The Seminal plasma proteins *Peptidyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase and KIAA0825* are linked to better motility post thaw in stallions

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ABSTRACT

Seminal plasma plays an important role in sperm physiology. Seminal plasma proteins vehiculated in microvesicles, carry RNAs and proteins with a potential role in early embryo development. Additionally, proteins present in seminal plasma participate in redox regulation and energy metabolism. In view of these facts, we hypothesized that differences in protein composition of the seminal plasma among stallions may help to explain differences in freeze-ability seen among them. Three independent ejaculates from 10 different stallions of varying breeds were frozen using standard protocols in our laboratory. Aliquots of the ejaculate were separated and stored at -80º C until further proteomic analysis. Semen analysis was performed using computer assisted sperm analysis and flow cytometry. Significant differences in proteome composition of seminal plasma were observed in the group of stallions showing better motility post thaw. 3116 proteins were identified, and of these, 34 were differentially expressed in stallions with better motility post thaw, 4 of them were also

differentially expressed in stallions with different percentages of linearly motile sperm post thaw and 1 protein, Midasin, was expressed in stallions showing high circular velocity post thaw.
Seminal plasma proteins may play a major role in sperm functionality; being vehiculated through extracellular vesicles and participating in sperm physiology. Bioinformatic analysis identifies discriminant proteins able to predict the outcome of cryopreservation, identifying potential new biomarkers to assess ejaculate quality.

Key words: stallion, spermatozoa, flow cytometry, CASA, proteomics, cryopreservation, seminal plasma

1.- Introduction

Seminal plasma is composed of secretions from the accessory sex glands and has immunosuppressive/immunoregulatory functions[1, 2]. This fluid harbors numerous microvesicles, mainly prostasomes[3], with different functions nourishing spermatozoa and probably carrying RNAs and proteins with a role in early embryo development[4]. Despite its importance, equine seminal plasma has received little attention in comparison to the spermatozoa. However, the composition of equine seminal plasma has been the subject of several studies, some of them in relation to the freezeability of the ejaculate[5-8]. A detailed description of the proteome of equine seminal plasma has recently been published [9]. In this study, reactome and KEGG pathway analysis revealed an important role of seminal plasma in metabolism and vesicle mediated transport. Moreover, a specific protein in the seminal plasma, Annexin A2 served as a discriminant variable for stallions that needed removal of seminal plasma to maintain the quality of their ejaculates when conserved by refrigeration. However, little information is available regarding the potential relationship between the proteins present in seminal plasma and the freeze-ability of the ejaculates. While cryopreservation of stallion spermatozoa is a reproductive technology of which use is increasing year after year, unresolved questions remain. One of the major drawbacks is the high stallion-to-stallion variability, that precludes many valuable stallions entering the international market for horse semen[10]. Undoubtedly, increasing knowledge around the factors that are involved in this variability will open new opportunities enabling its reduction. While sperm factors have received attention [11-14], the role of seminal plasma has not been so extensively investigated in relation with freeze-ability [6, 15, 16]. Previous reports indicate that equine seminal plasma plays important roles in hexose metabolism and is rich in antioxidants [6, 9, 17]. Cryopreservation causes osmotic induced necrosis in a high proportion of spermatozoa, while the surviving population experience a compromise in their energetic

metabolism and redox regulation [18, 19]. Since seminal plasma has roles in redox regulation and energetic metabolism through prostasome and other micro-vesicles that vehiculate, we hypothesized that differences in protein composition of the seminal plasma between stallions may help to explain differences in freeze-ability seen among them. The objective of this study was to identify proteins in seminal plasma that could be potential markers of freeze-ability using potent bioinformatic tools.

2.- Material and methods

2.1.- Reagents and media

All chemicals were purchased from Sigma-Aldrich (https://www.sigmaaldrich.com/spain), unless otherwise stated. JC-1, monochlorobimane (MCB), Annexin V 647 conjugated, CellEvent® Caspase 3/7 Green Detection Reagent, Hoechst 33342 and Ethidium homodimer (Eth-1) were purchased from Thermofisher (https://www.thermofisher.com/es/es/home.html) DRAQ7 was purchased from Beckman Coulter (https://www.beckmancoulter.com/es).

2.2.- Experimental design

Three independent ejaculates from 10 different stallions (n=30) were frozen using standard protocols in our laboratory [13, 19, 20]. Aliquots of the same ejaculate used for freezing semen were separated, and the seminal plasma removed by serial centrifugation (2 x 1500g 10') and stored at -80º C until proteomic analysis. The absence of spermatozoa or other contaminant cells in the samples was assessed under phase contrast microscopy. Stallions were classified according to the outcome of cryopreservation relative to total motility of their ejaculates post thaw (good > 35%). This threshold was based on current recommendations for minimum quality for commercial doses of equine semen *http://www.wbfsh.org/GB/Other%20activities/Semen%20standards.aspx)*, linear motility post thaw (good > 30%) circular velocity post thaw (good > 115µm/s), viability (good > 40% live spermatozoa at thawing) and mitochondrial membrane potential (> 40% of spermatozoa showing high mitochondrial membrane potential at thawing). Ejaculates were classified according of the number of good scores in the 5 different categories (0, 1, 2, 3, and 4), however there were no ejaculates which achieved the highest score in all five categories.

2.3.-*Semen collection and processing*

Semen was collected from stallions of different breeds maintained as indicated by specific institutional and European regulations for animal care (Law 6/2913 June 11th and European Directive 2010/63/EU). The ethical committee of the University approved this study. Ejaculates were collected using a warmed, lubricated Missouri model artificial vagina and the gel fraction of the ejaculate was removed with an inline filter. Upon arrival at the laboratory, the semen was processed through colloidal centrifugation [21, 22] and seminal plasma removed. The ejaculate was extended in freezing media and frozen using standard procedures that have been previously described by our laboratory [19]. In brief semen was diluted in the Cáceres freezing medium (University of Extremadura, Cáceres, Spain) containing 2% egg yolk, 1% glycerol, and 4% dimethylformamide to 100 $x10^6$ spermatozoa/ml. After loading the extended semen into 0.5-mL straws (IMV, L'Aigle, France), the straws were ultrasonically sealed with an UltraSeal 21® (Minitube of America MOFA, Verona, Wisconsin, USA) machine and immediately placed in an IceCube 14S (SY-LAB Neupurkersdorf, Austria) programmable freezer. The following freezing curve was used. Straws were kept at 20ºC for 15 min, and they were then slowly cooled from 20ºC to 5ºC at a cooling rate of 0.1 ºC/min. Thereafter the freezing rate was increased to -40ºC/min from 5ºC to -140ºC. The straws were then plunged into liquid nitrogen and stored until analysis. Frozen samples were thawed in a water bath at 37°C for at least 30 sec.

2.4.- *Protein solubilization*

Seminal plasma aliquots were solubilized in lysis buffer as previously described[9] and 20 microliters of lysis buffer was added, the solution was vortexed and incubated under constant rotation at 4ºC for 1 hour.

2.5.- *Protein quantification*

Protein quantification was performed using the 2-D Quant Kit [9]. All samples were normalized to obtain a final concentration of 100 μg of protein per sample.

2.6.- *In-solution trypsin digestion*

200 µL of the seminal plasma solution obtained from the previous stage were mixed with 100 µl of 25 mM ammonium bicarbonate buffer, pH 8.5 and the proteins were reduced and alkylated. Digestion was performed by adding 1 µL of Trypsin Proteomics Grade as previously described [9].

2.7.- *UHPLC-MS/MS analysis.*

Separation and analysis of the samples was performed following the protocol described in a previous study [9] with a UHPLC/MS system consisting of 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, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Control of the HPLC and Q-TOF was via MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.06.01).

2.8.- *Data processing*

Data processing and analysis was performed using Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA) following previously described protocols[9].

2.9.- *Computer-assisted sperm analysis (CASA)*

Sperm motility and velocity were assessed using a computer-assisted sperm analysis (CASA) system (ISAS Proiser, Valencia, Spain) in fresh and frozen and thawed spermatozoa according to standard protocols used at our center [23]. Semen samples were loaded into a Leja® chamber with a depth of 20 µm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37°C. Analysis was based on an evaluation of 60 consecutive digitized images obtained using a 10x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa with VAP > 35 µm/s were considered motile. Spermatozoa deviating < 45% from a straight line were classified as linearly motile.

2.10.- *Flow cytometry*

Flow cytometry (FC) analyses were conducted using a Cytoflex®Sflow cytometer (Beckman Coulter) equipped with violet, blue, yellow and red lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each

experiment. Files were exported as FCS files and analyzed using FlowjoV 10.7 Software (Ashland, OR, USA). Unstained, single-stained, and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory [24, 25].

2.10.1.- *Measurement of GSH, viability and mitochondrial membrane potential in stallion spermatozoa*

Intracellular GSH was measured adapting previously published protocols optimized for GSH detection using flow cytometry[26] tailored to equine spermatozoa in our laboratory [27]. Mitochondrial membrane potential and sperm viability were also simultaneously assessed. In brief, sperm aliquots (1-5 x 10^6 sperm/mL) were stained with JC-1 1μM, (30 minutes in the dark at r.t.), DRAQ7 3μM and monochlorobimane (MCB) 10 μM (10 minutes in the dark at r.t). After assessing flow quality, doublets and debris were gated out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates) and DRAQ7, at a peak excitation of 640, and emission of 690 nm.

2.10.2.- *Assessment of caspase 3 activity and phosphatidylserine (PS) translocation*

Annexin V 647 conjugated and CellEvent® Caspase 3/7 Green Detection Reagent were combined in a multiparametric test and evaluated by FC [20]. Samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent (2 μM) and incubated at room temperature for 15 minutes. Following this the samples were washed by a short centrifugation spin for 12" and suspended in 200 μl of Annexin binding-buffer (solution in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Five μL of Annexin V was added to 200 μL of sample. After 15 minutes of incubation in the dark at room temperature, 400 μL of 1 × Annexin binding-buffer was added before analysis using the flow cytometer (Cytoflex® S flow cytometer, Beckman Coulter). To gate dead spermatozoa, samples were stained with 0.3 μM of Eth-1 and incubated for 5 minutes before they were immediately evaluated in a flow cytometer (Cytoflex® flow cytometer, Beckman Coulter). CellEvent staining was validated as previously described [28].

2.11.- *Bioinformatic Analysis*

2.11.1 .- Variance filtering and PCA

Data were normalized and log_2 transformed using Qlucore Omics Explorer (https://qlucore.com). Principal Component Analysis (PCA) was used to visualize the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centering and scaling the remaining variables to zero mean and unit variance. The projection score [29] was used to determine the optimal filtering threshold.

2.11.2.- Identifying discriminating variables

Qlucore Omics Explorer (https://qlucore.com) was used to identify the discriminating variables that are most highly significantly different between good and poor freezers. The identification was performed by fitting a linear model for each variable with condition proteins in seminal plasma as a predictor of the outcome of cryopreservation and including the stallion nuisance covariate. P-values were adjusted for multiple testing using the Benjamini-Hochberg method [30, 31] and variables with adjusted *P*-values (q value) below 0.1 were considered significant.

2.12.- Statistical analysis

End points measured were, the percentage of total motile spermatozoa and circular velocity after thawing, and the percentages of live spermatozoa, caspase 3 postitive spermatozoa and the percentage of spermatozoa showing high mitochondrial membrane potential.

The normality of the motility, sperm velocity and flow cytometry data were 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).

3.- Results

3.1.- Sperm quality post thaw differed between stallions

3.1.1.- Motility and velocities

Significant differences were observed in total motility post thaw between the two groups of stallions, good and poor freezers, with good freezers showing a mean percentage of total motile spermatozoa of 37.7 ± 1.3 % while the group of poor stallions showed a percentage of total motility post thaw of

21 ± 0.71% (*P*<0.001) (Figure 1A). A similar trend was observed for the percentage of linear motile spermatozoa (Figure 1B). Sperm velocity (circular velocity) was also significantly different between both groups, with velocities of 125.6 ± 2.5 μm/s in good stallions, while VCL post thaw in poor freezers was 107.2 ± 2.5 μm/s (*P*<0.0001) (Fig 1C).

3.1.2.- Viability and mitochondrial membrane potential

The percentage of live spermatozoa after thawing in the group of good stallions was 51.1 ± 1.6 % while in the group of poor freezers it was 34.2 ± 1.6% (*P*<0.0001) (Fig 2A). Poor freezers showed higher percentages of caspase 3 positive spermatozoa (13.6 ± 0.6 vs 8.1± 04% in good freezers *P* <0.0001 Fig 2C). Good freezers showed a higher percentage of spermatozoa with high mitochondrial membrane potential (49.1 ± 1.5 % vs 34.5 ± 1.1 in poor freezers, P<0.0001, Fig 2B).

3.2.- Protein composition of seminal plasma differs in stallions with better motility post thaw

To determine possible differences in the composition of seminal plasma in stallions showing better values for motility, viability and mitochondrial activity post thaw, independent volcano plots were constructed in a first step comparing the proteome of the stallions showing significantly higher values in motility, viability and mitochondrial membrane potential with the proteome of the rest of the stallions (Fig 3 A-C). Volcano plots showed a different predominance of specific seminal plasma proteins among stallions showing better results after cryopreservation in the three categories considered (Fig 3 A-C). However, significant differences in the composition of the seminal plasma proteome were only observed between the group of stallions showing better motility post thaw. We identified 3116 proteins, and of these 34, were differentially expressed in stallions with better motility post thaw (Fig 3D), 4 of them were also differentially expressed in stallions with different percentages of linearly motile sperm post thaw and 1 protein, Midasin, was expressed in stallions showing high circular velocity post thaw (Fig 3 D-E). Next, bioinformatic analysis was conducted to identify discriminant variables; proteins in seminal plasma with potential to identify stallions that show good motility post thaw. Qlucore Omics Explorer (https://qlucore.com) was used to identify seminal plasma proteins that are most significantly different based on spectral counts in stallions with better motility post thaw. Proteins of which amounts differed in seminal plasma of stallions showing better motility after thawing were identified (Fig 4 C) with a fold change > 2, P=0.009 and q=0.098. A further filter was applied to the analysis to specifically identify proteins which were more abundant in stallions showing better motility post thaw, and then a much stricter criteria was applied to select

discriminant proteins to obtain the most powerful discriminant variables. Proteins were filtered by a fold change of at least 5 between both conditions, with a P=9.6e-04 and q=0.05. Six proteins were identified as more abundant in the seminal plasma of stallions with better motility post thaw (Fig 4D). These proteins were peptidyl arginine deiminase 2, rRNA adenine N (6)-methyltransferase, KIAA0825, Rho guanine nucleotide exchange factor 28, endoplasmatic reticulum protein 44, and two uncharacterized proteins F6SCY and A0A3Q2HPE3, one corresponding to the RAPGEF6 gene, with a guanyl nucleotide exchange factor activity as molecular function, involved in small GTPase mediated signal transduction. The second uncharacterized protein found corresponding to the ARHGEF18 gene, has guanyl-nucleotide exchange factor activity and participates in small GTPase mediated signal transduction processes.

In relation to the percentages of linear motile spermatozoa, significant differences were observed in the proteome of stallions showing higher percentages of linear motile spermatozoa post thaw (Fig 5). With a fold change >4.75, *P*= 8.7e-4 and q=0.071 four proteins in the seminal plasma were identified as discriminant variables for stallions showing different percentages of linearly motile spermatozoa post thaw (Fig 5 D). These were *Peptitdyl arginine deaminase 2, rRNA adenine N (6)-methyltransferase, KIAA0825 and an* Uncharacterized protein (A0A3Q2IAZ9), corresponding to the SCAF1 gene with RNA polymerase II C-terminal domain binding molecular function.

3.3.- Stallions showing an overall better outcome after cryopreservation show differences in the composition of seminal plasma

Bioinformatic analysis was performed to identify discriminant variables (proteins) in seminal plasma potentially able to identify stallions with a better overall outcome after cryopreservation and six proteins were identified as discriminant variables, being more abundant in ejaculates scored in category 4 (p = 8.7e-4, q = 0.052 fold change > 4). These proteins were the ATR serine/threonine kinase, peptidyldeiminase 2, rRNA adedine N(6) methyltransferase, an uncharacterized protein (A0A3Q2IAZ9), KIAA0825 and the Solute carrier family 25 member 37 (Fig 6).

4.- Discussion

In the present study the relationship between the proteome of seminal plasma in stallions and sperm quality post thaw were investigated. Post thaw sperm quality was defined in terms of the percentages of total and linear motility, circular velocity (VCL) in µm/s and percentages of viable sperm and spermatozoa showing high mitochondrial membrane potential. Three proteins were significantly

enriched in the seminal plasma of stallions showing better motility (total and linear) post thaw, *peptidyl arginine deiminase 2, rRNA adenine N (6)-methyltransferase and KIAA0825*. Other proteins were observed in stallions with better motility post thaw including th*e Rho guanine nucleotide exchange factor 28, endoplasmatic reticulum protein 44,* and two uncharacterized proteins *F6SCY and A0A3Q2HPE3, one corresponding to the RAPGEF6 gene,* with a guanyl nucleotide exchange factor activity as molecular function, involved in small GTPase mediated signal transduction. The second uncharacterized protein found corresponded to the *ARHGEF18* gene, which has guanyl-nucleotide exchange factor activity and participates in small GTPase mediated signal transduction processes. *Peptidyl arginine deiminase 2* catalyzes the deamination of arginine residues of proteins leading to citrulline. Citrullination is a poorly understood post translational modification that has been related to modulation of epigenetic events, immunity, and transcriptional regulation[32-34]. This protein has potential roles in fertility, since it has been reported that the female human knockout phenotype for the gene coding for a similar protein (PADI6) is sterile due to a cleavage failure of their fertilized eggs [35, 36]. The possibility that the spermatozoa vehiculates this protein to the oocyte must be considered and warrants further investigation, taking into account that a set of embryo proteins are exclusively of paternal origin [4]. The *Rho guanine nucleotide exchange factor 28* belong to the family of guanine nucleotide exchange factors, this family of proteins have key regulatory roles in embryo development [37], through regulation of differentiation, proliferation and morphogenesis. *KIAA0825* (A0A3Q2HFS8) was also more abundant in the seminal plasma of stallions showing better total and linear motility post thaw. This is a protein of unknown function, however the murine ortholog is known to be expressed during limb development. It has also been reported that variants of this gene are linked to post axial polydactyly in humans[38]. Other proteins enriched in the seminal plasma of stallions showing better motility post thaw were *rRNA adenine N(6)-methyltransferase*, this protein is involved in rRNA methylation as codified by the transcription factor B1 mitochondrial (TFB1M) gene. The knockouts for this gene show altered mitochondrial function, reduced ATP production and increased levels of reactive oxygen species (ROS) in response to cellular stress [39], providing a potential link between the presence of higher levels of this protein and resistance to cryopreservation. The protein A0A3Q2IAZ9 was also more abundant in the seminal plasma of stallions showing higher percentages of linearly motile sperm post thaw, this protein corresponds to the SCAF1 gene. This gene codifies for a protein with RNA polymerase II C-terminal domain binding. Potential functions in early embryo development could be attributed to this protein, considering recent developments indicating the importance of paternal proteins vehiculated in semen and seminal plasma for early embryo development [4]. We also searched for variables able to discriminate between stallions showing better overall performance post thaw. Four proteins were also

discriminant for good motility post thaw, peptidyl arginine deiminase 2, rRNA, adenine N(6) methyltransferase, KIAAA0825 and an uncharacterized protein corresponding to the SCAR-1 gene, as well as two specific proteins from this group consisting of an ATR serine/threonine kinase and the solute carrier family 25 member 37 (SLC25A37). The ATR serine/threonine kinase is activated in the presence of single stranded DNA[40], participating in DNA repair and playing an important role in meiosis in the male germinal epithelium [41], probably linking the higher presence of this protein with better sperm quality, and thus more resistance to the stresses of cryopreservation. The mitochondrial metal transporter mitoferrin1 (SLC25A37), plays a major role in mitochondrial iron homeostasis, as well as in the functionality of oxidative phosphorylation proteins[42], this protein is probably related with improved mitochondrial functionality and thus cryo-resistance.

In conclusion, seminal plasma proteins may play a major role in sperm functionality, in spite of limited time of contact between seminal plasma proteins and spermatozoa during semen processing, these proteins may be vehiculated through extracellular vesicles that rapidly attach to sperm membranes [43, 44], participating in sperm physiology. Bioinformatic analysis identifies discriminant proteins with potential to predict the outcome of cryopreservation, pointing out the potential for the development of new biomarkers reflecting the quality of the ejaculates after further validation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose

AUTHOR`S CONTRIBUTIONS

GG-P performed experiments and writing (native English speaker), FEM-C, JMO-R, AS-R, ES-R performed experiments, CG and CO-F supervision and data analysis, FJP conceived the study, data analysis and interpretation, writing and funding acquisition.

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Figures

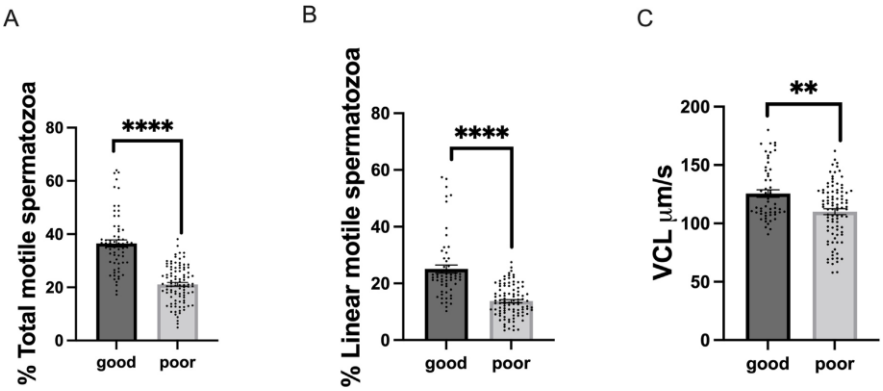

Figure 1.- Average values of motility (total and linear) and velocity (circular velocity) after freezing and thawing in ejaculates from 10 different stallions (3 replicates per stallion n=30), classified as good (>35% total motility post thaw) or poor (<35% total motility post thaw). Semen was collected and processed as indicated in material and methods and the percentage of total motile spermatozoa (A), the percentage of linear motile spermatozoa (B) and the circular velocity (VCL) µm/s (C) were measured using computer assisted sperm analysis (CASA). Data are presented as means ± s.e.m. and derived from 3 identical replicates from each of the stallions (n=30 ejaculates) ** P<0.01, ****P<0.00001.

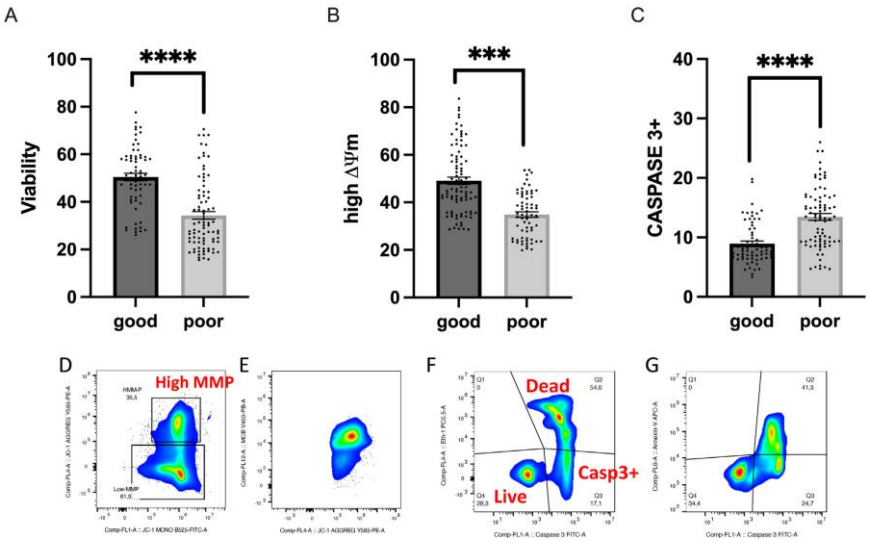

Figure 2.- Viability and mitochondrial membrane potential after freezing and thawing in ejaculates from 10 different stallions (3 replicates per stallion n=30), classified as good (>40 viability) or poor (<40% viability post thaw). Semen was collected and processed as indicated in material and methods and the percentage of live spermatozoa (A), percentage of spermatozoa showing high mitochondrial membrane potential (B) and the percentage of caspase 3 positive spermatozoa (C) were measured using flow cytometry. D-F are representative cytograms of the assays. Data are presented as means ± s.e.m. and derived from 3 identical replicates from each of the stallions (n=30 ejaculates) ***P<0.001, ****P<0.00001.

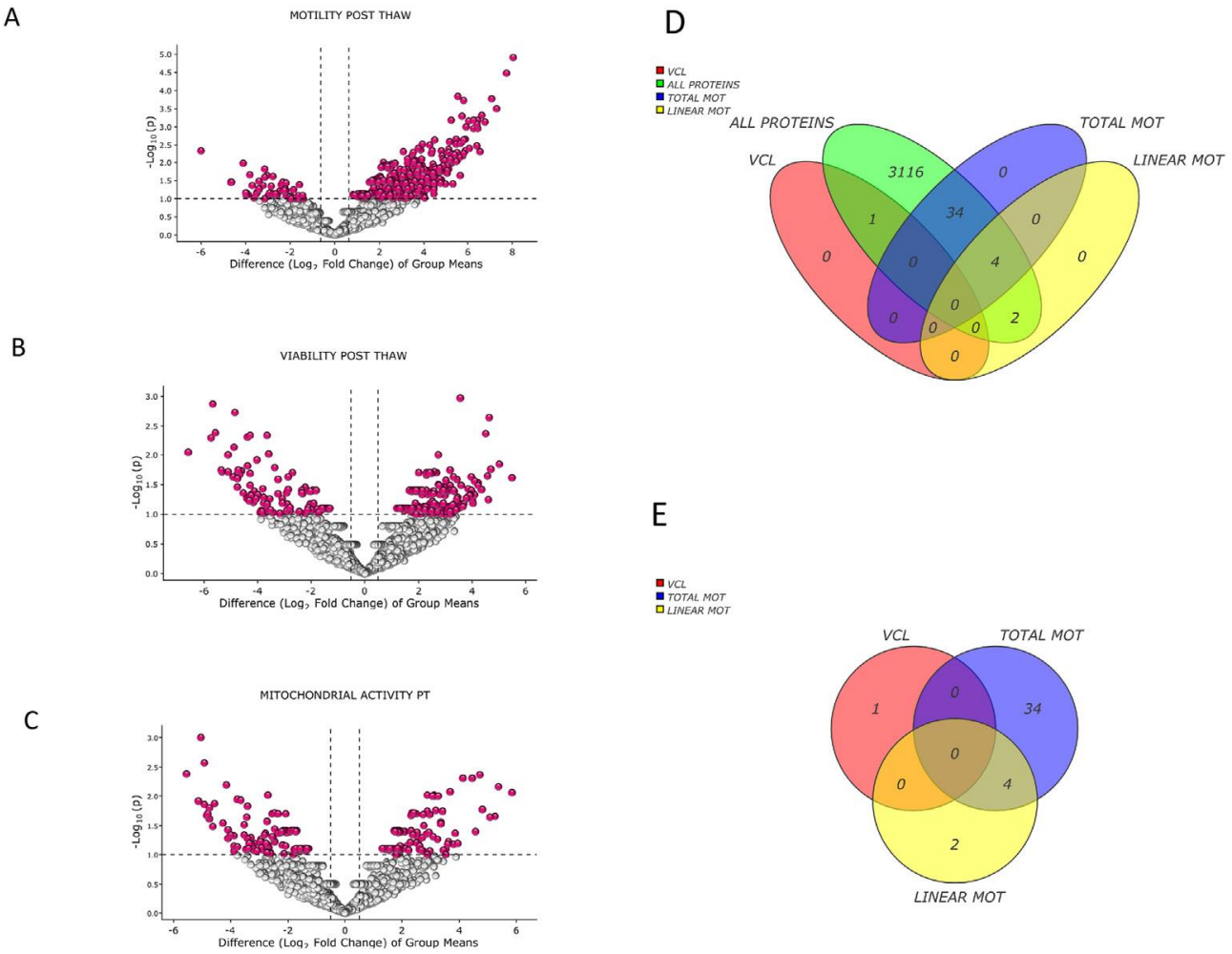

Figure 3.- Volcano plots showing seminal plasma proteins differentially expressed in ejaculates with better quality post thaw in terms of motility (A), viability (B) and mitochondrial membrane potential (C). Proteins which were more abundant are presented on the right-hand side of the volcano plot, proteins less abundant are presented on the left-hand side of the volcano plot. The difference in protein content (Log_2 fold change) is plotted against the significance of the difference $-Log_{10}$ (P) between the two conditions. D and E: Venn diagram showing different amounts of proteins in ejaculates showing better motility, linear motility and velocity post thaw (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis).

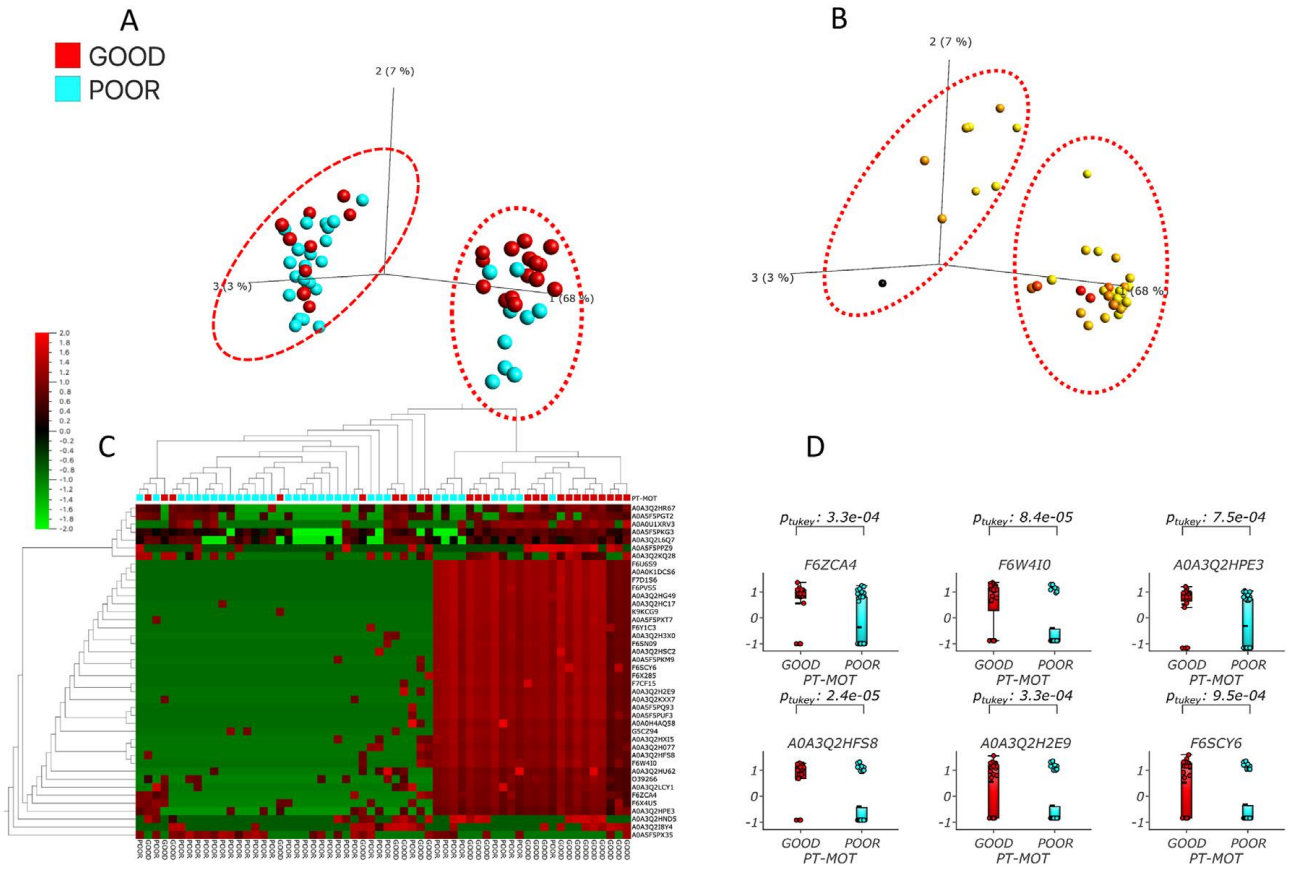

Figure 4.- Bioinformatic analysis of the proteins in stallion seminal plasma (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis), showing different amounts present in stallions with good and poor motility post thaw. A) Principal component analysis of the samples (ejaculates) B) Principal component analysis (variables) C) Heat map showing the different amounts of proteins in seminal plasma of stallions with good and poor motility post thaw D) Seminal plasma proteins identified as potential discriminant variables for stallions showing good (>35%) and poor motility (<35%) post thaw. Proteins were filtered by a fold change of at least 5 between both conditions, with a P=9.6e-04 and q=0.05.

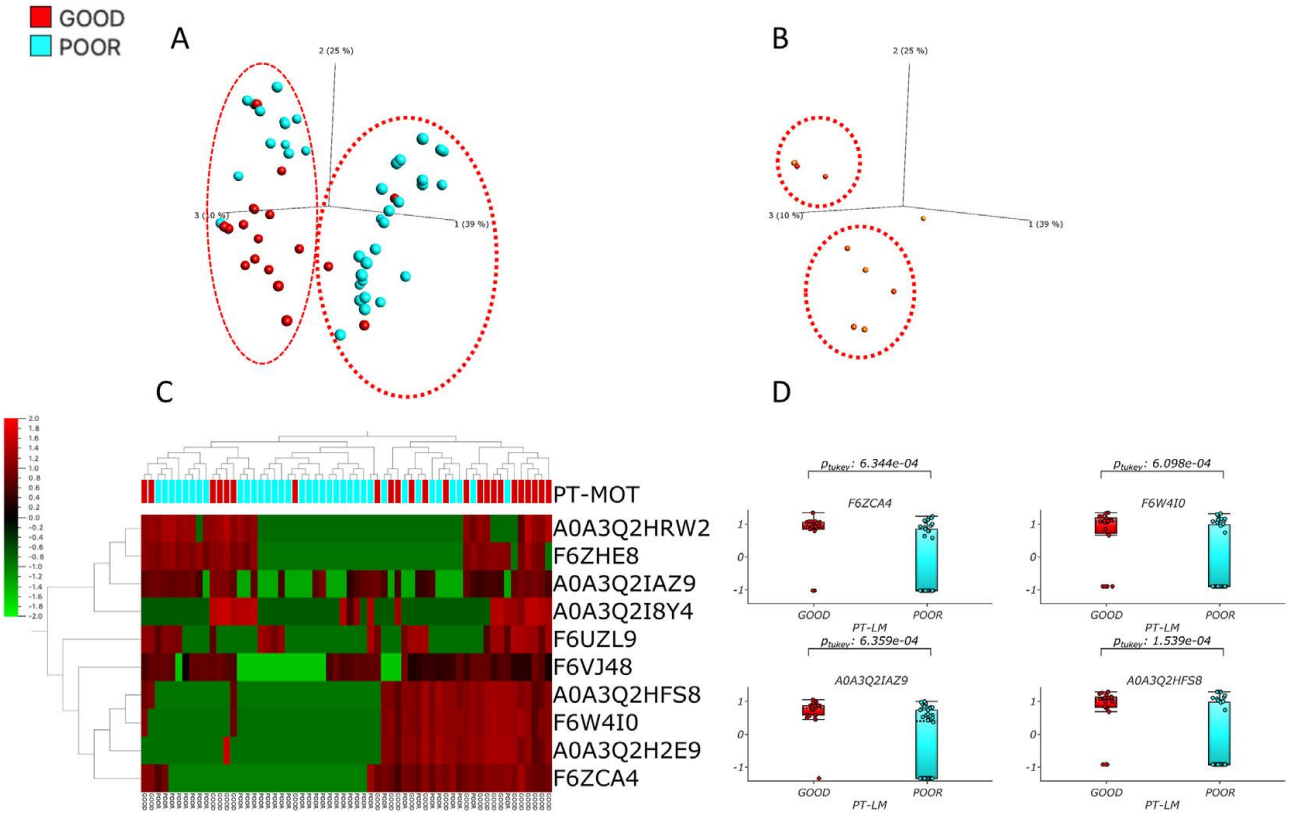

Figure 5.- Bioinformatic analysis of the proteins in stallion seminal plasma (3 independent ejaculates from 10 different stallions in addition to two technical replicates (n = 60 samples were used to derive results from proteomic analysis), showing different amounts in stallions with good and poor linear motility post thaw. A) Principal component analysis of the samples (ejaculates) B) Principal component analysis (variables) C) Heat map showing the different amounts of proteins in seminal plasma of stallions with good and poor linear motility post thaw D) Seminal plasma Proteins identified as discriminant variables for stallions showing good and poor linear motility post thaw. With a fold change >4.75, *P*= 8.7e-4 and q=0.071 four proteins in the seminal plasma were identified as discriminant variables.

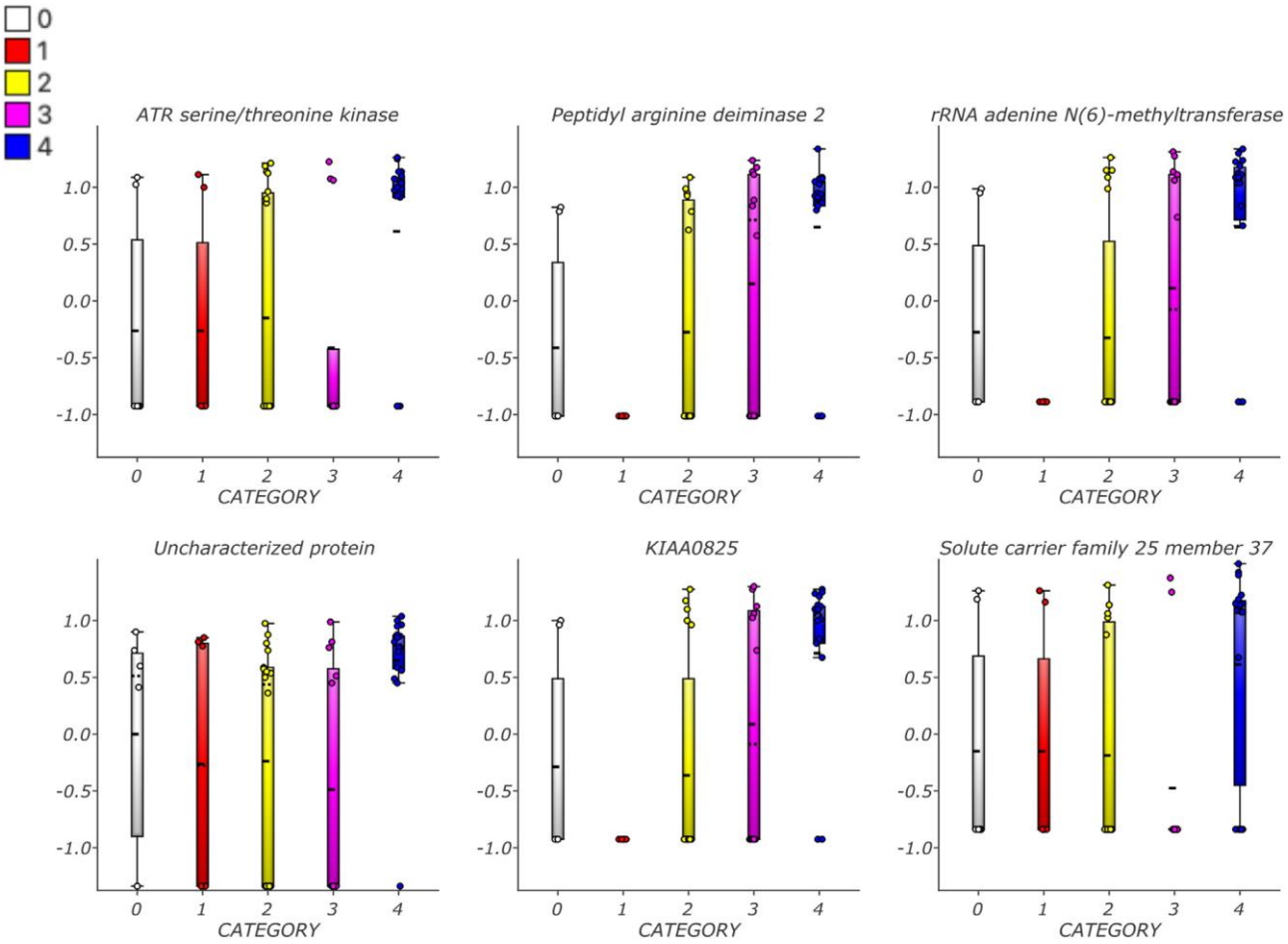

Figure 6.- Bioinformatic analysis of the proteins in stallion seminal plasma showing different amounts in stallions classified in 5 categories according to post thaw sperm quality, with 0 being the worst and 4 the best. Seminal plasma proteins identified as discriminant variables for stallions classified in category number 4. Six proteins were identified as discriminant variables, being more abundant in ejaculates scored in category 4 ($p = 8.7e\text{-}4$, $q = 0.052$, fold change > 4).