	3.10	8.73E-05
CC morphogenesis (GO:0032989)	2.82	1.03E-04
Regulation of response to stress (GO:0080134)	2.73	7.73E-06

PANTHER Overrepresentation Test (Released 2020-04-07) GO Ontology database Released 2020-02-21 Fisher's Test. ATP, adenosine triphosphate; ADP, adenosine diphosphate; FDR, false discovery rate; GO, Gene Ontology; CC, cellular component; UHPLC/MS/MS, ultra high-performance liquid chromatography with tandem mass spectrometry. FDR, P < 0.05.

Table 2. Panther overrepresentation test (GO CC complete) of proteins of interest identified by UHPLC/MS/MS and GO analysis of equine (*Equus caballus*) SP.

GO Cellular component (CC)	Fold-enrichment	P value
Immunological synapse (GO:0001772)	13.38	3.46E-04
Mitotic spindle (GO:0072686)	6.76	1.19E-04
Microtubule cytoskeleton (GO:0015630)	2.38	1.78E-04
Cytoskeleton (GO:0005856)	2.31	2.01E-06
Extracellular space (GO:0005615)	2.23	2.06E-04
Extracellular region (GO:0005576)	1.98	1.36E-04
Intracellular non-membrane-bounded organelle (GO:0043232)	1.91	1.94E-07
Nonmembrane-bounded organelle (GO:0043228)	1.91	1.96E-07

PANTHER Overrepresentation Test (Released 20200407) GO Ontology database Released 2020-02-21 Fisher's Test. CC, cellular component; FDR, false discovery rate; GO, gene ontology; SP, seminal plasma; UHPLC/MS/MS, ultra high-performance liquid chromatography with tandem mass spectrometry. FDR, P < 0.05.
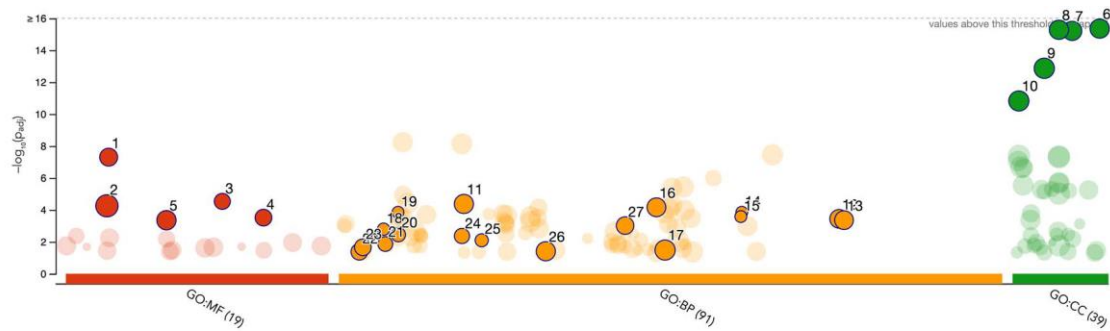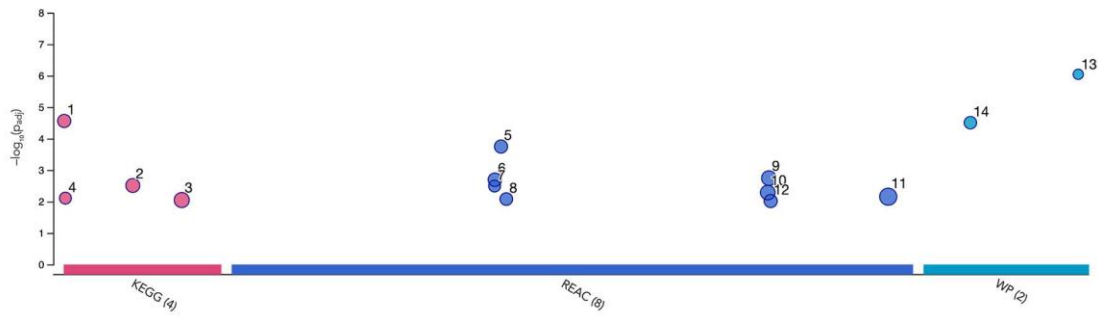
ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0008092	cytoskeletal protein binding	4.973×10^{-8}
2	GO:MF	GO:0005515	protein binding	5.547×10^{-5}
3	GO:MF	GO:0045296	cadherin binding	2.938×10^{-5}
4	GO:MF	GO:0050839	cell adhesion molecule binding	3.071×10^{-4}
5	GO:MF	GO:0019899	enzyme binding	4.437×10^{-4}
6	GO:CC	GO:1903561	extracellular vesicle	4.411×10^{-16}
7	GO:CC	GO:0070062	extracellular exosome	6.122×10^{-16}
8	GO:CC	GO:0043230	extracellular organelle	5.231×10^{-16}
9	GO:CC	GO:0031982	vesicle	1.354×10^{-13}
10	GO:CC	GO:0005615	extracellular space	1.505×10^{-11}
11	GO:BP	GO:0016192	vesicle-mediated transport	4.254×10^{-5}
12	GO:BP	GO:0120036	plasma membrane bounded cell projection organization	3.658×10^{-4}
13	GO:BP	GO:0140352	export from cell	4.389×10^{-4}
14	GO:BP	GO:0061718	glucose catabolic process to pyruvate	1.411×10^{-4}
15	GO:BP	GO:0061615	glycolytic process through fructose-6-phosphate	2.629×10^{-4}
16	GO:BP	GO:0046903	secretion	6.879×10^{-5}
17	GO:BP	GO:0048513	animal organ development	3.292×10^{-2}
18	GO:BP	GO:0006007	glucose catabolic process	1.626×10^{-3}
19	GO:BP	GO:0006735	NADH regeneration	1.411×10^{-4}
20	GO:BP	GO:0006757	ATP generation from ADP	3.563×10^{-3}
21	GO:BP	GO:0006090	pyruvate metabolic process	1.378×10^{-2}
22	GO:BP	GO:0002283	neutrophil activation involved in immune response	4.206×10^{-2}
23	GO:BP	GO:0002446	neutrophil mediated immunity	2.201×10^{-2}
24	GO:BP	GO:0016052	carbohydrate catabolic process	4.308×10^{-3}
25	GO:BP	GO:0019320	hexose catabolic process	8.009×10^{-3}
26	GO:BP	GO:0033554	cellular response to stress	3.915×10^{-2}
27	GO:BP	GO:0045055	regulated exocytosis	9.652×10^{-4}

version	e99_eg46_p14_f929183
date	20/4/2020 19:16:05
organism	hsapiens

g:Profiler

Figure 1.- g:GOST multiquery Manhattan plot showing enrichment analysis of whole equine seminal plasma proteins. The whole equine seminal plasma proteome was queried against the equine proteome database. Gene Ontology terms (GO) for molecular function (MF) are in red, for biological process (BP) in orange, and for cellular component (CC) in green. The P values are depicted on the y axis and in more detail in the results table below the image.

ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	KEGG	KEGG:00010	Glycolysis / Gluconeogenesis	2.747×10^{-5}
2	KEGG	KEGG:04066	HIF-1 signaling pathway	3.081×10^{-3}
3	KEGG	KEGG:05132	Salmonella infection	8.960×10^{-3}
4	KEGG	KEGG:00051	Fructose and mannose metabolism	7.784×10^{-3}
5	REAC	REAC:R-HSA-70171	Glycolysis	1.785×10^{-4}
6	REAC	REAC:R-HSA-70326	Glucose metabolism	2.028×10^{-3}
7	REAC	REAC:R-HSA-70263	Gluconeogenesis	3.217×10^{-3}
8	REAC	REAC:R-HSA-3214842	HDMs demethylate histones	8.338×10^{-3}
9	REAC	REAC:R-HSA-2990846	SUMOylation	1.804×10^{-3}
10	REAC	REAC:R-HSA-3108232	SUMO E3 ligases SUMOylate target proteins	5.166×10^{-3}
11	REAC	REAC:R-HSA-5653656	Vesicle-mediated transport	7.010×10^{-3}
12	REAC	REAC:R-HSA-4551638	SUMOylation of chromatin organization proteins	9.655×10^{-3}
13	WP	WP:WP4629	Computational Model of Aerobic Glycolysis	9.040×10^{-7}
14	WP	WP:WP534	Glycolysis and Gluconeogenesis	3.119×10^{-5}

version	e99_eg46_p14_f929183
date	19/4/2020 19:04:52
organism	hsapiens

g:Profiler

Figure 2.- g:GOST multiquery Manhattan plot showing comparative enrichment analysis of whole equine seminal plasma proteins. The whole equine seminal plasma proteome was queried against the equine proteome database. Kyoto Encyclopedia of Genes and Genomes are depicted in red, Reactome pathways in dark blue and Wikipathways in light blue (all using human orthologs). The P values are depicted on the y axis and in more detail in the results table below the image.
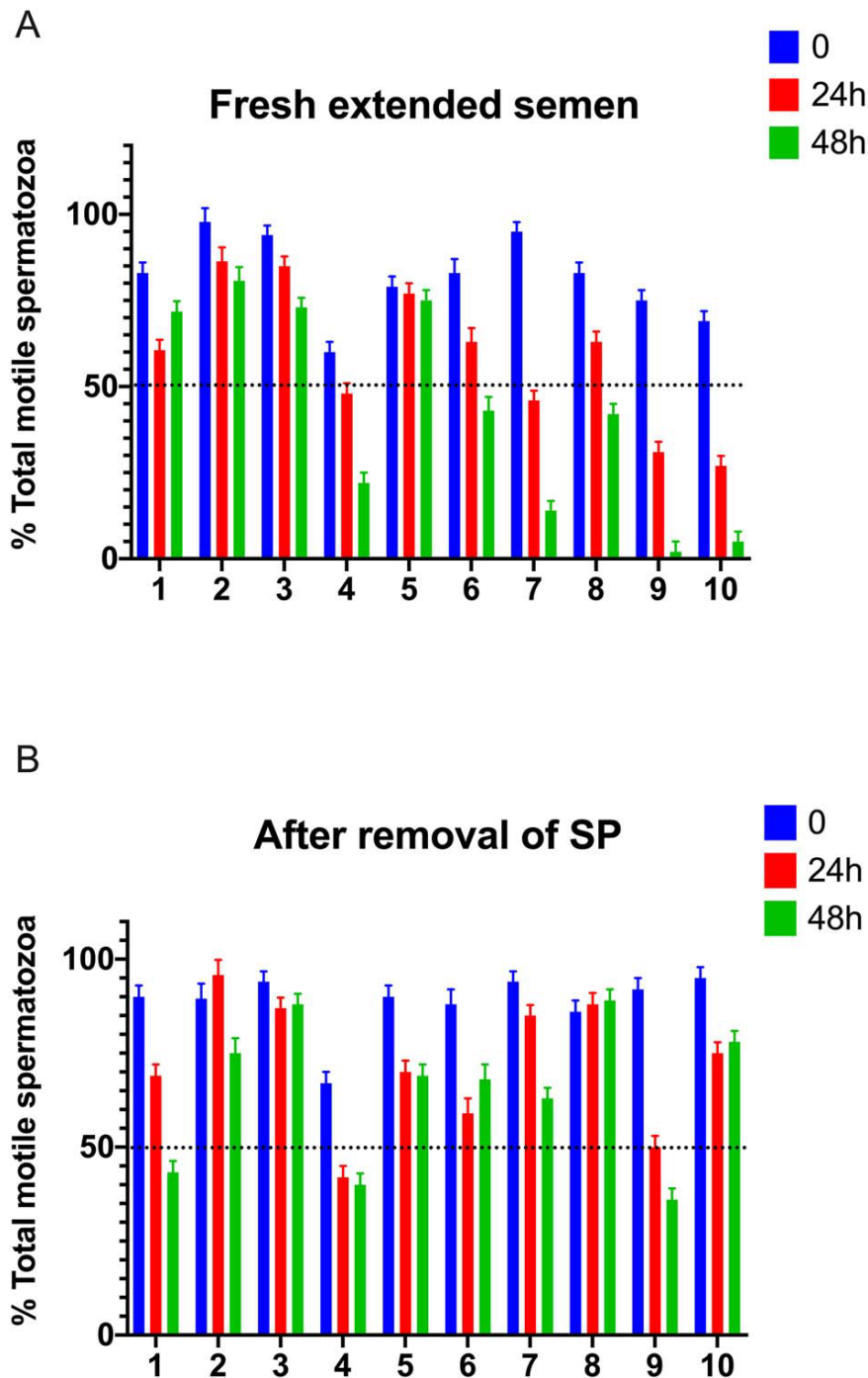
Figure 3.- CLueGO network analysis of proteins in equine seminal plasma. To reduce redundancy of GO terms, the fusion option was selected. GO/KEGG pathway functionally grouped networks with terms are indicated as nodes (Benjamini–Hochberg p value<0.05), linked by their kappa score level (≥0.35) where only the label of the most significant term per group is shown.

Figure 4.- Storage under refrigeration resistance test of the 10 stallions used in a commercial AI program used in the present study. Ejaculates were collected and split into two subsamples, one was extended in INRA 96 to 25 x10^6 spm/ml, and the other half was extended after removal of seminal plasma by centrifugation and chilled at 5°C for up to 48h. Sperm motility using computer assisted sperm analysis (CASA) classified stallions into three groups: *good* if motility after 48h was >50% and VCL was >100μm/s, *poor* if motility after 48h was <50% and VCL was < 100μm/s and *good-RSP*, if 48h after centrifugation and removal of the bulk of seminal plasma, motility was >50% and VCL was >100μm/s. In A) stallions 1, 2, 3 and 5 were *good* stallions, in B) stallions 6,7,8 and 10 were *good-RSP*, stallions 4 and 9 were *poor* and did not improve after removal of seminal plasma. In a to c the percentage of total motile spermatozoa after 24 and 48 horas of conservation by refrigeration at 5ºC are given in the three groups of stallions. In d to f, circular velocity is given. Data are means± sem. ** *P*<0.01, *** *P*<0.001, n.s. not significant.
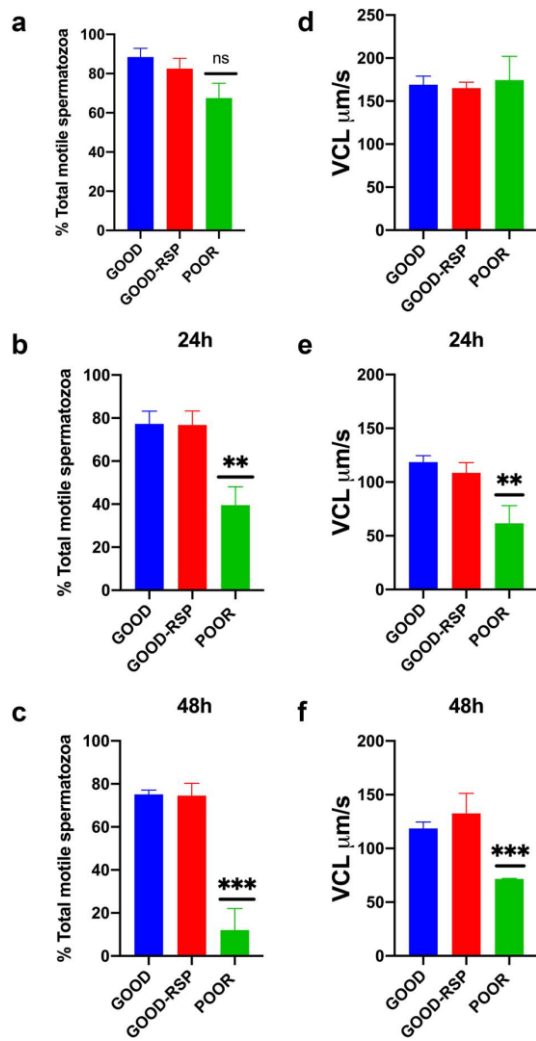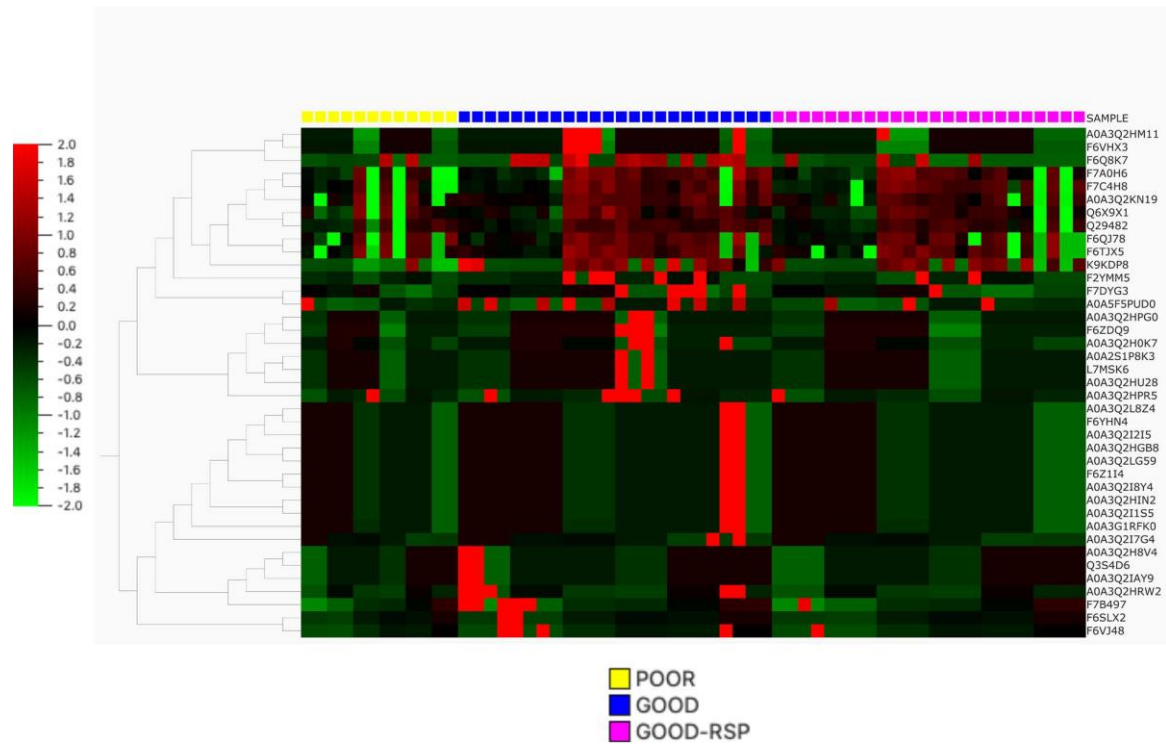
Figure 4.- Continued.

Figure 5.- Heat map showing the identification of discriminant variables (seminal plasma proteins) for the group of *good* stallions in comparison with *good-RSP* and *poor* with a fold change > 2, *P*<0.05 and q=0.1
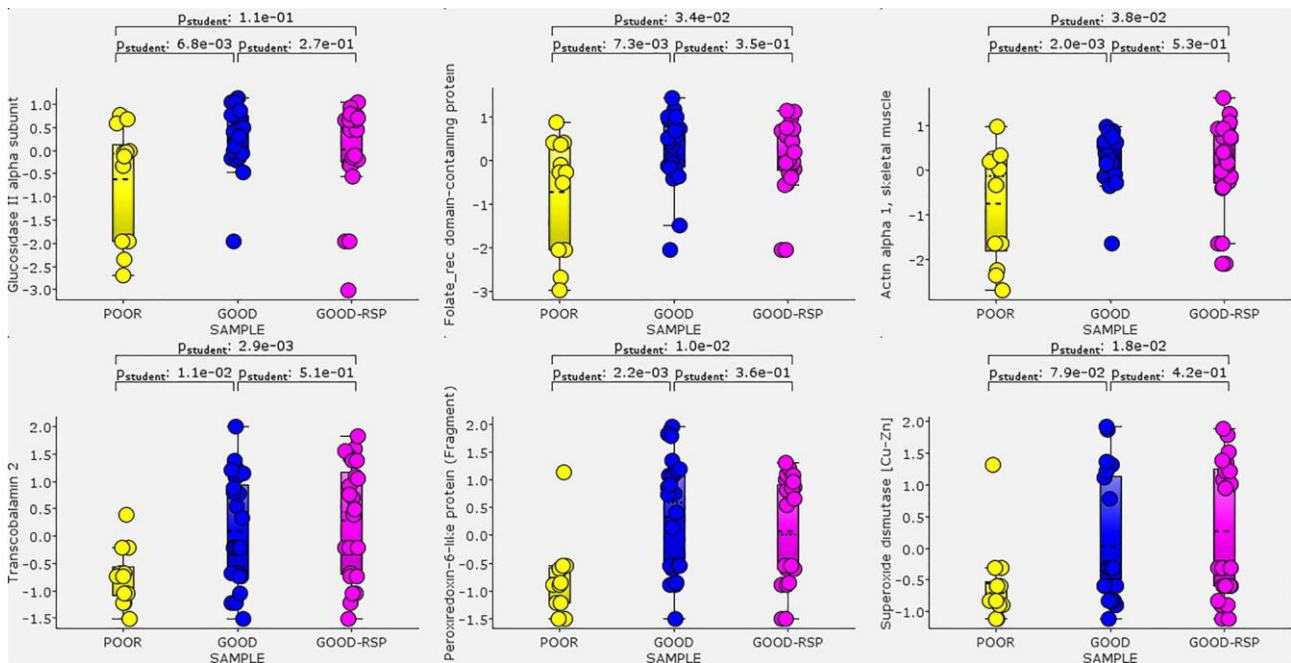

Figure 6. Differences in the amount of specific representative proteins in the three groups of stallions, *good*, *good-RSP* and *poor*. Qlucore Omics Explorer (Lund, Sweden https://qlucore.com) was used to compare differences in the relative amounts of proteins in the seminal plasma based on spectral counts among stallions classified in function of the period of time that their ejaculates can be stored under refrigeration. *Poor* samples are represented in yellow boxes, *good* samples in blue boxes, and *good-RSP* in pink boxes.
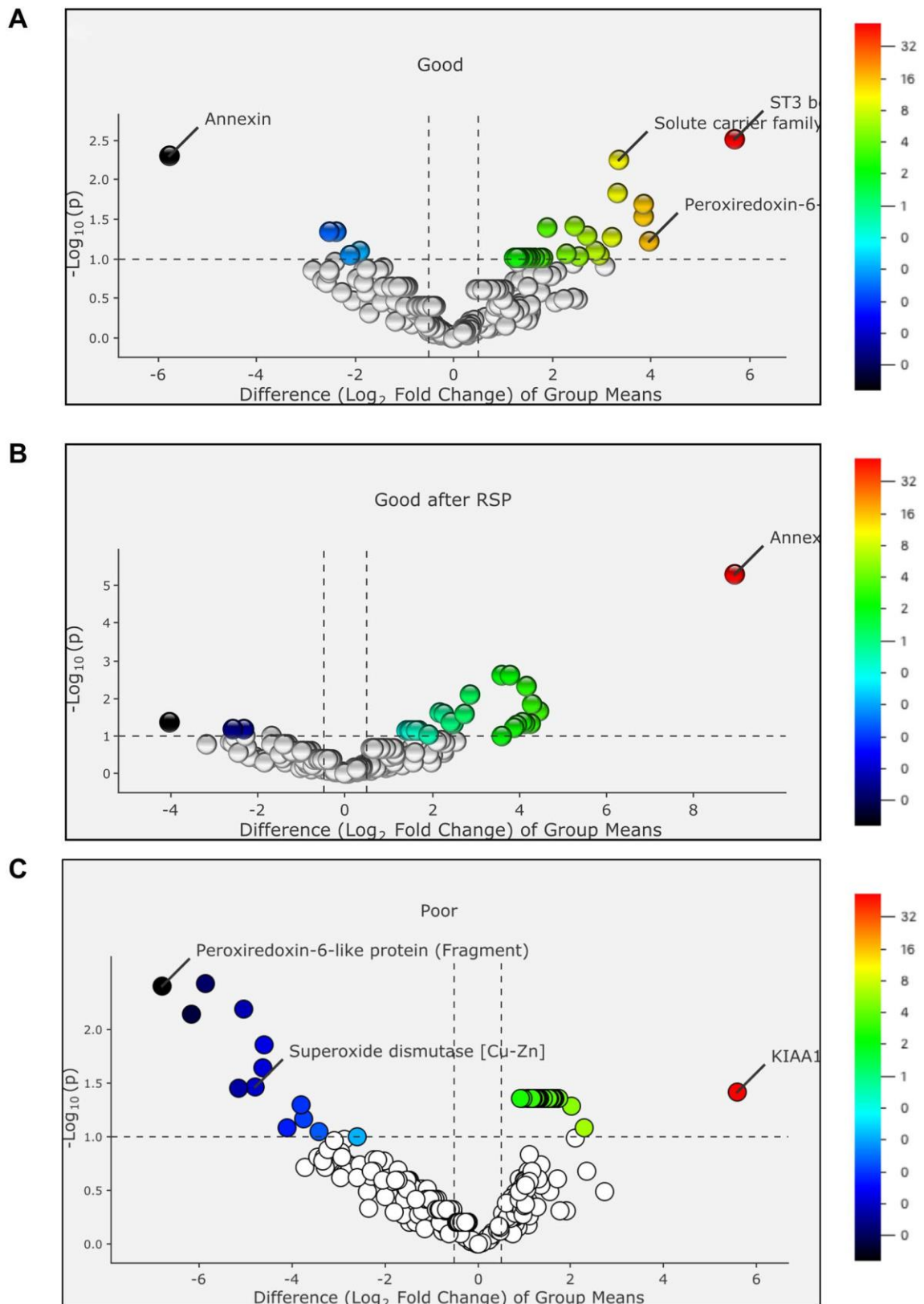
Figure 7.- Volcano plots showing proteins enriched or underrepresented in the three groups of stallions (A: *good*, B: *good-RSP* and C: *poor*). Representative proteins identified in each group are depicted.
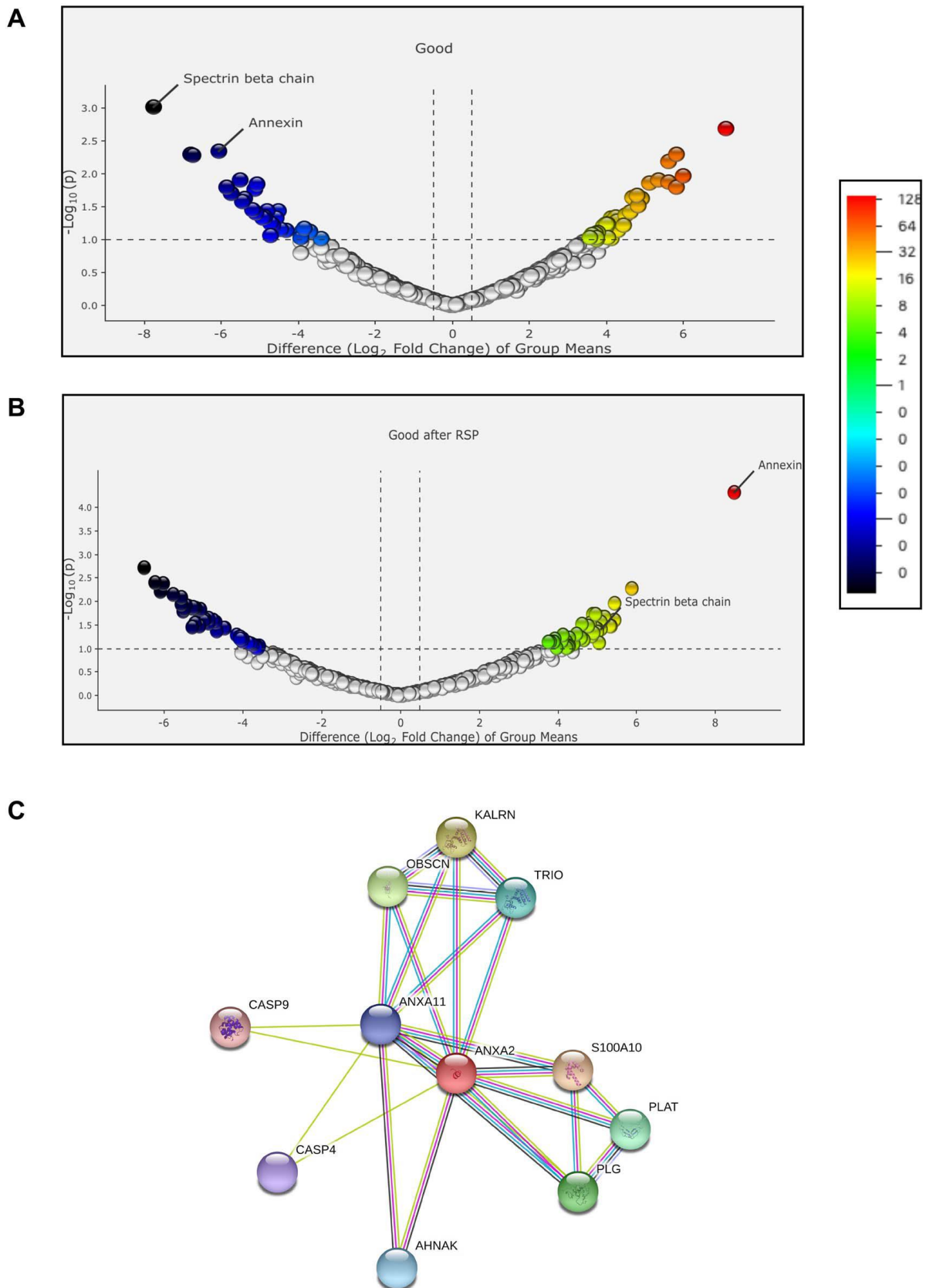
Figure 8.- Volcano plots showing proteins with the highest power as discriminant variables between *good* and *good-RSP* stallions. AnnexinA2 appeared as a powerful discriminant variable to identify stallions needing removal of seminal plasma prior to chilled preservation, with a p =0.002 and a q value= 0.005. C STRING analysis (https://string-db.org) was used to identify potential functional partners of this protein.
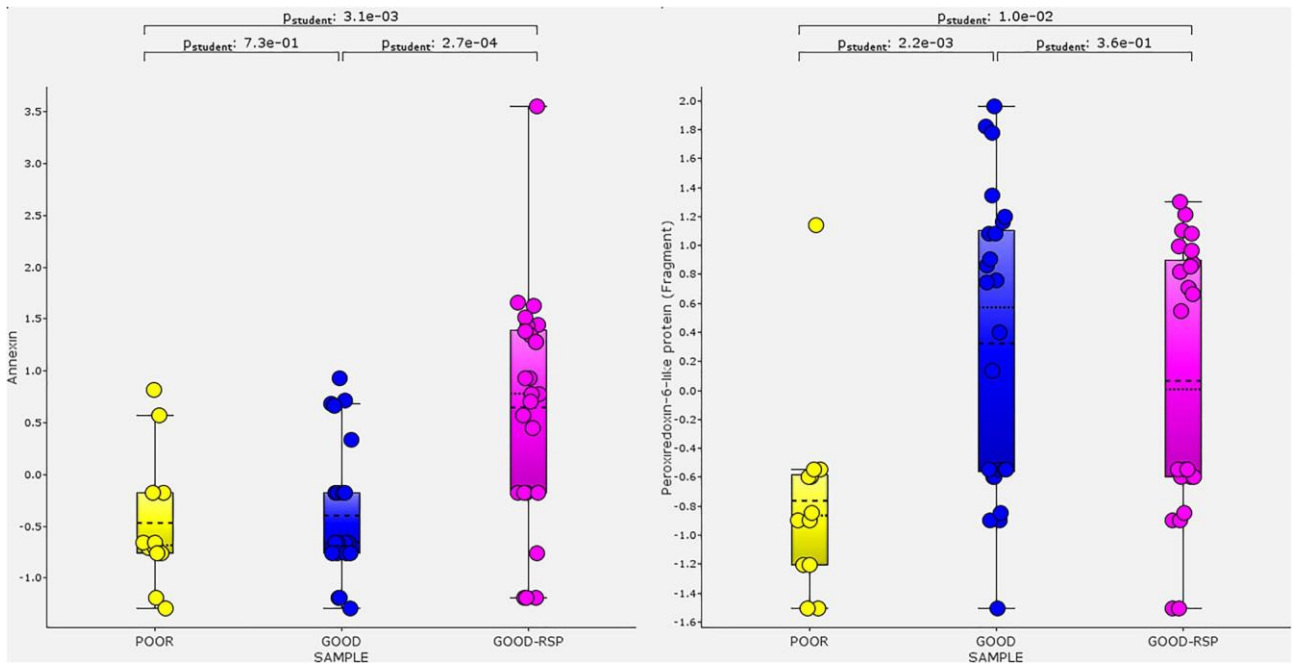
Figure 9. Amount of specific seminal plasma proteins in specific groups of stallions. Annexin A2 (A0A3Q2LPE6) and Peroxiredoxin-6 like protein (K9KDP8). Annexin was present in significantly larger amounts in the group of stallions requiring removal of seminal plasma (left panel), while the presence of Peroxiredoxin-6 like protein was significantly reduced in the seminal plasma of stallions classified as *poor* (right panel).